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The Acid Tolerance Response of Enteropathogenic *Salmonella* and *Escherichia coli* Strains: A Proteomic Characterisation and Novel Links with Motility and Virulence

By

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ABSTRACT

FACULTY OF SCIENCE
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Doctor of Philosophy

THE ACID TOLERANCE RESPONSE OF ENTEROPATHOGENIC *SALMONELLA* AND *ESCHERICHIA COLI* STRAINS: A PROTEOMIC CHARACTERISATION AND NOVEL LINKS WITH MOTILITY AND VIRULENCE

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Enteropathogenic strains of *Escherichia coli* (EPEC) and *Salmonella* are major sources of food- borne disease and cause significant mortality and morbidity world-wide. In order to survive within the host and cause disease, these bacteria must adapt to a wide range of hostile environmental conditions, including extremes of temperature, pH and osmolarity among others. This thesis concentrates on elucidating the mechanisms involved in the adaptation of exponentially growing EPEC and *Salmonella* to inorganic and weak organic acids. An understanding of these mechanisms, collectively known as the acid tolerance response (ATR), is of relevance to both the food production and medicinal industries.

A protocol has been established enabling the ATR survival kinetics of bacterial species and strains to be compared. This method was used to identify the conditions required for the optimal induction of ATRs in these micro-organisms using both inorganic and organic acids. Protein components of a maximally induced ATR stimulon of *S. typhimurium* were subsequently identified using a proteomic approach. Two-dimensional gel electrophoresis followed by tryptic mass fingerprinting and/or N-terminal sequencing has enabled 12 ATR proteins, 3 of which were previously of unknown function, to be grouped according to their probable roles in acid tolerance. Co-regulation studies suggest that 7 of these proteins fall within the virulence associated PhoPQ and/or RpoS regulons.

One of the most striking findings was that flagellin, the major structural component of the bacterial flagellum, is down-regulated at pH 3.0 in acid adapted *Salmonella*. Further studies using reporter gene fusions indicated that the entire flagellar apparatus is transcriptionally repressed under these conditions. PhoPQ was found to mediate this mechanism, which results in a loss of cell motility, by acting directly or indirectly at the level of the flagellar *flhCD* master operon.

Additional links between other virulence associated mechanisms and the ATR were also investigated. 2 ATR proteins regulated by PhoPQ were expressed similarly during the oxidative stress response. Correspondingly, survival assays indicate that an acid induced cross protection to oxidative stress in *Salmonella* is mediated by this global regulator. A further two regulators of pathogenesis (the BipA GTPase and the EAF plasmid) were characterised as negative regulators of the ATR in certain EPEC strains. It is concluded that ATR mechanisms are variable between different species and strains.

Based on these results, it is proposed that intimate connections exist between the regulation of the ATR and virulence associated processes, such as motility, that are essential for pathogenesis. The full elucidation of the mechanisms behind the regulation and implementation of these systems may pave the way for future treatments of food-borne disease. The global regulatory molecules involved constitute putative drug targets.

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ABBREVIATIONS.

ABC.	ATP Binding Cassette.
A/E.	Attaching and Effacing.
AIDS.	Auto Immune Deficiency Syndrome.
ASP.	Acid Shock Protein.
ATP.	Adenosine Tri-Phosphate.
ATR.	Acid Tolerance Response.
BCCP.	Biotin Carboxyl Carrier Protein.
BFP.	Bundle Forming Pili.
BSA.	Bovine Serum Albumin.
CAMP.	Cationic Anti-Microbial Peptides.
cAMP.	Cyclic Adenosine Mono-Phosphate.
CCW.	Counter Clockwise.
CW.	Clockwise.
CFAs.	Cyclopropane Fatty Acids.
CFTR.	Cystic Fibrosis Transmembrane Conductance Regulator.
CFU.	Colony Forming Units.
CRP.	cAMP Receptor Protein.
2D-PAGE.	Two-Dimensional Polyacrylamide Gel Electrophoresis.
DNA.	Deoxyribonucleic Acid.
DNP.	Dinitrophenol.
DPGA.	Diphosphoglycerate.
DTT.	Dithiothreitol.
EAEC.	Enteroaggregative *Escherichia coli.*
EAF.	EPEC Adherence Factor.
ECL.	Electrochemiluminescence.
EDTA.	Ethylenediaminotetraacetic acid.
EHEC.	Enterohaemorrhagic *Escherichia coli.*
EICs.	Extracellular Induction Components.
EIEC.	Enteroinvasive *Escherichia coli.*
EPEC.	Enteropathogenic *Escherichia coli.*
ESP.	*Escherichia coli* Secreted Protein.
ETEC.	Enterotoxigenic *Escherichia coli.*
FACS.	Fluorescence Activated Cell Sorter.
G+C.	Guanine + Cytosine.
GDP.	Guanosine Di-Phosphate.
GTP.	Guanosine Tri-Phosphate.
HSP.	Heat Shock Protein.
HUS.	Haemolytic Uraemic Syndrome.

IEF.	Isoelectric Focusing.
IPG.	Immobilised pH Gradient.
LB.	Luria Bertani.
LPS.	Lipopolysaccharide.
MCPs.	Methyl-Accepting Chemotaxis Proteins.
M_r.	Molecular Weight.
ONPG.	O-nitrophenyl-β-D-galactopyranoside.
OSR.	Oxidative Stress Response.
P.	Phosphate.
PDHC.	Pyruvate Dehydrogenase Complex.
PFU.	Plaque Forming Units.
pH_i.	Intracellular pH.
pH_o.	Extracellular pH.
pI.	Isoelectric Point.
Pips.	Pathogenicity Island Proteins.
PKC.	Protein Kinase C.
PLC-γ.	Phospholipase C-γ.
PMSF.	Polymethylsulphonylflouride.
ppGpp.	Guanosine 5'-diphosphate 3'-diphosphate.
PVDF.	Polyvinyldiflouride.
ROS.	Reactive Oxygen Species.
SDS.	Sodium Dodecyl Sulphate.
SDS-PAGE.	SDS-Polyacrylamide Gel Electrophoresis.
Sop.	*Salmonella* outer membrane protein.
SPI-1.	*Salmonella* Pathogenicity Island-1.
SPI-2.	*Salmonella* Pathogenicity Island-2.
SPI-3.	*Salmonella* Pathogenicity Island-3.
SPI-4.	*Salmonella* Pathogenicity Island-4.
SPI-5.	*Salmonella* Pathogenicity Island-5.
SSR.	Starvation Stress Response.
US.	United States.
X-GAL.	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside.

CHAPTER 1.

INTRODUCTION.

CHAPTER 1. INTRODUCTION.

Enteric bacteria such as the *Salmonella* serovars and *Escherichia coli* frequently encounter hostile conditions in their natural habitats. Examples of these potentially life-threatening stresses include high levels of acidity, reactive oxygen species and nutrient limitation to name but a few. An additional threat from host antimicrobial defences must also be circumvented by pathogenic bacteria if they are to survive and cause disease. Thus, these micro-organisms have evolved complex mechanisms enabling adaptation and survival in the many extreme environments encountered.

This thesis concerns the Acid Tolerance Response (ATR), an adaptive mechanism enabling bacteria to survive at extremes of low pH. The study focuses on the ATR employed by the enteric pathogens *Salmonella enterica* serovar Typhimurium (commonly known as *Salmonella typhimurium*) and enteropathogenic *Escherichia coli* (EPEC) during exponential growth. Therefore, prior to describing this research, it is appropriate to introduce these bacteria, their enteropathogenic behaviour and the current status of research into stress adaptation.

1.1. The Enteropathogenicity of *S. typhimurium* and *E. coli.*

1.1.1. Classification.

S. typhimurium and *E. coli* are both members of the Enterobacteriaceae family. They are Gram-negative, non-sporing, motile (peritrichously flagellate), facultatively anaerobic bacilli.

Closely related strains of *Salmonella* and *E. coli* are often distinguished by serotype. This is a representation of differences in cell-surface O and H antigens. Variations in the O antigens occur in the O-specific chains of the lipopolysaccharide in the outer membrane, whereas differences in the H antigens are due to variation in flagellin structure. There are about two thousand named *Salmonella* serotypes. The antigenic formula of *S. typhimurium* is 1,4,[5],12:i:1,2. Therefore, O antigens 1 (underlined to show that the antigen is present as a result of phage conversion), 4, 5 ([] indicates

variable presence) and 12 are present as well as phase 1 H antigen i, and phase 2 H antigens 1 and 2 (Singleton, 1999).

1.1.1.1. E. coli *Classification.*

Serotypes of *E. coli* that cause diarrhoea/ gastroenteritis have been classified into groups. The five main groups, enteroaggregative *E. coli* (EAEC); enterohaemorrhagic *E. coli* (EHEC); enteroinvasive *E. coli* (EIEC); enteropathogenic *E. coli* (EPEC); and enterotoxigenic *E. coli* (ETEC) are described in Table 1.1 (Singleton 1999).

Table 1.1. Groups of *E. coli* which cause diarrhoea/gastroenteritis including their pathogenic properties and examples of specific strains. Adapted from Singleton, 1999.

GROUP	PATHOGENESIS OF DISEASE
EIEC e.g. O124, O143, O152.	Usually food-borne. Adheres to, invades and destroys epithelial cells in the ileum/colon, causing dysentery. Can cause watery diarrhoea. At least one plasmid encoded enterotoxin is formed
EHEC e.g. O26, O157:H7.	Usually food-borne. Minimum infectious dose <100 cells. Forms one or both of two phage-encoded toxins (shiga-like toxins I and II or verotoxins I and II). Toxins can cause severe bloody diarrhoea (haemorrhagic colitis). Can also cause haemolytic uraemic syndrome (bloody diarrhoea followed by acute kidney failure). Many strains cause EPEC-like lesions *in vitro*.
ETEC e.g. O6, O8, O63, O115, O148.	Usually food- and/or water-borne. Common cause of travellers diarrhoea and diarrhoea of children in developing countries. Plasmid encoded fimbriae (colonisation factors I and II) with which it adheres to epithelia of small intestine. Forms heat stable toxins (STI and STII) and heat labile toxins (LTI and LTII). STI binds brush border and activates host signals resulting in ionic imbalance and watery diarrhoea.
EPEC e.g. O55, O114, O127.	Food- and/or water-borne. Causes diarrhoea that may result from reduction in absorption due to loss of microvilli. See text.
EAEC e.g. O111:H12	Cause persistent diarrhoea in children, particularly in developing countries. Characterised by aggregative pattern of adherence to epithelial cells. Mechanism of pathogenesis unknown. Forms adhesins and toxins.

This study is particularly concerned with bacteria belonging to the fourth of these groups, which are collectively a major cause of infant diarrhoea in the developing world. It is thought that the EHEC group arose from EPEC by acquisition of phage bearing genes for shiga-like toxins (Reid *et al*, 2000).

1.1.1.2. Salmonella *Classification.*

A collection of 72 strains representing 37 serovars of *Salmonella* have been classified by enzyme electrophoresis for allelic variation in 25 chromosomal genes (Boyd *et al*, 1993). The collection includes isolates of the host adapted serovars *S. choleraesuis*, *S. dublin*, *S. gallinarium*, *S. paratyphi A*, *S. paratyphi B*, *S. paratyphi C*, *S. pullorum*, *S. sendai*, *S. typhi* and *S. typhisuis* as well as strains of *S. enteritidis* and *S. typhimurium* commonly recovered from broad host ranges. 71 distinctive multilocus genotypes are represented. This study is particularly concerned with *S. typhimurium*, a significant cause of non-typhoid disease in man. Infection of the mouse by this bacteria represents a well established model of *S. typhi* pathogenesis in humans (see section 1.1.3.1.).

1.1.2. Life cycles.

Due to their close relatedness, the natural life cycles of *S. typhimurium* and EPEC outside of the host are very similar. The various environmental habitats of *Salmonella* and *E. coli* and modes of transmission between them are summarised as a flow diagram in Figure 1.1. Various evolved and acquired (through horizontal gene transfer) mechanisms have enabled these organisms to survive in a diverse range of natural environments. Their ability to survive harsh environments such as desiccation, nutrient limitation, and varying degrees of temperature, pH, oxygen and osmolarity ensures efficient transfer between environments. For example, transmission between stagnant, acidic water and food products via skin contact; or between fermentative survival in animal faeces and drinking water.

1.1.3. *Salmonella* and *E. coli* disease epidemiology and symptoms.

Potentially fatal *Salmonella* and *E. coli* infections are some of the most common causes of foodborne disease in man. Even in the most developed countries such as the United States (US), infection attributable to food affects 6 to 8 million people, causes 9,000 deaths and costs an estimated 5 billion US dollars a year (Altekruse *et al*, 1997). It is therefore pertinent to review the disease epidemiology and symptoms of these micro-organisms before describing the mechanisms of their enteropathogenicity.

1.1.3.1. Salmonella *Epidemiology and Symptoms.*

Salmonella infection, or Salmonellosis, is usually contracted through oral ingestion of the bacteria. A minimum number of cells of a specific strain is required before disease symptoms appear (the infectious dose). The infectious dose for *Salmonella* constitutes 10^3 to 10^5 bacteria. The major factor controlling infectious dose is the bacterium's ability to survive the acidic pH of the stomach. Salmonellosis can be contracted through foods such as eggs, poultry, meat and fresh produce as well as via untreated water and other human/animal carriers.

The incidence of non-typhoid Salmonellosis, of which *S. typhimurium* is a significant cause in man, is estimated to be increasing. With an estimated 2,000,000 cases and 500 - 2,000 deaths per year in the US alone, it is a major global health problem (Altekruse *et al*, 1997). Infection of otherwise healthy individuals by non-typhoid *Salmonella* usually results in gastroenteritis and enteric fever marked by abdominal pain and diarrhoea (Slauch *et al*, 1997). In most people, this occurs 12 to 72 hours after infection (National Centre for Infectious Diseases, http://www.cdc.gov/ncidod/ncid.htm). These symptoms are caused by intestinal inflammation and fluid secretion involving an activated host immune response, for example, the migration of polymorphonuclear leukocytes to invaded tissues. However, the disease can progress to infection of the liver, gall bladder and spleen (Slauch *et al*, 1997). In immunocompromised individuals such as Auto Immune Deficiency Syndrome (AIDS) patients, *S. typhimurium* can cause more serious, potentially life threatening systemic disease, for example, bacteremia (Spector, 1998).

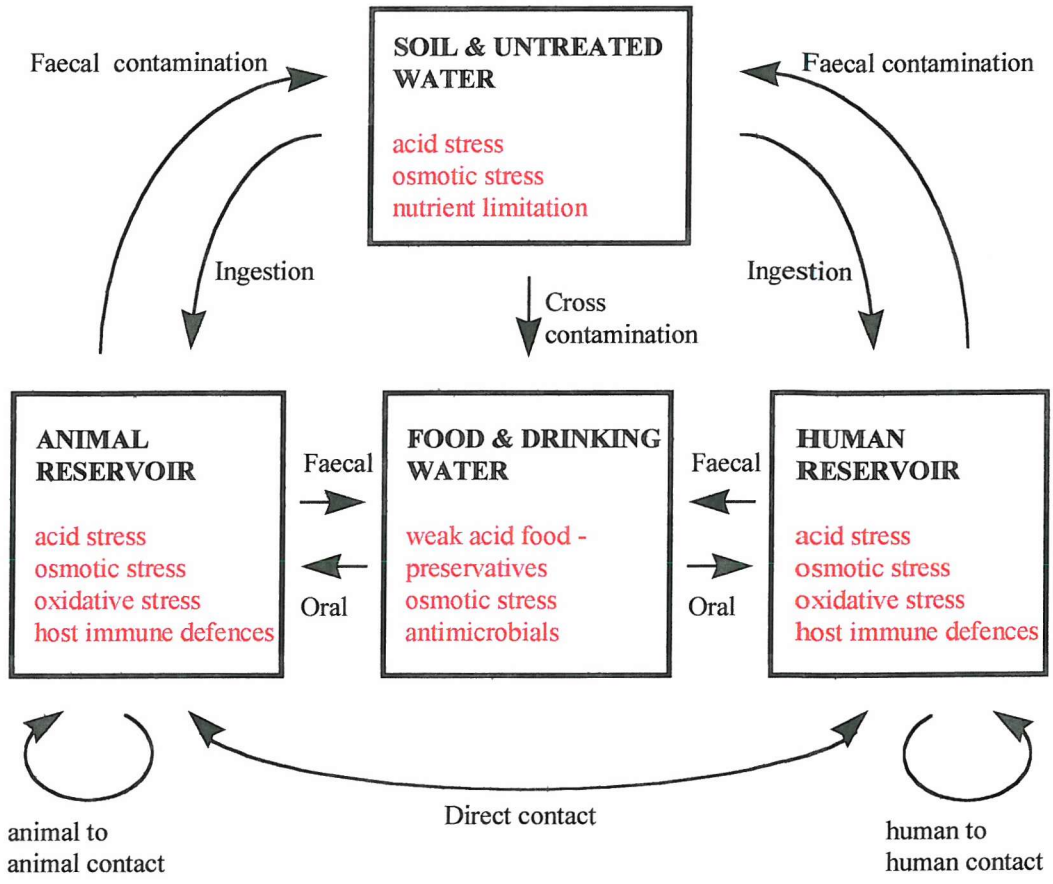


Figure 1.1. A flow diagram depicting various environmental habitats of *Salmonella* and *E. coli*, including modes of transmission between them . Examples of potentially lethal stresses contended with are given in red.

A more severe form of illness, Typhoid Fever, is caused by certain serovars of *Salmonella* such as *S. typhi*. During this disease, inflammation of the intestine can become so intense that it causes local necrosis of tissue with formation of typhoid ulcers and may cause perforation and haemorrhage (Sansonetti and Phalipon, 1999). People with typhoid fever usually have a sustained fever as high as 39° to 40°C. They may feel weak or have stomach pains, headache or loss of appetite. In some cases, patients have a rash of flat, rose coloured spots (National Centre for Infectious Diseases). *S. typhimurium* infection in mice mimics the systemic disease caused by *S. typhi* in humans. The mouse infection is well established as a model of systemic Salmonellosis as well as a general model system to study the molecular basis of host-pathogen interactions (Slauch *et al*, 1997). It is therefore important that the relatively less virulent (from a human perspective) *S. typhimurium* can be utilised to gain further insight into the stress tolerance mechanisms employed by *S. typhi*.

1.1.3.2. E. coli *Epidemiology and Symtoms.*

Enteropathogenic *E. coli* mediated food poisoning is contracted in much the same way as Salmonellosis. Vehicles for transmission include, for example, foods, untreated water, and human/animal to human contact (usually via faecal contamination). The mechanisms by which EPEC causes diarrhoea type are still unclear (DeVinney *et al*, 1999). They may result partly from reduction in absorption due to loss of microvilli (Singleton, 1999). The symptoms of EAEC, EHEC, EIEC and ETEC mediated disease are summarised in Table 1.1.

Of particular importance is the EHEC serovar O157:H7. This is a highly virulent foodborne pathogen, first recognised after two outbreaks in 1982 in the US associated with consumption of undercooked hamburgers (Riley *et al*, 1983). Outbreaks have since been reported in the UK, Canada, Africa and Japan. This strain alone is responsible for 725,000 cases and 250 deaths per year in the US (Altekruse *et al*, 1997). The bacteria causes haemolytic uraemic syndrome (HUS): typically a bloody diarrhoea followed by acute renal failure, particularly in children. It is verocytotoxic: a protein is produced that was identified as toxic to Vero cells (kidney cells of the African Green

Monkey). Outbreaks have been traced to ground beef, dry, fermented sausage and unpasteurised apple juices amongst others (Buchanan and Doyle, 1997).

E. coli O157:H7 has a very low infectious dose, thought to be associated with its extreme acid tolerance resulting in high levels of survival after exposure to stomach acid. This is important as the basic ATR mechanisms appear to be conserved across all *E. coli* strains (Lin *et al*, 1996). Thus, examining the relatively less virulent, but phylogenetically related EPEC strains may help in characterising the stress responses of EHEC O157:H7.

It is clear that foodborne disease is a severe global health problem in both developed and developing countries. Malnutrition and the lack of basic sanitary conditions in the third world obviously have serious implications for the spread of disease in these countries. Studies into the mechanisms behind 'food-poisoning' such as the ATR are therefore essential.

1.1.4. Determination of virulence and enteropathogenicity.

Enteropathogenic bacteria such as *S. typhimurium* and EPEC have developed specific systems that enable them to survive and replicate within the host. These systems are absent from non-pathogenic strains. Virulence factors, pathogenicity islands and Type III secretion systems are the main determinants of enteropathogenicity.

1.1.4.1. Virulence Factors.

Virulence factors can be defined as the properties of an organism that allow a pathogen to infect a host and cause a disease. Genes that encode virulence factors are referred to as virulence genes. These genes have been estimated to constitute five to ten percent of the *S. typhimurium* genome (Slauch *et al*, 1997). When they are lost, for example by mutation, there is a significant decrease in the virulence of the organism. Extracellular pathogens such as EPEC and intracellular pathogens such as *S. typhimurium* activate or repress the expression of virulence genes to enable them to survive unique microenvironments within the host. For example, when *Salmonella* is in close

proximity to the intestinal epithelia it must adapt to the environment in a way that enables it to invade these cells. Proteins regulated in expression during stress responses such as the ATR can also be termed virulence factors.

Many virulence factors are regulated by global regulatory systems. These systems control the expression of multiple genes simultaneously. The ferric uptake regulator protein, Fur, is an example of a global regulator that activates or represses transcription of a number of genes in relation to iron availability. This protein is discussed in more detail with respect to the ATR. Another example of a global regulator is the alternative sigma factor, σ^s, encoded for by *rpoS*. The role of RpoS in the ATR is detailed later.

Virulence genes are also regulated by a sub-set of global regulators termed two-component signal transduction systems. These systems have a membrane associated molecule which can respond to an extracellular signal. The signal is then relayed to an intracellular response regulator molecule. The latter component can cause an adaptive response, usually by directly binding deoxyribonucleic acid (DNA) to influence transcription (Hoch, 2000). The sensor molecule is a histidine kinase. This can undergo adenosine triphosphate (ATP) dependent autophosphorylation, at an active site containing a histidine residue, in response to the extracellular signal. The sensor can then activate the response regulator molecule by phosphorylation at a site containing an aspartate residue. Examples of two component regulatory systems are described in more detail in relation to stress responses.

1.1.4.2. Pathogenicity Islands.

Many virulence genes are clustered in localised regions of the chromosome called pathogenicity islands (Groisman and Ochman, 1996). Pathogenicity islands are present in pathogenic strains, but absent in their closely related non-pathogenic cousins. The guanine plus cytosine (G + C) content of these regions is often in contrast to the rest of the genome. This suggests they were acquired by horizontal gene transfer. An example of a pathogenicity island is SPI -1 (Groisman and Ochman, 1996) in *S. typhimurium*: a large region (40kb) containing multiple virulence genes. SPI-1 has a G + C content of

40 - 47%, whereas the average G + C content of the *S. typhimurium* genome is 52% (Slauch *et al*, 1997).

1.1.4.3. Type III Secretion Systems.

Type III secretion systems occur mainly or exclusively in pathogens and are encoded by genes located on pathogenicity islands. These protein secretion systems are activated by contact with eukaryotic host cells. The structure is composed of multiple protein subunits which span the bacterial cell envelope and the host cytoplasmic membrane, thus forming a channel connecting the cytoplasm of the two cells. Bacterial effector proteins which modulate host cell function are delivered into the eukaryotic cell via this pore. These proteins can then dictate the terms of the bacterial to host cell interaction. Interestingly, the secreted proteins do not contain a cleavable signal peptide as in *sec* dependent systems; many require chaperone proteins for correct transport (Galán and Collmer, 1999). A comprehensive review of type III secretion systems in bacterial pathogens of animals and plants is given by Hueck (1998). Examples of type III secretion systems are described below.

1.1.5. *Salmonella typhimurium* is a facultative intracellular pathogen.

Immediately after oral infection of the host, *Salmonella* and *E. coli* are subjected to the extreme acid environment of the stomach. The mean stomach pH is 2.0. However, this can range between 1.5 to 5.5, increasing to pH 6.0 upon ingestion of a Western-type meal (Konturek *et al*, 1994). Combating this potentially lethal environment involves induction of ATR mechanisms. Once through the stomach, both pathogens proceed to the slightly alkaline pH of the small intestine. Here, the murine model has shown that *Salmonella* can cross the intestinal barrier and cause a systemic infection. *Salmonella* preferentially penetrates the intestinal mucosa via M cells associated with lymphoid follicles called Peyer's patches (Jones *et al*, 1994). These areas, involved in antigen processing, are surrounded by a network of lymphatic capillaries. Macrophages, typically in close contact with the M cells are then induced by bacteria to phagocytose the invading *Salmonella* (for example see Tomita *et al*, 1981; Ivanoff *et al*, 1980). The pathogen is then transported to the spleen and liver via the lymphatic system. The

bacteria can replicate in the acidic environment of the macrophage phagosomes, as well as the lymph nodes, liver and spleen. Subsequent entry into the bloodstream can lead to septicaemia (Reviewed in Slauch *et al*, 1997). *Salmonella* can also progress to the gall bladder where it persists and from where it can re-enter the intestine. Although such chronic carriage only develops rarely, for example in roughly 1.4 percent of patients with typhoid (Hoffman *et al*, 1975), epidemiological considerations suggest it is a critical factor in the continued propagation of host-adapted *Salmonella* serotypes (Kingsley and Bäumler, 2000). Inflammation and ulceration of the Peyer's patches and intestinal epithelia results in diarrhoea (Slauch *et al*, 1997). The passage of *Salmonella* through the host is summarised in Figure 1.2.

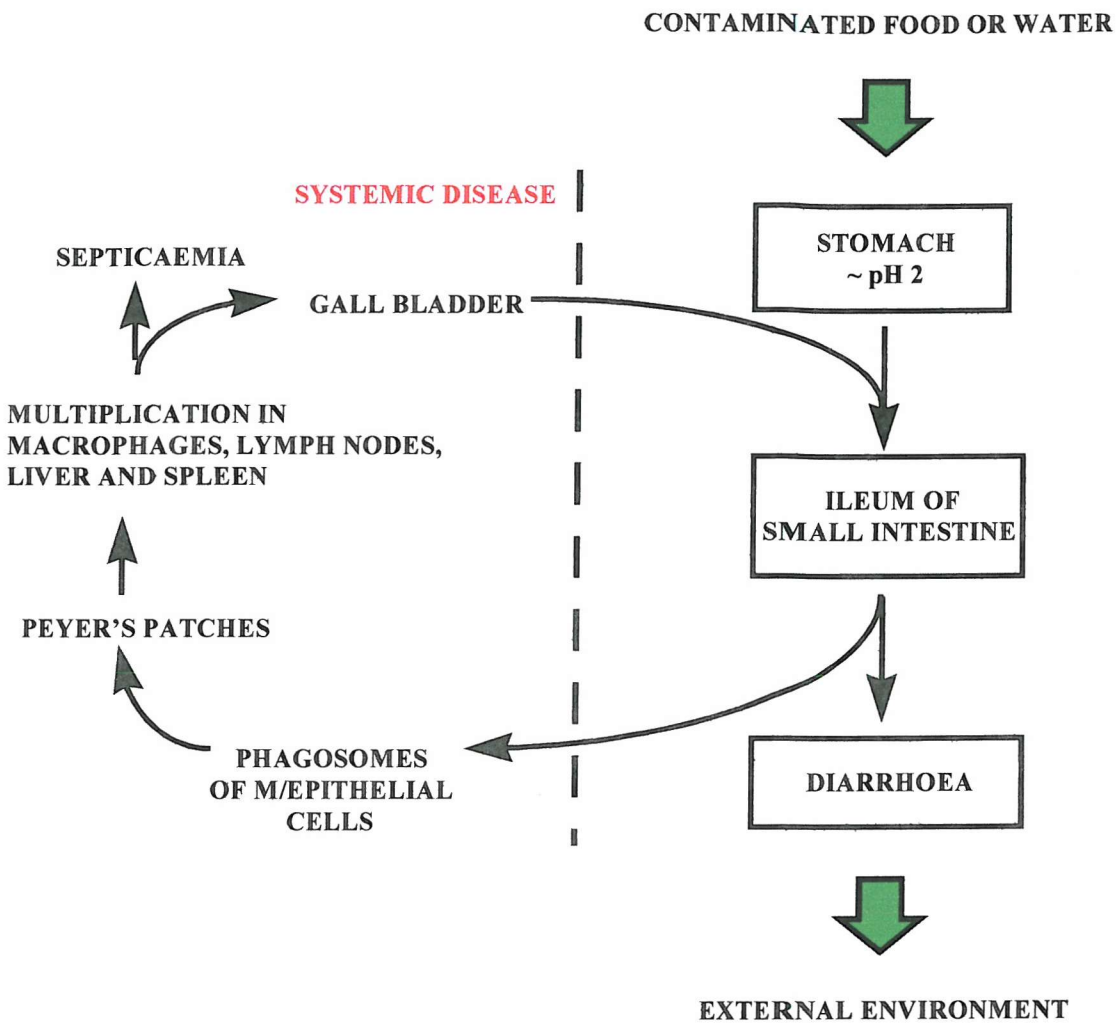
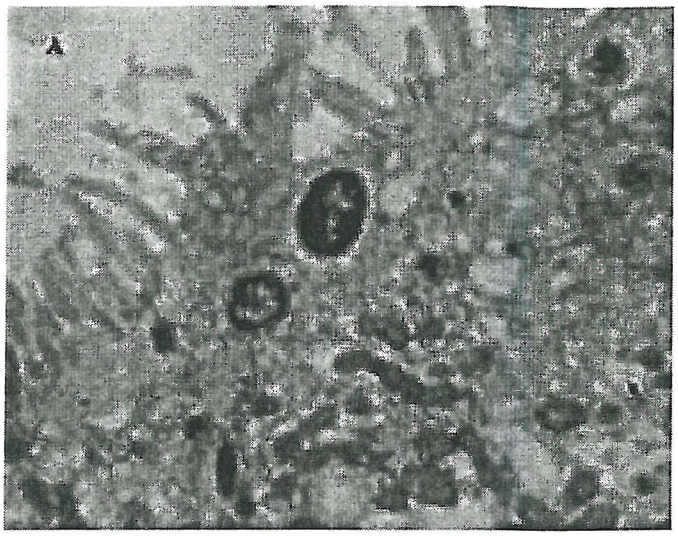


Figure 1.2. The passage of *Salmonella* through the BALB/c mouse. Adapted from Slauch *et al*, 1997.

1.1.5.1. Invasion of the Intestinal Epithelia.

Salmonella invasion of the intestinal epithelia constitutes a two step process of attachment and subsequent entry via the apical membrane of the host cell. Several micro-organisms selectively penetrate the intestinal epithelial layer via the relatively increased permeability of M cells. These cells have a poorly organised brush border with few microvilli present and lack a mucus coat. This together with the absence of a glycocalyx facilitates binding to the apical surface. However, *Salmonella* is also capable of invading standard, villous epithelia (Sansonetti and Phalipon, 1999). Attachment to M cells is mediated by the long, polar Lpf fimbrae which are specific towards this cell type in mice (Bäumler *et al*, 1996). Adherence to villous epithelia is via the plasmid encoded Pef fimbrae (Bäumler *et al*, 1997). *S. typhi*, but not *S. typhimurium* can also bind to the host epithelia via the cystic fibrosis transmembrane conductance regulator (CFTR) that facilitates submucosal translocation (Pier *et al*, 1998). Once in close contact with the epithelium, *Salmonella* induces a loss of microvillar structure followed by membrane ruffling localised to the area of bacterial-host cell contact (Finlay *et al*, 1991). A transmission electron micrograph of *Salmonella* induced membrane ruffling in epithelial cells is shown in Figure 1.3a. Membrane ruffling is accompanied by macropinocytosis, which leads to internalisation of the bacteria (Garcia-del Portillo and Finlay, 1994). The process is caused by bacterial mediated disruption of the host cell cytoskeleton (Goosney *et al*, 1999). Once inside the epithelial cell, *Salmonella* resides within membrane bound vesicles (Goosney *et al*, 1999). The environment of such phagosomes has been studied by monitoring the expression of *Salmonella* genes that are transcribed under particular environmental conditions (Garcia-del Portillo *et al*, 1992). Expression levels of *iroA* and *mgtB* (induced by Fe^{2+} and Mg^{2+} limitation respectively) in Madin-Darby canine kidney epithelial cells were monitored by determining the β-galactosidase activity derived from *lacZ* transcriptional fusions. High levels of expression suggested that the concentrations of free Fe^{2+} and Mg^{2+} in the *Salmonella* containing vacuole may be low. In a similar manner, expression of the *cadA* gene (induced by pH 6.0 in the presence of lysine, with enhanced expression when oxygen is limited) was found to be elevated, but only at early post-infection times. This suggests the vacuole may have a mildly acidic pH and contain lysine at this time (Garcia-del Portillo *et al*, 1992).

a.



b.

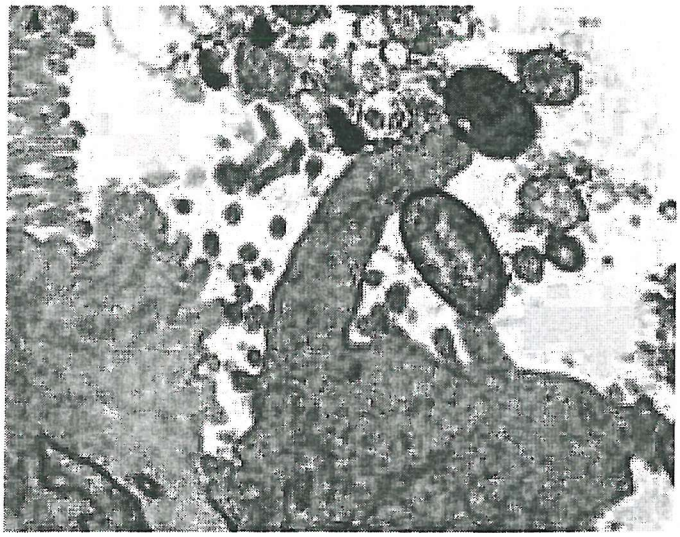


Figure 1.3. A comparison of the infection of intestinal epithelial cells by enteropathogenic *Salmonella* and *E. coli*. **a.** A transmission electron micrograph of *Salmonella* induced membrane ruffling in epithelial cells. *Salmonella* induces host cell cytoskeletal rearrangements leading to membrane ruffling and subsequent internalisation of the bacteria. **b.** A transmission electron micrograph of an A/E lesion formed by EPEC infecting intestinal epithelial cells. The bacterium binds to the external face of the epithelial cell by secreting its own receptor, Tir, into the host and binding to it via intimin in the outer membrane. Subsequent host cell cytoskeletal rearrangement form the characteristic pseudopod underneath the bacteria (Adapted from Goosney *et al*, 1999).

Many of the genes required for *S. typhimurium* invasion are located on a 40kb pathogenicity island termed *Salmonella* pathogenicity island - 1 (SPI-1). This is at a position of 63 minutes on the bacterial chromosome. Inactivation of these genes prevents *Salmonella* from invading epithelial cells *in vitro* (Galán and Curtiss, 1989). SPI-1 encodes structural components of the Inv/Spa type III secretion system and a number of secreted proteins, including the *Salmonella* invasion proteins (Sips). The pathogenicity island also expresses the associated regulatory proteins, InvF and HilA (Groisman and Ochman, 1997). SptP is an example of a SPI-1 encoded protein essential for *S. typhimurium* invasion and translocated into the host cell by the Inv/Spa type III secretion system. This protein modulates the host actin cytoskeleton through its tyrosine phosphatase activity (Goosney *et al*, 1999). Bacterial proteins secreted into the host cell also stimulate eukaryotic Cdc42 (Hardt *et al*, 1998) and phospholipase C (Ruschkowski *et al*, 1992), resulting in alteration of host Ca^{2+} levels and subsequent cytoskeletal rearrangements. This results in the disruption of host actin polymers, thereby facilitating membrane ruffling and subsequent invasion (Goosney *et al*, 1999). The SptP protein may be responsible for the reversal of these cytoskeletal changes once invasion is complete, again by acting on Cdc42 and another Rho GTPase termed Rac (Fu and Galán, 1999).

A second pathogenicity island involved in *S. typhimurium* invasion is *Salmonella* pathogenicity island - 5 (SPI-5). SPI-5 was discovered in *Salmonella dublin* and is conserved in all *Salmonella* serovars tested including *S. typhimurium*. The pathogenicity island encodes for *Salmonella* outer protein B (SopB), and pathogenicity island proteins (Pips) PipA, PipB, PipC and PipD (Wood *et al*, 1998). SopB is translocated into the eukaryotic target cell by the Inv/Spa type III secretion system encoded for within SPI-1. Here, it affects cellular responses leading to the influx of neutrophils into the intestinal epithelium and the induction of fluid secretion (Galyov *et al*, 1997). PipC is thought to be a specific chaperone, assisting secretion of SopB; PipB may have a role in glycolipid biogenesis and PipD may be a secreted peptidase (Wood *et al*, 1998).

The regulation of SPI-1 and SPI-5 and thus invasiveness is complex. Sensing of the bacterium's environmental conditions is linked to the expression of particular

pathogenicity islands. HilA is a transcriptional regulator encoded within SPI-1 that controls the expression of other SPI-1 genes. This protein lacks a phosphoryl acceptor domain as found in two component regulatory systems and is therefore assumed to be a constitutive activator. Thus, if HilA is present, it is active. HilA positively regulates the SPI-1 genes in response to environmental factors including osmolarity, pH, oxygen and divalent cations. (Bajaj *et al*, 1996). It has also been found to activate SPI-5 and an additional pathogenicity island, SPI-4 (Ahmer *et al*, 1999). The sensory proteins known to regulate the expression of *hilA* are the *Salmonella* invasion regulator, SirA, (Ahmer *et al*, 1999) and the two component transduction system, PhoPQ (Bajaj et al 1996). The PhoPQ system represses *hilA* after the intestinal stage of disease is completed. Thus, genes essential for invasiveness are repressed when no longer required. As PhoPQ responds to conditions of low pH and low levels of divalent cations, repression of *hilA* will occur in the macrophage, but not in the intestinal environment. In fact, SPI-1 genes are induced by pH8, the approximate pH of the gut lumen and repressed by low osmolarity, as in the macrophage (Lee *et al*, 2000). In contrast to PhoPQ, SirA was found to be required for *hilA* expression. The environmental conditions that SirA responds to are unknown (Ahmer *et al*, 1999). The regulation of SPI-1, SPI-4, and SPI-5 is summarised in Figure 1.4.

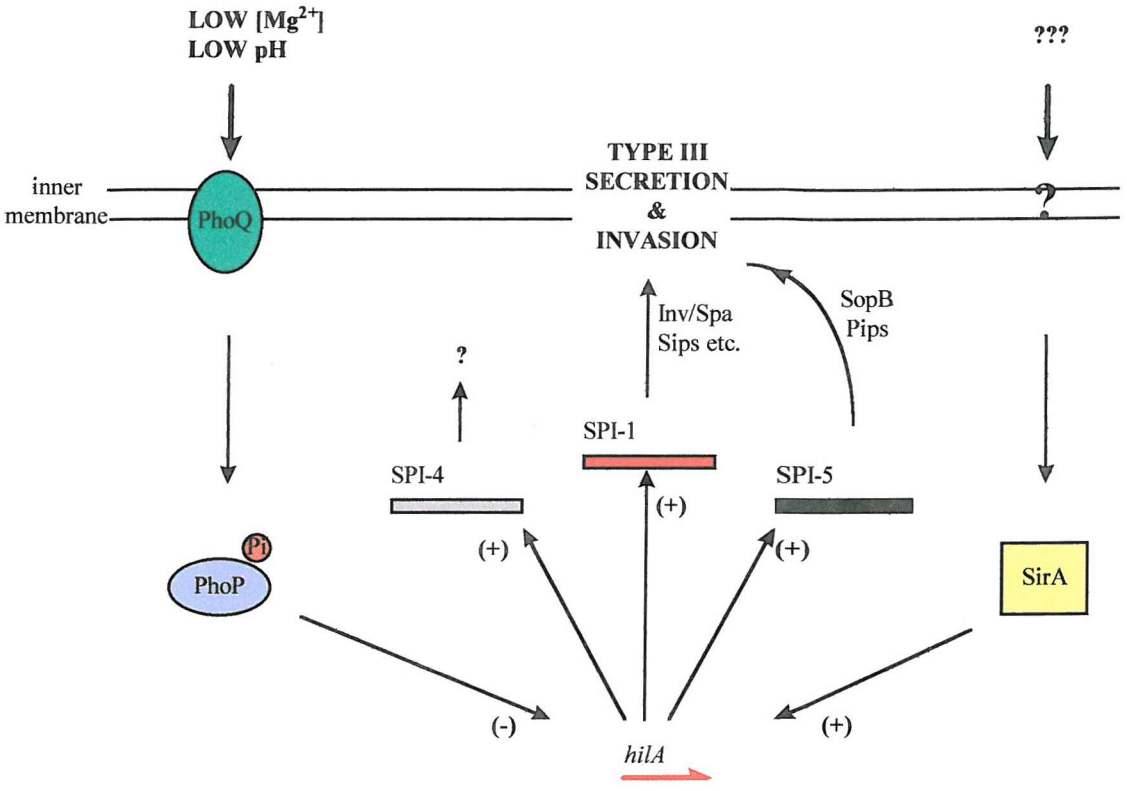


Figure 1.4. Regulation of *hilA* expression by PhoPQ and SirA. In an environment where invasion is not required (e.g. the macrophage), PhoP inhibits *hilA* expression. SirA positively regulates *hilA* expression in response to unknown environmental stimuli. HilA activates genes within its own pathogenicity island, SPI-1, as well as genes within SPI-4 and SPI-5. This results in *Salmonella* invasion of the epithelial cell.

A second route of *Salmonella* invasion from the intestine into the systemic circulation has recently been identified (Vazquez-Torres *et al*, 1999). CD18 expressing phagocytic cells are able to transmigrate into the intestinal lumen from the blood stream and engulf *Salmonella* cells. The bacteria, able to survive in mononuclear phagocytes (Fields *et al*, 1986), are then transported in the reverse direction to commence the systemic phase of disease. This result is important as it explains how SPI-1 deficient *S. typhimurium* strains can gain access to the spleen and cause a lethal infection in mice following oral administration (Galán and Curtiss, 1989) without invading M cells or localising in Peyer's patches.

1.1.5.2. Survival Within Macrophages.

When *Salmonella* emerges from the basal side of the intestinal epithelial cells it is most likely to be engulfed by the numerous macrophages which reside close to the Peyer's patches or that have been recruited to the area upon infection. Once phagocytosed, the bacteria reside inside vacuoles variously called phagosomes or 'spacious vacuoles or *Salmonella* containing vacuoles (for example see Rathman *et al*, 1997; Gomes *et al*, 1999; Pfeifer *et al*, 1999). The environment here is potentially lethal and the pathogen must elicit a number of tolerance responses in order to survive. One of the most extreme conditions that *Salmonella* endures within the phagosome is high acidity and, therefore, the ATR mechanisms are again called into play. The pH of the *Salmonella* containing phagosome drops within 30 minutes of formation and remains below pH 5.0 for at least five hours (Rathman *et al*, 1996). Other host defence responses within the phagosome, such as bactericidal cationic peptides, reactive oxygen intermediates and low osmolarity may also be brought into play against *Salmonella* in this environment. As will be seen, regulation of all of the processes employed by *Salmonella* to survive and proliferate within the phagosomal environment is closely linked.

An additional pathogenicity island, *Salmonella* pathogenicity island - 2 (SPI-2) is induced by the pathogen within macrophages. This 40 kb island mapped to 31 minutes on the *S. typhimurium* chromosome. It encodes a type III secretion system designated Spi/Ssa, secreted effector proteins as well as their specific chaperones, and a two component regulatory system, SsrAB. SPI-2 is essential for survival and replication

within macrophages (Ochman *et al*, 1996; Shea *et al*, 1996). The Spi/Ssa type III secretion system is unusual as it functions intracellularly. Bacterial effector proteins can thus be exported across the cell envelope and the phagosomal membrane into the host cell cytoplasm.

S. typhimurium can inhibit phagosome-lysosome fusion and survives within phagosomes that diverge from the normal degradative pathway of the macrophage (Rathman *et al*, 1997). Uchiya *et al* identified an SPI-2 encoded protein, SpiC, that is secreted into the macrophage cytosol via the Spi/SsA type III secretion system. It was discovered that a functional *spiC* gene is required to inhibit phagosome-lysosome fusion and to interfere with normal trafficking of vesicle compartments devoid of *Salmonella*. The ability of SpiC to interfere with intracellular membrane trafficking in this way was found to be essential for pathogenesis. A second SPI-2 encoded protein, SpiA, an outer membrane compartment of the Spi/Ssa type III secretion system was found to be required for SpiC export (Uchiya *et al*, 1999). The mechanism of action of SpiC is unknown.

The SsrAB two component regulatory system of SPI-2 is required for expression of the chromosomal *sifA* gene (Beuzón *et al*, 2000). As *sifA*$^-$ bacteria lose their phagosomes and are found in the macrophage cytosol, it is thought that SifA plays an important role in the maintenance of the vacuolar membrane surrounding wild type bacteria in this environment (Beuzón *et al*, 2000). Beuzón *et al* propose that SPI-2 effectors (including SpiC) divert the *Salmonella* containing phagosome from the endocytic pathway, and subsequent maintenance of vacuolar membranes that enclose replicating bacteria is mediated by translocation of SifA.

SPI-2 gene expression is positively regulated by the environmental conditions found within macrophages (Cirillo *et al*, 1998). These include limiting concentrations of divalent cations, phosphate starvation, low pH and low osmolarity. SsrAB, PhoPQ and OmpR-EnvZ (another two component regulatory system) all modulate SPI-2 expression in response to environmental stimuli (Deiwick *et al*, 1999; Lee *et al*, 2000).

Unlike SPI-1, SPI-2 is not repressed by PhoPQ. The low pH and divalent cation concentration within the phagosome activates the PhoPQ system which, in turn, stimulates SPI-2 gene expression (Deiwick *et al*, 1999). Thus, PhoPQ regulates invasion and systemic infection of *Salmonella* by repressing SPI-1 and inducing SPI-2 in the appropriate environmental conditions. As is discussed later, PhoPQ also induces the expression of further sets of proteins required for macrophage survival, including some involved in the ATR.

SsrAB also stimulates SPI-2 gene expression. SsrA, the sensor kinase, may react to low phosphate concentration resulting in activation of SsrB and transcription of SPI-2 genes (Deiwick *et al*, 1999). OmpR, the response regulator component of a two component signal transduction system has also been shown to stimulate the SsrAB system by directly binding to the *ssrA* promoter region (Lee *et al*, 2000). OmpR, in conjunction with its sensor kinase, EnvZ, has previously been implicated in the osmotic stress response of *E. coli* (see section 1.2.2.1.). EnvZ responds to changes in external osmolarity. As a change in osmolarity will occur upon entering the phagosome, it appears that SPI-2 expression can be induced via OmpR activation of SsrAB.

Salmonella pathogenicity island - 3 (SPI-3) is also essential for intramacrophage survival. SPI-3 consists of the *mgtCB* operon at 83 minutes on the *S. typhimurium* chromosome. This operon is positively regulated by PhoPQ. SPI-3 confers the ability to grow in Mg^{2+} limiting environments. The MgtB protein is located in the inner membrane and functions to transport Mg^{2+} into the cell. The function of MgtC is unknown. However, it is predicted to localise to the inner membrane and interact with MgtB. As a plasmid carrying $mgtC^{+}$ rescued the intramacrophage survival of an *mgtCB* mutant, the MgtC protein was thought to be a magnesium transporter that functions independently of MgtB and the similar MgtA (Blanc-Potard and Groisman, 1997). However, this has subsequently been disproved (Moncrief and Maguire, 1998).

An additional mechanism utilised by *Salmonella* in order to survive the potentially lethal effects of the macrophage is to induce the programmed cell death of this phagocyte (Monack *et al*, 1996). *S. typhimurium* SL1344 mutants with a non-invasive phenotype are unable to induce programmed cell death when engulfed by macrophages.

The wild type strain, however, stimulates ruffling of the macrophage membrane and is internalised at a rate ten fold higher than that of the mutants. Both strains are able to replicate intracellularly, but only those macrophages containing wild type, invasive *Salmonella* apoptose. In addition, invasive bacteria that were mutant for intracellular replication continued to induce macrophage apoptosis. Therefore, it is thought that invasion, rather than intracellular replication, triggers programmed cell death of the phagocyte (Monack *et al*, 1996). Monack *et al* suggest that this could be a result of the changes in host second messenger levels that are triggered upon *Salmonella* invasion. Correspondingly, the elevated Ca^{2+} levels associated with *Salmonella* invasion (Rushkowski *et al*, 1992) of host cells are also seen in T cells upon receptor ligation which proceed programmed cell death (Penninger *et al*, 1994).

In summary, to survive as an intracellular pathogen, *S. typhimurium* employs tolerance mechanisms to survive harmful environmental conditions and pathogenicity islands to mediate host - bacteria interactions. In the interests of conserving energy and vital nutrients, it would seem plausible that the bacteria would co-regulate the expression of tolerance response genes and pathogenicity island genes required in the same host environments. This indeed appears to be the case, with both PhoPQ and OmpR-EnvZ required for tolerance and pathogenicity.

1.1.6. EPEC is an extracellular pathogen.

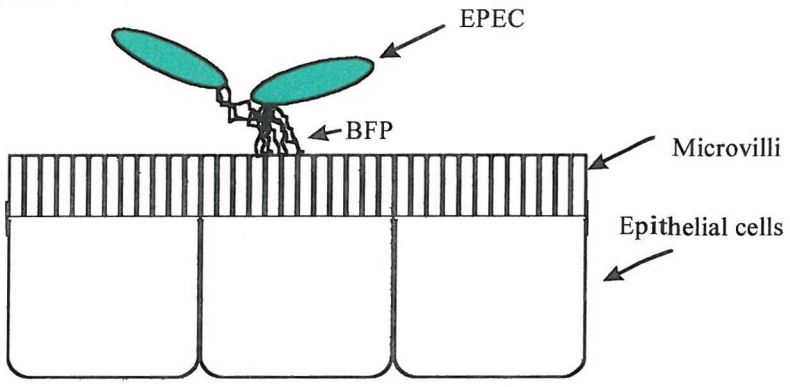
In contrast to *Salmonella* pathogenesis, the ability of EPEC to cause diarrhoeal disease is not thought to depend on invasion of host cells. Once EPEC has passed through the stomach, it requires intimate attachment to the host epithelia for full virulence. Intimate attachment leads to subsequent formation of attaching and effacing (A/E) lesions on the apical membrane of the intestinal cell. These are characterised by formation of cup-shaped, actin rich structures termed pedestals within the host cell. A/E pathogens reside on these cup-like pedestals which form a raised structure on the surface of the host cell. Pedestals may develop into more extended pseudopods that can elevate the attached bacteria >10μm above the surface of the host cell (Rosenshine *et al*, 1996). A transmission electron micrograph of an A/E lesion formed by an EPEC infecting epithelial cells is shown in Figure 1.3b. This can be compared to the distinctive

membrane ruffling of epithelial cells observed during *Salmonella* infection (Figure 1.3a). Ultimately, severe degeneration of the epithelial cell microvilli brush border occurs. Similar features are displayed during EHEC pathogenesis (Goosney *et al*, 1999). The formation of A/E lesions in EPEC pathogenesis can be divided into three stages. Firstly, localised adherence to the epithelial cell occurs via bundle forming pili (BFP); secondly, bacterial effector proteins are secreted into the host cell and thirdly, intimate attachment to the host cell takes place (Goosney *et al*, 1999). These mechanisms are described below and are summarised in Figure 1.5.

1.1.6.1. Localised Adherence.

The process by which initial contact with the host epithelial cell occurs is termed localised adherence. This is mediated by type IV fimbrae known as bundle forming pili (BFP). The corresponding *bfp* genes are located on the EPEC adherence factor (EAF) plasmid and, in addition to encoding the BFP structure, these genes also regulate its formation. In addition to bringing the pathogen into contact with the host cell, the BFP also functions in adherence between bacteria (DeVinney *et al*, 1999). This typically results in the formation of microcolonies of EPEC on the surface of an infected host cell.

A. Initial Adherence.



B. A/E Lesion Formation.

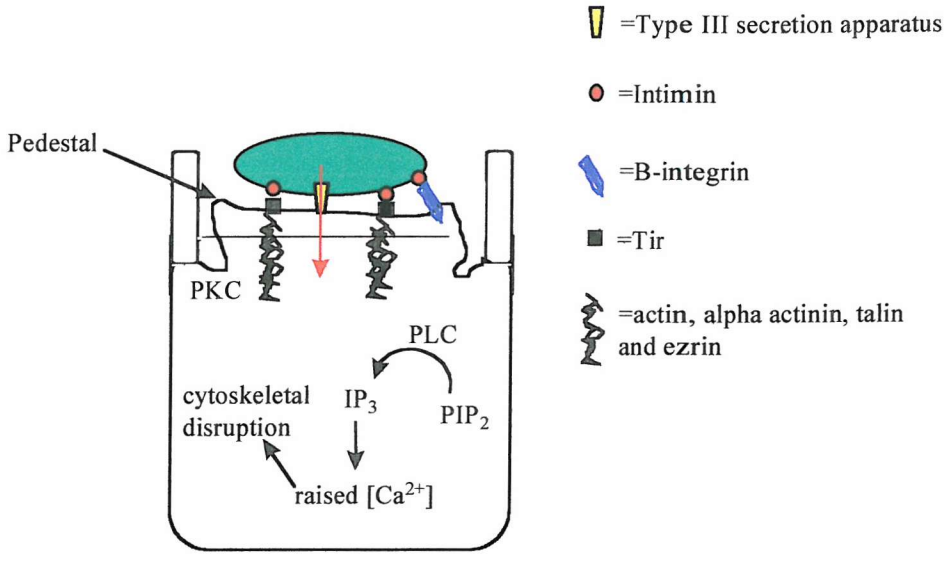


Figure 1.5. EPEC pathogenesis. **A**. Initial adherence mediated by bundle forming pili (BFP). **B**. Formation of A/E lesions with characteristic pedestal and loss of microvilli. Bacterial effector proteins are secreted into the host cell via a type III secretion apparatus. Intimate attachment is mediated by Intimin which binds phosphorylated Tir and β-integrin. Cytoskeletal rearrangements occur accompanied by the activation of phospholipase C-γ (PLC-γ), an increase in intracellular calcium concentration and activation of protein kinase C (PKC). IP_3: inositol triphosphate;
PIP: phosphatidylinositol 4,5-bisphosphate.

1.1.6.2. Attaching and Effacing Lesions.

A 35 kb pathogenicity island designated the locus of enterocyte effacement (LEE) encodes the entire molecular apparatus required for the formation of A/E lesions by EPEC. The sequencing of the LEE locus from EPEC strain E2348/69 has been completed and a standard nomenclature system for many of the encoded proteins has been adopted (Elliott *et al*, 1998). This pathogenicity island harbours genes which can be classified into three functional domains. The first encodes the Tir and intimin proteins required for intimate adherence. A second region encodes *E. coli* secreted proteins (ESPs) and their associated chaperones. The third domain encodes a type III secretion system. This constitutes the *esc* (*E. coli* secretion) and *sep* (secretion of *E. coli* proteins) genes (Goosney *et al*, 1999).

After localised adherence, EPEC protein secretion occurs. Five proteins encoded within the LEE and transported via the type III secretion system have been identified. These include EspA, EspB, EspC EspD and Tir (translocated intimin receptor). All except EspC are required for A/E lesion formation. Their secretion is inducible under the environmental conditions present in the gut lumen. The function of EspC at this stage of pathogenesis is unknown (DeVinney *et al*, 1999).

EspA functions on the external surface of the EPEC cell. It forms filamentous organelles which span between the bacteria and the host cell surface. The protein contains a pore like domain through which other secreted proteins could be transported. The EspA filaments are down regulated once A/E lesion formation commences (Knutton *et al*, 1998).

EspB is translocated into the host cell cytosol and membrane where it may effect changes in the epithelial cell's signalling pathways. EspB is also required for protein secretion during infection (Wolff *et al*, 1998).

Although required for A/E lesion formation, the exact function of EspD is unknown. A LEE encoded chaperone, CesD interacts with and is necessary for the secretion of

EspD. CesD also has a role in EspB secretion, but is not essential even though a *cesD* mutant showed reduced translocation of EspB (DeVinney *et al*, 1999).

The Tir protein is directly involved with intimate attachment to the host cell surface. It is produced in an unphosphorylated form which undergoes phosphorylation on tyrosine residues at its carboxyl terminus upon insertion into the epithelial cell membrane (Kenny *et al*, 1997). Tir functions primarily to bind intimin (see below) and thus lock the pathogen and host cell together. Phosphorylated Tir is also required as a focus of cytoskeletal rearrangements induced within the epithelial cell. This results in the distinct actin accumulation and consequent pedestal formation beneath the bound bacteria. After intimate attachment has occurred, Tir induces additional host signalling events including the activation of phospholipase C (DeVinney *et al*, 1999).

The intimate attachment phase of EPEC infection requires both Tir and intimin. Intimin is a bacterial outer membrane protein encoded by *eae* (*E. coli* attaching and effacing) within the LEE pathogenicity island. In addition to binding Tir for intimate attachment, the carboxy-terminal 280 amino acids may bind β1 integrins of eukaryotic cells. β1 integrins are found on the apical surface of M cells (DeVinney *et al*, 1999).

1.1.6.3. Modulation of Signal Transduction Within the Host Cell.

Within 3 hours of infection, host-cell actin and actin cross linking components (α-actinin, talin, erzin and villin) accumulate directly under the bacteria. These actin pedestals lengthen and shorten resulting in movement of EPEC along the epithelial cell surface. The processes are mediated via changes in host cell signalling events generated by bacterial effector proteins. EPEC infection induces inositol phosphate (IP) fluxes within the eukaryotic cell that are dependent on a functional bacterial type III secretion system and EspB. Increases in inositol triphosphate (IP_3) levels are also seen. The IP fluxes and increased IP_3 concentrations could result in the release of Ca^{2+} from intracellular stores. An increased intracellular Ca^{2+} concentration is dependent on intimate attachment of the bacteria to the host cell via intimin (Dytoc *et al*, 1994; Baldwin *et al*, 1991). EPEC infection has been shown to activate phospholipase C-γ (PLC-γ) (Kenny and Finlay, 1997). Thus, phosphorylation of PLC-γ could result in the

increases in IP_3 and Ca^{2+} levels mentioned above. In line with elevated concentrations of IP_3 and Ca^{2+}, protein kinase C (PKC) is also activated and subsequently translocates to the host cell cytoplasmic membrane. It appears that A/E lesion formation may require increases in calcium flux within the cell. For example, increases in calcium ion concentration can result in the depolymerisation of actin by Ca^{2+}-dependent villin (Goosney *et al*, 1999; DeVinney *et al*, 1999). However, other reports dispute that increases in Ca^{2+} concentration are required for EPEC infection. For example, Bain *et al* (1998) found no evidence for a change in intracellular Ca^{2+} levels at sites of A/E adhesion using several classical EPEC and EHEC strains. They were unable to detect any significant alterations in Ca^{2+} concentration in infected compared with uninfected host cells. Thus, the role of Ca^{2+} in EPEC pathogenesis is inconclusive. Bain *et al* suggest previously observed fluxes of this divalent cation may be due to EPEC induced cytotoxixity.

1.1.6.4. Regulation of A/E Lesion Formation.

Additional regulation of host cell pedestal/pseudopod formation is mediated via an EPEC tyrosine phosphorylated GTPase, BipA (Farris *et al*, 1998). BipA was first discovered in *Salmonella* when it was induced more than seven fold in response to exposure to bactericidal permeability increasing protein (BPI) (Qi *et al*, 1995). BPI is a cationic host defence peptide produced by human granulocytes. EPEC *bipA::cat* mutants fail to trigger cytoskeletal arrangements within host cells, although adherence still occurs. Thickening of epithelial microvilli also takes place. Additionally, EPEC overexpressing BipA cause the production of numerous pseudopods on the host cell surface. These pseudopods are associated with a 40% increase in actin accumulation over that induced by wild type EPEC cells (Farris *et al*, 1998). BipA is therefore thought to contribute to the regulation of actin reorganisation (and other associated cytoskeletal proteins) in host cells. This function is most probably related to the GTPase activity of BipA rather than its tyrosine phosphorylation, as host GTPases such as Cdc42 and Rho have been found to be essential for cytoskeletal rearrangements in *Salmonella* and *Shigella* (Adam *et al*, 1996; Chen *et al*, 1996) BipA also regulates a number of other processes likely to be important for EPEC infection as discussed in Chapter 7 (Farris *et al*, 1998).

1.2. Adapting to the Environment: Stress Responses of *S. typhimurium* and *E. coli.*

Bacterial stress responses are elicited by both *Salmonella* and *E. coli* in response to harsh environmental conditions. The basic mechanism involves the sensing of the particular stress condition(s), followed by a complex adaptation process involving the regulation of gene expression. This enables survival in particular niches within the external environment and, in the case of enteropathogenic strains, within the host. Although the virulence mechanisms described above are essential for enteropathogenicity, it is clear that bacterial stress response mechanisms are also an essential requirement for this process. In line with this fact, it is evident that not only is there a degree of co-regulation between different stress responses, but also between stress responses and other enteropathogenic mechanisms.

Although this study concentrates on the ATR, it is appropriate to first introduce a number of the other stress responses utilised by *S typhimurium* and *E. coli.*

1.2.1. The oxidative stress response.

The oxidative stress response (OSR) is elicited to contend with the potentially harmful effects of the exogenous or endogenous production of reactive oxygen species (ROS). Examples of ROS include superoxide anions ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2) and hydroxyl radicals ($HO^{\bullet}$) which can cause damage to proteins, nucleic acids and cell membranes. The OSR is an adaptive mechanism: upon sensing low concentrations of ROS (e.g. μM H_2O_2), the bacteria will induce or repress specific gene products enabling survival in much higher concentrations (e.g. mM H_2O_2) (Foster and Spector, 1995).

1.2.1.1. Oxidative Stress.

A primary source of oxidative stress is the endogenous formation of $O_2^{\bullet -}$ and H_2O_2 as by-products of aerobic respiration. For example, these two ROS are formed when NADH dehydrogenase II transfers electrons to molecular oxygen in the aerobic

respiratory chain. The constitutive expression of superoxide dismutase (SOD) maintains these relatively low $O_2^{\bullet-}$ levels below lethal concentrations during aerobic growth. The levels of H_2O_2 produced are relatively higher, although they do not exceed toxicity thresholds. However, if the rates of intracellular $O_2^{\bullet-}$ and H_2O_2 formation increase above these background respiratory levels, the oxidative stress response is required to protect the bacterial cell (Storz and Imlay, 1999).

When $O_2^{\bullet-}$ levels are not kept in check by the cell (e.g. in *E. coli* mutants lacking cytosolic superoxide dismutase), the superoxide anion can oxidatively destroy iron sulphur clusters found in specific dehydratases such as aconitase and fumarase. These enzymes use exposed 4Fe-4S clusters to bind and dehydrate substrates. The clusters disintegrate upon oxidation resulting in loss of enzyme activity. The release of iron from the 4Fe-4S clusters causes additional damage when combined with H_2O_2 (see below).

Deregulation of cellular metabolism can also result in increased levels of intracellular H_2O_2. Hydrogen peroxide can efficiently oxidise enzyme thiol groups thereby inactivating enzymes with active site cysteine residues, such as glyceraldehyde-3-phosphate dehydrogenase. The concomitant presence of excess iron and H_2O_2 can result in $HO^{\bullet}$ formation via the Fenton reaction. This can primarily result in $HO^{\bullet}$ mediated DNA damage as the intracellular iron localises along the phosphodiester backbone of the nucleic acid molecule (Storz and Imlay, 1999).

The greatest oxidative stress challenge to *S. typhimurium* might occur within the phagosome of host macrophages. NADPH oxidase and nitric oxide synthase within the phagosomal membrane produce $O_2^{\bullet-}$ and nitric oxide ($NO^{\bullet}$) respectively. This also results in the production of the harmful by-products, H_2O_2, $HO^{\bullet}$, peroxynitrate (HOONO) and nitrothiol (RSNO). All can cross the bacterial membrane at the acidic pH of the phagosome. Once inside the cell, $NO^{\bullet}$ inhibits bacterial respiration by binding the haem and/or copper sites of cytochrome oxidases. It also inactivates the 4Fe-4S containing enzyme, aconitase. $NO^{\bullet}$ reacts with $O_2^{\bullet-}$ in the phagosome to form the oxidant HOONO. This molecule diffuses into the bacterial cell where it attacks

cysteinyl residues and iron sulphur clusters. RSNOs are produced by the reaction of HOONO in the presence of thiols. These molecules are then able to stimulate thiol oxidation. Cell damage caused by H_2O_2 is as described previously (Storz and Imlay, 1999). *Salmonella* may be able to avoid the potentially lethal effects of NADPH oxidase. Recent research suggests that SPI-2 prevents this enzyme localising in *Salmonella* containing phagosomes by interfering with the trafficking of oxidase containing vesicles to the vacuoles (Vazquez-Torres *et al*, 2000).

1.2.1.2. Bacterial Defences Against Oxidative Stress.

The majority of the adaptive resistance to oxidative stress in *S. typhimurium* and *E. coli* can be divided into two sets of inducible proteins that constitute regulons. The first regulon is controlled by the SoxR-SoxS transcription factors in response to superoxide or nitric oxide generating compounds. The second regulon is governed by OxyR, activated by hydrogen peroxide generating species (Demple, 1999). Overall, the antioxidant capability of the cell is upregulated along with repair activities.

The SoxR protein exists as a dimer and is expressed constitutively. It contains two [2Fe-2S] centres per complex. These are converted from reduced to oxidised forms in the presence of $O_2^{\bullet-}$ or $NO^{\bullet}$, thereby activating SoxR. Activated SoxR induces the transcription of *soxS*. Elevated levels of SoxS then activate the transcription of the various *soxRS* regulon genes (Gaudu *et al*, 1997; Liochev *et al*, 1999a). The mechanism of SoxR repression is presently unknown.

The SoxRS inducible proteins include manganese superoxide dismutase (encoded by *sodA*) which degrades superoxide to hydrogen peroxide; endonuclease IV (*nfo*) involved in DNA repair; and $O_2^{\bullet-}$ resistant isozymes of fumarase (*fumC*) and aconitase (*acnA*) (Storz and Imlay, 1999). SoxRS activation also results in increased expression of glucose-6-phosphate dehydrogenase (*zwf*) which increases the reducing potential of the cell, and the ferric uptake regulator (*fur*) which decreases iron uptake, thus potentially decreasing $HO^{\bullet}$ formation (Storz and Imlay, 1999; Zheng *et al*, 1999). Entry of redox active compounds into the cell that could result in increased levels of $O_2^{\bullet-}$ is

prevented by increased levels of the TolC outer membrane protein, the AcrAB drug efflux pump and the MicF regulatory RNA (represses expression of an outer membrane porin, OmpF). Nitroreductase A (*nfsA*) is also upregulated by SoxRS. It is oxygen insensitive and functions by converting organic nitro compounds to stable products by divalent reduction. This renders them unavailable for redox cycling and $O_2^{\bullet-}$ production via oxygen sensitive nitroreductases (Liochev *et al*, 1999b). Roles for the SoxRS induced flavodoxin A (*fldA*), ferrodoxin/flavodoxin-$NADP^+$ reductase (*fpr*), GTP cyclohydrolase II (*ribA*), *inaA* and *pqi5* in the oxidative stress response have not been established (Storz and Imlay, 1999).

Proteins which protect against $O_2^{\bullet-}$ damage, but which are not regulated by SoxRS include the periplasmic copper-zinc superoxide dismutase (*sodC*) and the cytosolic iron superoxide dismutase (*sodB*) (Gort *et al*, 1999; Fridovich, 1995).

The OxyR protein is activated for DNA binding by the formation of an intramolecular disulphide bond between residues Cys199 and Cys208. This forms upon direct oxidation of OxyR by hydrogen peroxide or (less strongly) RSNO. Once hydrogen peroxide mediated oxidative stress has been sensed in this manner, the OxyR regulon is positively regulated in response. OxyR autoregulates its own expression by inducing glutathione reductase (*gorA*) and glutaredoxin I (*grxA*): these compounds act to reduce the disulphide bond of OxyR, resulting in deactivation (Storz and Imlay, 1999).

OxyR induces the expression of peroxide degrading enzymes such as hydroperoxidase I (or catalase, *katG*) and two subunits of alkyl hydroperoxide reductase (*ahpCF*) (Storz and Imlay, 1999). As with SoxRS, OxyR also induces the ferric uptake regulator protein (Fur) (Zheng *et al*, 1999). A second protein that may protect against DNA damage induced by Fe^{2+} is Dps. This is a DNA binding protein induced by OxyR that has homology to ferritin, and may thus sequester iron (Martinez and Kolter, 1997). The OxyR induced OxyS regulatory RNA is also thought to protect against DNA damage (Altuvia *et al*, 1997). The roles of the OxyR induced *hemF*, *rcsC*, and *f497* in the oxidative stress response have not been established (Mukhopadhyay and Schellhorn, 1997).

Proteins protecting the cell against hydroperoxide stress that are not induced by OxyR include the DNA repair enzymes exonuclease III (*xthA*), DNA polymerase I (*polA*) and RecA as well as the peroxide degradative hydroperoxidase II (*katE*) (Storz and Imlay, 1999).

In addition to OxyR and SoxRS control of the oxidative stress response, σ^s (RpoS) has also been identified as a regulator of *pqi5* (also SoxRS regulated) and *katG*, *gorA* and *dps* (all also OxyR regulated). Additionally, the *katE*, *xthA*, and *sodC* genes are all part of the σ^s regulon (Gort *et al*, 1999; Loewen *et al*, 1998). As will be seen, *rpoS* is an important general regulator of stress responses such as the starvation stress response, the osmotic stress response and the ATR and serves as a prime example of co-regulation.

Much of the research into the oxidative stress response has involved *E. coli*. However, recently, a parallel pattern of gene regulation has been observed in the *S. typhimurium* OxyRS regulon (Pomposiello and Demple, 2000). This research confirms an earlier study indicating that as in *E. coli*, AhpC expression is under the control of OxyR in *S. typhimurium*. The *Salmonella ahp* operon is induced in the macrophage and its disruption results in alkyl peroxide sensitivity (Francis *et al*, 1997).

1.2.2. The osmotic shock response.

S. typhimurium and *E. coli* are subjected to changes in external osmolarity as a result of the many different environments they inhabit. Increased external osmolarity (hyper-osmotic shock) tends to withdraw water from the cell, decreasing turgor pressure and raising the concentrations of intracellular solutes to levels which may inhibit growth. Upon hypo-osmotic shock, water can rapidly enter the cell causing increased turgor pressure and an imbalance in cytosol concentration. *Salmonella* and *E. coli* possess mechanisms, termed the osmotic shock response, enabling a constant cell volume and thus, survival, over a wide range of external osmolarity.

Under hyper-osmotic conditions, the bacteria accumulate specific solutes thereby counterbalancing the external solute concentration and minimising water loss. Rapid

release of the specific solutes occurs on encountering hypo-osmotic stress. Chemicals used in this way include potassium ions (K^+), glutamate, proline, glycine betaine and trehalose. These are defined as compatible solutes as they can be accumulated (and tolerated) to high levels via transport mechanisms or *de novo* synthesis without destabilising essential cellular processes. Compatible solutes with no overall charge are utilised in preference to ionic solutes such as K^+ and glutamate as they are less damaging to the cell at high concentrations and favour protein stability. The favoured compatible solute is glycine betaine. Following an initial fast adaptive response by the cell involving K^+ and its counterion, glutamate, these ionic solutes are displaced by glycine betaine accumulation (Foster and Spector, 1995; Singleton, 1999).

1.2.2.1. Response to an Upshift in External Osmolarity.

The initial stages of the bacterial response to an increase in external osmolarity involves the uptake of K^+ into the cell. This is balanced by the synthesis of the counterion glutamate, however, proton efflux may also contribute to electrical neutrality. K^+ uptake occurs via the constitutive Trk transport system and the Kdp transport system. The presence of the Trk and Kdp systems in the cytosolic membrane is increased or induced respectively by low turgor pressure.

The high affinity Kdp system is encoded by the *kdpABC* operon. It consists of a complex of the proteins KdpA, KdpB and KdpC. KdpB is an ATPase which hydrolyses ATP to provide energy for K^+ uptake. A two component system, KdpDE, regulates the expression of the *kdpABC* operon. The KdpD sensor is located in the cytoplasmic membrane. Increased membrane stretch induces autophosphorylation of KdpD. The subsequent transfer of the phosphoryl group to the cytosolic KdpE activates this transcription factor which results in increased expression of the *kdpABC* genes (Poolman and Glaaskar, 1998). The role that K^+ plays in this sensory process is unclear. Laimins *et al* (1981) claim that neither the extracellular nor intracellular K^+ concentration controlled *kdp* transcription per se. Instead, it is suggested that transcription is influenced indirectly via the ion's effect on turgor pressure. However, an alternative model is proposed in which KdpD responds to altered extracellular K^+ concentrations (Asha and Gowrishankar, 1993). This study also shows that KdpD does

not respond to increased intracellular concentrations of osmoprotectants (assumed to restore turgor pressure), suggesting *kdp* expression is affected by K^+ levels and not turgor. Malli and Epstein (1998) dispute these claims, and suggest that the data result from the effect of K^+ limitation on growth rate. They also indicate that the addition of glycine betaine does not affect turgor pressure.

The transport of the Trk potassium uptake system to the cytoplasmic membrane is also increased in response to membrane stretch as a consequence of decreased turgor pressure (Poolman and Glaaskar, 1998). The rapid accumulation of K^+ via the Kdp and Trk systems is the most important mechanism in the early response to increased osmolarity.

Uptake of other compatible solutes is comparatively slower and glycine betaine only displaces K^+ and glutamate later in the response. Trehalose cannot be transported into the cell and is thus synthesised. When present in the medium, glycine betaine and proline are taken up via the proton motive force driven ProP and the ATP binding protein ProU systems (Cairney *et al*, 1985; Crothe *et al*, 1986; Faatz *et al*, 1988). The hyper-osmotic activation of ProP depends on the presence of K^+, but the mechanism is unknown. ProP may sense an increase in internal pH upon K^+ uptake. However, once counterions are present, the pH returns to normal and this is not concurrent with a drop in activated uptake by ProP (Koo *et al*, 1991; Poolman and Glaasker, 1998). Hyper-osmotic activation of ProU takes several minutes and is dependent on the presence of glycine betaine (Faatz *et al*, 1988). The mechanism of activation is unclear: it is hypothesised (Csonka *et al*, 1994) that an increase in K^+-glutamate accumulation stimulates *proU* transcription as it represents a favourable environment for RNA polymerase-promoter interaction. As intracellular osmolarity increases, the steady state expression of *proU* would then increase. Thus, when the external osmolarity increases, it would take some time before ProU levels increase (Poolman and Glaasker, 1998).

An increase in external osmolarity also induces the EnvZ-OmpR two component regulatory system. The kinase activity of EnvZ, located in the inner membrane, is activated as a consequence of increased external osmolarity. EnvZ then transfers a phosphoryl group to the regulator protein, OmpR. Phosphorylated OmpR, which

oligomerises, then enhances the transcription of the *ompC* gene and inhibits the transcription of the *ompF* gene. The *ompCF* genes encode the major outer membrane porin molecules OmpC and OmpF respectively. OmpC has a relatively small pore size compared with that of OmpF. Thus, under conditions of high external osmolarity, the outer membrane contains higher levels of OmpC and lower levels of OmpF, resulting in reduced permeability. Such conditions are prevalent in the host intestine, where OmpC could aid in excluding harmful molecules such as bile salts from the bacterial cell (Foster and Spector, 1995). The same regulatory pattern of *ompCF* occurs during the ATR (Leyer and Johnson, 1993; Chart *et al*, 1994). Interestingly, *ompR* or *ompCF* double mutants of *S. typhimurium* SL1344 are attenuated *in vivo* (Dorman *et al*, 1989; Chatfield *et al*, 1991). Combined with the activation of SPI-2 by OmpR in the macrophage, the above mechanisms describe an excellent example of co-regulation.

1.2.2.2. Response to a Downshift in External Osmolarity.

When *Salmonella* and *E. coli* are exposed to a hypo-osmotic shock, rapid release of the compatible solutes, K^+, glutamate, glycine betaine and trehalose from the cell occurs (Wood, 1999). The low external osmolarity is sensed via an increase in turgor pressure. Unlike the uptake systems, the efflux of compatible solutes does not require metabolic energy.

Much of the K^+ released during osmotic down shift passes through mechanosensitive channels. The MscL channel of *E. coli* has been studied in greater detail. As a result of increased turgor pressure, tension in the lipid bilayer is conveyed to MscL (Sukharev *et al*, 1994). This increases the open probability of the channel several fold thus leading to an increase in compatible solute efflux. However, the MscL channel is not essential for excretion of compatible solutes: cells lacking the pore released K^+, glutamate, glycine betaine and trehalose in response to osmotic downshock (Ajouz *et al*, 1998).

1.2.2.3. Sigma S Regulation of the Osmotic Shock Response.

The *rpoS* encoded sigma factor, σ^s, has been implicated in the regulation of a number of osmotically controlled genes. σ^s is more commonly associated with regulation of

gene expression associated with entry into stationary phase (Hengge-Aronis, 1993). However, σ^s levels are also elevated in exponentially growing cells if undergoing an osmotic upshift (Hengge-Aronis, 1996). Osmotically controlled genes regulated by σ^s include *otsB*, *otsA* and *treA* involved in the synthesis and degradation of trehalose (Hengge-Aronis, 1996). Additionally two dimensional gel electrophoresis has identified sixteen σ^s controlled proteins whose expression changes in response to osmolarity (Hengge-Aronis *et al*, 1993). The addition of K^+ and glutamate *in vitro* has been shown to enhance transcription of *in vivo* σ^s regulated promoters and inhibit transcription mediated by σ^{70} (Ding *et al*, 1995). Conversely, the presence of glycine betaine strongly reduces σ^s levels. The increase in σ^s on osmotic upshift is due to a reduced turnover of the protein. *rpoS* mRNA levels are unaffected under these conditions suggesting transcription is not affected (Hengge-Aronis, 1996).

1.2.3. The starvation stress response.

It is important to distinguish between the starvation stress response (SSR) and the stationary phase of growth. Although stationary phase inducible loci are also SSR inducible, the two conditions occur in distinct populations of cells. Starved cell cultures have stopped growing in response to exhaustion of one or more defined nutrients, and will grow logarithmically if the defined nutrient(s) is added. Stationary phase cultures on the other hand, undergo growth arrest following exponential growth in rich or non-limiting media. The majority of studies on the SSR have concentrated on the response to carbon (C) starvation. However, the SSR is also elicited in response to phosphate (P) and nitrogen (N) starvation (Spector, 1998). C, N or P starvation can also induce an additional protective mechanism termed the stringent response (Cashel *et al*, 1996).

The SSR alters cellular metabolism to adjust cell growth to the limited nutrient availability. Over longer periods of starvation, the SSR results in a more resistant and energy efficient bacterial cell. For example, systems are induced to scavenge for particular nutrients, degradation of RNA and protein occurs in conjunction with a reduction in cytosolic ribosome concentrations, alterations in both inner and outer membrane constituents as well as in peptidoglycan structure increase the cells stress

resistance, and chromosomal DNA is condensed to protect it from damage (Spector, 1998).

Space constrictions only allow examples from the large SSR stimulon to be discussed here. To keep within the context of this report, systems which are related to other stress responses will be considered.

1.2.3.1. SSR Inducible Loci.

The KatE protein is induced upon long term C starvation conditions (Spector, 1998). As has already been mentioned, this H_2O_2 degrading catalase is also induced during the oxidative stress response and is positively regulated by σ^s. In fact, resistance to hydrogen peroxide can be induced by C starvation. Both KatE and σ^s are required for development of this cross resistance along with *stiC* (starvation inducible C). The *stiC* gene is induced by C, P or N stress and is also positively regulated by σ^s, however its function has yet to be deduced. Other SSR inducible loci that are involved in the oxidative stress response include *xthA*, *narZ* (*stiA*) and *dadA* (*stiB*). These genes are, however, not required for the SSR induced cross protection to H_2O_2 (Spector, 1998).

The *narZ* gene encodes the α subunit of nitrate reductase II. This is part of the nitrate reductase system encoded by the *narZYWV* operon and is induced as part of the SSR by C, N or P starvation. *narZ* expression is σ^s dependent and exhibits repression by the cyclic adenosine monophosphate (cAMP) receptor protein (CRP). It is also positively regulated by guanosine 5'-diphosphate 3'-diphosphate (ppGpp) in a *relA* dependent manner. Although not essential for SSR cross protection to H_2O_2, *narZ* is required for C starvation inducible thermotolerance and acid tolerance (Spector *et al*, 1999). The complex regulation of *narZ* points to a central role in the physiology of starved and stressed cells, however, its function here is unknown.

The *dadA* gene is induced by C or P starvation and encodes a subunit of D-amino acid dehydrogenase. Along with *dadB* which encodes an alanine racemase, it is a constituent of the *dadAB* operon. These proteins are required for the utilisation of L-alanine and

certain D-amino acids as carbon/energy sources. This catabolic function is important during starvation; likely amino acid sources include the turnover of bacterial cell wall components or proteins. *dadA* expression is negatively regulated by cAMP bound CRP and positively regulated by ppGpp (Spector, 1998).

A large virulence plasmid is possessed by several *Salmonella* serovars that is essential for systemic disease in the host. An 8kb region of the plasmid can restore virulence to plasmid cured strains (Gulig *et al*, 1993). This region contains the *spvABCD* operon and the *spvR* gene. SpvR is a positive regulator of *spvABCD* and its own expression. SpvA negatively regulates *spvR* and thus completes a negative feedback loop. The role of these genes in virulence is unknown. However, C, P or N starvation has been shown to induce the *spvABCD* operon and this is probably effected via induction of *spvR*. *spvABCD* and *spvR* are both positively regulated by σ^S and PhoP and negatively regulated by cAMP:CRP. The *spv* genes are also induced under low pH conditions (Spector, 1998).

A number of SSR induced loci also participate in the osmotic shock response. *otsA* expression is stimulated by C starvation (Spector, 1998). The protein product, OtsA, is involved in the synthesis of periplasmic trehalose that functions in osmoprotection. Additionally, the glycine-betaine transport system encoded for by *proP* is induced during the SSR (Spector, 1998).

1.2.3.2. Regulation of the SSR.

Two of the major regulators of the SSR are σ^S and the cAMP bound CRP, both of which exhibit increased expression in C starved cells.

RpoS positively regulates a group of genes within the SSR stimulon. This sigma factor is essential for long term starvation tolerance and is responsible for C starvation induced cross protection to H_2O_2, heat shock, omotic shock and acid shock. This cross protection is two fold. Firstly, *rpoS* itself is involved in other stress responses (e.g. osmotic shock and oxidative stress) and increased levels may induce these mechanisms.

Secondly, some σ^s dependent SSR loci are also involved in these stress responses (e.g. *narZ* and *otsA*).

Positive and negative regulation of SSR loci occurs through the cAMP:CRP complex. An increase in cAMP is seen during C starvation as adenylate cyclase is activated. Increased CRP levels are observed as a consequence of increased expression of the *crp* gene. cAMP:CRP positively regulates those SSR loci involved in scavenging for available nutrients and therefore hopefully avoiding long term starvation. Examples of cAMP:CRP induced loci are the two putative transport proteins encoded by *csiA* and *csiG* (Spector, 1998). Negative regulation by cAMP:CRP (e.g. *narZ*) is not dependent on the cellular concentration of this complex. Thus, although an increase in cAMP:CRP is seen during the SSR, essential SSR loci are not repressed. These loci are involved in long term starvation survival or stationary phase and are only repressed by cAMP:CRP under non-inducing conditions (log phase growth) (Spector, 1998).

Other SSR regulatory systems include PhoPQ which positively regulates the *spv* genes. This system, which also regulates invasion of host cells and a set of proteins during the ATR is induced during long term starvation (Behlau and Miller, 1993). Thus, the PhoPQ regulon could be part of the SSR stimulon. The OxyR regulator of the OSR and SPI-2, also plays a role in the activation of the *narZ* SSR locus. OxyR represses *narZ* transcription under non-inducing conditions (lack of exogenous H_2O_2 and exponential growth). *narZ-lac* fusions in the presence of H_2O_2 have only one third of the expression of those exposed to C starvation in *Salmonella*. It is therefore apparent that cAMP:CRP negatively regulates *narZ*, even in the presence of H_2O_2 during log phase growth (Spector, 1998).

1.2.3.3. The Stringent Response.

Starvation can trigger the stringent response in bacteria. This response which conserves energy and material, results in the decreased synthesis of proteins, ribosomal RNA and various cell components. DNA synthesis is also inhibited. The absence of a specific amino acid can result in an uncharged transfer RNA (tRNA) molecule binding to the A site of a ribosomal-messenger RNA translation complex. This stimulates the ribosome

bound pyrophosphotransferase (RelA) to catalyse the formation of ppGpp from ATP and guanosine triphosphate (GTP) (or guanosine diphosphate (GDP)). ppGpp is an example of an alarmone: a small molecule which accumulates under certain stress conditions and serves as a signal for redirecting the cell's metabolism. For example, it can inhibit protein synthesis by interacting with protein initiation factor, IF-2 and blocking the binding of the tRNA complex to the ribosome (Cashel *et al*, 1996). As has been described, ppGpp can also enhance transcription of certain genes (e.g. *narZ* and *dadA*) including *rpoS*.

1.2.4. The heat shock response.

A rise in temperature above that required for optimum growth induces the heat shock response in *Salmonella* and *E. coli* (Foster and Spector, 1995). The response includes the increased synthesis of heat shock proteins (HSPs) and confers the ability to resist additional, more extreme temperature rises (thermotolerance).

A major group of HSPs identified in *E. coli* include the molecular chaperones GroEL, GroES, DnaK, DnaJ and GrpE (Yura *et al*, 1993). Increases in GroEL and DnaK levels in response to heat challenge have also been documented in *S. typhimurium* (Foster and Spector, 1995). Under non-stressful conditions, the molecular chaperones facilitate the correct folding of newly translated proteins. Chaperones also aid the targeting of specific proteins (for example, the ESPs) to the correct intra- or extracellular locations. In response to heat shock, chaperones can bind to heat denatured proteins and facilitate refolding or protect them from further damage.

Other HSPs include the Lon and Clp proteases which degrade heat damaged and abnormal proteins. σ^{70} (RpoD), the housekeeping RNA polymerase sigma factor is also part of the HSP regulon.

The temperature dependent induction of the HSP regulon requires an alternative sigma factor, σ^{32} (RpoH). Increased levels of σ^{32} occur in response to heat shock. This sigma factor can then bind to RNA polymerase and promote increased transcription from the promoters of the HSP genes. The amount of active σ^{32} is regulated by several

mechanisms. Three *rpoH* promoters are transcribed by σ^{70} bound RNA polymerase; a fourth is preferentially transcribed in the presence of another sigma factor, σ^{24} (Foster and Spector, 1995). However, enhanced σ^{32} levels following heat shock are primarily a result of increased translation and stabilisation. Translation of σ^{32} may be blocked at growth optimising temperatures by a secondary structure in the *rpoH* transcript. This structure is probably modified at elevated temperatures allowing translation to occur (Singleton, 1999). Increased stabilisation of σ^{32} during the heat shock response involves the molecular chaperone component of the HSP regulon. A complex is formed between DnaJ, DnaK, GrpE and σ^{32} under non-stressful conditions. The formation of this complex prevents σ^{32} from promoting the transcription of the HSP genes. It also allows the rapid degradation of σ^{32} by the FtsH protease. During heat shock, the molecular chaperones preferentially bind the heat denatured proteins, thus leaving σ^{32} free to associate with RNA polymerase (Singleton, 1999).

1.3. The Acid Tolerance Response.

One of the most common stress situations encountered by micro-organisms in their natural habitats is acid pH. Survival in the many acidic environments encountered within the host is essential for the virulence of enteropathogenic *S. typhimurium* and *E. coli*. During oral infection, the first major stress encountered is extreme low pH in the stomach. Correspondingly, the infectious dose of different bacterial species correlates well with the micro-organisms' ability to resist acid stress (Waterman and Small, 1998). In addition, exposure to acid induces cross protection to other stresses encountered within the host (Leyer and Johnson, 1993). Thus, this initial acid encounter may prime the pathogen for subsequent host survival. After alkalinisation in the small intestine, fermentation processes in the bowel result in weak organic acid accumulation and pH levels of 4.5 to 6.0 (Baik *et al*, 1996). As already discussed, the intracellular pathogen, *S. typhimurium* is also capable of surviving phagosomal acidification. Examples of acidic environments outside of the host include acid rain, acid mine drainage and industrial slurries which serve to lower the pH of natural water systems (Baker *et al*, 1991). Additionally, many foods are acidic in nature or have weak acid preservatives, such as benzoate, added. Interestingly, fermented foods and acidic products have been implicated in *Salmonella* infections (Leyer and Johnson, 1997). The mechanisms of acid tolerance in enteric bacteria are many and complex. It is obvious that the characterisation of these systems will be of great benefit to both clinicians and the food industry.

Although still far from clear, the ATR has been most extensively studied in *S. typhimurium* and *E. coli* strains. Specific ATR mechanisms have been related to either *Salmonella* or *E. coli* and it is thus appropriate to discuss the two species separately here. However, the basic edict of the ATR is true for both micro-organisms: prior exposure to non-lethal levels of acidity invokes adaptive mechanisms enabling subsequent survival at more extreme levels of low pH (Foster and Hall, 1990; Goodson and Rowbury, 1989). It is also evident that both species of bacteria encounter two distinct types of acid stress: inorganic and organic. Inorganic acids cannot cross the bacterial cell envelope and thus, acidification of the cell cytoplasm is indirect (Foster, 1999). For example, increased external proton concentration can lead to an increase in

proton influx. Conversely, organic acids such as benzoate are capable of crossing the cell envelope (Foster, 1999). These weak acids can diffuse across the cell membrane in their undissociated forms. At lower external pH (pH_o) values, more undissociated weak acid is present. Therefore, less weak acid is required to produce the same amount of acid stress at lower pH values. Once inside the cell, the neutral cytoplasmic pH favours dissociation of the weak acid. Here, not only can released protons acidify the cell cytoplasm, but accumulating weak acid anions can interfere with cell metabolism. In their natural environment it is most likely bacteria will encounter a combinatorial effect of organic and inorganic acids rather than each individually.

Different experimental conditions used to study the ATR (for example, media type, growth phase, pH values used) impose difficulties in comparing data between laboratories. Therefore, the following mechanisms can not be expected to be utilised by the bacteria in all conditions encountered. In fact, the ATR constitutes a range of systems that are probably activated or repressed in relation to the particular environmental niche encountered.

A summary of proteins that are differentially expressed during the ATRs of *S. typhimurium* and/or *E. coli* is given in Table 1.2 on page 60.

1.3.1. The acid tolerance response of *S. typhimurium*.

S. typhimurium possesses two low pH inducible ATR systems specific for either the stationary or exponential phases of growth.

The stationary phase ATR is distinct from the *rpoS* dependent general stress resistance associated with stationary phase cells (Lee *et al*, 1994). Stationary phase *S. typhimurium* LT2 cells incubated in minimal glucose medium at an adaptive pH of 4.3 show a 1000-fold greater survival level at a challenge pH of 3.0 than unadapted cells. Maximum tolerance of pH 3.0 conditions is seen after two hours of adaptation at pH 4.3. However, unadapted stationary phase cells are still 1000-fold more acid tolerant at pH 3.0 than unadapted exponential phase cells in the same conditions. This acid tolerance of unadapted stationary phase cells is lost in *rpoS* minus strains. Conversely,

low pH induced stationary phase acid tolerance is fully functional in these mutants. The low pH induced stationary phase acid tolerance requires protein synthesis (Lee *et al*, 1994).

The ATR is best characterised in exponentially growing *S. typhimurium* cells. *S. typhimurium* in this growth phase rapidly dies if transferred from neutral pH directly to an acidic value below pH 4.0. However, if first incubated at a mild acidic pH above 4.0, these acid adapted cells show increased tolerance of extreme acid conditions. Protein synthesis is required for this adaptive ATR (Foster and Hall, 1990). Inducible pH homeostatic mechanisms are evoked during the adaptive phase of the ATR. These mechanisms are better able to maintain cytoplasmic neutrality at external pH conditions below 4.0. They can thus reinforce constitutive pH homeostatic systems at low pH_o values (Foster and Hall, 1991).

The cytoplasmic neutrality of acid adapted cells enables the bacteria to induce mechanisms at a low pH_o that are beneficial to acid survival. This stage of the ATR involves the increased or decreased expression of specific proteins, termed acid shock proteins (ASPs). ASPs function in such areas as the prevention or repair of acid damaged macromolecules. Examples include the heat shock chaperones DnaK and GroEL (Foster, 1991). ASPs also function in the stationary phase ATR (Lee *et al*, 1994). The identities and functions of many ASPs remain unknown.

The complex pH homeostatic and ASP mechanisms are described in more detail below. A summary of these systems is provided in Figure 1.6.

1.3.1.1. Constitutive pH Homeostasis.

pH homeostasis is the process whereby the cell maintains a constant intracellular pH (pH_i) over a broad range of pH_o values. Constitutive pH homeostasis is under allosteric control and can function down to a pH_o of 4.0. The molecular mechanisms involved include the primary proton pumping machinery of the cell. For example, constituents of the electron transport chain, sodium/proton antiporters and potassium/proton antiporters. These transport systems vary the flux of hydrogen ions into and out of the

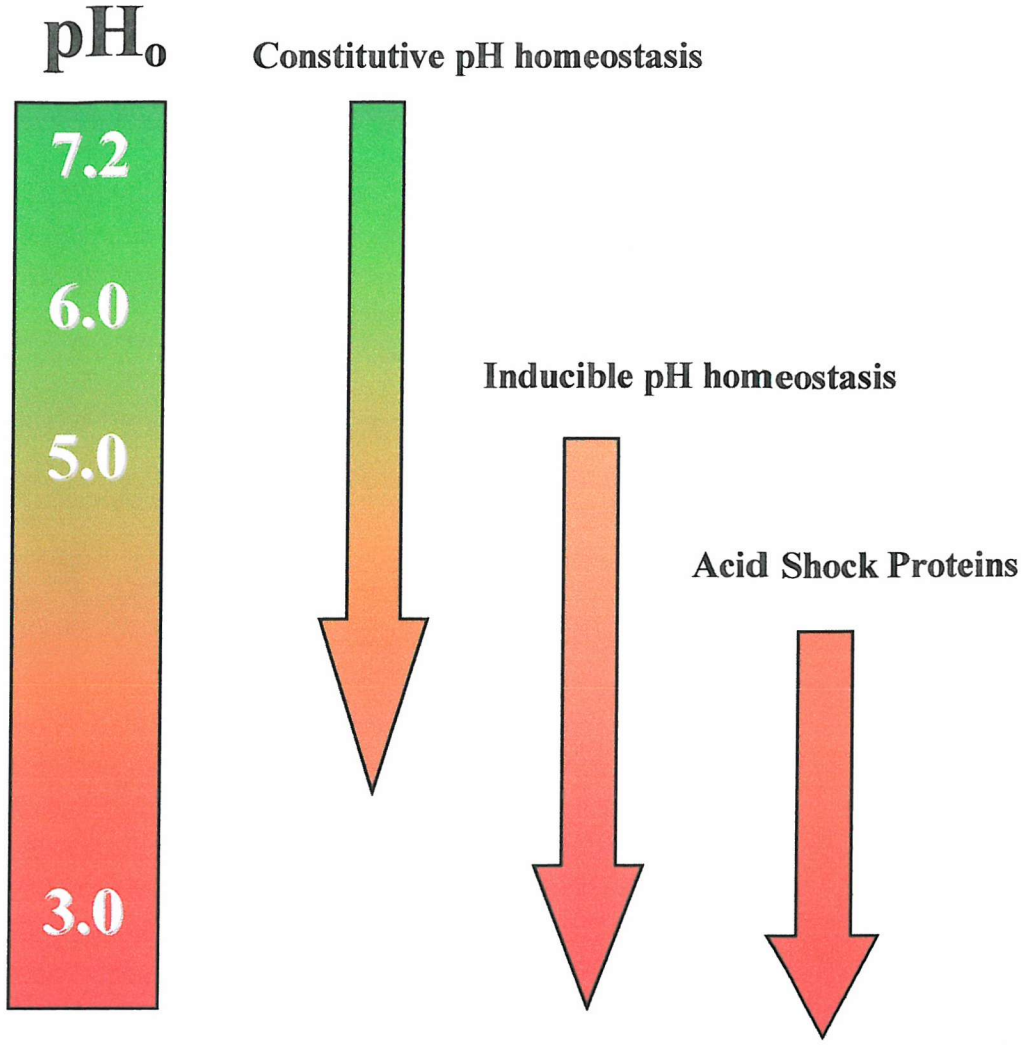


Figure 1.6. The ATR mechanisms employed by *S. typhimurium* and the external pH range over which they protect the cell. Constitutive pH homeostasis alone can only buffer against pH_o values down to pH 4.0. Below a pH_o of 4.0, inducible pH homeostasis (induced at pH_o 5.8 and below) reinforces the constitutive mechanism. Inducible pH homeostasis dependent ASPs are differentially regulated to protect against or repair acid induced damage at extreme pH.

cell, depending on pH_o (Foster and Hall, 1991). They are overwhelmed when the external proton concentration becomes too great.

1.3.1.2. Inducible pH Homeostasis.

Inducible pH homeostasis reinforces constitutive homeostasis at low pHo values. The mechanism is evoked during the adaptive phase of the ATR and requires protein synthesis (Foster and Hall, 1991). An experiment measuring the pH_i of *S. typhimurium* (using the distribution of radiolabelled weak acids or bases across the cell membrane) identified acid adapted cells as having a pH_i of 0.9 units higher than unadapted cells at a pH_o of 3.3. At pH_o values between 4.0 and 7.5, no difference in pH_i was observed between the two cell types (Foster and Hall, 1991). The same study isolated two constitutively acid tolerant *S. typhimurium* mutants, termed *atr-1* and *atr-12*. These bacteria had a dramatically improved pH homeostasis at a pH_o of 3.3.

The *atr-1* mutant was determined to be isocitrate dehydrogenase deficient. These cells accumulate intracellular citrate and isocitrate. The pKi of these acids is 6.4. They therefore help to buffer the pHi to this value. The *atr-1* mutants had a pH_i value 1.1. units higher than that of wild type cells at a pH_o of 3.3. Isocitrate dehydrogenase, citrate synthase double mutants lost this ability and failed to mount an effective ATR (Foster and Hall, 1991). It is not known if isocitrate dehydrogenase is actively repressed during the ATR.

The *atr-12* mutant was deficient in pyruvate dehydrogenase. The pH_i of these cells was also 1.1 units higher than that of wild type at a pH_o of 3.3. The mechanism of this increased pH homeostatic ability is unknown (Foster and Hall, 1991). The current study proves for the first time that pyruvate dehydrogenase is actively repressed during the ATR of *S. typhimurium* (Chapter 5).

The F_0F_1 Mg^{2+} dependent, proton translocating ATPase (Atp) is essential for inducible, but not constitutive, pH homeostasis: *atp* minus strains only exhibit pH homeostasis down to an external pH of 4.0. The F_0 subunit functions as an inner membrane pore for proton translocation. The F_1 subunit protrudes into the cytoplasm and functions as an

ATPase. The proton motive force enables Atp to generate ATP via proton entry into the cytoplasm. During the ATR, Atp probably utilises ATP to pump hydrogen ions out of the cell against the electrochemical concentration gradient (Foster and Hall, 1991). Inducible pH homeostasis also involves the stimulation of lysine decarboxylase (CadA) along with the lysine-cadaverine antiporter (CadB). The low pH induction of *cadA* may be mediated by H-NS. Results suggest that this DNA binding protein reduces *cadA* expression at non-inducing pH (Shi *et al*, 1993). A *cadA* null mutant renders the cell incapable of inducible pH homeostasis. Upstream of the *cadAB* operon is *cadC*. When present in the external environment, lysine can enter the bacteria via a lysine permease in the cytoplasmic membrane (LysP). Lysine, in addition to low pH induces the expression of *cadC*; the resulting gene product positively regulates *cadAB*. CadA subsequently decarboxylates intracellular lysine to form cadaverine, consuming a proton in the process. CadB then exports cadaverine from the cell in exchange for more lysine. This process helps to raise the pH_i of the cell. The pH optimum for CadA function is 5.7. Therefore, upon re-entering an alkaline environment this process is shut down. In addition, excess cadaverine levels inhibit CadAB function, although the mechanism is unknown. The CadABC apparatus is one of several inducible amino acid decarboxylase systems which can alkalinise pH_i in *S. typhimurium*. This suggests the bacteria can employ different systems depending on the amino acids available. Other inducible amino acid decarboxylases identified in this bacteria include those specific for ornithine, arginine and histidine (Park *et al*, 1996).

The ferric uptake regulator, Fur, is also required for inducible pH homeostasis. The role of this protein in the regulation of the ATR is detailed below.

1.3.1.3. Acid Shock Proteins.

Tolerance to severe acid conditions requires both inducible pH homeostasis and ASP regulation. Differential expression of 52 ASPs occurs at and below a pH_o of 4.5 in acid adapted *S. typhimurium* LT2 cells in minimal E glucose medium (Foster, 1991). Specific ASPs are either expressed or repressed at specific pH values. Subsets are regulated according to pH_o and others according to pH_i. Further ASPs are regulated in response to unknown signals (Foster, 1993). The majority of acid shock proteins

remain uncharacterised. However, some ASP regulators have been identified. These are detailed below in the context of both the log phase and stationary phase ATR.

1.3.1.4. Regulation of the ATR.

The ferric uptake regulator, Fur.

The Fur protein was initially characterised as a negative regulator of iron acquisition gene expression. When complexed with Fe^{2+}, Fur represses the transcription of membrane proteins that recover siderophore-iron complexes. Therefore, when internal Fe^{2+} concentrations are sufficient, iron scavenging is inhibited. In addition to this function, Fur is essential for the ATR in exponential phase *S. typhimurium* (Foster, 1991; Hall and Foster, 1996). Not only is Fur required for inducible pH homeostasis (Foster and Hall, 1991), but it is also an ASP that regulates a set of other ASPs (Foster and Hall, 1992). Fur is now known to be a positive as well as negative regulator of gene expression. Nine of thirty six proteins differentially regulated by Fur in an iron dependent manner are also ASPs regulated in response to low pH (Foster and Hall, 1992). The role of Fur in the ATR has been identified as physiologically and genetically separated from its role in iron acquisition (Hall and Foster, 1996). Firstly, the level of Fe^{2+} in the cell is irrelevant to the transcriptional control of specific ATR genes: only Fur and low pH are required. Secondly, a mutation in Fur converting the histidine 90 residue to an arginine (H90R mutation) severely diminishes iron associated regulation, but does not affect the role of Fur in acid tolerance (Hall and Foster, 1996). The H90R mutation effects the iron sensing ability of Fur: Fe^{2+} binding induces a conformational change at the N termini of Fur dimers. This permits binding of a 19 base pair DNA consensus sequence (called the Fur box) in the promoter region of iron acquisition genes. The mechanism of pH regulation of Fur is currently unknown. The protein has been implicated more strongly however, in organic as opposed to inorganic acid tolerance (Bearson et al, 1998). Correspondingly, Fur mutants are unable to mount an ATR induced by short chain fatty acids (Kwon and Ricke, 1998). As described earlier, Fur is also involved in the regulation of the OSR.

The alternative sigma factor, RpoS.

The alternative sigma factor, σ^s, competes for the binding of RNA polymerase with other sigma factors including the house keeping σ^{70}. Alternative sigma factors can direct the binding of RNA polymerase to specific gene promoters recognised by their -10 and -35 consensus sequences. Therefore, if σ^s levels rise, then transcription from genes with RpoS specific promoters will increase. In this way, alternative sigma factors can direct the expression of particular subsets of genes in response to different environmental conditions. Increased levels of σ^s have been primarily associated with the transition from exponential to stationary phase growth. Previously in this chapter, increased σ^s levels have also been detailed in relation to oxidative stress, osmotic shock and the SSR. In addition, σ^s is induced by up to four times normal levels when acid adapted exponential phase *S. typhimurium* transits to extreme acid conditions (Bearson *et al*, 1996). RpoS is, in fact, an ASP that is essential for a sustained ATR in log phase cells (Lee *et al*, 1995). It is also responsible for the up regulation of eight other ASPs of unknown function during log phase growth. As described earlier, σ^s is not required for the low pH induced stationary phase ATR, but is essential for the general acid resistance of stationary phase cells (Lee *et al*, 1994).

σ^s levels may be controlled at transcriptional, translational or protein stability levels (Hengge-Aronis, 1996). In the case of the ATR, σ^s concentrations are controlled by a 38kDa protein, MviA. This protein is associated with virulence in mice and has homology to the regulatory components of two-component signal transduction systems (Benjamin *et al*, 1996). It appears to constitute a direct connection between virulence and the ATR. MviA senses a change in environmental conditions via an unknown mechanism and in response, directs the turnover of RpoS post-translationally (Bearson *et al*, 1996). Insertions in the *mviA* gene lead to an increased level of RpoS and its associated ASPs. This in turn causes an increased tolerance to acid in acid adapted and unadapted cells. It is hypothesised that, in the absence of stress, MviA somehow stimulates the ClpX protease which degrades σ^s. The decreased virulence of *mviA* mutant strains is probably due to an inappropriate expression of σ^s dependent genes in locations, for example, requiring rapid growth (Bearson *et al*, 1996).

Experiments investigating the effects of organic acids at low pH suggest, although the log phase ATR can protect against organic and inorganic acids, RpoS is primarily involved in organic acid tolerance (Baik *et al*, 1996; Kwon and Ricke, 1998). Acid adapted cells required RpoS for enhanced survival of an organic acid challenge at pH 4.4. However, if the organic acids produced by fermentation are removed from a culture prior to acid adaptation and acid challenge, *rpoS* mutants still undergo a strong ATR.

The AtbR regulon.

ATR deficient mutants of *S. typhimurium* LT2 were screened for on the basis that they would succumb more quickly to the lethal effects of dinitrophenol (DNP) and low pH than ATR competent cells. Several mutants deficient in specific proteins were found. One such protein was termed AtrB. This protein, together with 9 others was found to be over expressed in a constitutively acid tolerant mutant. This mutant lacked an unidentified protein, termed AtbR. Thus, the AtbR protein was hypothesised to repress genes required for acid tolerance in non-stressful conditions (Foster and Bearson, 1994).

AtbR was subsequently identified as phosphoglucosisomerase (Pgi). It was also discovered that AtbR mutants in non-LT2 strains were not constitutively acid tolerant. It transpires that the acid sensitive phenotype of *rpoS* deficient strains can be suppressed by deleting the *pgi* gene. As *S. typhimurium* LT2 strains possess an altered *rpoS* allele (Wilmes-Riesenberg *et al*, 1997), this gave the appearance of constitutive acid tolerance in the *atbR* mutants. Pgi mutants are hypothesised to suppress *rpoS* minus acid sensitivity as they do not metabolise glucose. A lack of glucose metabolism results in the absence of organic acids produced via glucose fermentation. Therefore, the organic ATR, controlled primarily by σ^s is not required (Bearson *et al*, 1998).

An additional protein identified via DNP screening as essential for the ATR is the OSR induced DNA polymerase (PolA). As an ASP, PolA could repair the DNA damage induced by acid.

PhoPQ.

In contrast to Fur and σ^S, primarily implicated in organic acid tolerance, the PhoPQ two component signal transduction system provides protection specifically to inorganic acid (Bearson *et al*, 1998). PhoPQ is low pH inducible in exponentially growing *S. typhimurium* and *phoP* mutants are ten fold less tolerant of inorganic, but not organic acid. PhoP has been identified as an ASP that is required for the low pH induction of a further 4 ASPs (Bearson *et al*, 1998). PhoPQ is essential for survival within the macrophage where it sense low pH and reduced magnesium levels, subsequently inducing protective mechanisms (for example, SPI-2 gene expression (Deiwick *et al*, 1999) and host cationic peptide resistance (Guo *et al*, 1997)). The induction of the ATR during this phase of infection may thus depend primarily on PhoPQ regulation.

When subjected to elevated magnesium levels alone, the PhoQ membrane sensor component does not phosphorylate the PhoP regulatory protein. Thus, PhoP is not active and cannot bind DNA under these conditions. However, if high magnesium levels are present in a low pH environment, it is possible that PhoQ will phosphorylate and activate PhoP (Bearson *et al*, 1998). Evidence for this notion comes from the PhoPQ activated gene, *pagA*, which is induced in a PhoP dependent manner at low pH, even in the presence of high magnesium concentrations (Bearson *et al*, 1998). PhoQ could be sensing pH and magnesium levels independently, or alternatively, pH could affect the interaction between magnesium and PhoQ. PmrAB, another two component regulatory system, is activated by PhoP and is also required for *pagA* acid induction at both high and low magnesium levels (Bearson *et al*, 1998).

Three methods of acid induced expression of PhoP dependent genes have so far been identified. The first, as above, requires both PhoP and PmrA. The second is PmrA independent. For example, the low pH/magnesium induction of *mgtB* requires PhoP, but not PmrA. The third method requires PmrA and occurs in a *phoP* mutant, for example, induction of *pmrC* at pH 4.9 (Bearson *et al*, 1998). A model described by Bearson *et al* suggests PmrA independent genes respond to levels of phosphorylated PhoP (PhoP-P) induced by PhoQ sensing magnesium. Changes in proton concentration would then influence the amount of magnesium required to produce a given level of

PhoP-P. PmrA dependent genes would be regulated by a PhoP-PmrA cascade that amplifies the pH response.

Ada.

The Ada protein activates target genes involved in DNA repair in *S. typhimurium*. It is induced by low pH and aids in the organic ATR. Low pH caused by organic acid in the cell can facilitate inappropriate methylation of DNA. Ada removes O^6-methyl guanine residues and can induce other components of the Ada regulon. These may deal with organic acid damage (Bearson *et al*, 1998).

cAMP receptor protein and TyrR.

The cAMP receptor protein (CRP) and TyrR (a regulator of aromatic amino acid metabolism) are strong positive regulators of a set of genes that, although induced by low pH, also require anaerobiosis for expression. These genes include *aniC* which could encode an arginine/agmatine antiporter, functioning similarly to CadB; and HyaB (hydrogenase 1) of unknown function in respect to the ATR. Both AniC and HyaB are also negatively regulated by σ^s. AniC is also negatively regulated by H-NS (J. Hinton, personal communication). CRP also positively regulates the expression of *aniG* (Park *et al*, 1999). This gene is optimally expressed in complex medium at low pH in anaerobic conditions (Foster and Hall, 1990). Interestingly, AniG is also subject to negative control via the membrane associated regulator, EarA. EarA is inhibited by low pH resulting in increased *aniG* expression (Aliabadi *et al*, 1988). An additional gene that responds to acid only in anaerobic conditions, identified as *hyd* (Foster and Hall, 1990), does not appear to be regulated by CRP or TyrR. Park *et al* (1999) suggest CRP and TyrR may sense alterations in internal pH, which may be more significant under anaerobic conditions and in the presence of fermentation end products.

1.3.1.5. Other ATR Associated Mechanisms.

Several exponential phase acid inducible genes have been identified in *S. typhimurium* using a fluorescence activated cell sorter (FACS). Eight acid inducible proteins were characterised and divided into three categories. The first included cell surface structure and maintenance proteins. This group included 2-acylglycerophosphoethanolamine acyltransferase (2-acyl-GPE), a protein also induced under conditions of increased osmolarity and positively regulated by OmpR. It has a potential involvement in membrane repair. Interestingly, the increased production of OmpR and its subsequent phosphorylation has recently been associated with acid shock, but only in stationary phase *S. typhimurium* (Bang *et al*, 2000). The second group included stress-response proteins such as Dps. This non-specific DNA-binding protein is also induced by the SSR and the OSR. The third group contained generalised efflux pumps such as MarRAB which is induced in response to certain antimicrobial agents and during the OSR (Valdivia and Falkow, 1996).

Other studies have identified outer membrane constituents as members of the ATR stimulon. Firstly, OmpC expression increases and OmpF is repressed in acid adapted cells (Leyer and Johnson, 1993; Chart *et al*, 1994). This is analogous to Omp regulation during the osmotic shock response and could be a result of increased OmpR synthesis and activation at low pH (Bang *et al*, 2000). Secondly, an activated PhoPQ system regulates structural modifications of the lipid A component of LPS by the addition of aminoarabinose and 2-hydroxymyristate (Guo *et* al, 1998). The PmrAB system regulates the LPS structure in a similar manner (Gunn *et al*, 1998). These mechanisms protect the bacteria against the harmful effects of host cationic antimicrobial peptides (Guo *et al*, 1997; Guo *et al*, 1998) which bind lipid A with high affinity (Gazzano-Santoro *et al*, 1992). As both the PhoPQ and PmrAB systems are active during the ATR (Bearson *et al*, 1998), it is possible that the LPS composition is altered in acidic environments. However, Leyer and Johnson (1993) report that the LPS component of the outer membrane is unchanged in acid adapted cells. Finally, studies have shown decreased fimbriation at an acidic pH, but it is unclear whether this is an ATR induced effect (McDermid *et al*, 1996; Walker *et al*, 1999).

1.3.2. The acid tolerance response of *E. coli.*

The ATR mechanisms employed by *S. typhimurium* and *E. coli* are surprisingly divergent. Hickey and Hirshfield identified different patterns of ASP regulation between the two species and discovered that *E. coli* alone releases acid tolerance inducing factors into the medium. *S. typhimurium* is also more tolerant of low pH stress than *E. coli* (Lin *et al*, 1995). The differences could be due to the contrasting environmental niches of the two bacteria. For example, *E. coli* may be adapted more specifically to survive in the lower intestine where it would encounter a rich, complex medium. *Salmonella* is hardier outside of the intestine in nutrient dilute solutions (Hickey and Hirshfield, 1990). It is also important to point out that the majority of *E. coli* ATR studies have concentrated on the non-pathogenic K-12 strain. Thus, the ATR mechanisms deduced using this bacteria may not necessarily represent those of EPEC strains. To date, four distinct systems are known to constitute the *E. coli* ATR. The exponential phase acid tolerance system in *E. coli* has been termed acid habituation (Goodson and Rowbury, 1989). Three stationary phase mechanisms exist. These include one oxidative system and two fermentative systems.

1.3.2.1. Acid Habituation.

Acid habituation occurs when exponential phase cells are grown initially in nutrient broth at pH 4.5 to pH 5.0. These acid adapted cells will survive a challenge pH of 3.5 or 3.0 better than organisms initially grown at neutral pH (Goodson and Rowbury, 1989).

Differential regulation of specific ASPs occurs during acid habituation. Akin to *S. typhimurium*, particular ASPs are induced or repressed over defined pH ranges (Heyde and Portalier, 1990; Blankenhorn *et al*, 1999). A number of HSPs have been characterised as ASPs in *E. coli* including DnaK, GroEL, HtpG and HtpM which are induced at pH 4.3. The DNA binding protein, HNS, has been implicated in the regulation of DnaK and GroEL (J. Hinton, personal communication). In addition, the HSPs GrpE and LysU are induced at pH 5.0 (Heyde and Portalier, 1990). Additional ASPs divergently expressed at pH 4.5 have been identified using proteomic techniques

(Blankenhorn *et al*, 1999). The upregulation of alkyl hydroperoxide reductase (AhpC), a major component of the OSR is an example. This cross induction is theorised to be related to the increased production of oxygen radicals at low pH (Blankenhorn *et al*, 1999). Several induced ASPs function in sugar metabolism including the phosphotransferase system components, ManX and PtsH. This suggests that growth in acid may favour these pathways. The YfiD protein is also induced at pH 4.5. This protein shows homology to the anaerobically induced pyruvate formate lyase. However, YfiD is expressed in both aerobic and anaerobic conditions. Three ASPs are reduced in expression. These include the periplasmic maltose binding protein and two as yet unidentified proteins (Blankenhorn *et al*, 1999).

Inducible pH homeostasis mechanisms in *E. coli* are similar to those employed by *S. typhimurium*. The *E. coli* sodium/proton antiporter, NhaA, exhibits pH dependent induction in the presence of sodium ions (White *et al*, 1992). The replacement of histidine 226 with arginine markedly changes the pH dependence of the antiporter. This suggests that this residue may be part of a pH sensitive site that regulates the activity of NhaA (Gerchman *et al*, 1993). Interestingly, an *nhaA* null mutant prevented an acid induced alkali sensitisation in *E. coli* (Rowbury and Hussain, 1996). The DNA binding protein, H-NS, has been implicated in the transcriptional regulation of nhaA (Dover *et al*, 1996). A second sodium transporter, NhaB, is not thought to conduct protons during pH homeostasis as when this pump is inhibited, alkali sensitisation still occurs (Rowbury and Hussain, 1996). Potassium transport, as utilised during osmotic stress, may be a requirement for pH homeostasis. Strains deficient in the potassium transporters, *kdpABC*, *trkA* and *trkD* were less able to tolerate acid pH than wild type. The cell appears to require potassium at low pH, perhaps increasing the positive electrochemical charge of the cytoplasm to repel protons (White *et al*, 1992). Additional components of pH homeostasis in *E. coli* include the lysine and arginine decarboxylases, thought to function as in *S. typhimurium* (Guilfoyle and Hirshfield, 1996).

The mechanism of acid habituation is thought to involve the phosphate specific porin, PhoE. *phoE* mutants are acid resistant and high phosphate levels inhibit acid habituation. PhoE is hypothesised to constitute a major proton influx pathway during

external acidification (although, it has to be said that this seems unlikely: PhoE is specific for phosphate *anions* and proton influx is known to occur through many different routes, including other porins). The subsequent lowering of cytoplasmic pH would then stimulate acid habituation via an unknown mechanism. Phosphate ions are thought to block the PhoE pore preventing the access of hydrogen ions (Rowbury *et al*, 1992). Correspondingly, the presence of phosphate prevented the pH dependent induction of *cadA* (Rowbury and Goodson, 1993). PhoE has also been implicated in sodium chloride induced acid sensitivity (Rowbury *et al*, 1996a; Lazim *et al*, 1996). Low NaCl concentrations (150-200 mM) can induce acid sensitivity, related to increased PhoE synthesis. NaCl dependent acid sensitivity requires a functional *relA* gene (Rowbury *et al*, 1996a). Correspondingly, *relA* mutant strains produce lower levels of PhoE than wild type cells (Lazim *et al*, 1996). Glucose, which protects against NaCl induced acid sensitivity, can virtually abolish PhoE formation even in the presence of a functional *relA* gene (Lazim *et al*, 1996). Glucose has been implicated in the induction of acid tolerance at neutral pH in exponential phase cells. This unknown mechanism does not involve a reduction in pH_i induced by glucose metabolism. It is however, inhibited by high levels of NaCl, cAMP or weak acids (Rowbury and Goodson, 1998a).

L-leucine has also been found to induce acid sensitisation. A *phoE* mutant displays normal levels of L-leucine sensitivity, suggesting the mechanism utilised is distinct from that of NaCl. L-leucine induced acid senstivity is absent in strains lacking the OmpA outer membrane protein, suggesting it may constitute a proton channel (Rowbury *et al*, 1996b). Another outer membrane protein that functions in the ATR of *E. coli* is OmpC. As in the ATR of *S. typhimurium*, *ompC* expression is induced at acidic pH. This also parallels the regulation of OmpC during osmotic shock. However, unlike osmotic regulation, pH_o control of OmpC in *E. coli* was dependent on the presence of glucose as the sole carbon source. The addition of cAMP at acidic pH reduced the induction of OmpC (Thomas and Booth, 1992). Additionally, Thomas and Booth infer that DNA supercoiling becomes more relaxed under acidic conditions.

A marked shift in the fatty acid composition of both cell membranes is seen during acid habituation (Brown *et al*, 1997; Chang and Cronan Jr, 1999). Virtually all unsaturated

fatty acids are converted to cyclopropane fatty acids (CFAs) during acid adaptation. The cyclopropane ring of CFAs increases membrane rigidity, thus decreasing permeability to protons. Variations in the CFA content of non-habituated cultures correlates well with levels of intrinsic acid tolerance. Strains with a higher CFA content at neutral pH, display an increased acid survival. CFAs are formed by the addition of a methylene group, derived from the methyl group of *S*-adenosyl methionine, across the carbon-carbon double bond of unsaturated fatty acids. CFA synthase catalyses the increased synthesis of CFAs both during acid habituation and on entry to stationary phase. The gene for this enzyme has two promoter sites. The proximal promoter (P2) is σ^s dependent and is thus active during the growth phase transition. It is also induced by 10 to 25 fold during acid habituation, therefore suggesting increased CFA levels here are also σ^s dependent. The distal CFA synthase promoter is σ^{70} dependent and therefore active throughout the growth curve.

Weak acids have been shown to induce acid habituation in exponential phase *E. coli* cells. The presence of 0.1% butyrate or proprionate at pH 6.5 potentiates the ability of *E. coli* to tolerate lethal levels of acid (pH 3.5) in the order of 50 to 190 fold. The arginine and lysine decarboxylases are thought to contribute to survival in these weak acids (Guilfoyle and Hirshfield, 1996). Thirty three proteins were found to be induced by benzoic acid in *E. coli*, one of which may be the Fur protein associated with *S. typhimurium* acid tolerance. However, it is unknown whether induction was due to pH_i or anion accumulation (Lambert *et al*, 1997). Anion accumulation on exposure to the weak acid acetate, results in a reduction in the intracellular glutamate pool. This is thought to compensate for the osmotic challenge posed by the acetate anions. Interestingly, Na^+ and K^+ levels were not affected. The reduction in glutamate is thought to occur via increased efflux and entry into the tri-carboxylic acid cycle. Glutamate decarboxylase is not utilised as a *gadC* mutant exhibited normal levels of glutamate reduction on exposure to acetate (Roe *et al*, 1998).

A unique RNA component inducible by low pH and not weak acids, termed acid shock RNA (*asr*), has been identified (Suziedeliene *et al*, 1999). The transcription of *asr* is completely abolished in a *phoBR* deletion mutant. In addition, upstream of the -10 region of the *asr* promoter is a region corresponding to a Pho box which PhoB binds to

activate transcription. Therefore it is hypothesised that the two component signal transduction system PhoBR is a sensor of low pH_o, resulting in transcription of *asr*. PhoR constitutes the sensory component and is more commonly associated with activation upon phosphate starvation. Hydrogen ions are proposed to cause the autophosphorylation of PhoR here, resulting in PhoB activation and subsequent *asr* transcription.

Recently, Rowbury *et al* have published several reports pertaining to the existence of extracellular components secreted by *E. coli* that are involved in acid tolerance. Neutral filtrates from acid adapted cells are able to induce acid tolerance in cells previously grown at pH 7.0. The filtrates are thought to contain small heat stable proteins: the addition of protease inhibited the cross adaptation effect, whereas heating at 75°C did not (Rowbury and Goodson, 1998b; Rowbury *et al*, 1998). Production of these proteins, termed extracellular induction components (EICs), is claimed to arise from a stress sensing EIC precursor and is inhibited by cAMP and phosphate (Rowbury and Goodson, 1999a). The EICs are also activated by heat and UV irradiation (Rowbury and Goodson, 1999b). Rowbury *et al* state that EICs are evident at both exponential and stationary phases of growth. However, this phenotype has not been observed in stationary phase in other laboratories (Foster, 1999).

1.3.2.2. The Oxidative System of Acid Tolerance.

The oxidative system of acid tolerance in *E. coli* is induced by growth to stationary phase in Luria Bertani (LB) broth and is repressed by glucose. The system is σ^s dependent and enables the bacteria to survive a subsequent challenge at pH 2.5 in minimal media (Small *et al*, 1994; Lin *et al*, 1995). Interestingly, although σ^s is required in aerobically grown stationary phase bacteria for acid tolerance, anaerobically grown stationary phase cells exhibit σ^s independent tolerance (Small *et al*, 1994). Recently cAMP and CRP have been identified as an additional requirement of the oxidative system (Castaine-Cornet *et al*, 1999). An unidentified inhibitor present at pH 8.0 that could be removed by washing cells before acid challenge was also discovered (Castaine-Cornet *et al*, 1999). The LB medium requirement of the oxidative system was found to be due to the presence of glutamate and glutamine in the yeast extract

used. These are thought to activate a pre-formed *rpoS* dependent system that is produced during entry into stationary phase. Addition of glutamate and glutamine to the media was shown to restore acid resistance to *crp* mutants and to glucose repressed strains. This proved the *rpoS* dependent system could stand alone in protecting the cells from acid stress. The CRP dependent pathway may contribute to intracellular glutamate synthesis. Thus, in the absence of glucose, this pathway may produce enough glutamate for *rpoS* dependent acid resistance to function. Intracellular glutamate may serve as a counter ion for K^+ entering the cell via K^+/H^+ antiporters in minimal media at pH 2.5 (Castaine-Cornet *et al*, 1999).

1.3.2.3. The Fermentative Systems of Acid Tolerance.

Complex medium dependent fermentative acid tolerance constitutes the stationary phase inducible glutamate decarboxylase and arginine decarboxylase systems. They are thought to function in a similar manner to the lysine decarboxylase system as described for *S. typhimurium*. Protons are consumed during the decarboxylation of glutamate or arginine. The products, γ-aminobutyric acid and agmatine respectively, are then exported from the cell in exchange for new substrate. The specific antiporter systems utilised are GadC for glutamate and an unknown antiporter for arginine. Thus, it is envisaged that these systems maintain pH_i at low pH_o values, whereas the *rpoS* and oxidative dependent systems minimise damage to macromolecules (Lin *et al*, 1996).

The glutamate and arginine decarboxylase systems were first linked to the ATR as acid resistance mechanisms that required glutamate or arginine respectively for the increased survival of fermentative cells at pH 2.5. Glutamate dependent acid resistance also required *gadC* (Hersh *et al*, 1996), whereas arginine decarboxylase (encoded by *adiA*) was essential for arginine dependent acid tolerance (Stim and Bennet, 1993).

Arginine decarboxylase is induced by anaerobic growth at low pH in rich medium (Stim and Bennet, 1993). The anaerobic requirement may explain the *rpoS* independent stationary phase acid resistance in anaerobic conditions described above. Mutations in the *cysB* locus caused acid sensitivity and specifically inhibited the arginine dependent system. Thus, CysB has been characterised as a positive regulator of *adiA* (Shi and

Bennet, 1994). Additionally, AdiY specifically stimulates the expression of *adiA* (Stim-Herndon *et al*, 1996). *AdiA* is also regulated by H-NS. Results suggest that this DNA binding protein contributes to the acid regulation of *adiA* by reducing *adiA* expression at non-inducing pH (pH 8.0) (Shi *et al*, 1993).

Either of two glutamate decarboxylase isoforms (GadA or GadB) affords pH 2.5 acid resistance in the presence of GadC plus glutamate. The *gadB* and *gadC* genes form an operon where *gadB* is the first gene. Although stationary phase induction of the *gadBC* and *gadA* genes is dependent on *rpoS*, acid induction does not require this alternative sigma factor. *gadA* is predominantly regulated by acid pH, whereas, *gadB* is predominantly regulated by entry into stationary phase (Castanie-Cornet *et al*, 1999). Like *adiA* and *cadA*, *gadA* and *gadB* are also thought to be regulated by H-NS (DeBiase *et al*, 1999). Glutamate decarboxylase has an optimum pH of 4.0-4.6 in *E. coli* and is inactive above pH 6.0. The pH optima for other decarboxylases is relatively higher (for lysine and arginine decarboxylases this equals pH 5.2 and 5.7 respectively). Therefore, GadA and GadB may be the dominant decarboxylases involved in extreme acid protection (Small and Waterman, 1998). Glutamate decarboxylase is more effective at resisting benzoic acid challenge than either arginine decarboxylase or the oxidative acid tolerance system (Lin *et al*, 1996). Lin *et al* also compared the three stationary phase acid tolerance systems between different EHEC strains. They found all strains possessed the systems, but their effectiveness was strain specific (Lin *et al*, 1996).

1.3.2.4. An Acid Resistant Sub-Population of E. coli *Cells.*

A recent study focusing on *E. coli* O157:H7 cultures of stationary phase, log phase and acid adapted log phase cells found significant survival levels after three days of acid challenge at pH 3.0. This acid resistant sub-population of surviving bacteria were not mutants as acid tolerance was rapidly lost when cultures were transferred to conditions enabling growth to resume. It was found that the cells with greater initial acid resistance accumulated protons at a slower rate. This was correlated with complex differences in the expression patterns of cell envelope proteins between each population of cells. The long-term acid resistant "tail" of cells represents 0.1% of the

total number existing naturally within a population. This sub-population did not require protein synthesis or *rpoS* (Jordan *et al*, 1999a). It is not known whether the *adi* or *gad* systems are involved (Foster, 1999).

Interestingly, studies have shown *E. coli* O157:H7 to display an increased acid tolerance over other serotypes to both organic and inorganic acids (Garren *et al*, 1997; Diez-Gonzalez and Russell, 1997). This may be partly explained by the altered *rpoS* allele of the O157:H7 serotype (Ferreira *et al*, 1999). It has recently been discovered that the acid tolerance of *E. coli* O157:H7 can be overcome by the addition of lactate, ethanol or a combination of both to the acidic media. This effect was seen in stationary phase, log phase and acid adapted log phase cells (Jordan *et al*, 1999b).

Table 1.2. A summary of ATR regulated protein expression in *S. typhimurium* and *E. coli* (St = *S. typhimurium*; Ec = *E. coli*).

Protein	Positive (+) or Negative (-) Regulation	Known Regulators	References
AceA	+		Blankenhorn *et al*, 1999 Lambert *et al*, 1997 (all Ec)
AceB	+		Lambert *et al*, 1997 (Ec)
Ada	+	RpoS	Bearson *et al*, 1998 (St)
AdiA	+	CysB, AdiY, H-NS	Stim and Bennet, 1993 Stim-Herndon *et al*, 1996 Shi *et al*, 1993 Guilfoyle and Hirshfield, 1996 (all Ec) Park *et al*, 1996 (St)
AhpC	+		Blankenhorn *et al*, 1999 Lambert *et al*, 1997 (all Ec)
AniC	+	CRP, TyrR, H-NS	Park *et al*, 1999 (St)

AniG	+	CRP, EarA	Aliabadi *et al*, 1988 Foster and Hall, 1990 Park *et al*, 1999 (all St)
Atp	+		Foster and Hall, 1991 (St)
CadAB	+	CadC, H-NS	Park *et al*, 1996 (St) Shi *et al*, 1993 Guilfoyle and Hirshfield, 1996 (all Ec)
CAMP-CRP	+		Castaine-Cornet *et al*, 1999 (Ec)
CFA synthase	+	RpoS	Chang and Cronan Jr, 1999 Brown *et al*, 1997 (all Ec)
ClpB	+		Lambert *et al*, 1997 (Ec)
DnaK	+		Foster, 1991 (St) Heyde and Portalier, 1990 Lambert *et al*, 1997 (all Ec)
Dps	+		Valdivia and Falkow, 1996 (St)
EarA	-		Aliabadi *et al*, 1988 (St)
EICs	+	CAMP, phosphate	Rowbury and Goodson, 1999a (Ec)
Fur	+		Foster, 1991 Foster and Hall 1991 (all St) Lambert *et al*, 1997 (Ec)

GadAB	+		Hersh *et al*, 1996 Lin *et al*, 1996 Small and Waterman, 1998 Castaine-Cornet *et al*, 1999 Blankenhorn *et al*, 1999 (all Ec)
GatY	+		Blankenhorn *et al*, 1999 (Ec)
GroEL	+		Foster, 1991 (St) Heyde and Portalier, 1990 Lambert *et al*, 1997 (Ec)
GroS	+		Lambert *et al*, 1997 (Ec)
GrpE	+		Heyde and Portalier, 1990 Lambert *et al*, 1997 (all Ec)
H-NS	+		Lambert *et al*, 1997 (Ec)
HslU	+		Lambert *et al*, 1997 (Ec)
HtpG	+		Heyde and Portalier, 1990 Lambert *et al*, 1997 (all Ec)
HtpM	+		Heyde and Portalier, 1990 (Ec)
HyaB	+	CRP, TyrR	Park *et al*, 1999 (St)
Hyd	+		Foster and Hall, 1990 (St)
IbpAB	+		Lambert *et al*, 1997 (Ec)
LysU	+		Heyde and Portalier, 1990 (Ec)

ManX	+		Blankenhorn *et al*, 1999 (Ec)
MarRAB	+		Valdivia and Falkow, 1996 (St)
MBP	-		Blankenhorn *et al*, 1999 (Ec)
MgtB	+	PhoPQ	Bearson *et al*, 1998 (St)
NhaA	+	H-NS	White *et al*, 1992 Gerchman *et al*, 1993 Dover *et al*, 1996 (all Ec)
OmpC	+	OmpR	Leyer and Johnson, 1993 Chart *et al*, 1994 Bang *et al*, 2000 (all St) Thomas and Booth, 1992 (Ec)
OmpF	-	OmpR	Leyer and Johnson, 1993 Chart *et al*, 1994 Bang *et al*, 2000 (all St)
PagA	+	PhoPQ, PmrAB	Bearson *et al*, 1998 (St)
Pgi	+		Foster and Bearson, 1994 Bearson *et al*, 1998 (all St)
PhoPQ	+	PhoPQ	Bearson *et al*, 1998 (St)
PmrAB	+	PhoPQ	Bearson *et al*, 1998 (St)
PmrC	+	PmrAB	Bearson *et al*, 1998 (St)
PolA	+		Foster and Bearson, 1994 (St)
PtsH	-		Blankenhorn *et al*, 1999 (Ec)

RpoS	+	MviA, ClpX	Lee *et al*, 1994 Lee *et al*, 1995 Bearson *et al*, 1996 Bearson *et al*, 1998 (all St) Castaine-Cornet *et al*, 1999 (Ec)
TyrS	+		Lambert *et al*, 1997 (Ec)
UspA	+		Lambert *et al*, 1997 (Ec)
YfiD	+		Blankenhorn *et al*, 1999 (Ec)

1.3.3. The ATR and survival in acidic foodstuffs.

A number of studies have highlighted the significance of the ATR of *Salmonella* and *E. coli* in relation to the survival of these pathogens in acidic foodstuffs. *Salmonella* strains adapted to acidic growth media adjusted to pH 5.8 with HCl, were found to tolerate surface inoculation onto cheddar (pH 5.2), Swiss (pH 5.6) and mozzarella (pH 5.3) cheeses which were subsequently stored at 5°C. Acid adapted cells were able to survive in these conditions for at least twice as long as cells previously held at neutral pH. (Leyer and Johnson, 1992). This study also found acid adaptation to enhance survival during active milk fermentation in which several organic acids and other inhibitors were present. *Salmonella* strains also showed enhanced survival in acidic condiments after acid adaptation. The bacteria were exposed to ground beef at pH 5.5, before inoculation of ketchup (pH 3.6) for 6 hours at 23°C. No unadapted survivors were seen. *E. coli* O157:H7 cells under the same conditions survived better than the *Salmonella* strains (Tsai and Ingham, 1997). In the summer of 1995, Salmonellosis was contracted from unpasteurised orange juice (citric acid). Correspondingly, a study whereby *Salmonella* strains were acid adapted at pH 5.0 in orange serum, detected survival after 27 days in orange juice at pH 3.5 (Parish *et al*, 1997). Interestingly, inoculation of *Salmonella* and *E. coli* pathogens onto certain solid food surfaces was shown to protect the bacteria against extreme acid stress by producing a local buffering

activity. Pathogens added to ground beef that was subsequently soaked in acidic media (pH 2.5) survived better than bacteria added directly into the low pH conditions. The acid survival of *rpoS* mutants was also enhanced in this way. The surface pH of the ground beef after 1 hour at pH 2.5 was measured as pH 3.3. This has important implications on the survival of food contaminating pathogens in stomach acid. (Waterman and Small, 1998). Studies on the foodborne survival of *E. coli* have unsurprisingly concentrated on the O157:H7 serotype. It appears that this particular bacteria can survive well in acidic foods even without prior acid adaptation. For example, both unadapted and adapted micro-organisms were able to survive in dry cereal reconstituted with apple juice (malic acid). The O157:H7 serotype was able to survive longer at lower storage temperatures (Deng *et al*, 1998). Another study showed survival in apple juice and orange juice to be enhanced at lower temperatures (5°C) (Ryu and Beuchat, 1998). Storage in apple juice and orange juice also aided subsequent survival in synthetic gastric fluid. Acid tolerance was again enhanced at lower temperatures (Uljas and Ingham, 1998). These studies highlight the industrial significance of the ATR and suggest that traditional methods of food preservation are not sufficient for protection against pathogenic bacteria.

1.4 Cross Protection.

Cross protection induced by specific stress responses against other potentially lethal factors that may be present in particular environmental niches is an important survival strategy of enteropathogenic bacteria. The overlapping regulatory networks evident in the previous discussion may play a major role in this process. It would seem an inefficient use of energy and other resources if bacteria did not co-regulate certain processes, and thus cross protect in certain environments. For example, in the macrophage phagosome, *Salmonella* may have to contend with acid stress, oxidative stress, osmotic stress, nutrient limitation and other host anti-microbial factors concurrently. Regulation by components such as PhoPQ and σ^s may play a role in cross protection here.

The ATR is a major purveyor of cross protection within the cell. As acid is the first major stress encountered on entering the host, this response may prime the pathogen for

subsequent stressful environments. Acid adapted *S. typhimurium* cells are also tolerant of heat shock, osmotic shock, oxidative stress and the antibiotic polymyxin B sulphate. However, this cross protection is not reciprocated (Leyer and Johnson, 1993). Interestingly, *E. coli* O157:H7 has a reciprocated acid induced heat tolerance (Wang and Doyle, 1998; Rowe and Kirk, 1999) Acid adaptation may not cross protect to osmotic stress in this bacteria (Ryu *et al*, 1999). Another major stress condition is nutrient limitation. Carbon starved cells are tolerant of acid, heat, oxidative and osmotic conditions and also elicit antibiotic resistance (Leyer and Johnson, 1993; Spector, 1998). Oxidatively stressed cells can provide cross protection to polymyxin B via SoxRS (Storz and Imlay, 1999) and also protect against heat shock (Leyer and Johnson, 1993). The role of σ^S in the osmotic stress response suggests this system may also induce cross protection (Hengge-Aronis, 1996).

1.5. Summary.

Salmonella and *E. coli* strains are a major cause of foodborne disease world-wide, and in a significant number of cases, this can be fatal. Many mechanisms are essential for the virulence and enteropathogenicity of these micro-organisms. These systems involve complex overlapping regulation of such processes as pathogenicity island expression, type III secretion systems and bacterial stress responses. In fact, specific stress responses can induce cross protection to additional stresses that may be present in particular habitats. This results in the subsequent tolerance of and survival in many potentially lethal host and environmental niches. Not least of these survival strategies is the ATR. Although several ATR mechanisms have been described, it is clear that much is still to be learnt. Moreover, the studies to date have focussed on standard lab strains such as *E. coli* K-12. There is reason to believe that analysis of other types of *E. coli*, for example, EPEC, may provide additional information in view of the differences in genome sizes displayed by these bacteria. A more detailed knowledge of the regulation of the ATR system and the proteins differentially expressed under such conditions, may lead to the development of novel formulations or regimes that reduce the viability of these pathogens. Therefore, further study of the ATR is obviously of great importance to both medicine and the food industries.

1.6. Aims.

The aims of the present study are:

1. Analysis of the survival kinetics of enteropathogenic *E. coli* and *S. typhimurium* strains under specific organic and inorganic acid conditions.

2. The characterisation of novel ATR regulatory pathways and the identification of differentially regulated proteins primarily utilising proteomic techniques.

3. Comparison of the above components with other mechanisms essential for pathogenesis.

CHAPTER 2.

MATERIALS AND METHODS.

CHAPTER 2. MATERIALS AND METHODS.

2.1. Bacterial Strains.

The bacterial strains utilised in this study are detailed in Table 2.1.

Table 2.1. A description of the *Salmonella* and *E. coli* strains used, including relevant source or reference information.

a. *Salmonella* strains.

STRAIN	RELEVANT GENOTYPE/ PHENOTYPE	SOURCE/REFERENCE
SL1344	mouse virulent non-LT2 isolate of *S. typhimurium*.	Wray and Sojka, 1978.
SL1344K	SL1344 *rpoS*::*kan*.	Francis Norel, Pasteur Institute. Coynault *et al*, 1996.
SL1344 *ΔbipA*	*ΔbipA* derivative of SL1344	N. Kinsella and C. D. O'Connor, University of Southampton.
SL1344 *phoP12*	*phoP12* (*purB*::Tn*10*, *phoP*-derivative) allele transduced into SL1344 from LT2 strain RMA1000.	P. Adams and C. D. O'Connor, University of Southampton. Kier *et al*, 1979.
SL1344 *pho-24*	*pho-24* (*purB*::Tn*10*, $phoQ^C$ derivative) allele transduced into SL1344 from LT2 strain RMA1004.	P. Adams and C. D. O'Connor, University of Southampton. Kier *et al*, 1979.
KK1102	LT2 *flgB-lac* (class 2 flagellar gene). Ap^R.	Kutsukake and Iino, 1994.
KK1107	LT2 *flhD-lac* (class 1 flagellar gene). Ap^R.	Kutsukake and Iino, 1994.
KK1110	LT2 *fliC-lac* (class 3 flagellar gene). Ap^R.	Kutsukake and Iino, 1994.
KK1113	LT2 *fliF-lac* (class 2 flagellar gene). Ap^R.	Kutsukake and Iino, 1994.

SL1344 *flgB-lac*	*flgB-lac* allele transduced from KK1102.	This study.
SL1344 *flhD-lac*	*flhD-lac* allele transduced from KK1102.	This study.
SL1344 *fliC-lac*	*fliC-lac* allele transduced from KK1110.	This study.
SL1344 *fliF-lac*	*fliF-lac* allele transduced from KK1113.	This study.
SL1344K *flgB-lac*	*flgB-lac* allele transduced from KK1102.	This study.
SL1344K *flhD-lac*	*flhD-lac* allele transduced from KK1102.	This study.
SL1344K *fliC-lac*	*fliC-lac* allele transduced from KK1110.	This study.
SL1344K *fliF-lac*	*fliF-lac* allele transduced from KK1113.	This study.
SL1344 *pho-24 flgB-lac*	*flgB-lac* allele transduced from KK1102.	This study.
SL1344 *pho-24 flhD-lac*	*flhD-lac* allele transduced from KK1102.	This study.
SL1344 *pho-24 fliC-lac*	*fliC-lac* allele transduced from KK1110.	This study.
SL1344 *pho-24 fliF-lac*	*fliF-lac* allele transduced from KK1113.	This study.
LB5010	Restriction deficient *S. typhimurium* LT2 strain.	Bullas and Ryu, 1983
LB5010 (pRF001)	pRF001 transformed into LB5010 from DH5α (pRF001).	This study.
LB5010 (pRF002)	pRF002 transformed into LB5010 from DH5α (pRF002).	This study.
LB5010 (pRF003)	pRF003 transformed into LB5010 from DH5α (pRF003).	This study.
LB5010 (pRF004)	pRF004 transformed into LB5010 from DH5α (pRF004).	This study.
LB5010 (pRF005)	pRF005 transformed into LB5010 from DH5α (pRF005).	This study.

LB5010 (pRF006)	pRF006 transformed into LB5010 from DH5α (pRF006).	This study.
SL1344 (pRF001)	pRF001 transformed into SL1344 from LB5010 (pRF001).	This study.
SL1344 (pRF002)	pRF002 transformed into SL1344 from LB5010 (pRF002).	This study.
SL1344 (pRF003)	pRF003 transformed into SL1344 from LB5010 (pRF003).	This study.
SL1344 (pRF004)	pRF004 transformed into SL1344 from LB5010 (pRF004).	This study.
SL1344 (pRF005)	pRF005 transformed into SL1344 from LB5010 (pRF005).	This study.
SL1344 (pRF006)	pRF006 transformed into SL1344 from LB5010 (pRF006).	This study.

b. *E. coli* strains.

MAR001	Enteropathogenic isolate of *E. coli* pMAR$^-$, Str^R.	V. Norris, University of Leicester. Baldini *et al*, 1983.
MAR001 *bipA*::*cat*	MAR001 with *cat* gene inserted into chromosomal *bipA*, Cm^R.	C. D. O'Connor, University of Southampton.
MAR001 *bipA*::*cat* (pBipA)	MAR001 *bipA*::*cat* expressing, in trans, pT7T318U/MarBipA, Cm^R, Ap^R.	C. D. O'Connor, University of Southampton.
E2348/69	EPEC isolate (O127:H6).	M. Donnenberg, University of Maryland. Levine *et al*, 1978.
E2348/69 *bipA*::*cat*	E2348/69 with *cat* gene inserted into chromosomal *bipA*.	A. Grant and C. D. O'Connor, University of Southampton.
JPN15	E2348/69 cured of EAF plasmid.	Jerse *et al*, 1990.
JPN15 *bipA*::*cat*	JPN15 with *cat* gene inserted into chromosomal *bipA*.	A. Grant and C. D. O'Connor, University of Southampton.

HB101 (pRS415)	*E. coli* K-12 strain (*recA*$^-$, *hsdR*$^-$, *mcrA*$^+$, *mcrB*$^-$, *mrr*$^-$, *gyrA*$^+$, *deoR*$^+$, *lacZΔM15*$^-$) harbouring a pBR322 derivative containing a promotorless *lacZYA* operon.	R. Simons, UCLA. Boyer and Roulland-Dussoix, 1969. Simons *et al*, 1987.
DH5α	*E. coli* K12 F$^-$ϕ80d*lacZ*ΔM15, *recA*$^-$, *hsdR*$^-$, *mcrA*$^+$, *mcrB*$^+$, *mrr*$^+$, *gyrA*$^-$, *deoR*$^-$.	Hanahan *et al*, 1991.
DH5α (pRF001)	Random SL1344 promoter 1 ligated to pRS415 & transformed into DH5α. Lac$^+$.	This study. See section 2.4.5.
DH5α (pRF002)	Random SL1344 promoter 2 ligated to pRS415 & transformed into DH5α. Lac$^+$.	This study. See section 2.4.5.
DH5α (pRF003)	Random SL1344 promoter 3 ligated to pRS415 & transformed into DH5α. Lac$^+$.	This study. See section 2.4.5.
DH5α (pRF004)	Random SL1344 promoter 4 ligated to pRS415 & transformed into DH5α. Lac$^+$.	This study. See section 2.4.5.
DH5α (pRF005)	Random SL1344 promoter 5 ligated to pRS415 & transformed into DH5α. Lac$^+$.	This study. See section 2.4.5.
DH5α (pRF006)	Random SL1344 promoter 6 ligated to pRS415 & transformed into DH5α. Lac$^+$.	This study. See section 2.4.5.

2.2. Specialist Materials and Reagents.

The following specialist materials and reagents were used (with source):

Acrylamide (30% w/v), 0.8% w/v bisacrylamide stock solution, gas stabilised (National Diagnostics).
Agar N° 3 (Oxoid).
Borosilicate capillary tubes (Clark Electromedical Instruments).
Broad range molecular weight markers (New England Biolabs).
DNA ladder: 1kb (Gibco BRL).
Ethidium bromide (BCL).
EZ Mix LB Broth Base (Sigma).
Glass cover slips (BDH).
Nanoflow probes (Protana).
Nitrocellulose transfer membrane (Schleicher and Schuell).
Poros R2 Reversed Phase Packing (PerSeptive Biosystems).
Premium glass microscope slides (BDH).
PVDF transfer membrane (PALL).
QIAprep spin kits (Qiagen).
Restriction Enzymes and Buffers (Promega).
Sterile Disposable Filters: 0.22μm (Sera-lab).
Supersignal Western blotting analysis system (Pierce).
Trypsin, sequencing grade (Boehringer Manheim).
T4 DNA ligase and ligase buffer (Promega).
Ultra clear micro-tubes: 0.6ml (Camlab Ltd).
X-ray film (Amersham).
X-ray film developer and fixer (Kodak).
2D Gel equipment and reagents (Amersham-Pharmacia Biotech).

All other materials and reagents used were obtained from GibcoBRL, Sigma, Fisher Scientific or Fisons unless stated otherwise.

2.3. Microbiological Techniques.

2.3.1. Culture conditions.

Unless stated otherwise, the standard growth medium used was Luria-Bertani (LB) medium adjusted to pH 7.2 with NaOH. The LB medium contained 20g of EZ Mix LB Broth Base per litre of deionised water. It was sterilised by autoclaving at 121°C (15psi) for 20 minutes. Once cooled below 45°C, antibiotic and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-GAL) supplements, where necessary, were added. The antibiotic used was ampicillin, added in solution (100mg/ml in sterile, analytical grade water) to a final concentration of 0.1mg/ml. X-GAL was added to media to a final concentation of 0.1%. Bacteria were cultivated in LB and grown, with shaking, at 37°C. LB plates were made by supplementing LB with 1.5% weight/volume (w/v) agar (Agar N° 3, Oxoid). Motility plates consisted of N-minimal media (5mM KCl; 7.5mM $(NH_4)_2SO_4$; 0.5mM K_2SO_4; 1mM KH_2PO_4; 38mM glycerol; 0.1M Tris-HCl, pH 7.4; 0.2% glucose; 0.2% casamino acids; 0.02% thiamine. Garcia Vescovi *et al*, 1994) supplemented with 0.35% (w/v) agar and 1 mM Mg^{2+}. For phage transduction studies, top and bottom LB medium containing 10 mM $MgSO_4$ was supplemented with 0.4% (w/v) and 1.5% (w/v) agar respectively. Strains were stored at –70°C after addition of sterile glycerol (25% final concentration) to LB cultures and flash freezing.

2.3.2. ATR survival assays.

2.3.2.1. Induction of Inorganic Acid Tolerance.

Overnight cultures were diluted 1:40 into fresh, pre-warmed LB medium (pH 7.2) and grown to an absorbance at 600nm (A_{600}) of 0.5 optical density units. At this time point, cultures were diluted 1:40 into pre-warmed LB medium at either pH 5.0 (adjusted with HCl) or pH 7.2. After incubation at 37°C for a specified time period, cells from each pH condition were diluted, in parallel, 1:40 into pre-warmed LB medium at pH 3.0 (adjusted with HCl). The pH 3.0 cultures were incubated for a specified period of time at 37°C. Samples were then serially diluted into sterile

(filtered through 0.22μm sterile disposable filters) phosphate buffered saline (0.14 M NaCl; 2.7 mM KCl; 8.2 mM Na_2HPO_4; 1.1 mM KH_2PO_4) at 4°C. Induction of an acid tolerance response was monitored by plating cell samples onto LB agar plates (pH 7.2) and scoring survivors after overnight incubation at 37°C.

2.3.2.2. Induction of Acid Tolerance in the Presence of Organic Acid at pH 5.0.

Mid-exponential phase cells, produced as above, were diluted 1:40 into fresh, pre-warmed LB medium at pH 5.0 (adjusted with benzoic acid, final concentration 1.4 mM) or pH 7.2. After incubation at 37°C for a specified period of time, cells were incubated at pH 3.0 as previously described. Induction of acid tolerance was monitored as above.

2.3.2.3. Induction of Acid Tolerance in the Presence of Organic Acid at pH 3.0.

Mid-exponential phase cells were incubated in pre-warmed LB medium at pH 5.0 (adjusted with HCl) or pH 7.2 for a specified period of time as described in section 2.3.2.1. The cultures were then diluted, in parallel, 1:40 into pre-warmed LB medium at pH 3.0 (adjusted with HCl) containing 0.5mM sodium benzoate. After incubation at 37°C for a specified time period, induction of acid tolerance was monitored as above.

2.3.3. Cross-protection survival assays.

2.3.3.1. Investigation of H_2O_2 Tolerance Following Acid Adaptation.

Overnight cultures were diluted 1:40 into fresh, pre-warmed LB medium (pH 7.2) and grown to an A_{600} of 0.5 optical density units. At this point, the cultures were split into three 10ml aliquots. The first was adjusted to pH 5.0 with HCl; H_2O_2 was added to the second (final concentration, 0.3 μM) and the third was kept at pH 7.2. After incubation at 37°C for 60 minutes, H_2O_2 was added to each culture to a final concentration of 30 μM. The cells were then incubated for specified periods of time at 37°C. Samples were then serially diluted into sterile phosphate buffered saline at 4°C.

Tolerance to oxidative stress was monitored by plating cell samples on LB agar plates (pH 7.2) and scoring survivors after overnight incubation at 37°C.

2.3.3.2. Investigation of Acid Tolerance Following Adaptation to H_2O_2.

Mid-exponential phase cells were produced as above and split into three 10ml aliquots. Each aliquot was adjusted to pH 5.0, 0.3 μM H_2O_2 or kept at pH 7.2 and incubated at 37°C for 60 minutes as described. Subsequently, each of the three separate cultures was adjusted to pH 3.0 with HCl. The cells were then incubated for specified periods of time and serially diluted as above. Acid tolerance was monitored by plating cell samples onto LB agar plates (pH 7.2) and scoring survivors after overnight incubation at 37°C.

2.3.4. Motility assays.

2.3.4.1. Motility Plates.

The motility plate procedure used was essentially as described by Silverman and Simon (1973). As the motility plates were not dried or inverted, the agar (see 2.3.1.) was left to cool before pouring to minimise the formation of condensation. Bacteria were stabbed into the agar using a platinum wire dipped into mid-exponential phase cultures. Plates were then incubated overnight at 37°C and the diameter of the subsequent growth was measured.

2.3.4.2. Phase Contrast Microscopy.

Phase contrast microscopy was used to assess the motility of bacteria during the acid tolerance response. Overnight cultures were diluted 1:40 in to pre-warmed LB medium (pH 7.2) and grown to an A_{600} of 0.5 optical density units. At this point, cultures were diluted 1:20 into fresh pre-warmed LB medium at either pH 5.0 (adjusted with HCl) or pH 7.2 and incubated at 37°C for 60 minutes. Culture pH was then adjusted to either pH 3.0, pH 4.0 or pH 5.0 using HCl or pH 7.2 with NaOH

before incubation at 37°C for 5 minutes. At this time point, 100µl of each culture was placed onto a glass slide and cell motility (expressed as a percentage of motile cells) was determined using a phase contrast microscope (Zeiss). At least 200 cells were counted under each set of pH conditions.

2.4. Genetic Techniques.

2.4.1. DNA preparation.

Plasmid DNA was purified using commercially available kits (Qiagen) and following the manufacturer's instructions. These procedures are based on the alkaline lysis of bacterial cells (Birnboim and Doly, 1979) followed by lysate clearing and adsorption of DNA onto silica gel membranes. DNA adsorption onto silica occurred in the presence of high salt-buffer. After washing, DNA was eluted in low salt and neutral pH conditions with sterile, analytical grade water.

Purified DNA was prepared from 10ml of overnight culture using the Qiagen QIAprep Spin Miniprep Kit as per the manufacturer's instructions. A Heraeus Biofuge 15 was used for centrifugation. The DNA was eluted in 50µl of sterile, analytical grade water.

2.4.2. Restriction enzyme digests.

Restriction endonuclease digestion of DNA was based on the procedure described by Sambrook *et al* (1989). Specifically, pRS415 DNA was digested with *Bam*HI in buffer E (6 mM Tris-HCl; 6 mM $MgCl_2$; 100 mM NaCl; 1 mM dithiothreitol (DTT)) for 3 hours at 37°C. *S. typhimurium* SL1344 chromosomal DNA was partially digested with *Sau*3A in buffer B (6 mM Tris-HCl; 6 mM $MgCl_2$; 50 mM NaCl; 1 mM DTT) for 1 hour at 37°C. Digests were stopped by the addition of ethylenediaminotetraacetic acid (EDTA) (final concentration, 0.5 M). DNA digests were purified using the Qiagen QIAquick PCR Purification Kit protocol. The DNA was eluted in 50µl of sterile, analytical grade water.

2.4.3. DNA ligation.

The DNA ligation procedure was based on that described by Sambrook *et al* (1989). Specifically, digested pRS415 DNA was ligated with partially digested *S. typhimurium* SL1344 chromosomal DNA using T4 DNA ligase overnight at 16°C. Ligations were purified using the Qiagen QIAquick Nucleotide Removal Kit protocol. DNA was eluted in 30µl of sterile, analytical grade water.

2.4.4. Agarose gel electrophoresis.

Horizontal agarose gels were prepared as described by Sharp *et al*, 1973 and McDonnell *et al*, 1977. 0.8% (w/v) agarose was made up in TAE buffer (0.04 M Tris-acetate; 1 mM EDTA) containing 0.6µg/ml of ethidium bromide. DNA was mixed with equal volumes of gel loading buffer (30% volume/volume (v/v) glycerol; 0.3% w/v bromopohenol blue made up in TE buffer (10 mM Tris-HCl pH 8.0; 1 mM EDTA)) and loaded onto the gel. A 2.5µl volume of 1kb DNA ladder was used as a size marker (see Table 2.2). Electrophoresis was carried out at a constant 100V for approximately 60 minutes in TAE buffer containing 0.6µg/ml of ethidium bromide. Gels were visualised and photographed using a UV transilluminator camera system (UVP).

Table 2.2. Protein molecular weight markers and DNA size markers used in SDS-PAGE and agarose gel electrophoresis respectively.

PROTEIN (broad range M.wt marker, NEB)	MOLECULAR WEIGHT (kDa)	DNA BAND (BRL, 1kb)	SIZE (kb)
myosin	212000	1	12.21
MBP-β-galactosidase	158194	2	11.19
β-galactosidase	116351	3	10.18
phosphorylase B	97184	4	9.16
serum albumin	66409	5	8.14
glutamate dehydrogenase	55561	6	7.12
maltose binding protein-2	42710	7	6.10
lactose dehydrogenase	36487	8	5.09
triose phosphate isomerase	26625	9	4.07
trypsin inhibitor	20040-20167	10	3.05
lysozyme	14313	11	2.03
aprotinin	6517	12	1.63
insulin A, B chain	2340-3400	13	1.01
		14	0.51
		15	0.50
		16	0.39
		17	0.34
		18	0.29
		19	0.22
		20	0.20
		21	0.15
		22	0.13
		23	0.04

2.4.5. Transformation.

2.4.5.1. Transformation into Electrocompetent Cells.

Electrocompetent cells for transformation were prepared by growing cultures to an A_{600} of 0.5 optical density units, harvesting (3500rpm, Beckman JLA 10500 rotor, 10 minutes, 4 °C) and washing twice with sterile, ice-cold, analytical grade water. Cells

were then resuspended in sterile, ice-cold, 10% v/v glycerol and pelleted (3500rpm, Beckman JLA 10500 rotor, 10 minutes, 4°C). At this point, cells were again resuspended in 10% v/v glycerol before they were aliquoted into 40µl batches, frozen with dry ice and stored at -70°C (Sambrook *et al*, 1989).

DNA to be transformed was added to 40µl of electrocompetent cells in 0.1ml capacity electroporation cuvettes before mixing and incubating on ice for 1 minute. The cells were then shocked at 1.25mV for 4.5-5 milliseconds. At this point, the cells were resuspended immediately in 1ml of sterile SOC medium (10 mM NaCl; 2.5 mM KCl; 10 mM $MgCl_2$; 10 mM $MgSO_4$; 20 mM Glucose; 2% w/v Bacto-tryptone; 0.5% w/v Bacto-yeast extract) and incubated at 37°C for 1 hour prior to plating.

2.4.5.2. Transformation into Calcium Competent Cells.

Calcium competent cells for transformation were prepared from cells grown to an A_{600} of 0.6 optical density units. The cells were then pelleted (3500rpm, Beckman JLA 10500 rotor, 10 minutes, 4°C) and resuspended in an equal volume of ice cold 0.1 M $CaCl_2$. The suspension was the re-centrifuged as above and resuspended in a 4% volume of ice cold 0.1 M $CaCl_2$. The calcium competent cells were then aliquoted into batches of 100µl, flash frozen and stored at -70°C (Sambrook *et al*, 1989).

DNA to be transformed was added to 100µl of calcium competent cells and left on ice for 45 minutes. The DNA/cell mixture was then heat shocked at 42°C for 1 minute prior to incubation on ice for a further minute. The cells were then added to 1ml of LB medium and incubated at 37°C for 2 hours prior to plating on appropriate selective media.

2.4.6. Transduction using P22.

Overnight cultures of donor cells were grown at 37°C with shaking. Amplification of P22 HT*int* on the donor strain was carried out by incubating 200µl of donor cells with an equal volume of P22 in LB broth containing 10mM $MgSO_4$ at 37°C with shaking.

After 16 hours, the phage lysate was centifuged at 5000rpm (Minor benchtop centrifuge) for 10 minutes to remove any remaining donor cells and cell lysates. The cleared lysate was then filter sterilised (0.2μm sterile, disposable filter). The transducing lysate was then serially diluted using 10 mM $MgSO_4$. 50μl of host cells (from an overnight culture) and 100μl of each phage dilution were then added to 5ml of molten LB top agar at 45°C before pouring over an undried LB bottom agar plate. The plates were incubated overnight base down at 37°C. The titre of the P22 transducing lysate was then calculated in plaque forming units (pfu) per ml. If the titre of the phage transducing stock was below 10^9 pfu/ml, it was reamplified on the donor strain (Sambrook *et al*, 1989).

For phage transduction, overnight cultures of recipient cells were grown up at 37°C with shaking. These were then pelleted (8000rpm, 10 minutes, Beckman JLA 10500 rotor) and resuspended in 5ml of 10 mM $MgSO_4$, 5 mM $CaCl_2$. Recipient cells were subsequently mixed with the P22 transducing lysate over a range of multiplicity of infection ratios (pfu/colony forming units (cfu)) and incubated at room temperature for 15 minutes. 1 M sodium citrate was then added to each tube before immediately spreading on LB agar plates containing the required chemicals for selection and 20 mM sodium citrate. Sodium citrate prevents the binding of phage to bacterial cells and thus further infection by through the chelation of Mg^{2+}. The plates were left overnight at 37°C.

The transductants were single colony purified on LB agar plates containing the required selection ingredients and 20 mM sodium citrate. This was repeated at least three times and until colony morphology displayed no signs of phage to remove any remaining P22 contamination.

2.5. Molecular Biology Techniques.

2.5.1. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE).

SDS-PAGE was performed using the Bio-Rad Mini-Protean II electrophoresis apparatus as per the manufacturer's instructions. The procedure is based upon the discontinuous polyacrylamide gel and buffer system for the molecular weight fractionation of proteins (Laemmli, 1970). Acrylamide (30% w/v), 0.8% w/v bisacrylamide stock solution (30% acrylamide/bis) was used for both the resolving and stacking gels.

The resolving gel (12% acrylamide/bis; 0.375 M Tris-HCl pH 6.8; 0.1% w/v SDS; 0.03% w/v ammonium persulphate; 0.0016% v/v TEMED) was poured and overlaid with water saturated butanol until polymerisation. At this point, the stacking gel (5% w/v acrylamide/bis; 62.5 mM Tris-HCl pH 6.8; 0.1% w/v SDS; 0.03% w/v ammonium persulphate; 0.0016% v/v TEMED) was poured and left to polymerise with a comb inserted.
Protein samples for electrophoresis were resuspended in an appropriate volume of final sample buffer (62.5 mM Tris-HCl pH 6.8; 2% w/v SDS; 10% v/v glycerol; 5% v/v 2-mercaptoethanol; 0.02% w/v bromophenol blue for visualisation) and boiled at 100°C for 5 minutes prior to electrophoresis. After sample loading, gels were run at a constant 100V until completion of electrophoresis. Gel running buffer include 25 mM Tris; 25 mM glycine and 0.001% w/v SDS.

Protein samples were visualised by staining the gels with Coomassie Brilliant Blue (0.25% w/v Coomassie Brilliant Blue R-250 dissolved in 9% v/v glacial acetic acid and 45% v/v methanol) for 30 minutes. Gels were then destained (7.5% v/v glacial acetic acid; 45% v/v methanol) until visualisation. A broad range protein marker (detailed in Table 2.2) was used to estimate the molecular weight of samples.

2.5.2. Western Blotting of proteins.

This procedure was based on the method first described by Towbin *et al*, 1979. Proteins to be immunoblotted together with broad range molecular weight markers were resolved on a 12% SDS-polyacrylamide gel (see section 2.5.1.). Prior to blotting, the gel was equilibrated in transfer buffer (192 mM glycine; 25 mM Tris; 20% v/v methanol). The BioRad Mini Trans Blot Electrophoresis Transfer Cell was used to transfer proteins onto a nitrocellulose membrane as per the manufacturer's instructions. Transfer was carried out at a constant 100V for 120 minutes. As transfer efficiency drops markedly above 30°C, a cooling block was inserted into the transfer apparatus to ensure the temperature remained below this value.

Following transfer, the nitrocellulose membrane was incubated in 200ml of blocking buffer (10 mM Tris pH 8.0; 1.8 mM Na_2EDTA pH 8.0; 133 mM NaCl; 0.5% v/v Triton X-100; 0.5% w/v bovine serum albumin (BSA)) overnight at 4°C. The membrane was then incubated in 20ml of a suitable primary antibody (see Table 2.3 for dilutions used) at room temperature for 2 hours with gentle agitation. After this time, the membrane was washed (3 × 30 minutes) with blocking buffer to remove any primary antibody left unbound. The membrane was then incubated at room temperature for 2 hours with gentle agitation in a suitable dilution (see Table 2.3) of secondary antibody conjugated to horse radish peroxidase. Unbound secondary antibody was the removed by washing the membrane (3 × 10 minutes) in Tris-buffered saline (50mM Tris-HCl pH 7.5; 100 mM NaCl) at room temperature. Binding of antibodies to the immobilised proteins was then visualised by electrochemiluminescence (ECL) using the Pierce SuperSignal kit in conjunction with X-ray film to detect the emitted light. Developer and fixing reagents were used to permanently embed the light image into the X-ray film.

After development of the X-ray film, transfer efficiency was checked by staining the nitrocellulose membrane with Amido Black (0.15% w/v Amido Black; 25% v/v methanol; 10% v/v glacial acetic acid) prior to destaining (50% v/v methanol). This also allowed estimation of the molecular weights of immunoblotted proteins through visualisation of the broad-range molecular weight markers. Additionally, a duplicate

of the SDS-PAGE gel used for transfer was stained with Coomassie Brilliant Blue prior to destaining (see section 2.5.1.) to check protein loadings.

Table 2.3. Specific Primary and Secondary Antibody Dilutions.

ANTIBODY TYPE	DILUTION
Anti-flagellin phase 1 (FliC) mouse monoclonal. Primary antibody (Gift, CVL, Surrey).	1:400 in blocking buffer.
Anti-HNS mouse monoclonal. Primary antibody (Gift, CVL, Surrey).	1:400 in blocking buffer.
Rabbit anti-mouse. Secondary antibody (DAKO, Denmark).	1:400 in blocking buffer.

2.5.3. Identification of lipopolysaccharide chemotypes using silver stained polyacrylamide gels.

This procedure, which allows visualisation of size fractionated bacterial lipopolysaccharide (LPS) chemotypes on polyacrylamide gels was performed essentially as described by Hitchcock and Brown (1983).

Bacteria were harvested from LB agar plates and washed in 10ml of phosphate buffered saline. 1.5ml of the cell suspension was then centrifuged (Heraeus Biofuge 15, 15000rpm, 15 minutes) before the pellets were collected and resolubilised in 50µl of lysing buffer (4% v/v 2-mercaptoethanol; 10% v/v glycerol; 1 M Tris pH 6.8; 2% w/v SDS and a few grains of bromophenol blue for visualisation). The lysate was then heated at 100°C for 10 minutes. At this point, the lysate was digested with 25µg of proteinase K (10µl of 2.5mg/ml proteinase K in lysing buffer) at 60°C for 1 hour. Undigested proteins can alter the migration pattern of LPS molecules through the polyacrylamide gel, resulting in incorrect separation patterns. A control was performed by adding 10µl of lysing buffer to a second lysate prior to incubation at 60°C for 1 hour. Digested whole cell lysates and undigested controls were then subjected to polyacrylamide gel electrophoresis.

Polyacrylamide gel electrophoresis was carried out as described in section 2.5.1. with two exceptions. Firstly, SDS, which can interfere with LPS molecules and affect migration was omitted from both the resolving and stacking gels. Secondly, the gel was run at a constant 35mA current for 2.5 hours to maximise the size separation of different LPS molecules. Gels were run in duplicate. One was stained for protein with Coomassie Brilliant Blue (see section 2.5.1.) and the other was visualised with a modified silver stain for LPS.

Gels for LPS staining were immersed in silver stain fixer solution (25% v/v isopropanol; 7% v/v glacial acetic acid) and incubated overnight at room temperature. They were then oxidised in 0.7% w/v periodic acid; 2.6% v/v fixer solution) for 5 minutes at room temperature. At this point, the gels were washed (8 × 30 minutes) in sterile, analytical grade water prior to incubation in silver stain (0.1 M NaOH; 29.4% v/v ammonium hydroxide; 20% w/v silver nitrate) for 10 minutes. After 4 × 10 minute washes in sterile, analytical grade water, gels were immersed into silver stain developer solution (0.2 mM citric acid; 0.05% formalin) until LPS bands were visualised. At the correct staining intensity, developer stop solution (0.35% acetic acid) was added and the gel was further incubated for 1 hour. The gel was then washed in 200ml of sterile, analytical grade water.

2.5.4. β-Galactosidase assays in permeabilised cells.

The experimental procedure used is based on that described by Miller (1972).

2.5.4.1. Preparation of Bacterial Cells.

β-galactosidase assays were performed on bacterial cells previously exposed to ranges of pH conditions. Samples were prepared from overnight cultures diluted 1:40 into fresh, pre-warmed LB medium (pH 7.2) and grown to an A_{600} of 0.5 optical density units at 37°C with shaking. At this point, the cultures were diluted 1:10 into fresh, pre-warmed LB medium at either pH 5.0 (previously adjusted with HCl prior to

sterilization) or pH 7.2. These cultures were then incubated at 37°C with shaking for 110 minutes. The pH of the cultures at pH 5.0 was then adjusted to pH 3.0 with HCl (monitored using a sterile pH meter). Both the pH 3.0 and the pH 7.2 cultures were then incubated at 37°C with shaking for 10 minutes. Cells were harvested by centrifugation (Beckman J2-21, 8000rpm, 20 minutes, 37°C) and resuspended in working buffer. Working buffer comprised 0.4 mM DTT in β-galactosidase assay buffer, pH 7.0 (61 mM Na_2HPO_4; 39 mM NaH_2PO_4; 10 mM KCl; 10 mM $MgSO_4.7H_2O$). The cell suspensions were equilibrated in working buffer to an A_{660} of 0.4 optical density units.

β-galactosidase assays were also performed using wild type and isogenic mutant strains at neutral pH during both the exponential and stationary phases of growth. Exponential phase cells were prepared from overnight cultures diluted 1:40 into fresh, pre-warmed LB medium (pH 7.2) and grown at 37°C with shaking to an A_{600} of 0.5. Stationary phase cells were prepared from overnight cultures in a similar manner, but incubated at 37°C with shaking to an A_{600} of 1.6. These cells were harvested (8000rpm, Beckman JA-20 rotor, 15 minutes, 4°C) and resuspended in working buffer to an A_{660} of 0.4 as above.

1ml samples of the A_{660} = 0.4 suspensions were used in the assays. 20µl of chloroform and 10µl of 0.1% w/v SDS were added to the 1ml suspensions to permeabilise the cells. After a 5 minute incubation at room temperature, O-nitophenyl-β-D-galactopyranoside (ONPG) was added to a final concentration of 2.2 mM. The suspensions were then incubated at 28°C until a yellow colour of between 0.1 and 0.4 optical density units at an absorbance of 420nm was visible. At this point, the colouration reaction was stopped by the addition of 1 M Na_2CO_3 to a final concentration of 0.3 M. A_{420} and A_{550} readings of the cell suspensions were then taken. The β-galactosidase activity (amount of ONPG hydrolysed per minute as a function of cell density) was then calculated using the formula:-

$$\text{Units} = 1000 \times \frac{A_{420} - (1.75 \times A_{550})}{t \times V \times A_{660}}$$

Where t = time of the colouration reaction (minutes) and V = volume of cell suspension in the assay (ml).

2.6. Proteomic Techniques.

2.6.1. Two-dimensional polyacrylamide gel electrophoresis.

Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) was carried out essentially as described by Görg *et al*, 1985.

2.6.1.1. Sample Preparation.

ATR induced bacteria.

Samples for two-dimensional polyacrylamide gel electrophoresis were prepared from overnight cultures diluted 1:40 into fresh, pre-warmed LB medium (pH 7.2) and grown to mid-exponential phase (A_{600} = 0.5) at 37°C with shaking. At this point, the cultures were diluted 1:40 into fresh, pre-warmed LB medium at either pH 5.0 (previously adjusted with HCl prior to sterilisation) or pH 7.2. These cell suspensions were then incubated at 37°C with shaking for 110 minutes. The pH of the acid adapted cultures was then adjusted to pH 3.0 with HCl (monitored using a sterile pH meter). Cultures at pH 3.0 and those remaining at pH 7.2 were then incubated at 37°C for 10 minutes with shaking. Cells were then harvested via centrifugation (Beckman J2-21, 8000rpm, 20 minutes, 37°C), washed with 5ml of phosphate buffered saline (4°C) and resuspended in 5ml of lysis buffer (2.5% v/v Triton X 100; 2.5% v/v 2-mercaptoethanol; 2.5% v/v ampholine pH 3.5-10; 8 mM polymethylsulphonylflouride (PMSF)) prior to sonication. The cells were sonicated (MSE Soniprep 150) on ice for 10 × 20s on, 20s off cycles. To complete lysis, urea was added to a final concentration of 9M. After centrifugation (8000rpm, Heraeus Biofuge 15, 1 minute) to pellet

insoluble material, protein concentration was estimated at an A_{280}. Samples were then flash frozen and stored at -70°C.

Induction of other stress responses.

Mid-exponential phase cells, produced as above, were treated with either 3 μM of H_2O_2 or 0.5 M of NaCl for 60 minutes at 37°C with shaking to induce an oxidative stress response or an osmotic shock response respectively. Cells were then harvested and samples prepared for 2D-PAGE as above.

Sample preparation from isogenic mutant and respective wild type strains.

Overnight cultures of the SL1344K mutant and respective SL1344 wild type strains were diluted 1:40 into fresh, pre-warmed LB medium (pH 7.2) and grown to stationary phase (A_{600} = 1.6) at 37°C with shaking. At this point, cells were harvested (8000rpm, Beckman JA-20 rotor, 15 minutes, 4°C), washed with 1ml of phosphate buffered saline (4°C) and resuspended in 500μl of lysis buffer prior to sonication. Sonication and storage was carried out as above.

All other mutant and respective wild type strains were grown to mid-exponential phase from overnight cultures, either in pre-warmed LB media (pH 7.2) as described previously, or in pre-warmed N-minimal media (containing 10μM or 1mM Mg^{2+}). At this point, sample preparation was carried out as for the stationary phase cultures detailed above.

2.6.1.2. First Dimension Isoelectric Focusing.

Proteins were separated in the first dimension according to their isoelectric point using the IPGphor™ Isoelectric Focusing System (Amersham-Pharmacia Biotech). The apparatus was set up as per the manufacturer's instructions utilising 18cm immobilised pH gradient (IPG) strips, pH 4-7.

An appropriate volume of prepared sample was diluted with rehydration solution (8 M urea; 2% w/v CHAPS; 2% w/v IPG-buffer pH 4-7L; 20 mM DTT; orange G to aid visualisation) to a desired protein concentration (4mg) in a final volume of 400µl. This was then added to an 18cm long IPG strip holder. The IPG strip was then inserted, gel side down, ensuring the rehydration/sample solution covered the strip and no air bubbles were present. The IPG strip was then overlaid with 2ml of Dry Strip Cover Fluid to prevent desiccation and urea crystallisation. A plastic lid was then placed onto the IPG strip holder before transfer to the IPGphor unit. It was ensured that the positive and negative electrodes of the IPGphor were in contact with the appropriate termini of the IPG strip holder. The IPGphor unit was set to rehydrate the IPG strips at 20°C for 12 hours, followed by an isoelectric focusing step for a total of 75kV hours at the same temperature. Up to twelve IPG strips could be run simultaneously at a maximum current of 50µA each. After isoelectric focusing, IPG strips were blotted to remove excess Dry Strip Cover Fluid. They could then be stored for up to 1 week at -70°C prior to the second dimension run.

2.6.1.3. Horizontal Second Dimension Polyacrylamide Gel Electrophoresis.

Prior to the second dimension, the IPG strips were equilibrated in 2 × 10ml of equilibration solution (50 mM Tris-HCl pH 6.8; 6 M urea; 30% v/v glycerol; 1% w/v SDS) for 10 minutes with agitation. The first volume of equilibration solution contained 16 mM DTT to reduce disulphide bonds and help solubilise the proteins. The second volume contained 0.2 M iodoacetamide to counteract any gel streaking caused by DTT. A few grains of bromophenol blue were also added to produce a dye front to aid in the monitoring of electrophoresis. After equilibration, the IPG strips were left to dry on their sides on filter paper for between 3 and 20 minutes.

During the second dimension, the proteins were separated by size fractionation on a 12-14% gradient polyacrylamide gel (ExcelGel XL SDS, gradient 12-14, Amersham-Pharmacia Biotech) utilising a Multitemp II Thermostatic Circulator and Cooling Plate (Amersham-Pharmacia Biotech). The apparatus was set up as per the manufacturer's instructions.

The Multitemp II Thermostatic Circulator and Cooling Plate were set to run the second dimension at 15°C. An ExcelGel XL SDS 12-14 gel was placed onto the cooling plate which had been previously coated in paraffin oil and set to 15°C. Buffer for this system was provided by anodic and cathodic ExcelGel SDS buffer strips placed at opposite ends of the gel. An equilibrated IPG strip was positioned, gel side down, approximately 1mm from the cathodic buffer strip. Small pieces of filter paper were inserted at either end of the IPG strip such that they touched the edge of the isoelectric focusing (IEF) gel. This prevented capillary action from transporting buffer around the edges of the gel. To ensure good contact between the IEF and SDS gels, and to remove air bubbles, a pair of forceps were run gently over the plastic backing of the IPG strip. Electrodes were then positioned to make the appropriate connections with the buffer strips. The second dimension was then run according to the following steps (all are constant current):-

i. 1000V, 20mA, 40W for 45 minutes.

The IPG strip and filter paper were then removed.

ii. 1000V, 40mA, 40W for 5 minutes.

The cathodic buffer strip and electrode were then moved to cover the position previously occupied by the IPG strip on the SDS gel.

iii. 1000V, 40mA, 40W for 135 minutes.

Once the dye front entered the anodic buffer strip, electrophoresis was stopped and the gel was visualised.

For densitometric analysis, gels were stained for 1 hour with Coomassie Brilliant Blue (0.25% w/v Coomassie Brilliant Blue R-250 dissolved in 9% v/v glacial acetic acid and 45% v/v methanol) and destained overnight in 7.5% v/v glacial acetic acid, 45% v/v methanol.

Gels subsequently used for in-gel digestion were stained for 20 minutes with modified Coomassie Brilliant Blue (0.2% w/v Coomassie Brilliant Blue R-250 dissolved in 0.5% v/v glacial acetic acid and 20% v/v methanol) and destained until spots were visualised in 30% v/v methanol.

2.6.2. Image analysis of two-dimensional gels.

Densitometric analysis was used to estimate the relative amounts of specific proteins present on individual 2D-PAGE gels. The gels were scanned into a computer at 600dpi using an Epson GT 8000 scanner. All gels were scanned using identical parameters. The software package, Phoretix 2D Software v.5.1 (Phoretix International, Newcastle-Upon-Tyne) was used for densitometric analysis. Protein expression patterns were compared between gels of equal sample loading. A particular protein was judged to be differentially regulated between distinct environmental conditions or stain types if its respective levels altered by ≥ 2 fold in at least 4 independent sample preparations. Images were exported to Adobe Photoshop v.5.0 for annotation and printing.

2.6.3. In-gel digestion of proteins separated by 2D-PAGE and identification by nanospray mass spectrometry and peptide mass fingerprinting.

The following procedures were performed essentially as described by Wilm *et al*, 1996.

2.6.3.1. In-Gel Digestion.

Where appropriate, all solutions were sterilised via 0.22μm sterile disposable filters. Two-dimensional gels were rinsed using sterile, analytical grade water before protein spots of interest were excised into separate 0.6ml Ultra-Clear microtubes (Camlab Ltd). Excised protein spots were cut into 1mm × 1mm gel particles within the microtubes using sterile scalpel blades. The tubes were checked visually at all times to ensure they were free from contaminating dust fibres. The gel particles were rinsed

again in sterile, analytical grade water prior to dehydration in 100% acetonitrile. Gel pieces were subsequently immersed in sterile 10 mM DTT; 0.1 M NH_4HCO_3 for 30 minutes at 56°C to allow protein reduction. At this point, the gel particles were again dehydrated in 100% acetonitrile before protein alkylation in 55 mM iodoacetamide; 0.1 M NH_4HCO_3 for 20 minutes at room temperature in the dark. The gel pieces were then washed in 0.1 M NH_4HCO_3 for 15 minutes before dehydration again in 100% acetonitrile. If the gel particles retained Coomassie Blue stain at this point, they were incubated overnight in 0.1 M NH_4HCO_3/acetonitile (1:1 v/v) before dehydrating once more in 100% acetonitrile. Protein digestion was initiated by rehydrating the gel pieces in 50 mM NH_4HCO_3; 5 mM $CaCl_2$; 12.5ng/µl trypsin at 4°C for 45 minutes. After this time, the supernatant was removed and 50 mM NH_4HCO_3; 5 mM $CaCl_2$ was added for overnight digestion at 37°C.

2.6.3.2. Peptide Extraction.

After overnight digestion, the gel particles were incubated in 25 mM NH_4HCO_3 at 37°C for 15 minutes with shaking. A volume of acetonitrile twice that of the gel particles was then added to this solution prior to further incubation for 15 minutes under the same conditions. After this time, the gel particles were centrifuged (Heraeus Biofuge 15, 8000rpm, 1 minute) and the supernatant collected into fresh 0.6ml Ultra-Clear microtubes. The gel particles were then vortexed in 5% v/v formic acid for 15 minutes at 37°C. As before, a volume of acetonitrile twice that of the gel particles was then added prior to incubation at 37°C for 15 minutes with shaking. The gel particles were again centrifuged (Heraeus Biofuge 15, 8000rpm, 1 minute) and the respective supernatants pooled.

2.6.3.3. Desalting of Peptide Solutions Prior to Nanospray Mass Spectrometry.

1µl of Poros R2 Resin (PerSeptive Biosystems) was loaded into 1.2mm OD × 0.69mm ID borosilicate tubes (pulled to a fine point at one end so that resin could not escape) to form micro-affinity purification columns and equilibrated with sterile, analytical grade water. The supernatants containing eluted peptides were subsequently loaded

onto individual columns and washed three times in sterile, analytical grade water. Peptides were then eluted in 1μl of 50% v/v methanol; 5% v/v formic acid directly into a nanospray capillary tube (Nanoflow probe tips, Protana).

2.6.3.4. Nanoelectrospray Mass Spectrometry.

The nanoelectrospray capillary tubes containing desalted peptides in 1μl of 50% v/v methanol; 5% v/v formic acid were inserted into a LCT orthogonal acceleration - Time of Flight instrument (Micromass). Mass spectrometry was carried out with the following settings: capillary voltage, 1200V; cone voltage, 46-52V; desolvation gas, 100-200 litres/hour; source temperature, 80°C. Data was collected in the 400-1700 mass range for up to 30 minutes using internal caesium chloride calibration. To resolve multiply charged peaks onto a singly charged axis, the MaxEnt Sequence software programme (Micromass) was used.

2.6.3.5. Database Searching and Protein Identification.

The experimentally derived masses of tryptic peptides were used to search an on-line non-redundant NCBI database for matching proteins using MS-FIT in the Protein Prospector suite of programmes (Internet address: http://prospector.ucsf.edu/). Protein identification was judged as correct according to the following parameters:-

i. ≥ 25% of peptide masses entered matched the predicted digest fingerprint.

ii. The matched peptides covered ≥ 25% of the protein sequence in the database.

iii. The predicted isoelectric point and molecular mass (Predict pI/M.wt tool, Expasy) matched those observed experimentally to within 1.0 pH units and 8.0kDa respectively.

2.6.4. Protein identification via *N*-terminal sequencing.

Protein spots of interest that were too low in concentration to be identified using the above methods were sent for *N*-terminal sequence analysis (A. Moir, University of Sheffield). Proteins were transferred onto polyvinyldiflouride (PVDF) membranes (PALL) from 2D gels using the Western Blotting procedures described in section 2.5.2. After visualisation using Amido Black stain, spots of interest were cut from the membrane using sterile scalpel blades and transferred into Ultra-Clear 0.6ml microtubes. Proteins were identified using the MS Edman programme at the Protein Prospector Internet site to match experimentally derived *N*-terminal sequences with those in the non-redundant NCBI database. Matched proteins had a 100% amino acid homology over ≥ 9 residues.

CHAPTER 3.

CHARACTERISATION OF THE SURVIVAL KINETICS OF *S. typhimurium* AND *E. coli* DURING THE ATR.

CHAPTER 3. CHARACTERISATION OF THE SURVIVAL KINETICS OF *S. typhimurium* AND *E. coli* DURING THE ATR.

3.1. Introduction.

Within the last decade it has become apparent that many enteric bacteria, including *Salmonella* and *E. coli*, posses inducible mechanisms for survival in acid. Much of this research has utilised classical acid adaptation survival assays, as first described by Goodson and Rowbury (1989) studying *E. coli*, or by Foster and Hall (1990) using *S. typhimurium*. Such assays involve the adaptation of bacteria to non-lethal levels of acidity before exposure to extremely low pH conditions. The survival of acid adapted bacteria at low pH can be compared to that of bacteria previously adapted to neutral pH conditions. These studies have provided important information, establishing the groundwork for future ATR research. However, variations in the specific assay conditions used has resulted in an abundance of data which cannot be easily compared between laboratories. These data not only includes such details as acid survival kinetics, but also involves the identification of different ATR stimulons, dependant on specific conditions used. Variables can include the type of growth media used; the particular growth phase of the bacteria studied; different adaptive and challenge pH conditions and the use of different strains amongst others.

The discovery of an altered *rpoS* allele in *S. typhimurium* LT2 has highlighted the problems associated with the use of variable strain types to study the ATR of a particular species (Wilmes-Riesenberg *et al*, 1997). As the RpoS sigma factor is a key component of the ATR (Lee *et al*, 1995), it can be expected and, in fact, has been proved that the *S. typhimurium* LT2 strain exhibits a much weaker acid tolerance than more virulent strains such as SL1344 (Wilmes-Riesenberg *et al*, 1996). As many of the early ATR studies of *S. typhimurium* used the LT2 strain (for example, Foster and Hall, 1990; Lee *et al*, 1994), the validity of the results obtained must be considered. The LT2 linked problems encountered while defining the AtbR regulon have already been discussed in Chapter 1 (see section 1.3.1.4.).

In addition to variations between strains, the ATR is also dependent on the presence of particular compounds which may be present in the external environment (see, for example, Foster 1999; Rowbury *et al*, 1996a; Rowbury and Goodson, 1988a). These can include, for example, amino acids, weak organic acids, glucose and sodium chloride. It is thus particularly important to be aware of the consequences of the use of different growth media. For instance, while the majority of Foster's ATR studies involve the use of minimal media, other investigators have used more complex solutions (for example, the use of Luria medium by Valdivia and Falkow, 1996). The specific ATR mechanisms that are induced depend on the growth phase of the bacteria, and it is thus also important to ensure the micro-organisms in use are present at a defined population density.

It is also apparent that there is a lack of information regarding the survival kinetics of ATR induced versus non-induced bacteria in the literature. Much of the research has restricted its investigation of the ATR to one or, at best, a few time points per survival assay. Therefore, the effect of varying the acid adaptation time and/or the time of exposure to extreme acid challenge has not been sufficiently investigated. The variation in adaptation and challenge times is another obstacle that has to be overcome when comparing work from different laboratories. In addition, the specific challenge and adaptive pHs used often differ throughout the literature (see, for example, Foster and Hall, 1991; Park *et al*, 1996; Goodson and Rowbury, 1989; Heyde and Portalier, 1990).

To combat the above complications, the present study aimed to establish a defined set of ATR survival assay conditions that could be used to readily compare the survival kinetics of various *S. typhimurium* and *E. coli* strains. Ultimately, for each strain investigated, specific acid adaptation and challenge times known to induce a full ATR under the defined assay conditions could be applied. This is important as it is vital to ensure that bacteria used to subsequently study specific ATR mechanisms are fully ATR induced.

Defined ATR conditions were ensured by restricting all assays to mid-exponential phase cells in complex LB medium as described in Chapter 2. Furthermore, acid

adaptation and challenge environments were fixed at pH 5.0 and pH 3.0 respectively. Control cultures were adapted to pH 7.2 prior to acid challenge at pH 3.0 as detailed in Chapter 2. Adaptation and challenge times were varied to monitor the survival kinetics of specific species and strain types. These defined assay conditions were also used to monitor survival in organic compared with inorganic acid environments, which previous research has suggested may involve separate, specific tolerance responses (see section 1.3.1.4. Chapter 1). Organic acids were incorporated at either the acid adaptation or challenge phases of the survival assays (see Chapter 2 for details).

3.2. Acid Tolerance Increases Concomitantly with Adaptation Time at pH 5.0 in Inorganic Acid.

The effect of adaptation time on the induction of an inorganic acid tolerance response by *S. typhimurium* SL1344 and EPEC strains MAR001 and E2348/69 was characterised. In each case, mid-log phase cells were adapted for various lengths of time at pH 5.0 (for ATR induced bacteria) or pH 7.2 (control bacteria) under defined assay conditions prior to exposure to an acid challenge of pH 3.0. The *Salmonella* strain was subjected to pH 3.0 conditions for 30 minutes, whereas the less tolerant EPEC strains (see section 3.4) were exposed to pH 3.0 for 15 minutes. All assays were carried out in duplicate and each experiment was repeated at least twice. Figures show single representative experiments.

Figures 3.1 and 3.2 show the effect of adaptation time on the acid tolerance of *S. typhimurium* SL1344 and the two EPEC strains respectively. In each case, for the first 30 minutes, the acid tolerance of each strain increased concomitantly with adaptation time at pH 5.0. If adaptation time was equal to or greater than 30 minutes, the concentration of ATR induced bacteria surviving the pH 3.0 challenge remained relatively constant. It was therefore concluded that a pH 5.0 adaptation time of at least 30 minutes was required to induce a maximal ATR in the *S. typhimurium* and EPEC strains studied under the defined assay conditions. In comparison, all control cultures adapted at pH 7.2 displayed no acid tolerance over the range of time points tested.

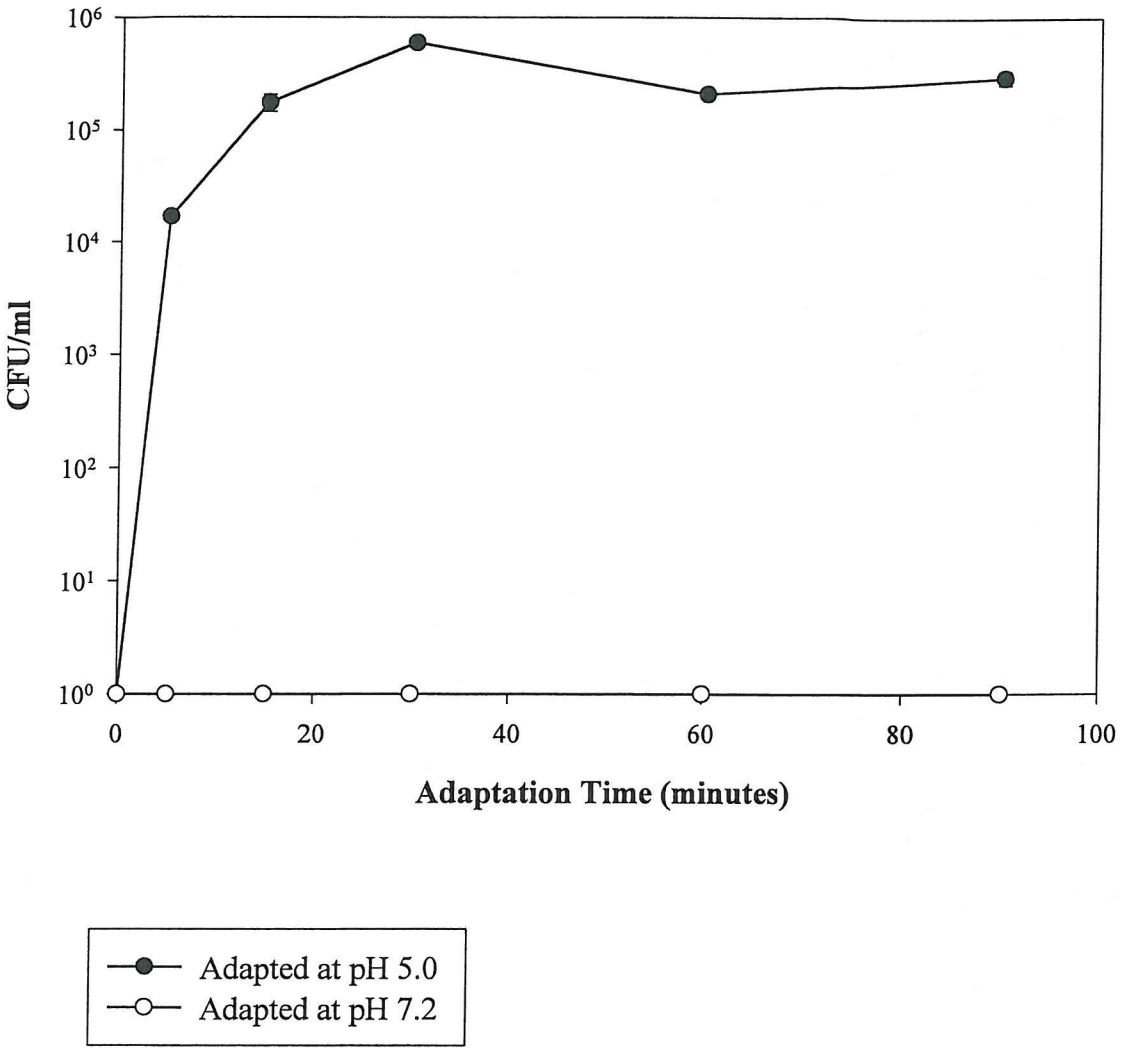


Figure 3.1. The effect of adaptation time at pH 5.0 and pH 7.2 on the induction of inorganic acid tolerance by mid exponential phase *S. typhimurium* SL1344 subsequently exposed to pH 3.0 conditions for 30 minutes in LB medium.

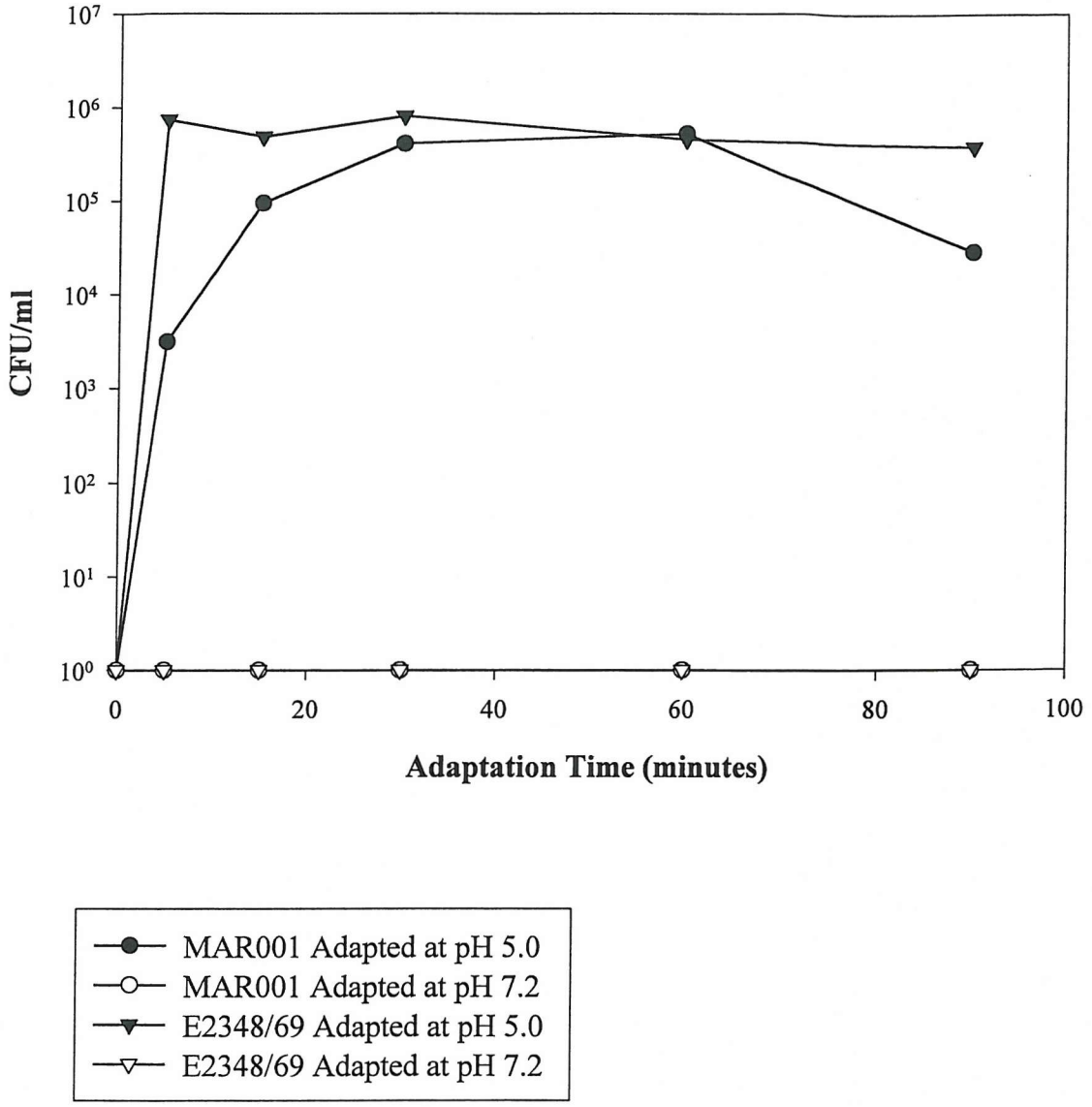


Figure 3.2. The effect of adaptation time at pH 5.0 and pH 7.2 on the induction of inorganic acid tolerance by mid exponential phase EPEC MAR001 and E2348/69 subsequently exposed to pH 3.0 conditions for 15 minutes in LB medium.

3.3. Tolerance to Organic Acid at pH 5.0 is Greater in *E. coli* Than in *S. typhimurium*.

The effect of the presence of 1.4mM benzoic acid (a commonly used food preservative) during adaptation at pH 5.0 on the induction of acid tolerance in *S. typhimurium* SL1344 and *E. coli* MAR001 was characterised. All assays were carried out in duplicate and each experiment was repeated at least twice. Figures show single representative experiments.

Figure 3.3 depicts the kinetics of induction of acid tolerance by *S. typhimurium* SL1344 following adaptation at pH 5.0 in the presence of organic acid for various times. Cells adapted to pH 5.0 with benzoate for 5 or 15 minutes showed a similar degree of survival at very low pH as cells adapted with inorganic acid (Figures 3.1 and 3.3). However, after prolonged adaptation at pH 5.0 with benzoate, subsequent survival at pH 3.0 rapidly declined and was non-existent after an adaptation of $\geq$60 minutes. It is concluded that when adapted in the presence of organic acid, an adaptation time of about 15 minutes is required for the induction of an optimal ATR in *S. typhimurium* SL1344 under the defined assay conditions. This adaptation time is necessarily shorter than that required to establish a maximal ATR in an inorganic environment as the presence of organic acid at low pH is deleterious to the bacterial cells.

Figure 3.4 details the results of similar experiments with *E. coli* MAR001. Cell survival was monitored after exposure to acid challenge at pH 3.0 for 15 minutes. Unlike *S. typhimurium* SL1344, the acid tolerance of the EPEC strain increased concurrently with its time of adaptation in pH 5.0 benzoate at all intervals monitored. It is concluded that the EPEC strain can tolerate acid adaptation with benzoate to a greater degree than *S. typhimurium* SL1344. However, *E. coli* MAR001 requires a longer adaptation time in benzoate to achieve maximal acid tolerance (60 minutes) compared with adaptation using inorganic acid (30 minutes).

For both the *S. typhimurium* and EPEC strains tested, the maximal acid tolerance (measured as cell survival after extreme acid exposure) was not dependent on the acid

type. Moreover, cells adapted at pH 7.2 displayed no acid tolerance over all time intervals tested.

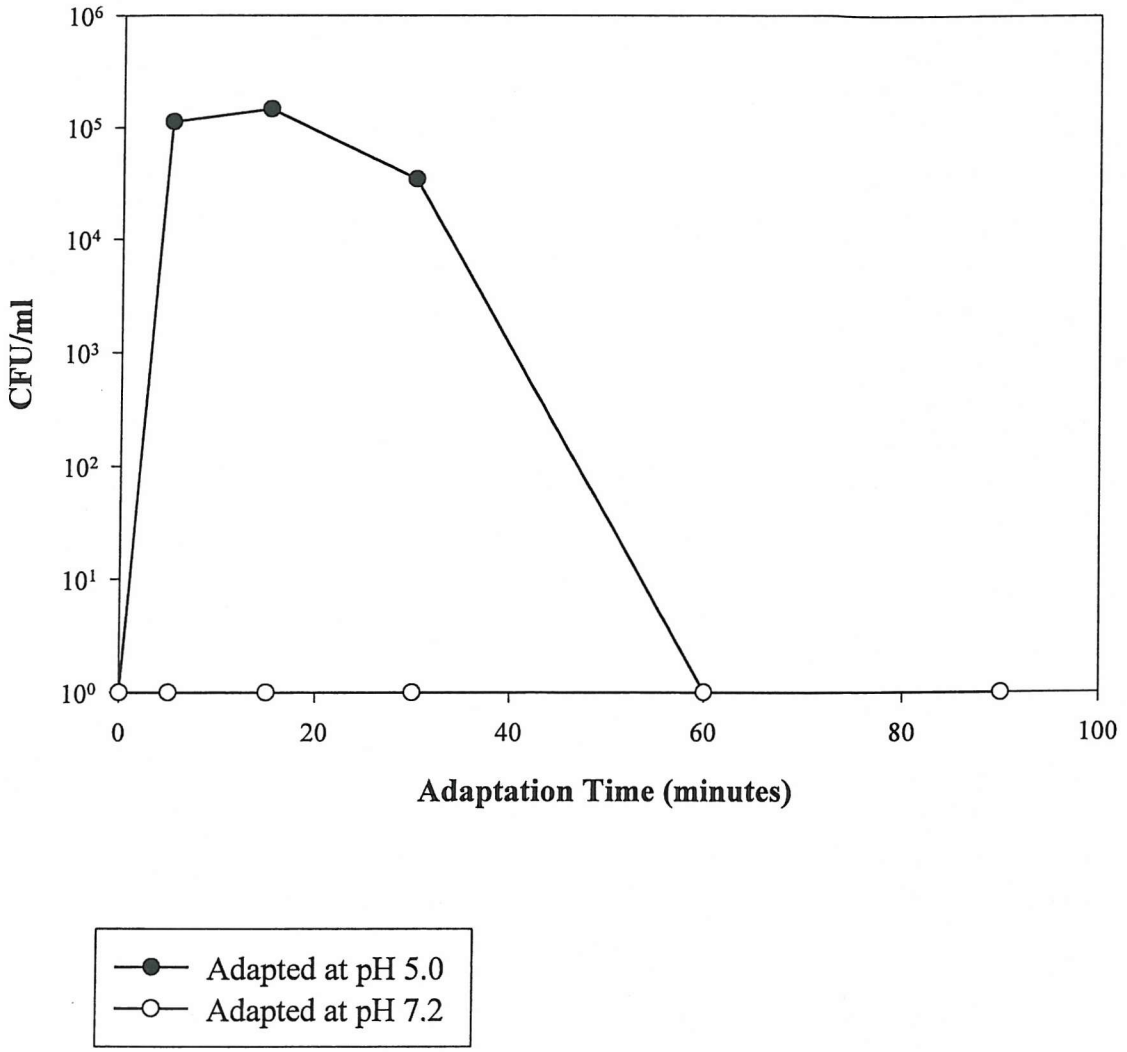


Figure 3.3. *S. typhimurium* SL1344 cells adapted at pH 5.0 (adjusted with benzoic acid, final concentration 1.4 mM) or pH 7.2 for various times prior to extreme acid exposure (HCl) at pH 3.0 for 30 minutes in LB medium.

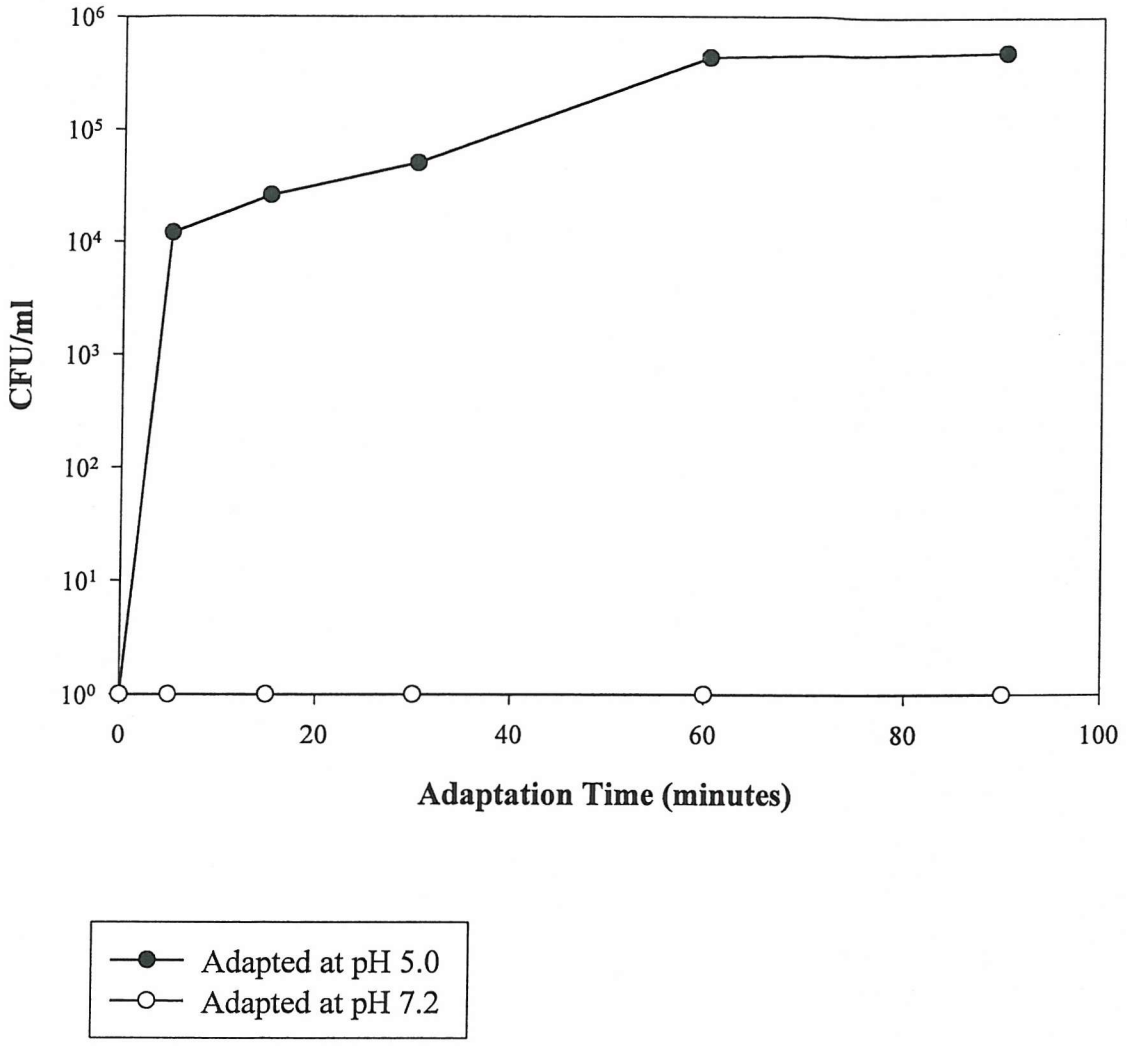


Figure 3.4. *E. coli* MAR001 cells adapted at pH 5.0 (adjusted with benzoic acid, final concentration 1.4 mM) or pH 7.2 for various times prior to extreme acid exposure (HCl) at pH 3.0 for 30 minutes in LB medium.

3.4. *Salmonella* Survival Time at Extreme pH in Inorganic Acid is Greater than that of *E. coli.*

The ability of pH 5.0 or pH 7.2 adapted *S. typhimurium* SL1344 and EPEC strains MAR001 and E2348/69 to tolerate extreme inorganic acid challenge at pH 3.0 for various times was characterised. It was ensured that bacteria were fully adapted by exposing them to inorganic acid at pH 5.0 or to pH 7.2 LB medium for 60 minutes (60 minutes was chosen to increase the confidence that all pH 5.0 incubated bacteria were fully acid adapted) prior to pH 3.0 challenge. As before, mid-exponential phase cells were studied under defined assay conditions and the ATR assays were carried out in duplicate and each experiment was repeated at least twice. Figures show single representative experiments.

As depicted in Figure 3.5, acid adapted *S. typhimurium* SL1344 was capable of tolerating extreme inorganic acid challenge at pH 3.0 for up to 60 minutes. Over the first 60 minutes of exposure to pH 3.0, the number of culturable acid adapted cells remained essentially constant, i.e. growth was stationary. After 60 minutes, however, viability declined steeply and at 90 minutes no culturable cells were present.

In contrast to the *Salmonella* strain, both of the EPEC strains tested could only survive for relatively short periods at pH 3.0, even when their ATR had been optimally induced by previous growth at pH 5.0 for 60 minutes (Figure 3.6). The acid adapted MAR001 strain appeared more acid tolerant than E2348/69 after 15 minutes at pH 3.0, with 100 percent versus one percent of zero minute survival levels remaining, respectively. In both cases, however, viable cells were undetectable after 30 minutes of exposure to pH 3.0. These results suggest that *S. typhimurium* SL1344 has a greater capacity for sustained survival at very low pH than the EPEC strains.

In all cases, uninduced control cells adapted at pH 7.2 for 60 minutes failed to demonstrate an effective tolerance to extreme acid conditions at pH 3.0 after more than 5 minutes of exposure.

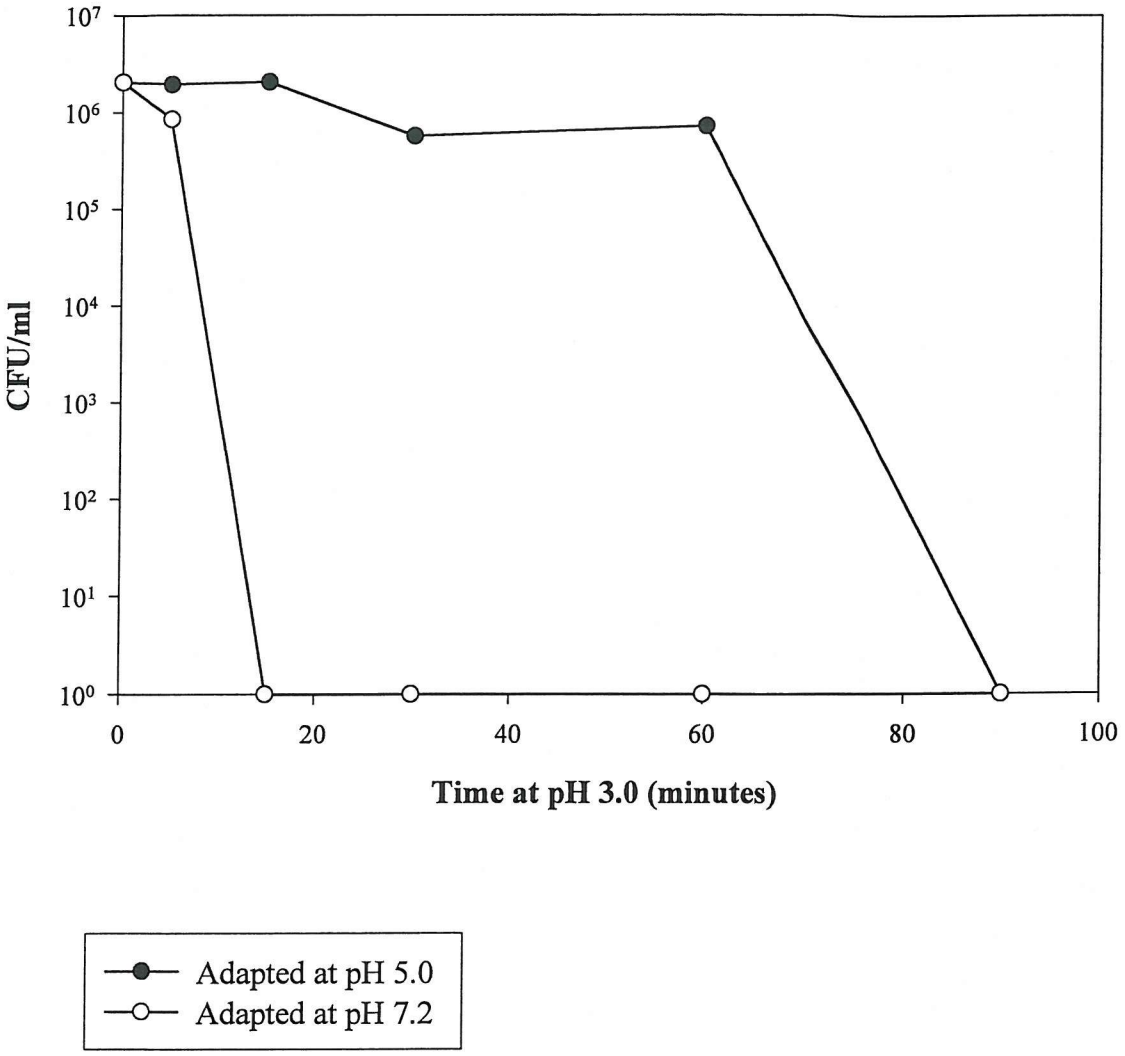


Figure 3.5. *S. typhimurium* SL1344 was adapted to inorganic acid at pH 5.0 or to pH 7.2 for 60 minutes prior to extreme inorganic acid exposure (pH 3.0) for various lengths of time. Surviving culturable cells were counted after plating on LB agar (pH 7.2).

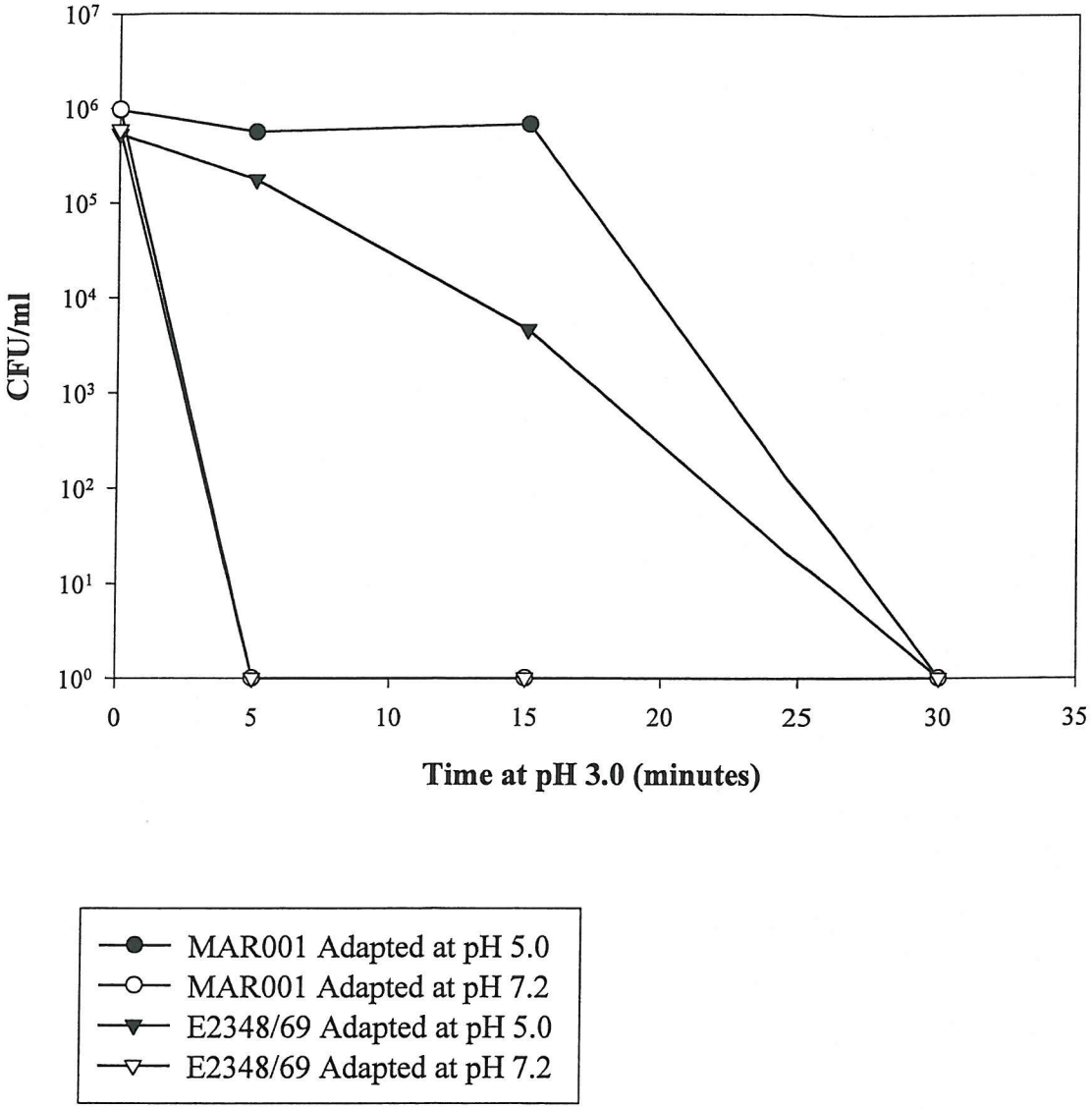


Figure 3.6. EPEC strains MAR001 and E2348/69 were adapted to inorganic acid at pH 5.0 or to pH 7.2 for 60 minutes prior to extreme inorganic acid exposure (pH 3.0) for various lengths of time. Surviving culturable cells were counted after plating on LB agar (pH 7.2).

3.5. The Presence of Organic Acid at Extreme pH Reduces the Survival Time of *Salmonella* and *E. coli.*

The ability of pH 5.0 or pH 7.2 adapted *S. typhimurium* SL1344 and *E. coli* MAR001 to tolerate extreme acid conditions at pH 3.0 in the presence of 0.5mM sodium benzoate over time was characterised. Bacteria were exposed to inorganic acid at pH 5.0 or to pH 7.2 conditions for 60 minutes prior to extreme acid challenge to ensure full adaptation. As before, mid exponential phase cells were studied under the defined assay conditions and experiments were performed in duplicate and repeated at least twice. Figures show single representative experiments.

As detailed in Figure 3.7, acid adapted *S. typhimurium* SL1344 was tolerant of extreme acid at pH 3.0 in the presence of sodium benzoate for up to 15 minutes. Over this time period, the number of viable acid adapted cells remained close to that present prior to pH 3.0 challenge. However, after 30 minutes at pH 3.0, no viable cells were found. The presence of sodium benzoate at pH 3.0 reduced the survival time of the pH 7.2 adapted cells to less than 5 minutes. In conclusion, the addition of 0.5 mM sodium benzoate to LB medium at pH 3.0 caused a four-fold decrease in the survival time of ATR induced SL1344 cells. Therefore, an optimal ATR is induced here by exposing mid log phase cells to pH 5.0 conditions for 60 minutes prior to a pH 3.0 plus organic acid environment for 15 minutes.

Figure 3.8 shows that acid adapted *E. coli* MAR001 is able to tolerate exposure to extreme acid conditions (pH 3.0) in the presence of 0.5 mM sodium benzoate for a relatively short period of time. After a 5 minute exposure time, survival levels were equivalent to those prior to pH 3.0 challenge. However, the cell viability then rapidly decreased, with no survival after 15 minutes of extreme acid exposure. In comparison with adapted cell survival at pH 3.0 in inorganic acid alone, the presence of sodium benzoate has reduced the survival time of the MAR001 strain by two thirds. As before (Figure 3.6.), cells adapted at pH 7.2 survive for less than five minutes at pH 3.0 in the presence of sodium benzoate. In conclusion, when 0.5mM sodium benzoate is present at pH 3.0, an optimal acid tolerance response is induced in mid exponential phase

MAR001 cells if they are acid adapted for 60 minutes prior to extreme acid challenge for 5 minutes.

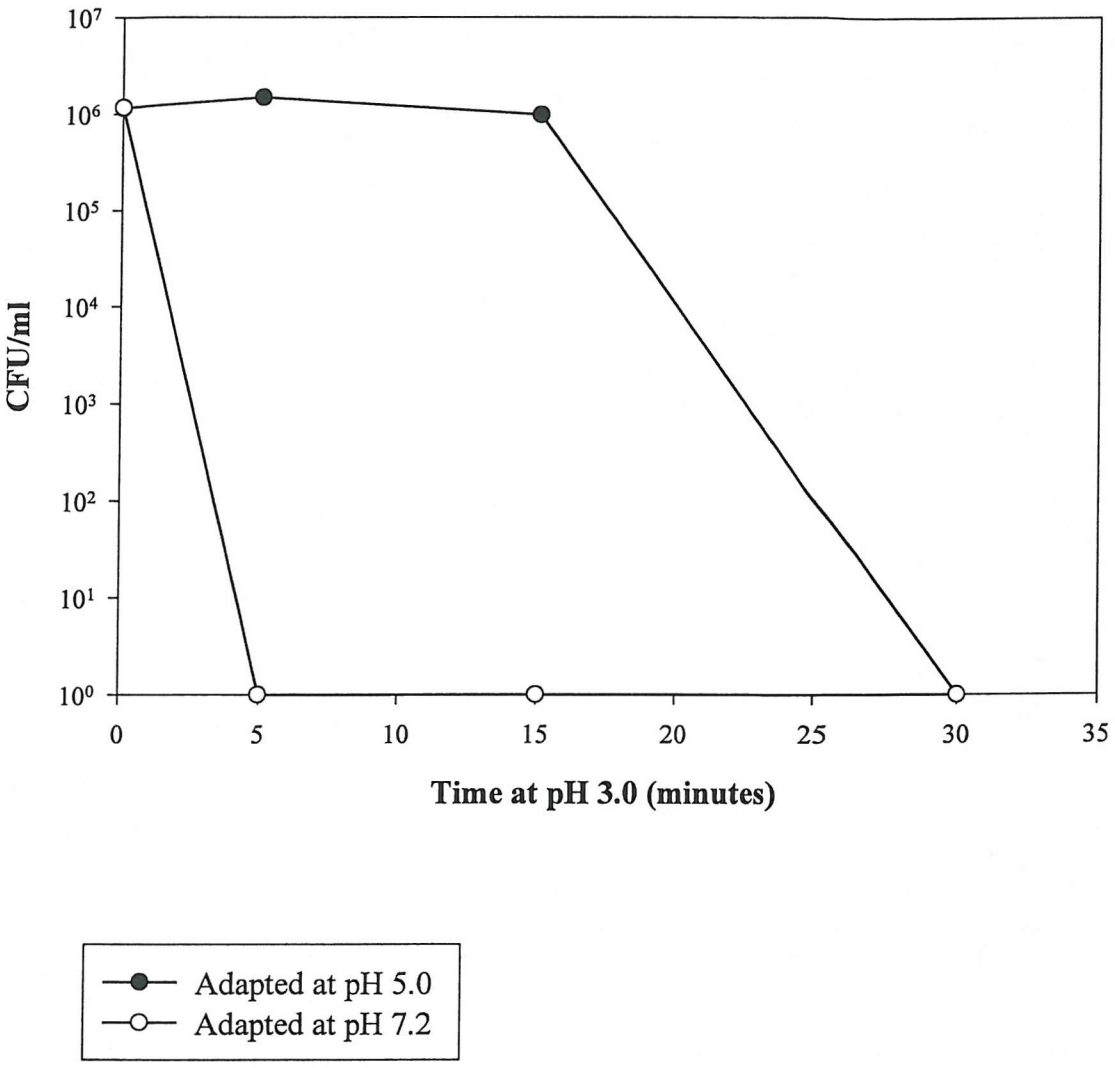


Figure 3.7. *S. typhimurium* SL1344 was adapted to inorganic acid at pH 5.0 or to pH 7.2 conditions for 60 minutes prior to pH 3.0 exposure in the presence of 0.5 mM sodium benzoate for various lengths of time. Surviving culturable cells were counted after plating dilutions onto LB agar (pH 7.2).

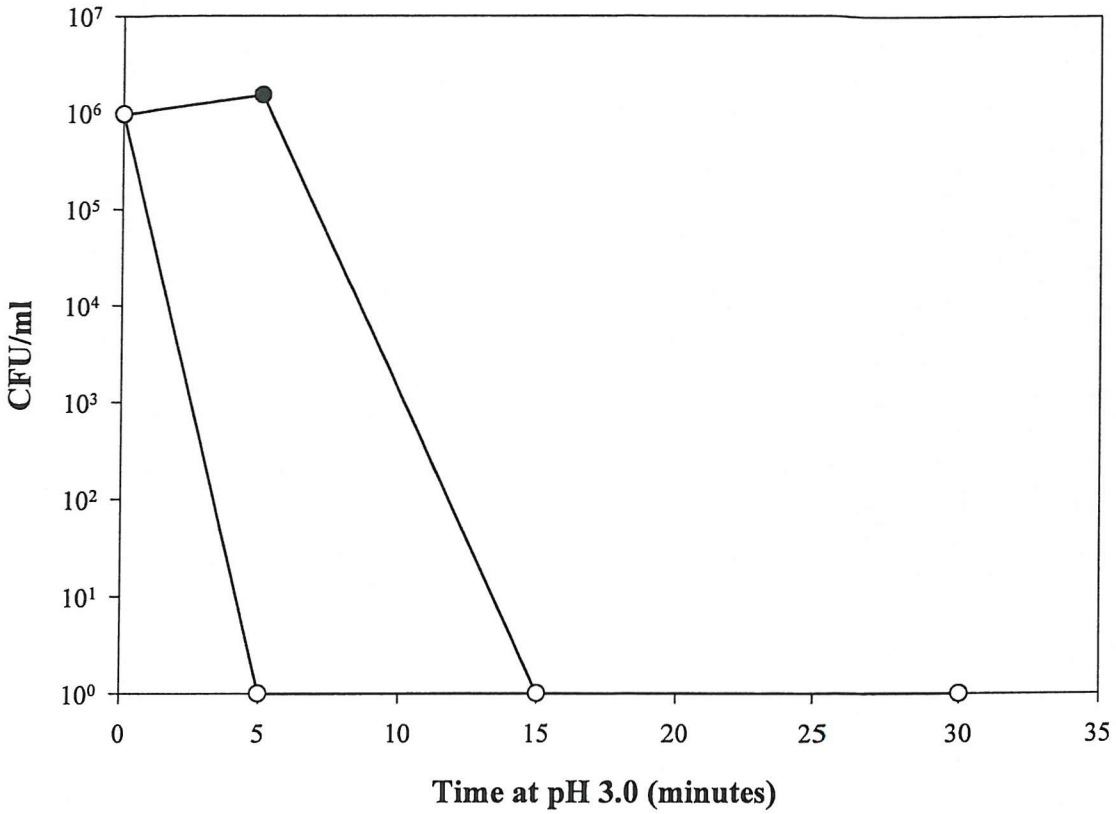


Figure 3.8. *E. coli* MAR001 was adapted to inorganic acid at pH 5.0 or to pH 7.2 conditions for 60 minutes prior to pH 3.0 exposure in the presence of 0.5 mM sodium benzoate for various lengths of time. Surviving culturable cells were counted after plating dilutions onto LB agar (pH 7.2).

3.6. Discussion.

In the past it has been extremely difficult to compare ATR survival kinetics established in different laboratories. This is due to inconsistencies in the assay conditions used. Additionally, much of the research has only identified acid tolerance after single acid adaptation and acid challenge time points. Thus, the ability of the bacteria to survive low pH conditions over time has not been fully investigated.

The studies presented in this chapter have established a protocol using a defined set of assay conditions that enables the ATR survival kinetics over time to be easily compared between bacterial species and strains in identical growth phases. This includes monitoring the effect of acid adaptation time and extreme acid exposure time on bacterial survival. In addition, the influence of organic in comparison with inorganic acid on the ATR has been characterised under these conditions.

After equivalent acid adaptation periods, it is clear that the non-LT2 *S. typhimurium* SL1344 isolate is able to withstand extreme acid exposure for at least 3 times longer than the EPEC strains studied. This is true in both organic and inorganic acid conditions. An explanation may involve the different environmental niches occupied by the bacteria within the host. Perhaps, the intracellular life style of *S. typhimurium* (see chapter 1) requires the bacteria to be able to withstand harsher environments than EPEC, including longer periods of acid exposure. For example, *Salmonella* can reside in the acid pH of the macrophage phagosome for extended periods, whereas *E. coli*, present on the luminal face of the intestinal epithelia, contends with more neutral pH conditions. Unsurprisingly, acid tolerance has been found to increase concomitantly with adaptation time at pH 5.0 in inorganic acid for both *Salmonella* and *E. coli*. However, once a sufficient acid adaptation period has occurred, any further increase does not evoke an even greater ATR. Both bacterial species appear to require at least 30 minutes for full induction of the ATR mechanisms required during acid adaptation. In all the bacterial strains investigated, acid adaptation was clearly required for the induction of a long term ATR. However, *S. typhimurium* SL1344 adapted to pH 7.2 conditions was able to tolerate extreme acid pH for at least 5 minutes without significant loss of viability, whereas viable *E. coli* cells were totally undetectable

under the same conditions. This level of intrinsic acid tolerance again suggests the *Salmonella* strain is more hardy of an acidic environment than the *E. coli* strains studied. Interestingly, these results corroborate a previous study indicating that *S. typhimurium* was more tolerant of a low pH environment in supplemented minimal medium than *E. coli* (Hickey and Hirshfield, 1990). Using radiolabelled weak acids, it was found that unlike *S. typhimurium*, the pH_i of *E. coli* falls below pH 7.0 after long term incubation at pH 5.0. Correspondingly, from equivalent growth rates (expressed as doubling time) at pH 7.0, that of *E. coli* is reduced to 140 minutes after long term incubation at pH 5.0, whereas that of *S. typhimurium* is reduced to 80 minutes. However, as the published research used the non-pathogenic *E. coli* K-12 strain, it is perhaps, unsurprising that this bacterium was less acid tolerant than *Salmonella*. The present research shows that even EPEC strains have a relatively lower tolerance of inorganic acid than *S. typhimurium*.

Unfortunately, it is difficult to compare these results with those of previous studies, unless such research has compared the two species under similar ATR environmental conditions. This allows relative acid survival rates of *E. coli* and *Salmonella* to be compared as above. However, most studies concentrate on a single bacterial species and hence, acid survival kinetics cannot easily be compared due to variations in experimental protocols. Nevertheless, it *is* clear that EHEC such as O157:H7 are much more tolerant of acid stress than EPEC or *S. typhimurium* strains such as those studied here. O157:H7 can survive for days at or below pH 3.0, perhaps related to a high level of intrinsic acid tolerance (see for example Jordan *et al*, 1999a; Brown *et al*, 1997; Cheville *et al*, 1996). This could be associated with ATR processes specific to EHEC, such as an altered *rpoS* gene (Ferreira *et al*, 1999). However, Lin *et al* (1996) have found little variation between the ATR mechanisms employed by EHEC and non-pathogenic lab strains.

Such variations in ATR mechanisms might explain the different levels of acid tolerance observed between *S. typhimurium* and EPEC here. As detailed in Chapter 1, a number of differences in the ATR mechanisms of *E. coli* and *S. typhimurium* exist. For example, components of the *E. coli* ATR such as increased CFA synthesis (Brown *et al*, 1997; Chang and Cronan, 1999), acid shock RNA (Suziedeliene *et al*, 1999),

extracellular induction components (Rowbury and Goodson, 1999a) and the oxidative system of acid tolerance (Castaine-Cornet *et al*, 1999) have, as yet, not been reported in *S. typhimurium*. Similarly, a number of regulatory components of the *Salmonella* ATR have not been described in *E. coli* (Foster, 1999). However, these differences could be a result of variations in the experimental conditions used rather than variations in bacterial metabolism.

Addition of organic acid to the pH 3.0 challenge medium considerably accelerated the loss of viability for both *Salmonella* and *E. coli*. Survival time decreased to, at most, one third of that in inorganic acid alone. The deleterious effect on cell survival produced by organic acid at acid pH is used to advantage in food preservation. For example, weak acids such as benzoate are added to foods to help eradicate bacterial contamination (Brul and Coote, 1999). The majority of acid stress encountered in the environment by bacteria can be accounted for by the combined biological effect of low pH and the presence of organic acids such as food preservatives or fermentation by-products. As described in section 1.3, weak acids in their uncharged, protonated forms can diffuse across the cell membrane and dissociate inside the cell, lowering internal pH in the process. As a consequence of their dissociation constants, the lower the external pH, the more undissociated weak acid will be available to cross the membrane and acidify the cell cytoplasm. In addition to cytoplasmic acidification, the accumulation of weak acid anions is also harmful to the cell.

At less acidic pH values, it follows that organic acids have a weaker effect on the cell. This is indeed the case for *E. coli*, which tolerated benzoate at pH 5.0 for at least 90 minutes: the presence of organic acid at pH 3.0 reduced survival to zero within 15 minutes. In contrast, *S. typhimurium* is severely compromised by the presence of organic acid at pH 5.0. If adapted for longer than 30 minutes in these conditions, no survival is detected after a pH 3.0 challenge. These surprising inter species differences may perhaps be explained by the specific environmental niches occupied by the bacteria within the host. *E. coli* on the luminal side of epithelial cells might require a greater long-term tolerance of the organic acid fermentation by-products present in the gut at slightly acidic pH than *S. typhimurium*. Indeed, the stress-related proteins, UspA

and H-NS are induced in *E. coli* by 20mM benzoate at pH 6.5 (Lambert *et al*, 1997). This induction has yet to be reported in *S. typhimurium*.

In defining their ATR survival kinetics, this study has characterised specific assay conditions for optimal induction of an ATR in each bacterial strain studied. This knowledge is essential for further elucidation of ATR mechanisms as presented in later chapters. For example, proteomic studies (as described in Chapters 4 and 5) to distinguish the ATR stimulon require the preparation of whole cell lysates from cells that are under maximal ATR inducing conditions. The ability to compare the induction of an optimal acid tolerance between strains is also necessary for the clarification of the effect of specific components on the ATR. For example, an ATR regulated gene can be removed from a particular strain by mutation. Subsequently, ATR survival assays can be used to compare mutant and wild type strains and monitor the effect of the missing component. Such studies are described in Chapters 5 and 7.

CHAPTER 4.

THE USE OF PROTEOMICS TO IDENTIFY COMPONENTS OF THE ATR STIMULON IN *S. typhimurium* SL1344.

CHAPTER 4. THE USE OF PROTEOMICS TO IDENTIFY COMPONENTS OF THE ATR STIMULON IN *S. typhimurium* SL1344.

4.1. Introduction.

As detailed in Chapter 1, a number of ATR components and their associated mechanisms of action have been identified. This includes the grouping of certain elements of the ATR stimulon into separate regulons, such as those controlled by RpoS and PhoPQ. However, it is clear that the majority of proteins that are differentially expressed during this bacterial stress response remain unknown. For example, only a small proportion of the 52 *S. typhimurium* proteins whose expression profile is modified at pH 3.3 in minimal media (Foster, 1991) have been identified. Additionally, although 2D PAGE studies have enabled the numbers of proteins controlled by specific regulatory molecules to be estimated, for example, at least 8 ASPs by Fur (Foster and Hall 1992), few of their identities are known. There have been a number of problems associated with the identification of the ATR stimulon. First, as described in Chapter 3, complications arise from the use of a variety of different ATR inducing conditions. Minor differences in experimental protocols can have major effects on the protein expression profiles observed on 2D PAGE gels. Secondly, the majority of studies have used genetic techniques such as transposon mutagenesis which have inherent limitations. For instance, the screening of collections of random transposon insertions for altered levels of acid tolerance suggested that cells deficient in pyruvate dehydrogenase were constitutively tolerant of low pH (Foster and Hall, 1991). However, it is unknown if this enzyme is actually repressed during the ATR, or whether the effect is just an artefact of the enforced disruption of the pyruvate dehydrogenase gene. Transposon mutagenesis also tends to uncover only a subset of the genes of interest and will not uncover any genes that are also essential for viability. Such procedures also tend to uncover the same mutants repeatedly and the use of reporter gene fusions can only detect large changes in gene expression.

The research described here circumvents some of these problems with the use of sensitive proteomic techniques to define components of the ATR stimulon of *S.*

typhimurium SL1344 under the optimal inducing conditions described in Chapter 3. The term, "proteome" describes the complete set of expressed proteins specified by the genome of a cell or tissue type (Wilkins, 1996ab). Current proteomic technology precludes the ability to define the entire set of proteins expressed in a cell at any one time. The most frequently used and more manageable procedure identifies sets of proteins that are differentially expressed under conditions of interest to researchers. Here, the proteomic approach uses two-dimensional gel electrophoresis in combination with sensitive protein identification techniques to identify proteins implicated in the ATR. The identification of such stimulons and associated regulons is one area where proteomics is at its most powerful. Two dimensional gels can readily separate hundreds of proteins on the basis of isoelectric point and molecular weight fractionation and can display the expression profiles of entire stimulons in single experiments (Görg *et al*, 2000). When combined with genetic techniques such as the production of mutant strains where a specific global regulatory protein is missing or constitutively active, proteomics can define the regulons within these stimulons. In this way, proteomics has the potential to reveal unexpected links between different regulatory circuits that might otherwise escape detection (O'Connor *et al*, 2000). Genome sequencing projects have suggested that as many as 40 percent of the genes in a particular organism may be novel. Again, when combined with these genetic approaches, proteomics can establish if such genes are really expressed, and if so, under which conditions (Hinton, 1997). Additional applications of proteome studies include the detection of post-translational modifications; the validation of genome sequences; the identification of immunogenic proteins for vaccine studies; the high-resolution separation of multiple proteins for purification; and the investigation of mechanisms of action of antimicrobial agents and identification of novel antimicrobial targets. It is clear that proteomics is of great use to pharmaceutical researchers and can make a major contribution towards a comprehensive description of the physiology of a microbial cell (O'Connor *et al*, 1998).

A second class of technique that has been used to study changes in gene expression in bacteria is transcriptomics. These procedures use methods such as DNA chip technology to investigate gene expression at the level of transcription (Hautefort and Hinton, 2000). Like proteomics, these procedures are reliant on genome sequencing

projects. DNA oligonucleotides or PCR products corresponding to every predicted open reading frame of a particular genome can be constructed on, for example, glass microscope slides. Hybridisation of labelled mRNA to this so-called microarray allows the relative level of expression of each gene from a bacteria to be measured. This method is particularly sensitive when compared with proteomic techniques. It can also measure gene expression within a single bacterial cell. Gene expression resulting only in RNA products can also be identified. However, proteomics is at an advantage in that it can identify expression at a level that takes into account any post-transcriptional and post-translational modifications. It can also identify novel proteins which previously existed only as hypothetical unknowns identified through genome sequencing. Proteomics is however restricted by the limited amount of proteins which can be visualised on 2D gels. Additionally, unlike micro-array technology, protein expression in individual cells cannot be measured because of the quantities of protein currently required for 2D gel analysis.

Microbial proteomes are relatively small (typically containing less than 4000 proteins) resulting in an increased resolution of proteins on 2D gels compared with more complex, eukaryotic organisms. *S. typhimurium* in particular presents a number of advantages for proteomic ATR studies. Firstly, as mentioned above, the small genome size makes protein identification relatively simple. Secondly, due to its close evolutionary relationship with *E. coli, Salmonella* proteins with homologues in this organism usually have similar sequences and hence can be reliably assigned. Thirdly, post-translational modifications which may hinder protein identification by *N*-terminal sequencing are relatively rare. Finally, the wealth of genetic procedures available for use with this bacteria, and the relative ease of construction of mutant strains lacking or over expressing particular regulatory components, facilitates the identification and further characterisation of proteins (O'Connor *et al*, 1997).

The proteomic experiments used in these studies can be divided into four stages. It is appropriate to introduce each, in turn, here.

The first stage involves sample preparation. A whole cell lysate is prepared from unstressed, wild type cells. In parallel, a second lysate from wild type cells under

inducing conditions (for example, acid stress), or from mutant cells, is produced. To ensure optimal protein solubilisation and disaggregation, detergents and reducing agents are included in the lysis buffer. Protease inhibitors are also included to avoid proteolysis during sample preparation. Care must also be taken to ensure the cells used are not subjected to any undesired stimuli that could alter the protein expression profile.

Once the whole cell lysates are prepared, their protein expression profiles are visualised using 2D PAGE (O'Farrell, 1975). Modern IPG technology has increased the reproducibility and resolving power of this procedure (Görg *et al*, 2000). It also enables relatively large amounts of protein to be loaded onto the gels, thus facilitating subsequent spot identification. IPG gels are prepared from a set of acrylamide derivatives with different pK values. An extremely stable pH gradient is produced as the buffering groups are covalently linked to the polyacrylamide gel matrix (Görg *et al*, 2000). Once proteins have been separated by isoelectric point on IPG strips, they are fractionated by size on SDS PAGE gels.

The third stage of the proteomic procedure involves spot detection and subsequent comparison of the protein expression profiles of each whole cell lysate. This is carried out using gel analysis software packages which can detect spots, measure them and match them between 2D gels. Ultimately, the percentage change in protein expression for every spot on a gel can be calculated.

Finally, the identification of protein spots of interest is carried out. Typically, this involves protein microsequencing or peptide mass fingerprinting using mass spectrometry. For microsequencing, proteins are transferred from 2D gels onto PVDF membranes prior to excision and subsequent *N*-terminal sequence analysis by Edman degradation (Bauw *et al*, 1989). Alternatively, protein spots are cut directly from 2D gels and digested *in-situ* with trypsin. The resulting peptide fragments are eluted from the gel onto micro-columns containing reverse phase resin. The peptides are subsequently washed, desalted and concentrated prior to nanoelectrospray or matrix assisted laser desorption/ionisation time of flight (MALDI-TOF) mass spectrometry (Wilm *et al*, 1996; Henzel *et al*, 1993). The peptide masses are then calculated and

used to search for matching values in a database containing a theoretical digest of known protein sequences . To enhance confidence, the predicted isoelectric point (pI) and molecular weight (M_r) of each identified protein can also be compared with the corresponding spot position on the original 2D gel. The identification of differentially expressed ATR proteins from 2D gels by tryptic mass fingerprinting is summarised in Figure 4.1.

The research described in this chapter uses proteomics to identify and construct a 2D PAGE reference map of the proteins which are differentially regulated during the *S. typhimurium* ATR. To help elucidate the ATR mechanisms employed, this reference map was compared with that produced using isogenic mutant strains that lack or constitutively express known ATR regulators. In this way, proteins can be assigned to specific regulons. As described in Chapter 1, many of the bacterial stress responses have overlapping regulatory mechanisms. Therefore, the ATR reference map was also compared with those specific for other stress responses to identify co-regulated proteins.

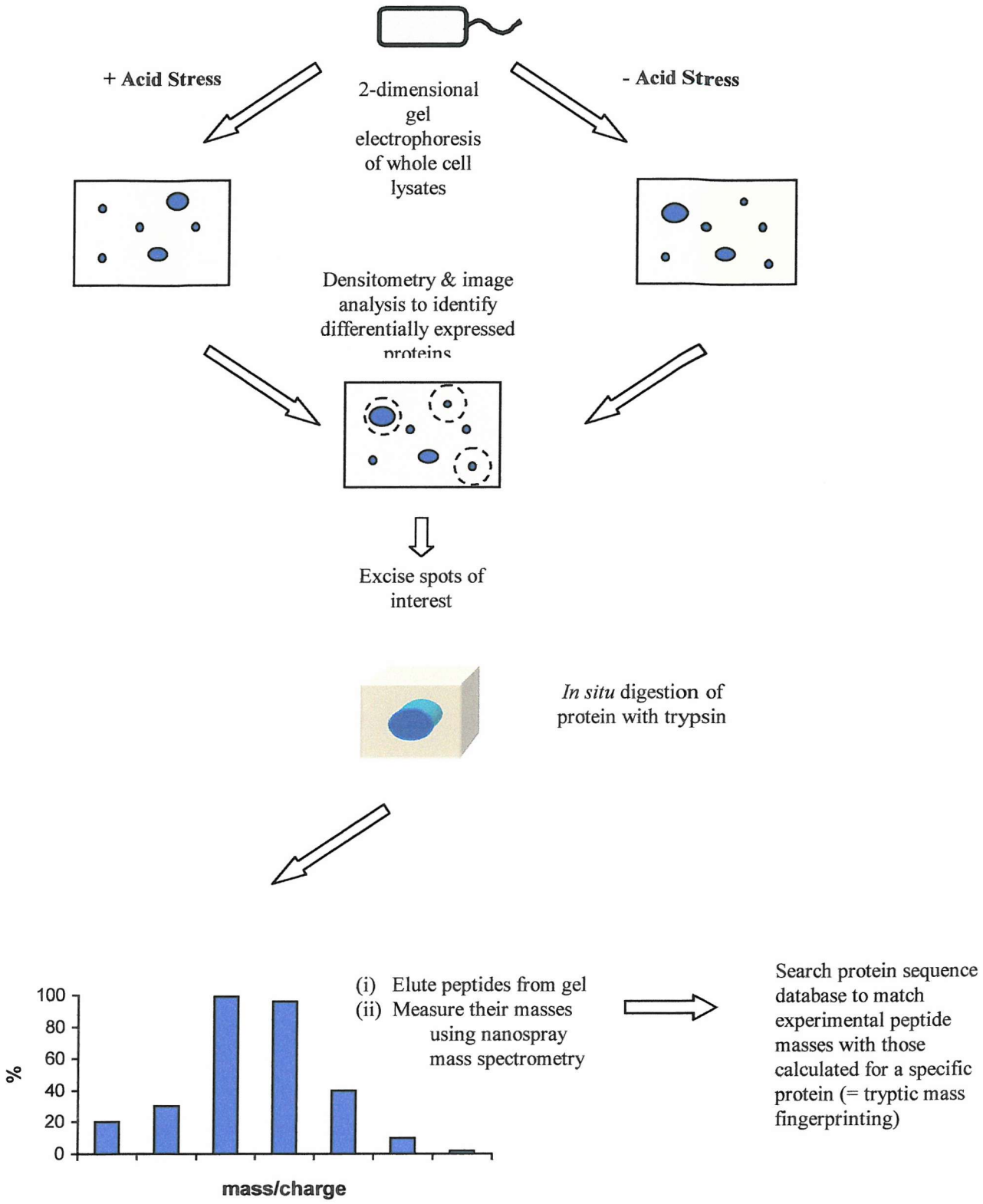


Figure 4.1. The proteomic approach: a summary. The identification of ATR regulated proteins via 2D PAGE of whole cell lysates and subsequent tryptic mass fingerprinting.

4.2. Analysis of Whole Cell Lysates using a pH 4-7 IPG Gradient Shows at Least 26 Proteins are Altered During the log-phase ATR in *S. typhimurium* SL1344.

Using whole cell lysates and a first dimension pH gradient of pH 4-7, the exponential phase ATR stimulon of *S. typhimurium* SL1344 under optimal inducing conditions was studied. Following image analysis of gels obtained from at least 4 separate batches of cells, it was concluded that at least 26 protein spots on 2D gels were reproducibly altered during the ATR. Figure 4.2 depicts a typical 2D gel profile obtained from an exponential phase *Salmonella* SL1344 whole cell lysate. Protein spots with an increased expression level during the ATR are boxed in red and those that are repressed are circled in yellow. It is clear that the ATR stimulon involves proteins throughout the pI and M_r range analysed. Also of note is that ATR repressed proteins (21 in total) far outnumber those that are up regulated (5 in total).

4.3. Identification of 16 Proteins of the ATR Stimulon of *S. typhimurium* SL1344.

Several proteins of the ATR stimulon of *S. typhimurium* SL1344 were identified using a combination of tryptic mass fingerprinting and *N*-terminal sequencing. The 2D gel profiles of those proteins successfully identified are depicted in Figure 4.3. The identities of the proteins are displayed in Table 4.1. Where tryptic mass fingerprinting was used, the extent to which the retrieved peptides covered the whole protein is listed (percent protein coverage). In each case the value is at least 25 percent. In addition, there was close agreement between the experimentally observed and predicted protein pIs and M_rs. *N*-terminal sequences obtained are shown in Table 4.2. The remaining 10 of the 26 proteins shown here to be differentially expressed during the ATR of SL1344 could not be identified using the above procedures. A subset of these unidentified proteins provided peptide masses or *N*-terminal sequence data that did not match any known proteins in databases. It is therefore possible that these proteins represent the products of genes that have yet to be sequenced. The peptide mass or N-terminal sequence data provided is thus included in Appendix A for future reference.

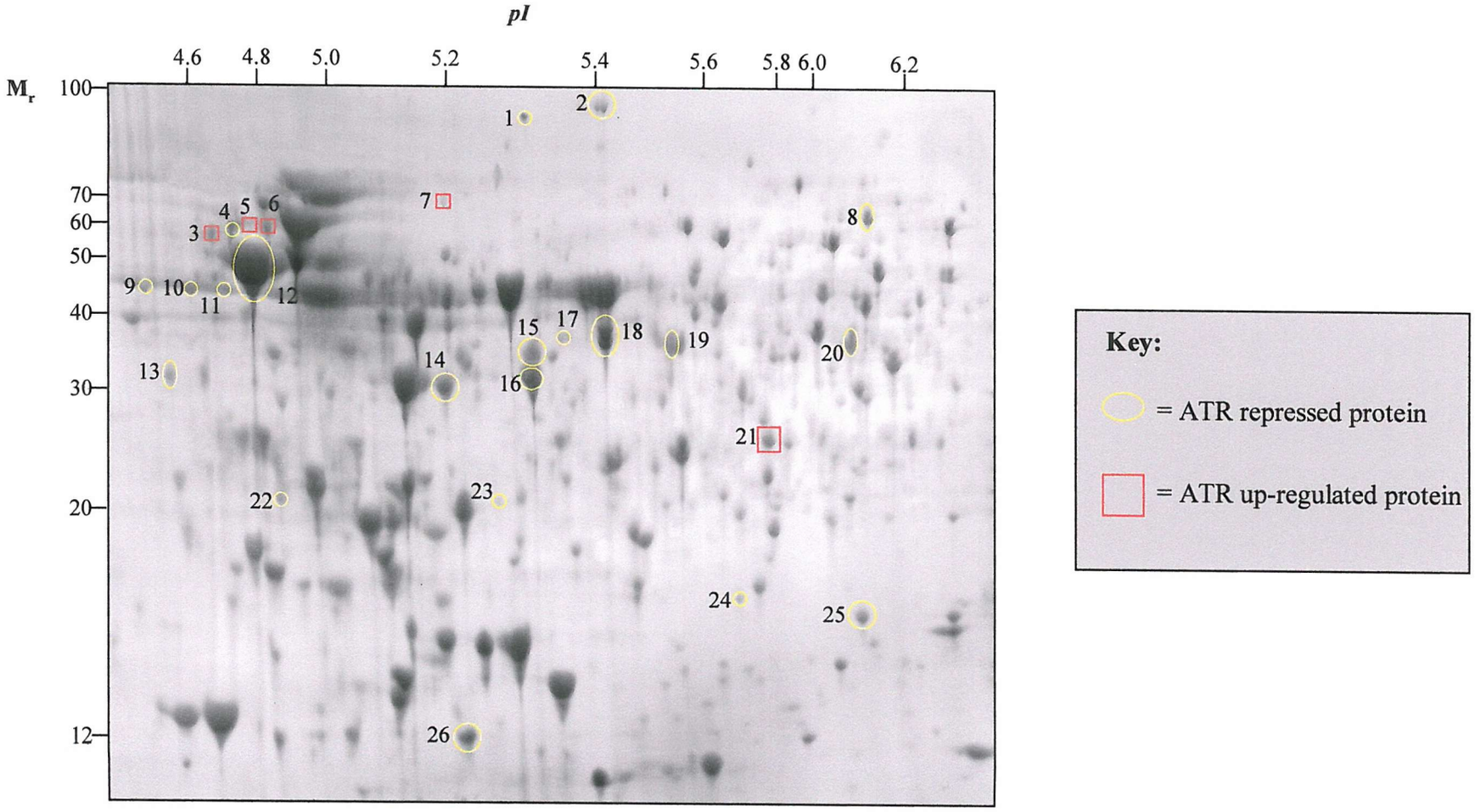


Figure 4.2. A proteome analysis of differentially expressed proteins of the *S. typhimurium* SL1344 ATR stimulon. Whole cell lysates were fractionated in the first dimension using an IPG of pH 4-7 and in the second using an SDS-polyacrylamide gradient gel (12-14%). Proteins were visualised by staining in Coomassie Brilliant Blue. The pH and M_r (in Kda) scales are shown on the top- and left-hand sides of the gel respectively. In this gel, a whole cell lysate of SL1344 was run and the locations of proteins that are reproducibly up- or down-regulated during the ATR are numbered. Figure 4.3 shows "zoomed in" details together with the corresponding ATR sample.

Figure 4.3. (next page). Enlarged portions of Coomassie stained 2D gels depicted in pairs with corresponding pI and M_r ranges. In each case, the left hand panel corresponds to whole cell lysates from mid exponential phase *S. typhimurium* SL1344 at pH 7.2, whereas the right hand panel shows samples from whole cell lysates from mid exponential phase *S. typhimurium* SL1344 undergoing an optimal ATR at pH 3.0. Protein spot numbers coincide with those listed in Table 4.1 and Figure 4.2. Only protein spots with reproducible changes in expression during the ATR are marked.

pI

5.3 5.4

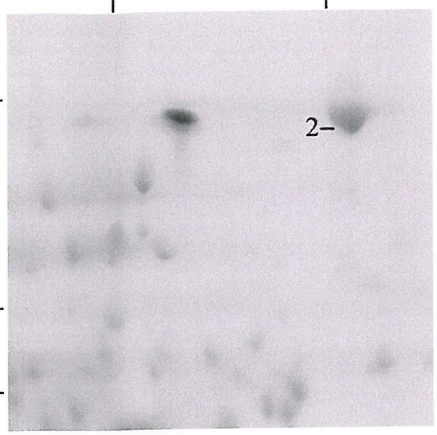
1.a. M_r

100 –

70 –

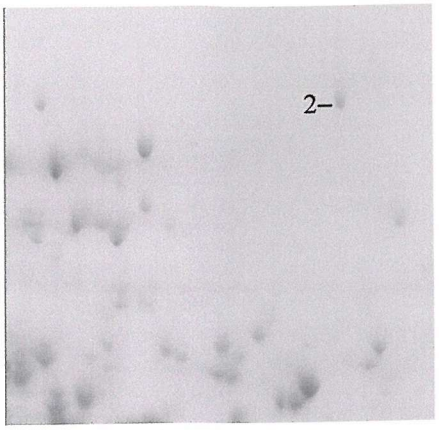
60 –

2–



1.b.

2–



pI

4.7 4.8 4.9

2.a. M_r

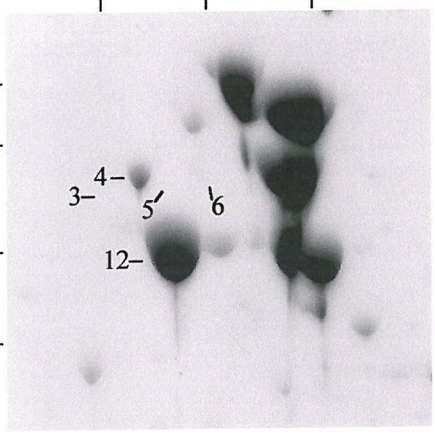
70 –

60 –

50 –

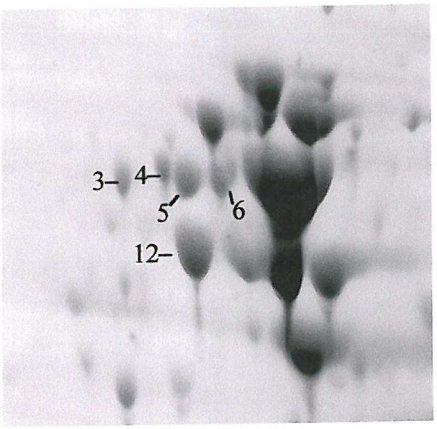
40 –

3– 4– 5 6 12–



2.b.

3– 4– 5 6 12–



pI

6.0 6.1 6.2

3.a. M_r

60 –

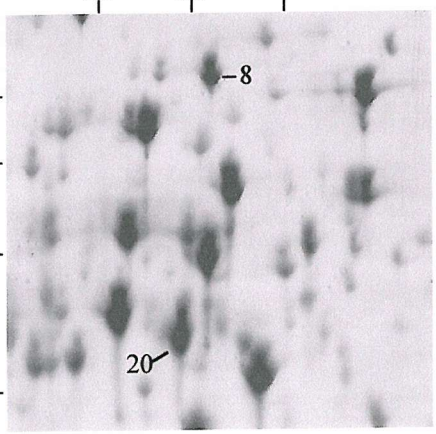
50 –

40 –

33 –

8

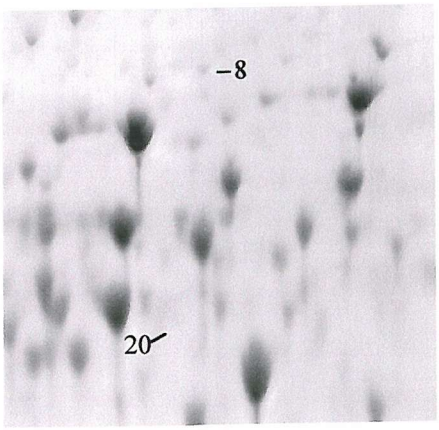
20



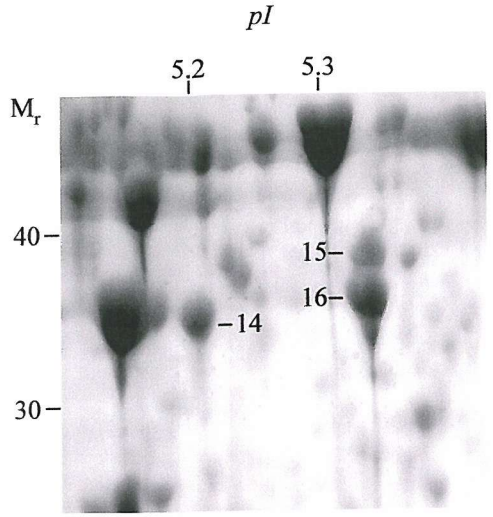
3.b.

8

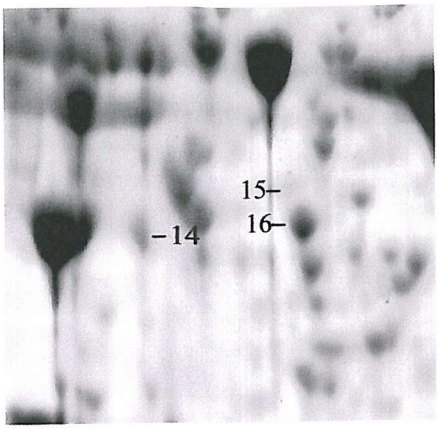
20



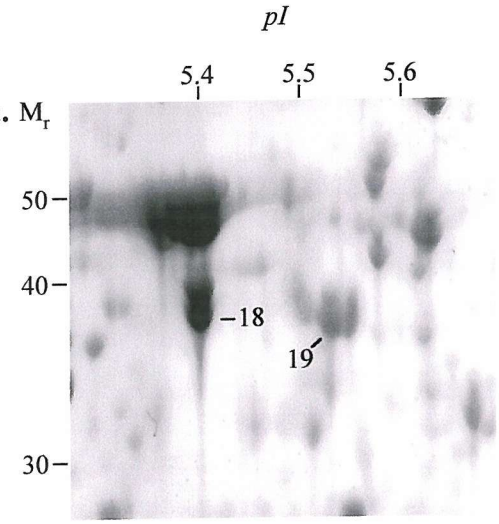
4.a.



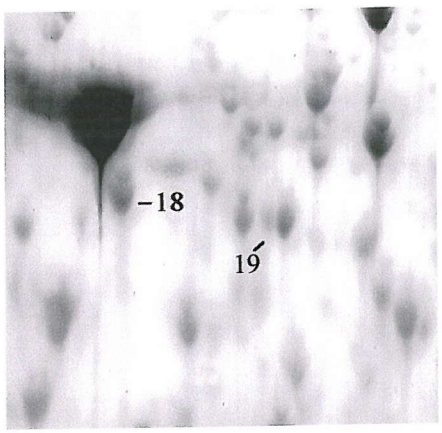
4.b.



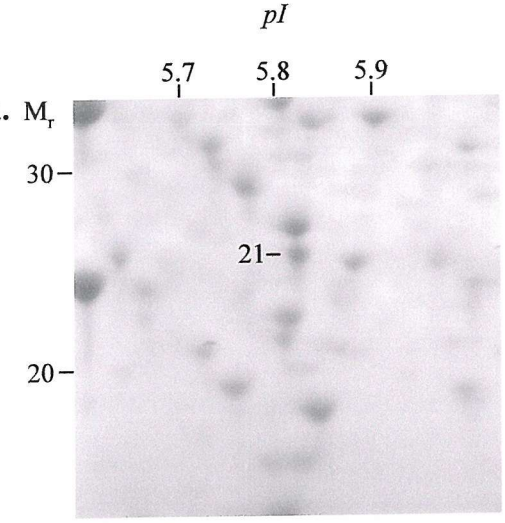
5.a.



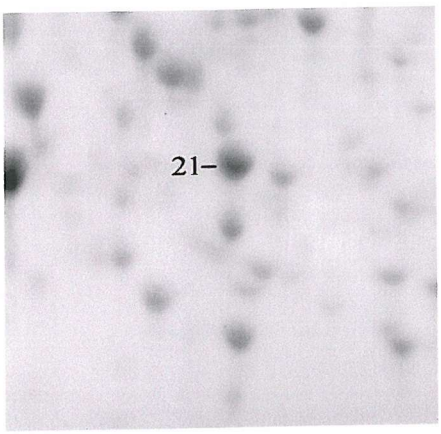
5.b.



6.a.

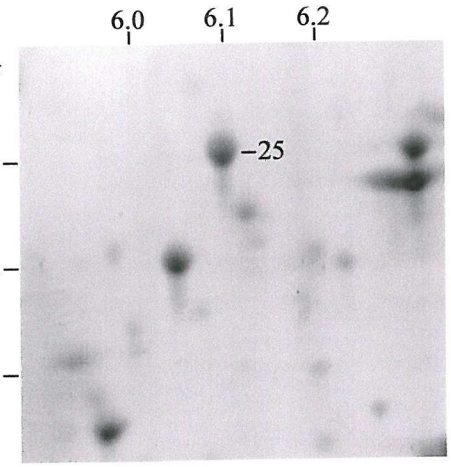


6.b.

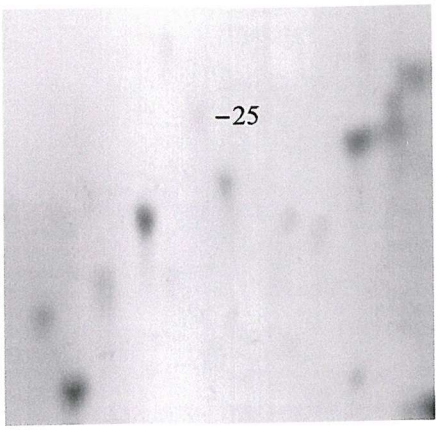


pI

7.a. M_r

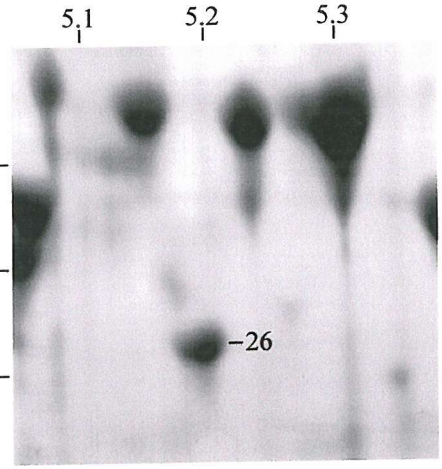


7.b.



pI

8.a. M_r



8.b.

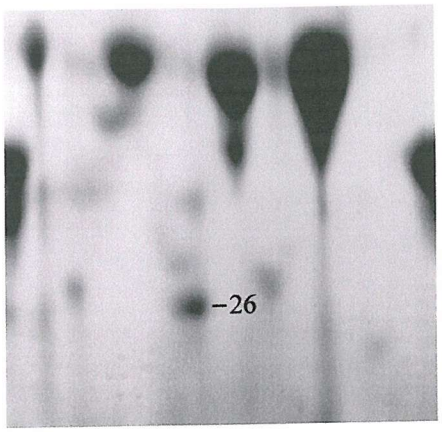


Table 4.1. (next page). Identification of proteins that are differentially expressed during the exponential phase ATR of *S. typhimurium* SL1344. Tryptic mass fingerprinting or *N-terminal* sequencing was used to identify proteins from 2D gels using the MS-FIT software in the Protein prospector suite of programmes at http://prospector.ucsf.edu/ucsfhtml3.2/msfit.htm. Protein spot numbers correspond to those in Figures 4.2, 4.3, 4.5 and 4.7. If tryptic mass fingerprinting was used, the extent to which the retrieved peptides covered the whole identified protein is given as percent protein coverage. The experimentally observed M_r and pI for each protein is compared with that predicted by the Expasy "Search pI/M_r" tool at http://www.expasy.ch/tools/pi_tool.html. The SwissProt protein database accession number is given for each protein.

a. The *N*-terminal sequences are shown in Table 4.2.

Spot Number	Protein Coverage (%)	Molecular Mass (kDa)		Isoelectric Point		Top Ranking Protein in MS-FIT Search	SwissProt Accession Number
		Experimental	Predicted	Experimental	Predicted		
2	(N-term)[a]	99.00	99.67	5.41	5.46	Pyruvate dehydrogenase E1, AceE	P06958
3	37	57.00	57.29	4.72	4.85	N-terminally truncated GroEL	P48217
4	44	58.00	52.40	4.75	4.75	Flagellin phase 2, FljB	P52616
5	47	57.00	57.29	4.78	4.85	N-terminally truncated GroEL	P48217
6	37	57.00	57.29	4.80	4.85	N-terminally truncated GroEL	P48217
8	35	60.10	60.11	6.12	5.88	Fumarase B, FumB	P14407
12	37	49.00	51.48	4.79	4.79	Flagellin phase 1, FliC	P06179
14	(N-term)	35.00	35.75	5.20	5.69	D-galactose binding protein, MglB	P23905
15	32 (+N-term)	39.00	35.24	5.34	5.76	Acetyl-CoA carboxylase carboxyl transferase s.u. α, AccA	P30867
16	(N-term)	37.00	29.70	5.34	5.19	YidA	P09997
18	(N-term)	39.00	40.84	5.38	5.38	Glycerophosphodiester phosphodiesterase, GlpQ	P09394
19	25	37.00	36.85	5.55	5.96	L-Asparaginase, AnsA	P18840
20	29	35.20	35.23	6.10	5.75	Threonine dehydratase (catabolic), TdcB	P05792
21	(N-term)	26.00	28.56	5.84	5.85	Phosphoglycerate mutase 1, GPM	P31217
25	78	16.00	16.02	6.10	5.60	YnaF	P37903
26	27	12.30	14.28	5.19	5.10	YfiD	P33633

Table 4.2. *N*-terminal sequence data obtained, compared with the corresponding *N*-terminal sequences from the matched proteins. Mismatched amino acids are highlighted in bold.

Spot Number	Protein	*N*-terminal sequence	
		Experimental	Actual[a]
2	Pyruvate dehydrogenase E1 (AceE)	SERF**Q**NDVDPIE	SERF**P**NDVDPIE
14	D-galactose binding protein (MglB)	ADIRIGVAIY**??**DDN	ADTRIGVTIY**K**YDDN
15	Acetyl-CoA carb-oxylase carboxyl transferase subunit α (AccA)	**R**LNFLDFEQPIAE	**S**LNFLDFEQPIAE
16	YidA	MNRTILVPI	MNRTILVPI
18	Glycerophospho-diester phospho-diesterase (GlpQ)	AEK**V**VIAHRGASGY	NEK**I**VIAHRGASGY
21	Phosphoglycerate mutase 1. (GPM)	AVTKLVLVRH	AVTKLVLVRH

a. Experimental *N*-terminal sequences were matched to those listed in the SwissProt database using the Protein Prospector MS-Edman database searching tool at http://prospector.ucsf.edu/ucsfhtml3.2/msedman.htm.

Figure 4.4 depicts the expression levels of the identified proteins during an optimal ATR as a percentage of that identified in unstressed cells. In conclusion it can be seen that the following proteins are all repressed by at least 50 percent during the ATR: pyruvate dehydrogenase E1 subunit (spot number 2), flagellin phase 2 (#4), fumarase B (#8), flagellin phase 1 (#12), D-galactose binding protein (#14), acetyl-CoA carboxylase carboxyl transferase subunit α (#15), YidA (#16), glycerophosphodiester phosphodiesterase (#18), L-asparaginase (#19), catabolic threonine dehydratase (#20), YnaF (#25) and YfiD (#26). In contrast, phosphoglycerate mutase 1 (#21) is up-regulated as part of the ATR. Protein spots 3, 5, and 6 are not shown in Figure 4.4. as they constitute *N*-terminally truncated forms of GroEL which only appear during the ATR. They are not, therefore, differentially expressed *per-se*, but appear to be substrates for a protease that is up-regulated or activated in response to acid stress. The down regulation of flagellin phases 1 and 2 is covered in detail in Chapter 5 and therefore will not be discussed further here.

Figure 4.4. (next page). The expression levels of identified proteins that are induced or repressed during the mid exponential phase ATR of *S. typhimurium* SL1344. Expression level is depicted as a percentage of that in unstressed wild type cells, calculated using the Phoretix 2D Software v.5.1. Protein expression levels were deduced by obtaining densitometry readings of respective spots from 2D-gels. The whole cell lysates run on the gels were from exponential phase bacteria that had either been incubated at pH 7.2 or in which an optimal ATR had been induced. Any background staining of the gel surrounding the protein spots was subtracted from the spot volumes, calculated using the Phoretix software. Spots representing the same protein in both environmental conditions were then matched between the gels. Respective spot volumes were then compared and those obtained from ATR induced samples were expressed as a percentage of that seen in samples from wild type, unstressed cells. Protein spots that changed in volume by ≥ 2 fold when compared between the two conditions were judged to have an altered expression profile if this was repeatable across four independent sample batches. The volumes of control spots (those that consistently showed no change in volume between environmental conditions in four independent sample batches) were compared between ATR induced and pH 7.2 samples. The spot volumes of proteins judged to display altered expression were then adjusted accordingly to account for changes in sample loading between gels. For each adjusted protein spot, two control spots were used from an adjacent region of the gel. Error was calculated as standard deviation.

expression level as a percentage of that in unstressed cells
0
100
200
300
400
500
600
YfiD
YnaF
Phosphoglycerate mutase 1
Threonine dehydratase
L-Asparaginase
Glycerophosphodiester phosphodiesterase
YidA
Acetyl-CoA carboxylase carboxyl transferase subunit alpha
D-galactose binding protein
FliC (phase 1 flagellin)
Fumarase
FljB (phase 2 flagellin)
Pyruvate dehydrogenase E1

4.4. Seven Proteins of the *S. typhimurium* SL1344 ATR Stimulon are Co-Regulated.

To further understand the mechanisms governing the differential expression of the identified proteins within the ATR stimulon, their co-regulation in a number of conditions was investigated. This included the examination of 2D gel profiles produced from PhoPQ constitutive and RpoS null mutants, to find out if any proteins fell within either of these two known ATR regulons. 2D gel profiles from other stress responses were also examined for co-regulated proteins. In all, 7 ATR proteins were found to be co-regulated by either PhoPQ, RpoS or the oxidative stress response. Two of these proteins, flagellin phase 1 and phase 2, are covered in detail in Chapter 5 and will thus not be discussed further here. The expression patterns of the proteins in question are considered in detail in the following sections and are illustrated in Figures 4.5, 4.6 and 4.7. Figure 4.8 depicts the expression levels of the co-regulated proteins as a percentage of that seen in wild type, unstressed cells.

4.4.1. The PhoPQ two component regulatory system and the oxidative stress response are co-repressors of glycerophosphodiester phosphodiesterase and D-galactose binding protein.

Panels a-c of Figure 4.5 depict the 2D gel expression profile of glycerophosphodiester phosphodiesterase from exponential phase samples of unstressed wild type cells, oxidatively stressed cells and PhoPQ constitutive bacteria respectively. Likewise, panels d-f of figure 4.5 display the expression profile of D-galactose binding protein under these three conditions. When the expression level of the two proteins is depicted as a percentage of that seen in exponential phase wild type unstressed cells (Figure 4.8), it is clear that PhoPQ^c, the OSR and the ATR generally repress the expression of 2 proteins. When the stationary phase expression profiles of the two proteins were compared between samples from RpoS null mutants and wild type cells on 2D gels, no significant difference was seen (Figure 4.8). Thus, RpoS is not thought to regulate the expression of glycerophosphodiester phosphodiesterase or D-galactose binding protein.

The PhoPQ^c strain was used here in preference to the PhoPQ null mutant for two reasons. First, the continuous activation of PhoPQ is thought to be more representative of the ATR as this two-component system is induced (not repressed) in acid conditions. Second, a PhoPQ null mutant would not allow the detection of proteins induced by an activated PhoPQ system when compared with WT, uninduced bacteria.

The *pho-24* allele used here (section 2.1) results in continuous signal transduction by PhoPQ due to an alteration of the membrane sensor-kinase component (PhoQ) that renders the system active in the absence of extracellular stimuli (Kier *et al*, 1979).

4.4.2. The RpoS sigma factor is a co-repressor of L-asparaginase and YnaF.

Panels a and b of Figure 4.6 show the 2D gel expression profile of L-asparaginase in samples from wild type and RpoS null mutant cells respectively, both in early stationary phase. Likewise, panels c and d of Figure 4.6 show the 2D gel expression profile of YnaF in samples from wild type and RpoS null mutant cells respectively, both in early stationary phase. When compared with expression in wild type cells (Figure 4.8), it is clear that the absence of RpoS in the null mutant results in an increased level of these two proteins (albeit, by only 2-fold). Therefore, RpoS and the ATR co-repress L-asparaginase and YnaF. When the exponential phase expression profiles of asparaginase were compared between samples from PhoPQ constitutive and wild type bacteria on 2D gels, no significant difference was seen (Figure 4.8). Thus, PhoPQ is not thought to regulate the expression of this protein. The expression profiles of asparaginase in exponential phase samples of oxidatively stressed bacteria were extremely variable on 2D gels. Therefore, results indicating the effect of the OSR on asparaginase expression are inconclusive. Likewise, the variable expression profiles of YnaF in exponential phase samples from both oxidatively stressed and PhoPQ constitutive bacteria meant that the effects of these two conditions on YnaF were unclear.

4.4.3. RpoS and PhoPQ are co-inducers of the *N*-terminally truncated GroEL isoforms.

Panels a and b of Figure 4.7 depict the 2D gel profile of the *N*-terminally truncated GroEL isoforms in samples from exponential phase wild type and PhoPQ constitutive bacteria respectively. Panels c and d of Figure 4.7 display the profile of the same isoforms in samples from stationary phase wild type and RpoS null mutant cells respectively. The GroEL isoforms are not present in unstressed wild type cells during exponential phase. However, it is clear that they are induced during the ATR and by a PhoPQ constitutive strain. The isoforms can be seen during stationary phase (Figure 4.7 c.), but are present in reduced levels in an RpoS null mutant strain (Figure 4.8). Therefore, it is concluded that RpoS, PhoPQ and the ATR are all co-inducers of the *N*-terminally truncated GroEL isoforms. The expression profile of the GroEL isoforms in samples from exponential phase oxidatively stressed bacteria was extremely variable. Therefore, the effect of the OSR on the formation of these proteins is unknown.

Figure 4.5. (next page). Details of 2D-gels showing the abundance of glycerophosphodiester phosphodiesterase (spot #18) and D-galactose binding protein (spot #14) under different conditions. Panels **a-c** show the 2D gel profile of glycerophosphodiester phosphodiesterase in samples from wild type unstressed, oxidatively stressed (2.3.3.1) and PhoPQ constitutive *S. typhimurium* SL1344 cells respectively. Likewise, panels **d-f** show the 2D gel profile of D-galactose binding protein in samples from wild type unstressed, oxidatively stressed and PhoPQ^{c} *S. typhimurium* SL1344 cells. All whole cell lysates were prepared from mid exponential phase bacteria. Protein spot numbering corresponds to that in Table 4.1.

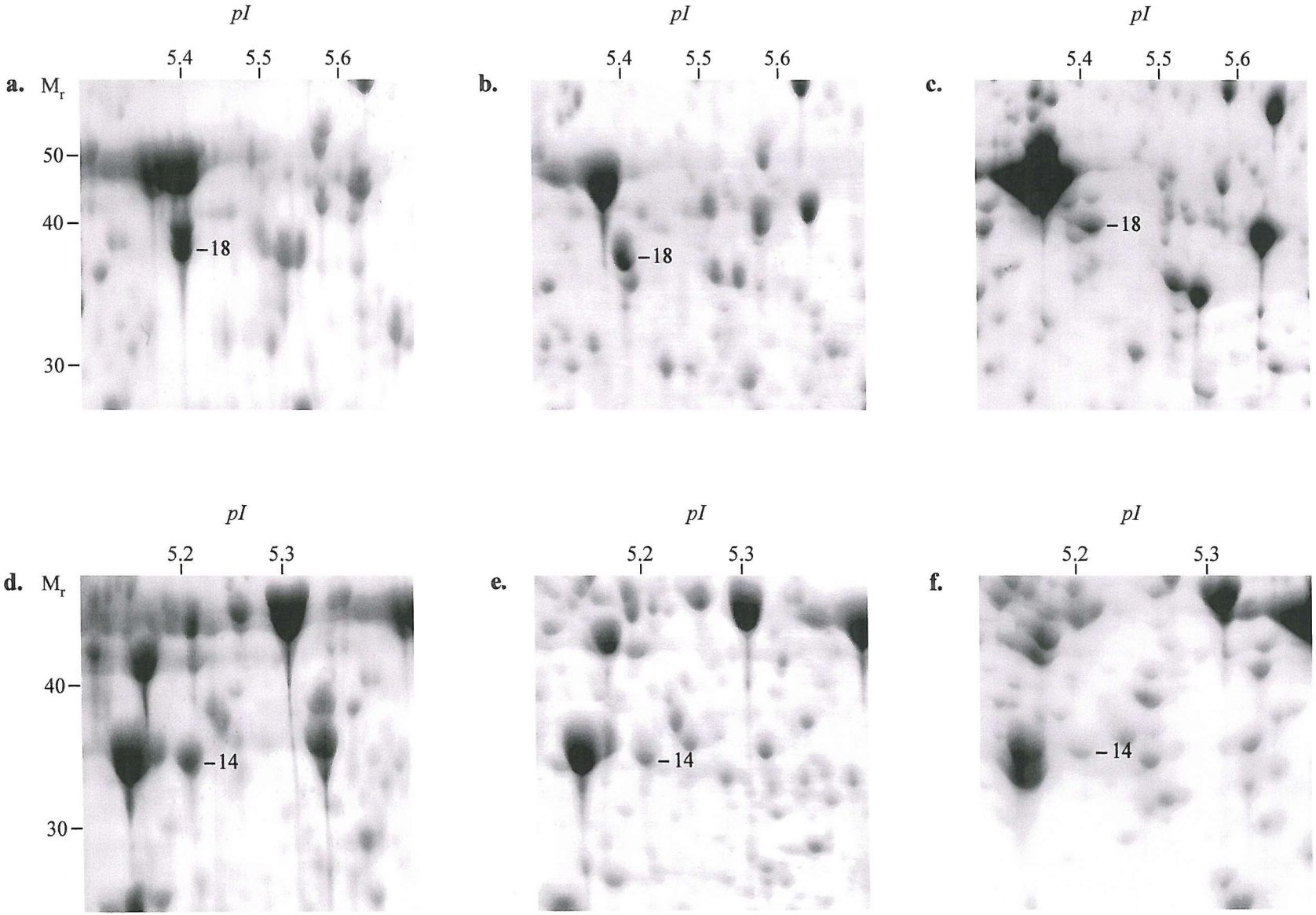

a.
b.
c.
d.
e.
f.
pI
5.4
5.5
5.6
5.2
5.3
Mr
50
40
30
18
14

Figure 4.6. (next page). Details of 2D-gels showing the abundance of L-asparaginase (spot #19) and YnaF (spot #25) under different conditions. Panels **a** and **b** show the 2D gel profile of L-asparaginase in samples from wild type unstressed and RpoS null mutant *S. typhimurium* SL1344 cells respectively. Likewise, panels **c** and **d** show the 2D gel profile of YnaF in samples from wild type unstressed and RpoS null mutant *S. typhimurium* SL1344 cells. All whole cell lysates were prepared from early stationary phase bacteria. Protein spot numbering corresponds to that in Table 4.1.

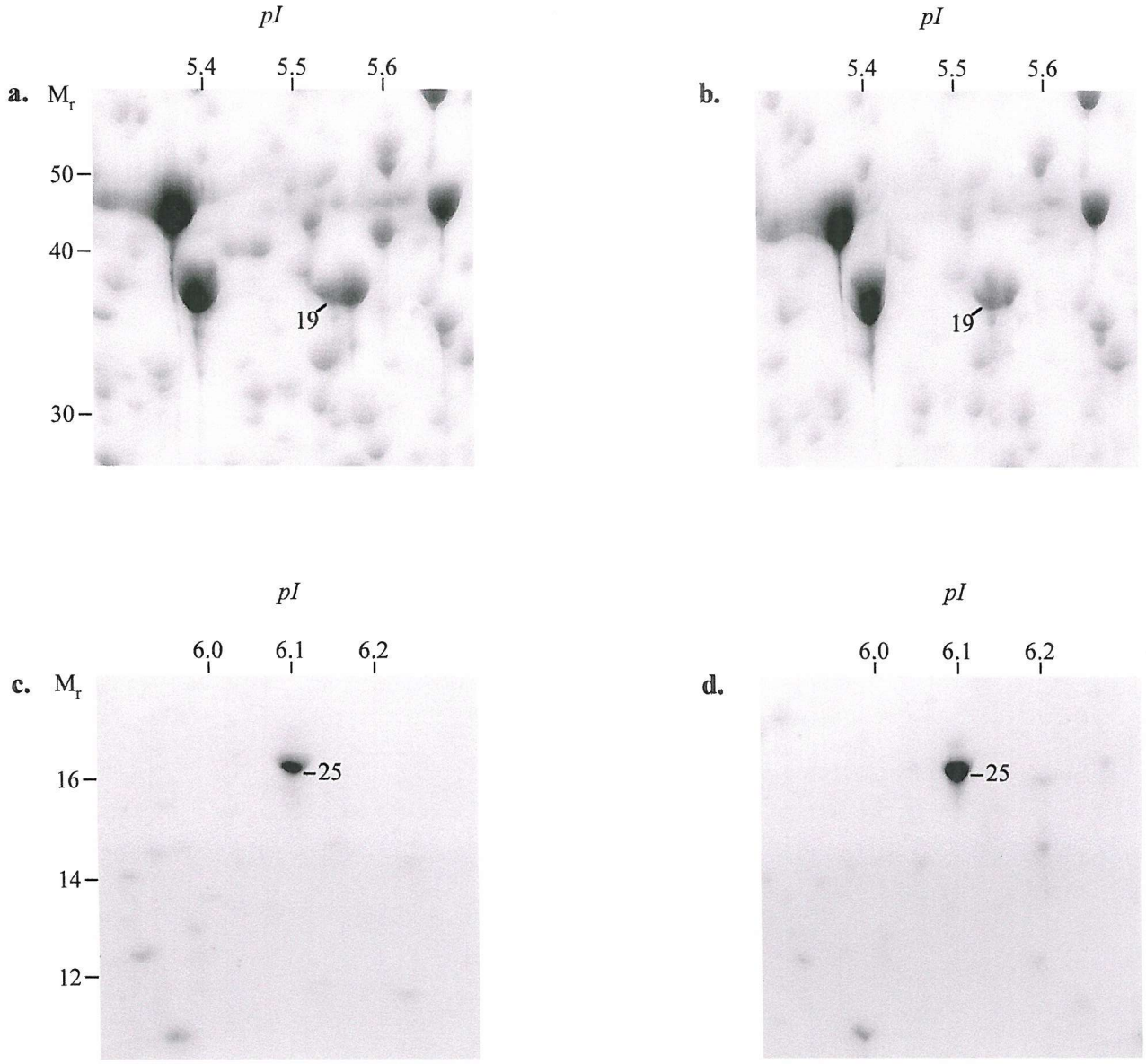

pI
5.4
5.5
5.6
a.
M_r
50
40
30
19
pI
5.4
5.5
5.6
b.
19
pI
6.0
6.1
6.2
c.
M_r
16
14
12
25
pI
6.0
6.1
6.2
d.
25

Figure 4.7. (next page). Enlarged portions of Coomassie stained 2D gels in pairs with corresponding pI and M_r ranges showing the abundance of the GroEL isoforms under different conditions. Panels **a** and **b** show the 2D gel profiles of the GroEL isoforms in samples from wild type and PhoPQ constitutive *S. typhimurium* SL1344 cells respectively. Whole cell lysates were prepared from mid exponential phase bacteria. Panels **c** and **d** depict the 2D gel profiles of the GroEL isoforms in wild type and RpoS null mutant *S. typhimurium* SL1344 cells respectively. Whole cell lysates were prepared from early stationary phase bacteria. Protein spot numbering corresponds to that in Table 4.1.

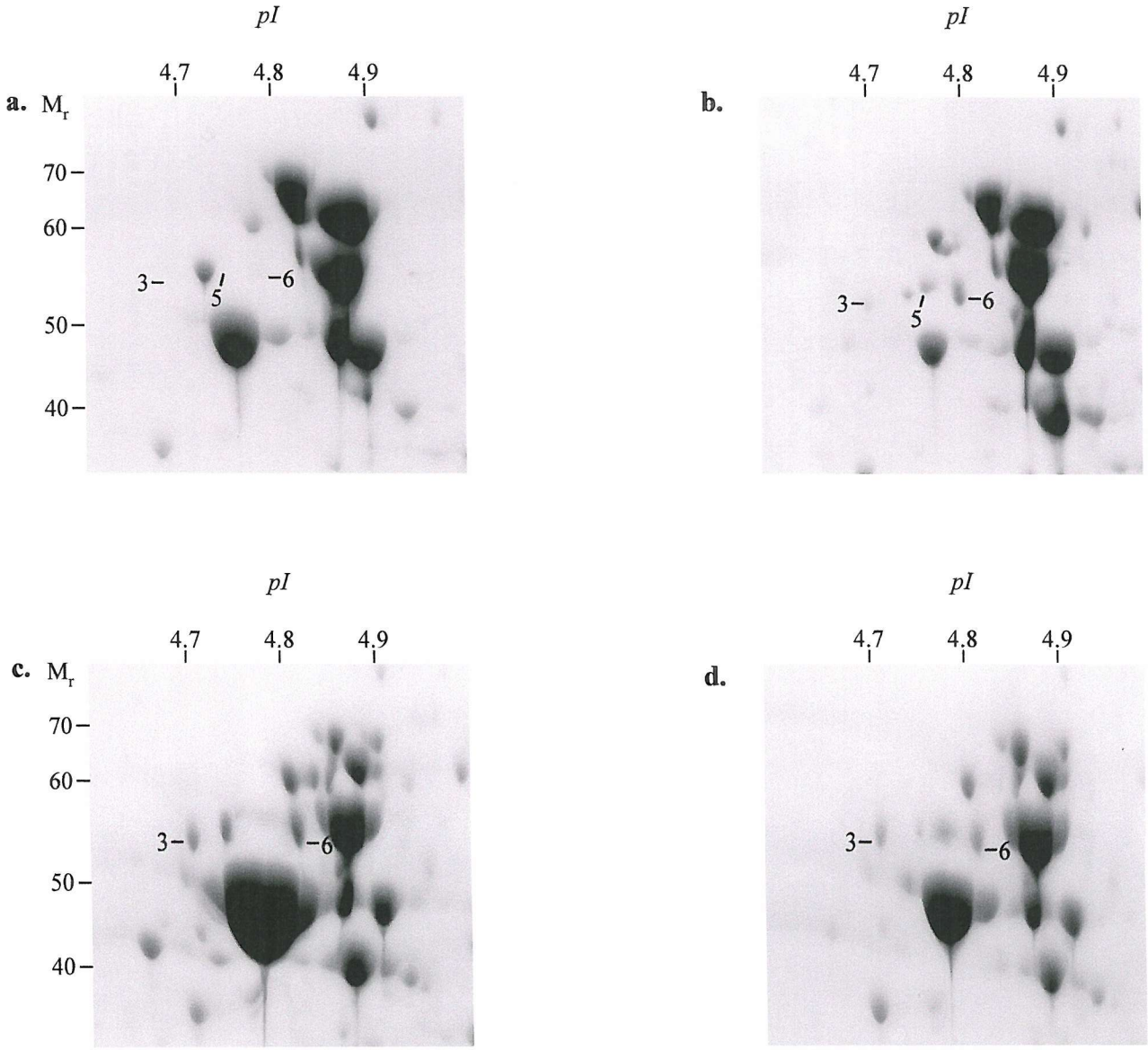
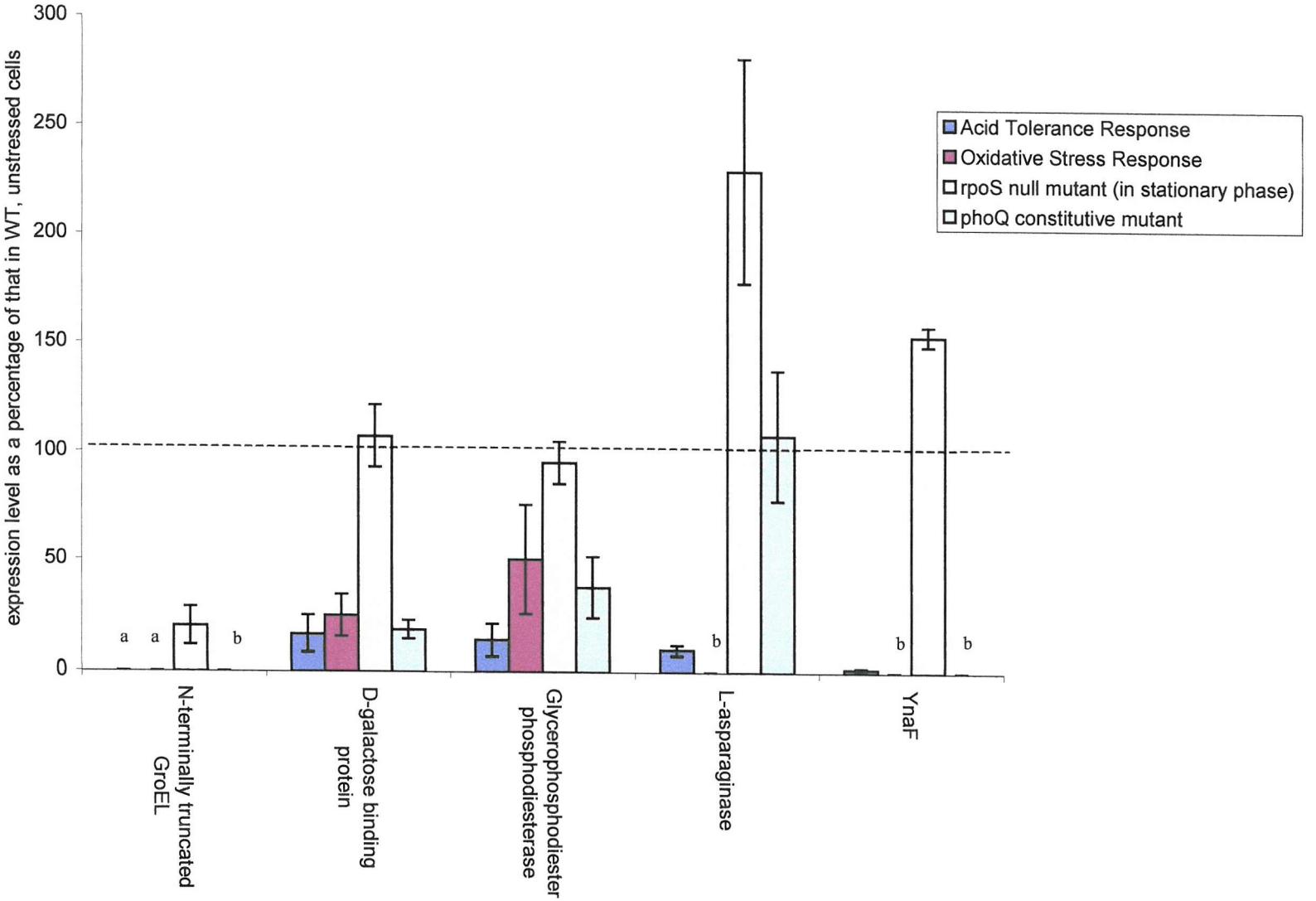

a.
b.
c.
d.
pI
4.7
4.8
4.9
M_r
70
60
50
40
3
5
6

Figure 4.8. (next page). The expression levels of proteins that are co-induced or co-repressed by the mid exponential phase ATR, the mid exponential phase OSR and PhoPQ in exponential phase and/or RpoS in stationary phase. Expression level is depicted as a percentage of that seen in unstressed wild type *S. typhimurium* SL1344 cells. Relative protein levels from 2D gels were calculated using the Phoretix 2D Software v.5.1 in a similar manner to that described in Figure 4.4.

a. The GroEL isoforms are absent in unstressed wild type log phase cells.
b. The spot densitometry readings in these conditions were inconsistent; therefore, expression levels as a percentage of wild-type, unstressed cells could not be obtained.


expression level as a percentage of that in WT, unstressed cells
300
250
200
150
100
50
0
Acid Tolerance Response
Oxidative Stress Response
rpoS null mutant (in stationary phase)
phoQ constitutive mutant
a
a
b
b
b
b
N-terminally truncated GroEL
D-galactose binding protein
Glycerophosphodiester phosphodiesterase
L-asparaginase
YnaF

4.5. Discussion.

The research presented here has characterised the exponential phase ATR stimulon of *S. typhimurium* SL1344 under optimal ATR inducing conditions and using whole cell lysates with an IPG of pH 4-7. A reference map of the proteins that are differentially regulated in this environment has been constructed and the majority of these proteins have been identified using tryptic mass fingerprinting and/or *N*-terminal sequencing techniques. The PhoPQ system and the RpoS sigma factor constitute known regulators of the ATR in *S. typhimurium*. With the help of mutant strains, it has been possible to define the co-regulation of a number of the ATR proteins. Additionally, their regulation by the exponential phase OSR has also been examined.

It is appropriate to discuss each identified ATR regulated protein in turn. Possible regulatory mechanisms will be discussed and the proteins' possible role in acid tolerance will be considered. A summary, including a comparison of the protein profile obtained here with that in the literature will then be described.

4.5.1. Down-regulated protein products of the ATR.

Pyruvate dehydrogenase E1 subunit (AceE).

The pyruvate dehydrogenase complex catalyses the oxidative decarboxylation of pyruvate in the following reaction:

$$\text{pyruvate} + \text{CoA} + \text{NAD}^+ \rightarrow \text{acetyl-CoA} + \text{CO}_2 + \text{NADH} + \text{H}^+$$

The complex is composed of three subunit types: E1, pyruvate dehydrogenase (AceE), E2, dihydrolipoamide transacetylase (AceF) and E3, lipoamide dehydrogenase (Lpd) in a 24:24:12 ratio respectively. *AceEF* and *lpd* are members of the same operon. The E1 subunit is a homodimer that binds thiamine diphosphate and Mg^{2+} as co-factors in a β-turn-α-turn-β motif (Dekok *et al*, 1998). It is transcriptionally repressed by the PdhR protein, which is transcribed from the same promoter as *aceEF.lpd* (Quail *et al*, 1994; Quail and Guest, 1995).

As the E1 subunit is down-regulated during the ATR, it is therefore probable that the activity of the pyruvate dehydrogenase complex (PDHC) is severely curtailed. As the reaction catalysed by the PDHC liberates protons, the reduced expression of this complex during the ATR may help maintain pH_i. Evidence for the involvement of PDHC in the *S. typhimurium* ATR has previously been described (Foster and Hall, 1991). Screening for spontaneous acid-tolerant mutants resulted in the discovery of a constitutively acid tolerant strain. Co-translation placed the mutation within the *aceEF.lpd* operon and hence in the PDHC. However, an actual ATR induced down-regulation of PDHC was not proved before the present study. Interestingly, AceE also shows reduced expression during heat shock at 42°C in the facultative intracellular pathogen, *Brucella melitensis* (Teixeira-Gomes *et al*, 2000). This bacterium, like *Salmonella*, can survive and multiply within macrophages (reviewed in Corbel, 1997). It thus may encounter similar stress conditions (for example, oxidative damage, cationic peptides and acid pH). The inhibition of E1 subunit expression during heat shock and the ATR may therefore point to the involvement of PHDC in the general stress responses of *B. melitensis* and *S. typhimurium*.

In the absence of PDHC, the pyruvate oxidase (PoxB) enzyme can support the growth of *E. coli* by converting pyruvate directly to acetate in the following reaction (Green and Guest, 1998):

pyruvate + ferricytochrome B1 + $H_2O \rightarrow CO_2$ + acetate + ferrrocytochrome B1

PoxB is associated with the stationary phase of growth (Green and Guest, 1998) and may be regulated by RpoS and hence the ATR. Research into the acid tolerance of *E. coli* has shown that an increase in acetate levels can induce extreme acid resistance. In fact, anaerobic induction with glucose resulting in increased acetate and lactate concentrations and a concomitant fall in pH_o produced a 1000-fold increased survival in extreme acid conditions (Gonzalez and Russel, 1999). Buchanan and Edelson (1996) also showed that *E. coli* can acidify its environment via glucose fermentation and subsequently tolerate extreme acid conditions. It is therefore possible that a reduction in PDHC levels could result in acetate accumulation which the bacteria could use to self-induce an acid tolerance response to subsequent extreme acid

exposure. In support of this hypothesis, two studies have described elevated levels of isocitrate lyase A (AceA) and B (AceB) in *E. coli* in response to acid conditions (Lambert *et al*, 1997; Blankenhorn *et al*, 1999). These are glyoxylate shunt enzymes that are required for growth on acetate and fatty acids.

The above mechanisms suggest that redox potential may play a role in acid tolerance. Previous research has also pointed towards a link between the two systems. For example, the acid induction of the amino acid decarboxylases described in Chapter 1 is enhanced by anaerobiosis (Blankenhorn *et al*, 1999). Additionally, the acid inducible *aniC* and *hyaB* require anaerobiosis for full expression (Aliabadi *et al*, 1988). It is thought that the cAMP receptor protein (CRP) and TyrR, both strong positive regulators of these two genes may sense alterations in internal pH. These alterations may be more pronounced under anaerobic conditions and in the presence of organic acid fermentation end products (Park *et al*, 1999). Interestingly, FNR, a global regulator of anaerobiosis, negatively regulates *narZ*, a gene that is positively regulated during the ATR, by hydrogen peroxide stress and by the starvation stress response (Spector *et al*, 1999).

Fumarase B (FumB).

Fumarase B functions in the fermentative pathway that leads to the production of succinate. It catalyses the formation of fumarate from L-malate for use as an anaerobic electron acceptor:

L-malate $\rightarrow$ fumarate + H_2O

FumB shares 79% identity with fumarase A (FumA), an aerobic citric acid cycle enzyme. Both enzymes bind 4Fe-4S clusters as cofactors.

As iron is an essential component of the FumA and FumB activities, it is not surprising that it is involved in the regulation of fumarase gene expression. Although primarily anaerobic, FumB levels are elevated under aerobic conditions in complex media and when iron concentrations are high. Iron limitation results in a 7 fold

decrease in FumB levels (Tseng, 1997). As FumB levels also drop markedly during the ATR, it can be hypothesised that perhaps the Fur protein is regulating the enzyme's expression in response to both conditions. Previous research has already implicated Fur in the co-regulation of unidentified proteins in response to both iron and acid levels (Foster and Hall, 1992). In addition, Fur has been identified as a positive regulator of FumA expression (Tseng, 1997). As FumA and FumB work antagonistically, it would seem appropriate that the up-regulation of one enzyme be coupled to the down-regulation of the other.

As an anaerobic enzyme elevated under aerobic conditions, FumB may play an additional role in the bacterial cell. Indeed, fumarate, the product of FumB activity, has been implicated in the chemotactic response (Prasad *et al*, 1998). Fumarate targets the switch-motor complex of the flagellar apparatus, subsequently increasing the probability of clockwise rotation and, thus inducing tumbling. Reducing FumB levels and increasing FumA would therefore increase the likelihood of smooth swimming. However, as the expression of the flagellar apparatus is partially repressed during the ATR (see Chapter 5), *fumB* may be repressed as the chemotactic function of fumarate would not be required.

FumC, a third form of fumarase is a tetrameric and more stable enzyme than FumA or FumB. It does not require a 4Fe-4S cluster as a cofactor. As described in Chapter 1, the OSR induces the expression of *fumC* via the SoxRS regulon in response to $O_2^{-}\cdot$ (Liochev *et al*, 1999a). FumC thus maintains fumarase activity under conditions that are likely to inactivate FumA or FumB. It is thus possible that the more stable form is also induced in response to other stress conditions. This could explain the fall in FumB levels during the ATR.

It is of interest to note that FumB regulation suggests an additional link between anaerobiosis and acid stress. Unlike previous discoveries, FumB is an anaerobic protein which is down-, as opposed to up-regulated, during the ATR. Thus, if cross regulation exists, it appears to be a complex process involving many regulators.

D-galactose binding protein (MglB).

D-galactose binding protein (MglB) is a component of the ATP-binding cassette (ABC) importer, galactose permease. This complex is responsible for the active uptake of galactose and glucose. MglB serves as the periplasmic receptor protein. The two other components are MglC, the membrane translocator, and MglA, the membrane associated ATPase. MglB binds substrate and transfers it to the membrane complex where the hydrolysis of ATP provides energy for transport. Newly synthesised D-galactose binding protein is translocated from the cytoplasm to the periplasm via the *sec*-dependent type II secretion system (Harayama *et al*, 1983; Muller *et al*, 1985; Bassford, 1990).

An apparent reduction in the quantity of MglB has previously been associated with a defect in MglB export via the type II secretion system (Yaagoubi *et al*, 1996). This could explain the reduced levels of MglB seen during the ATR. The type II secretion system is powered by the proton motive force and ATP hydrolysis. Therefore, during acid stress, a reduction in secretion levels would arrest a potentially harmful site of proton entry into the cell and release energy in the form of ATP for use in survival mechanisms. The resulting decrease in galactose import would release additional ATP for the same purpose, albeit at the expense of non-availability of a useful energy source.

MglB, like other periplasmic binding proteins, also functions in promoting chemotaxis towards its specific ligands. When bound to glucose or galactose, it can complex with the Trg methyl-accepting chemotaxis protein and regulate the frequency of tumbling via a phosphorylation cascade (Manson *et al*, 1998). However, the flagellar machinery is partially repressed during the ATR, hence this chemotactic ability is not required. Perhaps this may also contribute to the reduction in MglB levels under these conditions. Co-regulation experiments have also placed MglB within the oxidative stress stimulon and the PhoPQ regulon (see Figures 4.5 and 4.8). Thus it is clear that, whatever its mechanism of action, MglB is likely to play a role in a more general stress response.

Interestingly, *in-vitro*, the periplasmic binding proteins MglB, MalE and OppA have chaperone-like properties in *E. coli* and *S. typhimurium*. They form stable complexes with certain proteins and prevent aggregation of others during heat shock conditions (Richarme and Caldas, 1997). It would appear that, for MglB at least, these chaperone-like properties are not used *in vivo* during acid or oxidative stress. Otherwise, an increase or, at least a stabilisation of MglB expression, would be expected under these conditions.

Acetyl-CoA carboxylase carboxyl transferase subunit α (AccA).

The acetyl-CoA carboxylase complex consists of three major components. One component is the carboxyl transferase $\alpha_2\beta_2$ tetramer. Here, the carboxyl transferase α subunit was found to be down regulated during the ATR. The acetyl CoA carboxylase complex catalyses the first step in long chain fatty acid synthesis. First, a carboxylase component catalyses the carboxylation of a carrier protein (**i**), then the transcarboxylase transfers the carboxyl group to form malonyl-CoA (**ii**):

(**i**) Biotin carboxyl carrier protein (BCCP) + Mg-ATP + HCO_3^- ⇌ BCCP-CO_2^- + Mg-ADP + Pi

(**ii**) BCCP-CO_2^- + acetyl-CoA ⇌ BCCP + malonyl-CoA

The stringent response, as described in Chapter 1 involves the production of ppGpp and pppGpp in response to amino acid starvation. These alarmones act to redirect the cell's metabolism and to inhibit protein synthesis. Fatty acid synthesis is also reduced during amino acid starvation. This decrease is mediated by the inhibition of the carboxyl transferase component by ppGpp and pppGpp (Palakis *et al*, 1973). It is possible that this mechanism is also in operation during the ATR. RpoS-dependent promoters are known to require ppGpp for induction (Kvint *et al* 2000). As RpoS is a major regulon of the ATR, ppGpp levels must be elevated here as well. In fact, in *Lactococcus lactis*, activation of the stringent response and raised levels of (p)ppGpp have been implicated in this bacteria's ATR (Rallu *et al*, 2000).

YidA.

YidA is currently classified as a hypothetical 29.7kDa protein situated in the *ibpA-gyrB* intergenic region (Burland *et al*, 1993; Adachi *et al*, 1987). It is down-regulated during the ATR of *S. typhimurium* SL1344. YidA belongs to a family of *E. coli* proteins of unknown function to which it shares sequence homology (COF/YbhA/YidA/YigL). All except COF are classified as hypothetical.

Glycerophosphodiester phosphodiesterase (GlpQ).

GlpQ is a periplasmically located component of the *glp* regulon which encodes the major uptake system for glycerol-3-phosphate (G3P). GlpQ hydrolyses deacylated phospholipids to G3P and the corresponding alcohols in the following reaction:

a glycerophosphodiester + $H_2O \rightarrow$ an alcohol + G3P

Glycerophosphodiesters hydrolysed by GlpQ include glycerophosphoethanolamine, glycerophosphocholine, glycerophosphoglycerol and bis(glycerophospho)glycerol. G3P is then taken up by the G3P transporter, GlpT. Subsequently, the compound can be directed to the glycolytic pathway via G3P dehydrogenase (GlpABC) or utilised in phospholipid synthesis (Hengge *et al*, 1983; Boos, 1998; Larson, 1982; Larson, 1983). GlpTQ and GlpABC constitute two operons (Erhmann *et al*, 1987), both repressed by GlpR. Thus, the down-regulation of GlpQ suggests the co-repression of other components of the *glp* regulon. The repression of *glpT* expression could be of benefit during acid stress as the GlpT transport of G3P is proton motive force dependent. Thus, another route of proton entry would be abolished.

This study has also identified *glpQ* as a member of the PhoPQ regulon and established its place within the OSR stimulon of *S. typhimurium*. Thus, like D-galactose binding protein (also part of a permease mechanism), GlpQ probably has a role in the general stress response of the bacteria. The repression of D-galactose binding protein during the ATR suggested that the type II secretion system required for its transport to the periplasm may have been disrupted. This would be of benefit at low pH as the type II

secretion system is powered by the proton motive force and requires ATP. The down-regulation of GlpQ in these conditions might also be linked to this secretion system. However, more evidence is required to substantiate a link between type II secretion and the ATR.

L-asparaginase 1 (AnsA).

L-asparaginase 1 is a cytoplasmic enzyme that catalyses the following reaction:

L-asparagine + $H_2O \rightarrow$ L-aspartate + NH_3

The rate of synthesis of this enzyme in *E. coli* has previously been found to be dependent on the pH of the culture and the amount of oxygen dissolved in the medium (Barnes *et al*, 1977). Asparaginase activity diminished when the pH was lower than 7.5 or when the dissolved oxygen content was greater than zero. The present study has shown a significant drop in asparaginase levels as part of the ATR of *S. typhimurium*. Interestingly, there again appears to be a link between anaerobiosis and acid tolerance.

Co-regulation studies have shown asparaginase to be down-regulated by RpoS. It is therefore likely that this expression control mechanism is employed during the exponential phase ATR of *S. typhimurium*. No previous evidence of asparaginase regulation by RpoS exists.

Threonine dehydratase catabolic (TdcB).

TdcB catalyses threonine and serine catabolism in the reaction shown below. It is a component of the TdcABC operon which is involved in the transport and metabolism of threonine and serine during anaerobic growth. TdcB expression is induced in complex medium and during anaerobic growth.

L-threonine + $H_2O \rightarrow$ 2-oxobutanoate + NH_3 + H_2O

TdcB synthesis increases in *E. coli* in the presence of the DNA gyrase inhibitors nalidixic acid and coumermycin (Sumantran *et al*, 1989). DNA gyrase functions in promoting the negative supercoiling of DNA. *tdcB* may thus be a "supercoiling sensitive" gene. Therefore, it is possible that alterations in the level of DNA supercoiling, potentiated by fluctuations in environmental conditions (such as acid) could alter the expression of *tdcB*.

Alterations in DNA supercoiling have been implicated in the transcriptional regulation of bacterial stress responses. For example, an increase in the amount of negative supercoiling is seen when *E. coli* is exposed to an upshift in external osmolarity (Higgins *et al*, 1988). This altered DNA topology has been linked to the increased expression of *proU* and *ompC* during osmotic shock (Bhriain *et al*, 1989). Interestingly, both genes require anaerobic growth for optimal expression. OmpC levels also increase during the ATR of *E. coli* (Thomas and Booth, 1992). It is therefore possible that these and some other stress related genes are regulated by a common mechanism in response to different environmental signals. This regulatory overlap could be mediated by changes in DNA supercoiling levels in response to environmental stresses. The DNA topology of *E. coli* is also altered during heat shock. Here, a transient decrease in negative supercoiling is seen prior to supercoiling to original levels. The DnaK protein (induced during heat shock and the ATR) acts to increase the supercoiling ability of DNA gyrase (Ogata *et al*, 1996). DNA supercoiling also alters during the OSR where a transient decrease is followed by an increase which continues above normal levels (Tkachenko *et al*, 1999). The transient decrease in supercoiling during heat shock and oxidative stress is mediated by increased toperisomerase I (TopA) expression. During oxidative stress, H_2O_2 interacts with the DNA binding protein Fis. Fis then binds to the promoter region of *topA* and activates transcription (Weinstein-Fischer *et al*, 2000).

It is therefore plausible that an overall increase in negative DNA supercoiling during the ATR of *S. typhimurium* could produce the observed down-regulation of TdcB expression. The mechanism of action may be via an increased DnaK level which enhances the activity of DNA gyrase. Altered levels of DNA supercoiling may also partly explain the observed links between the ATR stimulon and anaerobiosis. It has

to be made clear however, that DNA supercoiling can only partially explain the mechanisms described above. If this process had a direct effect on gene expression during different stress responses, we would expect to see all "supercoiling sensitive" genes regulated by every stress response. This is simply not the case.

TdcB expression during the ATR may be reduced as a result of the general decrease in metabolism observed. However, overexpression of TdcB can result in the diversion of 70% of the carbon available for lysine metabolism into the isoleucine pathway (Guillouet *et al*, 1999). As the decarboxylation of lysine is a major acid survival mechanism (see Chapter 1), maybe a reduction in TdcB expression results in the increased availability of this amino acid for this purpose.

YnaF.

YnaF is an unknown protein previously identified via 2D PAGE. It belongs to a family of universal stress protein A (UspA) like proteins. If YnaF is assumed to be under the same regulatory control as UspA, its decreased expression during the *S. typhimurium* ATR can be explained. Co-regulation studies here have shown *ynaF* expression to increase in an RpoS null mutant. It is known that the similar *uspA* gene is transcribed by σ^{70} programmed RNA polymerase and is also superinduced in an *rpoS* mutant (Farewell *et al*, 1998). It is therefore possible that an increase in RpoS levels during the ATR results in decreased levels of YnaF and UspA as a result of sigma factor competition.

UspA over expression predisposes *E. coli* cells to the effects of environmental stresses (Diez *et al*, 1997). If YnaF has the same effect, reduced expression from the *ynaF* gene could possibly result in increased stress tolerance. Hence the observed fall in cellular YnaF concentrations during the ATR.

UspA has been identified as benzoate inducible (Lambert *et al*, 1997). Thus, perhaps YnaF could also be upregulated in the presence of this weak acid. As the present study has shown YnaF to be repressed during the ATR in response to inorganic acid, perhaps this protein is specific to organic acid tolerance.

Interestingly, UspA is a serine threonine phosphoprotein whose phosphorylation is dependent on BipA (also known as TypA) (Freestone *et al*, 1997). As described in Chapter 1, BipA regulates a number of virulence associated processes. The phenotypes of UspA and BipA mutants are similar. Therefore, a decreased level of BipA may also be beneficial to acid tolerance (see Chapter 7).

YfiD.

YfiD is a 14.3 kDa protein of unknown function. It is positively regulated by FNR, the regulatory protein associated with anaerobic growth (Green *et al*, 1998). However, 2D PAGE analysis has demonstrated that *yfiD* expression levels do not alter in an oxygen dependent manner (Blankenhorn *et al*, 1999). This same study identified an increase in *yfiD* expression in response to acidic pH in *E. coli*. This is in contrast to the data presented here and could be attributable to differences in the ATR inducing conditions used (a greater than 10 fold difference in acid incubation time and a difference of 2.5 pH units in acid challenge conditions).

4.5.2. Up-regulated protein products of the ATR.

_N_-terminally truncated GroEL isoforms.

This study identified three ATR upregulated protein spots (numbered 3, 5 and 6) as the molecular chaperone GroEL. These have since been identified as *N*-terminally truncated isoforms of this protein (P. Adams, unpublished). It thus appears in this case that the apparent up-regulation of *groEL* expression is, in fact an artefact of proteolytic cleavage, which causes a shift in pI, thereby producing "new" spots on the 2D gel. It has previously been reported that the constitutive activation of the PhoPQ system in *S. typhimurium* SL1344 results in the stimulation of at least two distinct proteases (Adams *et al*, 1999). The proteases, which are not affected by standard protease inhibitors, produce truncated forms of specific proteins visible on coomassie stained 2D PAGE gels. The proteolytic cleavage sites of one protease were defined by mapping peptides from truncated proteins to the amino acid sequences of the full length forms. The protease was thus found to recognise a dibasic amino acid motif

characteristic of the omptin protease family. Such proteins, which typically reside at the bacterial cell surface, may contribute to the PhoPQ mediated resistance of *Salmonella* to cationic antimicrobial peptides. As the PhoPQ system is a major regulon of the ATR in *Salmonella*, it is not surprising that such proteases may be active during this response, resulting in the artefactual GroEL isoforms seen. Indeed, the co-regulation studies depicted in Figure 4.7 show the three GroEL isoforms are present in a PhoPQ constitutive strain. Interestingly, as shown in Figure 4.7, all three GroEL isoforms are also present in stationary phase cells. A reduction in isoform spot number 6 is seen in an RpoS null mutant in stationary phase. This suggests that RpoS in addition to PhoPQ may contribute towards the activation of the protease during the exponential phase ATR.

Phosphoglycerate mutase 1 (GPM).

Phosphoglycerate mutase catalyses the following reaction in the glycolytic pathway:

2-phosphoglycerate + 2,3-DPGA $\rightarrow$ 3-phosphoglycerate + 2,3-DPGA
(2,3-DPGA = 2,3-diphosphoglycerate).

The role of GPM in the ATR of *S. typhimurium* is unknown. However, its expression increases in response to hypochlorous acid stress in *E. coli* (Dukan *et al*, 1998). Levels are also elevated in response to the stationary phase of growth (Nystrom *et al*, 1996). The stationary phase-like phenotype of ATR induced *S. typhimurium* may thus explain the upregulation of GPM in response to acid.

4.5.3. Summary.

Within the limitations of the techniques used, this study has characterised the exponential phase ATR stimulon of *S. typhimurium* SL1344 under defined assay conditions. Overall, the up-regulation of 5 protein products has been established, 4 of which have been identified. The majority of ATR regulation results in protein repression. 12 of 21 down-regulated proteins have been identified.

For the most part, the distinctive ATR 2D gel profile obtained is the result of changes in protein expression rather than post-translational modification. If the latter were responsible, the resultant shifts in pI and M_r would produce similar levels of up and down-regulation. Additionally, tryptic mass fingerprinting would identify the same protein from more than one gel location. On the whole, this is not the case. The obvious exception is the *N*-terminal truncation of GroEL by an ATR induced protease resulting in three additional protein spots.

Co-regulation studies have placed a number of the ATR regulated proteins within known ATR regulons. D-galactose binding protein and glycerophosphodiester phosphodiesterase are repressed during the ATR by the PhoPQ system. The repression of these proteins during oxidative stress implicates them in a more general stress response. RpoS may be responsible for the down-regulation of L-asparaginase and YnaF during the ATR. Additionally, the protease that cleaves GroEL in ATR induced cells may be under positive regulatory control by both PhoPQ and RpoS.

Overall, a diverse array of protein types have been identified for which many potential ATR mechanisms can be proposed. However, a number of related acid tolerance strategies seem to be applied. First, the repression of a number of proteins may serve to reduce proton influx and conserve ATP in acid conditions. The proteins involved (MglB, GlpQ and AccA) are all components of active mechanisms requiring a proton motive force and/or ATP hydrolysis. The down-regulation of these and other proteins such as AceE and AnsA associates the ATR with a less metabolically active, more hardy stationary phase-like phenotype. The involvement of RpoS and components of the stringent response makes this quite probable. Another group of identified proteins links the ATR with mechanisms of anaerobic regulation. This connection has been made previously, for example in the optimum induction of the acid inducible *aniC* (Park *et al*, 1999), *aniG* (Aliabadi *et al*, 1988) and glutamate decarboxylase (Blankenhorn *et al*, 1999) genes during anaerobic growth. In contrast, the acid inducible *narZ* is repressed by FNR, a global regulator of anaerobiosis (Spector *et al*, 1999). The ATR repressed anaerobically regulated proteins identified here include TdcB, AnsA and FumB. The mode of regulation is complex and may involve FNR and changes in DNA topology amongst others. Proteins of unknown function with no

previously defined roles have also been identified here as constituents of the ATR. These are YidA, YnaF and YfiD.

When the research presented here is compared with equivalent studies in *S. typhimurium* and *E. coli* only a small degree of overlap is found. This is also true of comparisons between previously published research. Three main reasons for this anomaly are proposed. First, only a small percentage of the *S. typhimurium* and *E. coli* ATR stimulons has so far been fully characterised. Therefore, the likelihood of identifying additional new ATR components is relatively high. Second, the lack of a standard set of ATR inducing conditions results in variations in the ATR protein profiles reported. As has been seen, the particular pH responses that become engaged depend in a large measure on the chemical composition of the environment and the acid induction times used. Finally, limitations in particular experimental techniques may alter the protein expression profiles visualised. For example, not every ATR regulated protein can be detected using the 2D PAGE procedure. Current gels are limited in the pI and M_r ranges that can be used; gel visualisation procedures can differ in protein sensitivity; and also many proteins are not expressed in sufficient concentrations to be detectable. If visualisation is possible, protein spot concentrations can still be too low for identification via tryptic mass fingerprinting or *N*-terminal sequencing, even if samples are pooled.

In contrast with the literature, which has concentrated on the up-regulation of expression during the ATR (for example see Valdivia and Falkow, 1996; Blankenhorn *et al*, 1999; Dukan *et al*, 1998), the research presented here has identified many ATR repressed proteins. Other studies have reported an overall repression of expression levels during the ATR, but have chosen to examine genes that are induced (for example see Hickey and Hirshfield, 1990). It is clear that repression plays an important role in the ATR mechanism of *S. typhimurium*. This is highlighted by the possible induction of the stringent response (as in *L. lactis*, Rallu *et al*, 2000) together with up-regulation of the stationary phase associated RpoS.

CHAPTER 5.

THE FLAGELLAR APPARATUS CONSTITUTES A SUBSET OF ASPs THAT ARE REPRESSED DURING THE ATR VIA THE PhoPQ TWO-COMPONENT REGULATORY SYSTEM IN S. typhimurium.

CHAPTER 5. THE FLAGELLAR APPARATUS CONSTITUTES A SUBSET OF ASPs THAT ARE REPRESSED DURING THE ATR VIA THE PhoPQ TWO-COMPONENT REGULATORY SYSTEM IN *S. typhimurium*.

5.1. Introduction.

As described in Chapter 4, a reduction in the expression of phase-1 and phase-2 flagellin has been identified in ATR-induced *S. typhimurium* (Figures 4.3 and 4.4; table 4.1).

A down-regulation of flagellin synthesis in acid adapted cells at low pH is potentially of great interest for several reasons. First, a reduction in flagellin levels has not been previously linked to the ATR. Second, flagellin forms part of the flagellar apparatus which is synthesised in a complex regulatory cascade. A repression of flagellin transcription could thus be mediated by a number of controlling factors and involve the altered synthesis of additional flagellar components. Third, flagella expression together with associated bacterial motility and chemotaxis have been implicated in pathogenicity (Ottemann and Miller, 1997). The flagellum's resemblance to Type III secretion systems (Kubori *et al*, 1998) and its ability to export proteins required for virulence (Young *et al*, 1999) enhance this association.

This chapter reports the characterisation of the ATR mechanism involved in reduced flagellin synthesis at low pH. However, it is first important to introduce the flagellar apparatus, its biosynthesis and its mode of action.

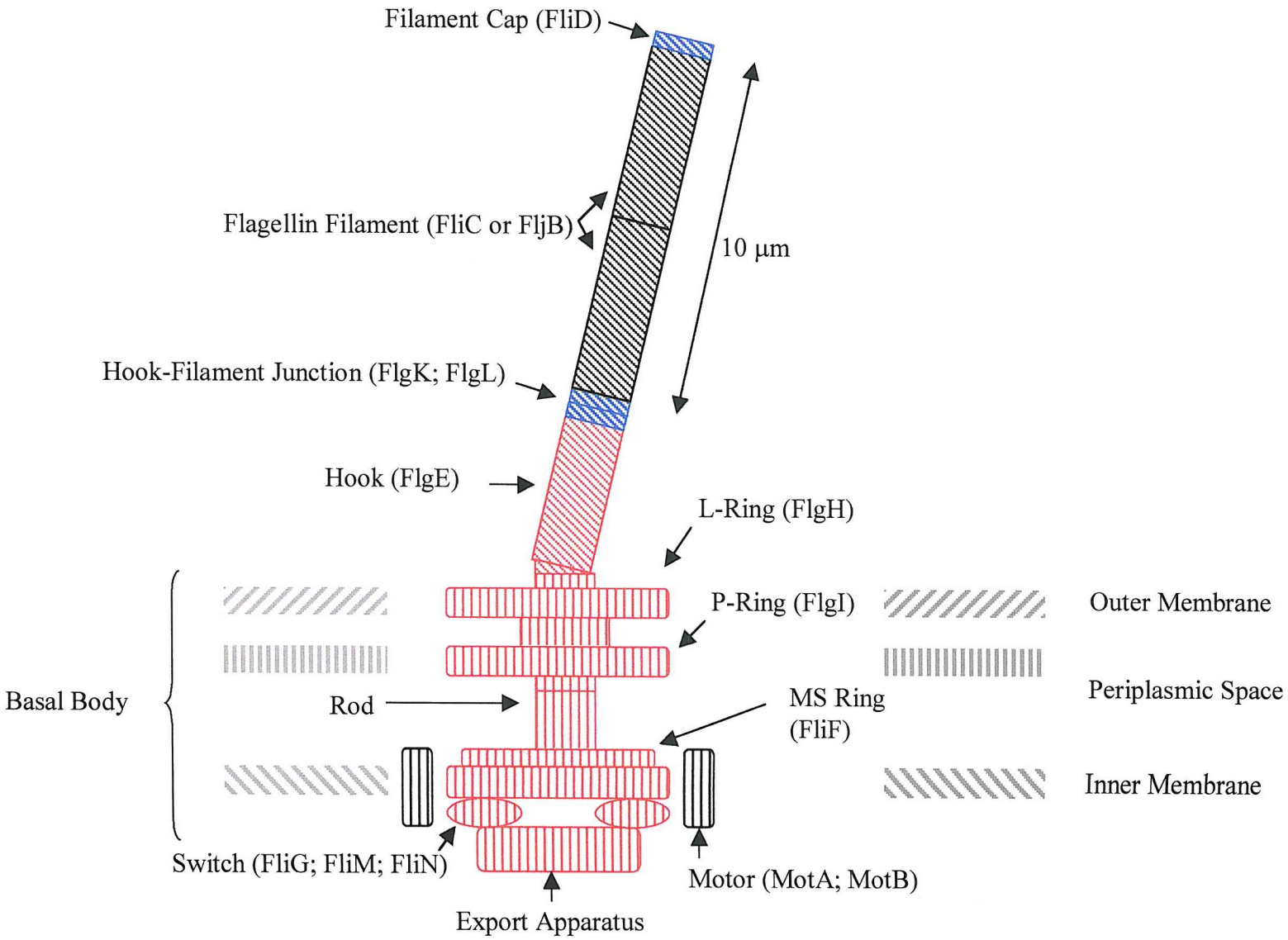
5.1.1. Flagellar structure.

S. typhimurium and *E. coli* use organelles called flagella for locomotion. Several individual flagella protrude from random points on the surfaces of the bacteria (peritrichous flagellation) and the cells swim by rotating these flagella (see section 5.1.4). A single flagellum is diagrammed in Figure 5.1.

The organelle consists of a filament, a hook and a basal body (reviewed in Macnab, 1992; Shapiro, 1995; Manson *et al*, 1998; Macnab, 1999). The filament is a thin helical

tube, comprised principally of flagellin, which can extend to ten times the length of the cell. It is positioned extracellularly and serves as a propeller. The hollow flexible hook joins the flagellar filament to the basal body. The basal body consists of a rod, to which the hook is attached, surrounded by three rings: the MS ring is situated in the cytoplasmic membrane, the P ring is in the peptidoglycan layer and the L ring is embedded in the outer membrane. The flagellum is rotated by a reversible motor made up of two proteins (MotA and MotB) which surround the MS ring. The motor is powered by the proton motive force (Manson *et al*, 1977). An inward proton current flows through a conducting channel formed between the MotA and MotB proteins (Manson *et al*, 1998). The motor interacts with a switch complex (the C-ring) mounted on the cytoplasmic face of the MS ring. The C ring complex consists of three proteins termed FliG, FliM and FliN (Macnab, 1999). It rotates along with the MS ring, rod, hook and filament and is involved in determining the turning direction of the flagellum. Also at the cytoplasmic face of the MS ring is the export apparatus, required for the correct assembly of the flagellar structure during synthesis. The entire basal body structurally resembles a type III secretion system and certain components share sequence similarity to those assumed to drive protein export in the latter. This similarity is reinforced by the recent finding that the flagellar apparatus may also function in *Yersinia* to export non-flagellar related proteins (Young *et al*, 1999).

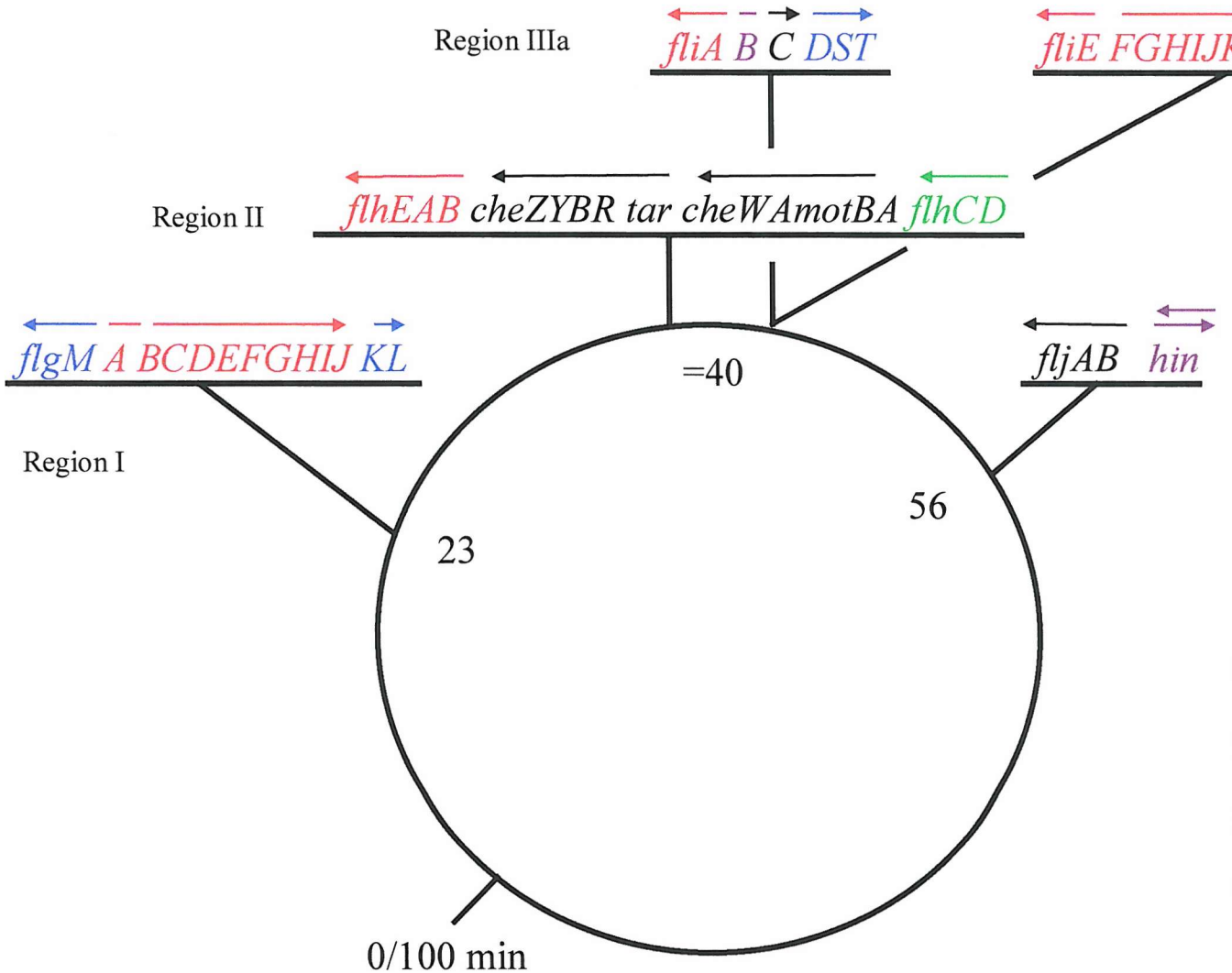
Figure 5.1. (next page). The structure of the *S. typhimurium* flagellum and its position in the cell envelope. The organelle consists of an external filament attached to a hook which in turn is linked to a basal body within the cell envelope. The cores of the filament, basal body and hook protein are hollow permitting transport of molecules by the export apparatus. The basal body is composed of a motor switch complex involved in flagella rotation and chemotaxis, a central rod and three parallel rings. The filament is composed of flagellin and the filament cap. The principal protein components in each sub-structure are given in parentheses. Colour coding refers to the gene class from which each protein product is transcribed (see text and Figure 5.2). Adapted from Macnab (1992).


Filament Cap (FliD)
Flagellin Filament (FliC or FljB)
10 μm
Hook-Filament Junction (FlgK; FlgL)
Hook (FlgE)
L-Ring (FlgH)
P-Ring (FlgI)
Outer Membrane
Basal Body
Rod
MS Ring
(FliF)
Periplasmic Space
Inner Membrane
Switch (FliG; FliM; FliN)
Motor (MotA; MotB)
Export Apparatus

5.1.2. The flagellar regulon and flagellar biosynthesis.

The flagellar genes are located at four main positions on the *E. coli* and *S. typhimurium* chromosomes named regions I, II, IIIa and IIIb respectively (Macnab, 1992). Within each region exist several operons containing between one and nine different genes. The operons constitute the flagellar regulon and are expressed in a hierarchical manner. *S. typhimurium* has an additional region containing the *flj* operon and the *hin* gene involved in flagellin phase variation. The chromosomal organisation of flagellar associated genes in *S. typhimurium* is depicted in Figure 5.2. The five main regions are shown together with their respective operons. The colour coding in Figures 5.1 and 5.2 helps to identify the positions of the structural gene products in the assembled flagellum. It can be seen that the hook and the majority of the basal body components are expressed from chromosomal regions I and IIIb. Filament components are scattered through regions IIIa, I and the 56minute *flj* position. The motor components are expressed from the same region as proteins involved in chemotaxis (CheA CheB, CheR, CheW, CheY and CheZ).

Figure 5.2. (next page). The chromosomal organisation of flagellar associated genes in *S. typhimurium*. The genes are located in four main regions (I, II, IIIa and IIIb) at the approximate map positions depicted. At 56 minutes is a group of genes involved in *Salmonella* flagellin phase variation (see text). Most of the flagellar genes are grouped into operons which are transcribed in the directions shown. The classification of each operon/gene within the flagellar hierarchy is shown in colour (green: class 1; red: class 2; blue: class 3a; black: class 3b). Adapted from Macnab (1992).



Class 1 : master operon

Class 2 : early/mid assembly

Class 3a: late assembly

Class 3b: late assembly

The hierarchical manner in which the flagellar genes are expressed results in the co-ordinated assembly and biogenesis of the flagellum (Macnab, 1992). The genes can be arranged into four classes depending on their position in the regulatory hierarchy. These classes are depicted by colour in Figure 5.2. Where appropriate, structural gene products are displayed in the same colour scheme in Figure 5.1. Class 1 genes (green) constitute the master operon (*flhCD*) and are required for the expression of all other flagellar genes. The FlhC and FlhD proteins form a transcriptional activator that interacts with class 2 (red) and class 3a (blue) gene promoter regions (Macnab, 1992). Class 2 genes encode structural and accessory proteins required for assembly of the hook and basal body components of the flagellum. They also encode FliA (σ^{28}), a sigma factor which regulates class 3a and class 3b (black) gene expression (Macnab, 1992). Class 3a and 3b genes thus have σ^{28} dependent promoters. Class 3a operons have appreciable levels of expression in the absence of FliA, presumably due to FlhCD dependent transcriptional activation. The class 3 genes generally encode proteins involved in the later stages of flagellum biosynthesis and in chemosensory mechanisms. In addition, a regulatory protein, FlgM, is expressed from a class 3a gene. FlgM synthesis is promoted by FlhCD and acts to limit σ^{28} activity (by sequestering it) until the synthesis and positioning of class 2 gene products is complete. Once the class 2 components have been correctly assembled FlgM is secreted, thereby increasing the level of free σ^{28} in the cytosol (Kutsukake, 1994). In this way, synthesis and assembly of late flagellar components cannot occur before the completion of the hook and basal body complex.

The flagellar export apparatus secretes the proteins of the rod, hook and filament in the correct temporal sequence such that they diffuse through the hollow centre of the flagellum to their appropriate assembly sites at the tip of the growing structure. This mechanism of protein export is akin to that of the type III secretion systems. The regulatory hierarchy of the four flagellar gene classes is depicted in Figure 5.3 alongside flagellum biosynthesis. The gene products are colour coded according to the scheme depicted in Figure 5.2.

At the top of the hierarchy are the inducers of the class 1 genes (orange). These include the housekeeping sigma factor σ^{70}, the cAMP/CAP complex (Yanagihara *et al*, 1999)

and the H-NS regulatory protein (Hinton *et al*, 1992; Bertin *et al*, 1994). Glucose exerts catabolite repression on flagellar biosynthesis by lowering cAMP levels. The presence of glucose can indicate a favourable environment where motility and chemotaxis would not be required. H-NS is a DNA binding protein involved in the organisation of the bacterial chromosome by affecting the level of DNA condensation. It is known to affect the expression of several unrelated genes including those of the flagellar master operon. *h-ns* mutants of *E. coli* and *S. typhimurium* are non-motile as a result of a complete lack of flagella (Hinton *et al*, 1992; Bertin *et al*, 1994). The positive control of *flhCD* expression by H-NS occurs via a mechanism that is independent of the cAMP/CAP complex (Bertin *et al*, 1994). In *E. coli*, H-NS negatively regulates the expression of *hdfR*. The *hdfR* gene product binds the *flhCD* promoter, indicating that *hdfR* is a transcriptional regulator of the flagellar master operon (Ko and Park, 2000a). Therefore, perhaps H-NS positively regulates flagellar synthesis by decreasing the levels of HdfR, a protein that may block transcription from the *flhCD* operon. It is believed that a region downstream of the +1 transcriptional start site of the *flhCD* operon is involved in H-NS dependent flagellar synthesis (Soutourina *et al*, 1999).

Interestingly, H-NS has also been implicated in the control of flagellar mediated motility via the binding of FliG, a torque generating rotor protein (Donato and Kawula, 1998). Enhanced binding of H-NS to FliG increased flagellar rotational speed and hypermotility in *E. coli*. *E.coli hns* mutants display reduced motility without a reduction in the intracellular levels of the flagellar motor components, MotA, MotB and FliG (Ko and Park, 2000b). These results suggest that H-NS affects flagellar function as well as biogenesis.

Figure 5.3. (next page). The hierarchical flagellar biosynthetic pathway. Inducing signals (orange) activate transcription of the class 1 genes (green). The FlhC and FlhD proteins are required for the expression of the class 2 (red) and class 3 genes. The class 2 gene product FliA is a sigma factor that directs transcription of the class 3a (blue) and 3b (black) genes. Class 3a genes are expressed in the absence of FliA as they are also under the transcriptional control of the FlhCD complex. The class 3a gene product FlgM represses FliA activity. This repression is removed upon the completed assembly of the hook-basal body complex via secretion of FlgM. This ensures late assembly does not commence until class 2 gene product synthesis and structural organisation is complete.

Inducing Signals

H-NS
cAMP/CAP
σ^{70}

Class 1

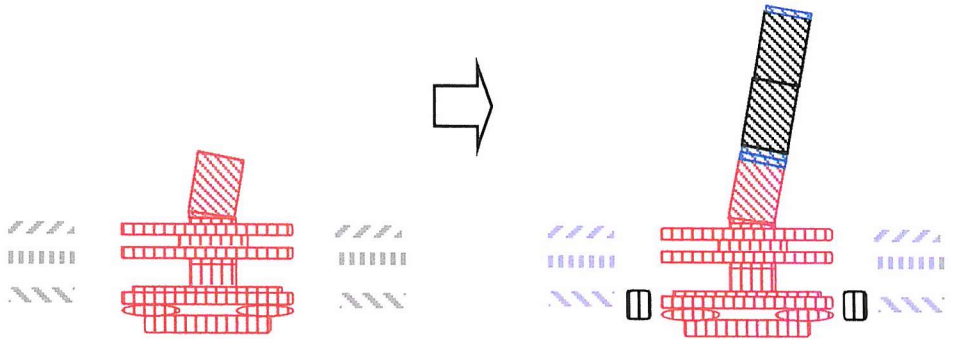
FlhDC

Class 2

FlgA
FlgBCDEFGHIJ
FlhBAE
FliA (σ^{28})
FliE
FliFGHIJK
FliLMNOPQR

Class 3a + Class 3b

FlgM
FlgKL
FliC
FliDST
FljBA
MotAB, CheAW
Tar, CheRBYZ



5.1.3. Phase variation of the flagellar filament.

S. typhimurium expresses either *fliC* or *fljB* to form phase-1 or phase-2 flagellin respectively (Macnab, 1992). The type of flagellin expressed is denoted by a phase variation mechanism. Any one *Salmonella* cell expresses only one type of flagellin at a time. In a growing population of cells, spontaneous phase switching ensures a mixture of the two flagellin phenotypes. The upstream control region of the *fljAB* operon is subject to site-specific inversion. This is catalysed by the product of the *hin* gene, a recombinase. As shown in Figure 5.2, the *hin* gene is situated within the upstream invertible region. In the 5' to 3' orientation, the region generates the promoter for the *fljAB* operon, resulting in the synthesis of phase-2 flagellin and FljB. FljB acts as a repressor of *fliC* expression, therefore, no phase-1 flagellin is produced. In the 3' to 5' orientation, the *fljAB* promoter is disrupted. Therefore, phase-2 flagellin and the *fliC* repressor protein are not expressed. This enables the synthesis of phase-1 flagellin. Flagellin phase variation is thought to benefit *Salmonella* during pathogenesis. The two flagellin phases constitute two antigenic markers. Thus, if host antibody is targeted against one antigen, the population of cells expressing the second antigen will remain resistant, ensuring survival. This may help to extend the period of infection.

5.1.4. Chemotaxis.

When flagella filaments rotate counter clockwise (CCW), they form into a bundle which propels the cell forward. This results in smooth uni-directional swimming. The clockwise (CW) rotation of flagella results in disruption of the filament bundle and a tumbling swimming motion. Bacterial cells suppress tumbling to head up concentration gradients of attractants or down gradients of repellents. Concentration gradients are sensed using a memory mechanism. The amount of a given compound at a point in time can be measured by percent receptor occupancy. This can be compared to its previous concentration determined by the level of receptor methylation (Manson *et al*, 1998).

Transmembrane receptor proteins function in chemotaxis containing cytoplasmic methylatable glutamate residues. The receptors are known as methyl-accepting chemotaxis proteins (MCPs). MCPs bind ligands directly or via periplasmic binding

proteins such as galactose binding protein. The flagellar switch, involved in determining the direction of flagella rotation is linked to the MCps via four proteins (CheA, CheW, CheY and CheZ). A protein complex consisting of two CheA and two CheW molecules associates with the cytoplasmic domains of MCP dimers (Manson *et al*, 1998). This conformation favours autophosphorylation of CheA resulting in the transfer of phosphate to cytoplasmic CheY. Phosphorylated CheY (CheY-P) binds to FliM in the flagellar switch complex to cause CW rotation. Cytoplasmic CheZ prevents the accumulation of CheY-P by accelerating the decay of the unstable phosphate group.

The binding of an attractant to the MCP receptor induces a conformational change which suppresses CheA activity. The resultant fall in CheY-P levels produces a reduction in tumbling frequency (Manson *et al*, 1998). Thus, cells increase smooth swimming run lengths in areas of attractant. To continually respond to increasing levels of attractant, methylation is necessary. Two additional cytoplasmic proteins, CheR and CheB are responsible for this mechanism. CheR is a methyltransferase that methylates glutamate residues in the MCP cytoplasmic domains. CheB is a methylesterase that removes phosphate groups from the MCPs (Manson *et al*, 1998). The protein is activated by the transfer of a phosphate group from CheA. Under steady state conditions, methyl addition by CheR balances methyl removal by CheB-P resulting in an intermediate level of MCP methylation. If CheA activity is inhibited by the binding of attractant, CheB-P levels fall slower than those of CheY-P which are repressed by CheZ. A reduction in CheB-P results in increased MCP methylation. Increased methylation results in the ability of the MCP to activate CheA, counteracting the attractant induced inhibition. As CheA activity rises, the intracellular CheY-P concentration returns to steady state conditions and flagellar rotation returns to its prestimulus Cw to CCW switching frequency.

The chemotaxis methylation system is involved in the migration of *S. typhimurium* and *E. coli* away from acid pH (pH 5.5) and membrane permeant weak acids (Kihara and Macnab, 1981). The acid conditions increase the frequency of CW flagella rotation and thus tumbling. Taxis away from acid pH is thought to be mediated by a pH sensitive component located in the cytoplasm or on the cytoplasmic side of the membrane. However, the precise mechanism used is unknown (Kihara and Macnab, 1981).

5.1.5. Flagella and pathogenicity.

Flagellar gene expression has been linked with the pathogenic properties of bacteria. In particular, it is essential for the pathogenesis of *Campylobacter jejuni* and *Helicobacter pylori* (Ottemann and Miller, 1997). *C. jejuni*, a cause of diarrhoea, is defective in intestinal colonisation when lacking flagella. This is considered to be the result of a lack of motility. When human volunteers were fed mixtures containing equal amounts of motile and non-motile *C. jejuni*, only motile bacteria were recovered from stool samples. *H. pylori* is associated with peptic ulcers, gastritis and gastric cancer. Insertional inactivation of the flagellin filaments in this micro-organism results in an inability to colonise the host. The lack of pathogenesis in both *C. jejuni* and *H. pylori* may involve a deficiency in chemotaxis. A motile *C. jejuni* variant expressing flagella was non-pathogenic if its chemotaxis system was disabled. The gram negative pathogen *Yersinia enterocolitica* uses the flagellar apparatus to export a phospholipase that is required for pathogenesis via a type III secretion mechanism (Young *et al*, 1999). The structure of the basal body of the flagellum and its role in protein export is similar to type III secretion systems (Kubori *et al*, 1998), which are also associated with pathogenesis.

The direction of flagellar rotation has been associated with pathogenicity in *S. typhimurium*. Smooth swimming Che$^-$ mutants (*cheA*, *cheW*, *cheR*, and *cheY*) were found to possess increased invasiveness for cultured mammalian cells. In contrast, a "tumbly" *cheB* mutant and Mot$^-$ strain were found to have decreased levels of tissue culture invasiveness (Jones *et al*, 1992).

5.1.6. Flagellar expression and the ATR of *S. typhimurium*.

This chapter identifies components of the *S. typhimurium* flagellar system as acid shock proteins and characterises their mode of regulation by low pH. The PhoPQ two component signal transduction system appears to transcriptionally down-regulate the flagellar genes at the level of the *flhCD* master operon. This is of interest as it establishes for the first time that the PhoPQ system, implicated in virulence, controls the motility of this pathogen. The role of other global regulators (RpoS and H-NS) in flagella synthesis in low pH conditions is also described. In addition, the effect of a

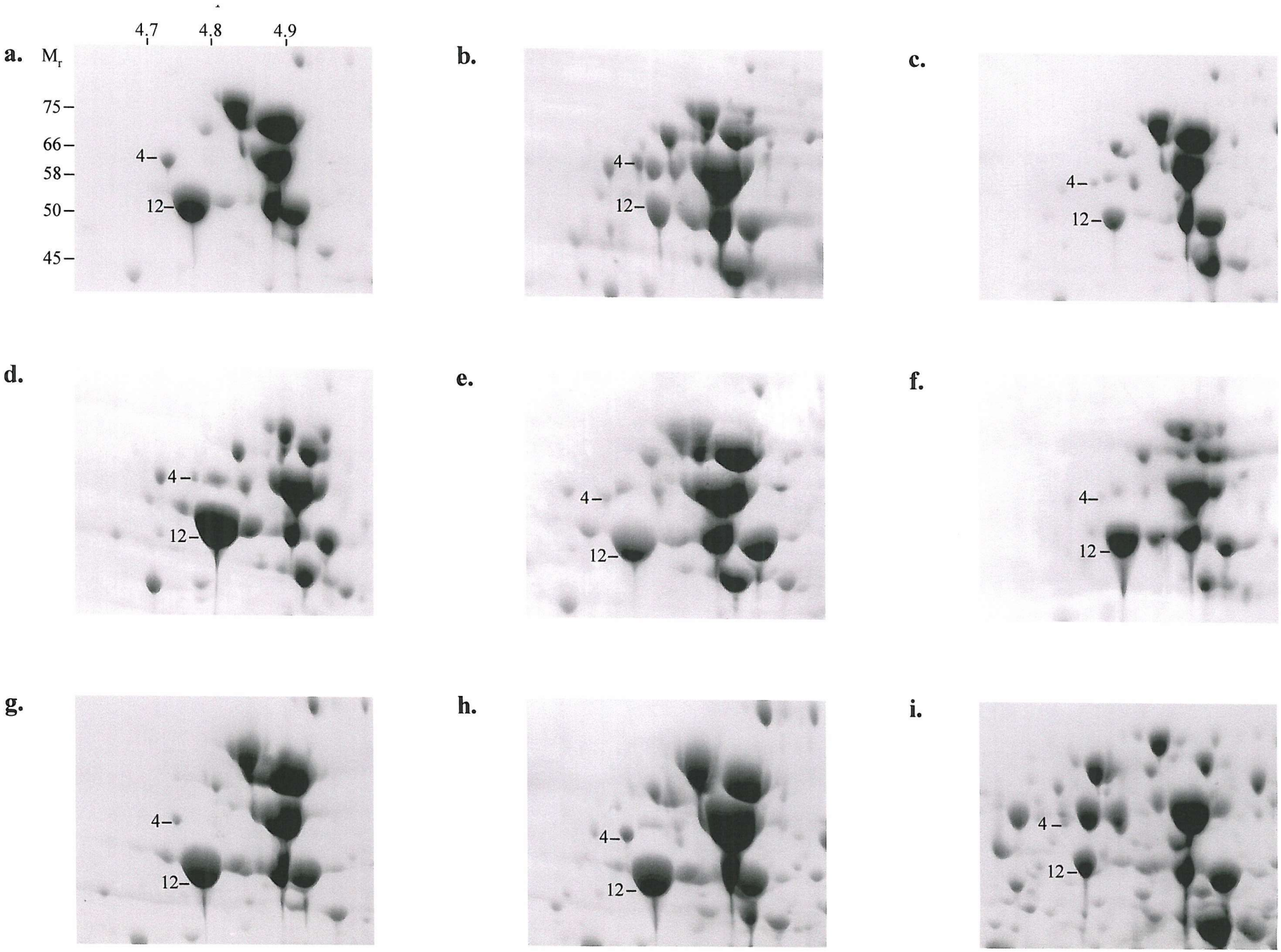
flagellar *flhD* gene (class 1) gene deletion on the ATR survival kinetics of *Salmonella* is investigated.

5.2. Phase-1 and Phase-2 Flagellin Are ASPs that are Down-regulated in Exponential Phase *S. typhimurium* Cells During the ATR.

As detailed in Chapter 4, a proteomic approach was used to detect proteins that are induced or repressed in mid-exponential phase *S. typhimurium* cells as a consequence of the ATR. The pattern of protein expression in *Salmonella* cells induced to exhibit an optimal ATR was examined by 2D-PAGE and compared with that of cells grown at pH 7.2 (Figure 5.4a and b). One of the most notable differences was the reduced expression of two acidic proteins (spot numbers 4 and 12 in Figure 5.4) of 58 and 49 kDa after acid adaptation and exposure to pH 3.0.

The identities of proteins 4 and 12 were determined by tryptic mass fingerprinting in conjunction with nanospray mass spectrometry. Database searching using the tryptic peptide masses derived from each protein showed good matches to phase-2 (FljB) and phase-1 (FliC) flagellin respectively (Table 4.1). The predicted pI and M_r values for both flagellin proteins were in close agreement with the experimental values determined from 2D-gels.

Figure 5.4. (next page). Sections of 2D-gels displaying the expression levels of phase-1 and phase-2 flagellin (spot numbers 12 and 4 respectively) by *S. typhimurium* SL1344 under different conditions. Spot numbers are consistent with the scheme used in Table 4.1. The *pI* and M_r ranges of each gel section are equivalent. Gels were loaded with whole cell lysates from bacteria exposed to the following conditions (all cells are wild type unless shown): **a.** Unstressed cells; **b.** Acid adapted cells exposed to pH 3.0; **c.** *pho-24 cells*; **d.** *rpoS::kan* cells; **e.** H_2O_2 stressed cells; **f.** *ΔbipA* cells; **g.** Osmotically stressed cells; **h.** Cells grown on minimal medium; **i.** Cells grown in Mg^{2+} depleted minimal medium.


4.7
4.8
4.9
a.
M_r
75
66
58
50
45
4
12
b.
c.
d.
e.
f.
g.
h.
i.

The synthesis of FliC and FljB has not been previously reported as pH regulated. Therefore, to confirm this finding, immunoblots of whole cell lysates from log phase cells grown under different pH conditions were probed with anti-flagellin antibodies. The expression levels of phase-1 flagellin in cells grown at pH 5.0 and pH 7.2 were not detectably different. However, phase-1 flagellin was markedly decreased in samples from acid adapted cells exposed to pH 3.0 (Figure 5.5a). Similar results were found when the samples were probed with antibodies specific for phase-2 flagellin (data not shown). Substantiating these results, quantification of phase-1 and phase-2 flagellin levels by densitometric analysis of 2D-gels also indicated their reduced expression in acid adapted cells grown at pH 3.0 (Figure 5.6). These results suggest that flagellin is either down-regulated or lost from cells during the exponential phase acid tolerance response. The data also indicate that, if repressed, flagellin is likely to constitute an ASP as opposed to a component of the inducible pH homeostasis mechanism.

5.3. Flagellin is Repressed During the ATR Through the PhoPQ System.

As detailed previously, the PhoPQ two-component regulatory system, which is activated by low pH and by depletion of extracellular Mg^{2+}, controls the expression of a subset of ASPs during the exponential phase ATR (Bearson *et al*, 1998). The levels of phase-1 and phase-2 flagellin in a *S. typhimurium pho-24* mutant, where PhoQ is constitutively expressed, were thus examined. The concentrations of FliC and FljB in log phase wild type cells grown in normal or Mg^{2+} depleted minimal media were also compared. In both experiments, a significant decrease in phase-1 and phase-2 flagellin levels was seen upon activation of the PhoPQ system (Figure 5.4a, c, h and i; Figure 5.6).

The alternative sigma factor, RpoS also regulates a subset of ASPs during the exponential phase ATR (Small *et al*, 1994; Lee *et al*, 1995). Therefore, the amounts of phase-1 and phase-2 flagellin expressed in a *rpoS* null mutant (*rpoS::kan* cells) were examined. A decrease in the expression of both flagellin phases was identified (Figure 5.4d; Figure 5.6). In contrast with the PhoPQ system, it appears that RpoS is therefore normally required for their high level expression.

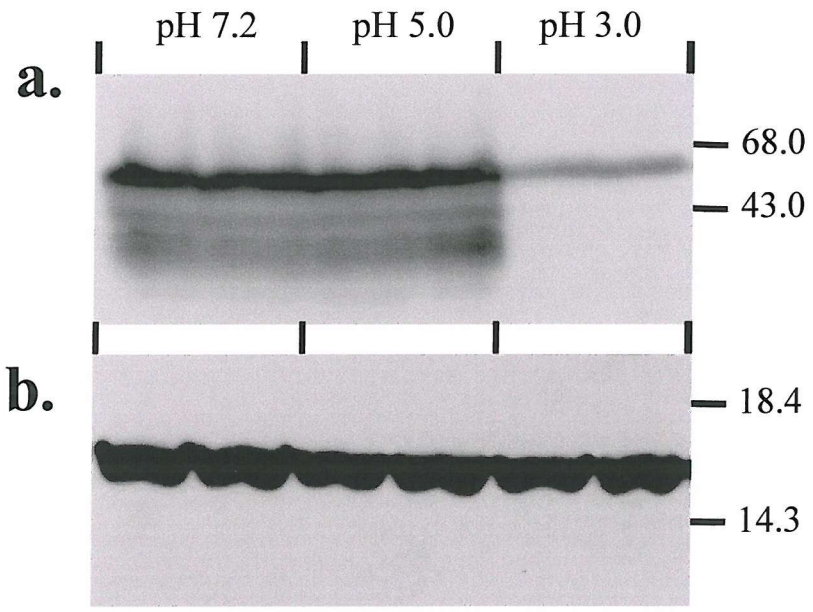


Figure 5.5. Immunoblots of whole cell lysates showing levels of: **a.** Phase-1 flagellin, and **b.** H-NS in *S. typhimurium* SL1344 cells. Cells were adapted to pH 7.2, pH 5.0 or pH 5.0 followed by exposure to pH 3.0. In **a.** the top band corresponds to intact flagellin and the lower bands are breakdown products that also cross react with the monoclonal antibody. Each lane contained an equal amount of sample.

The BipA GTPase is known to negatively regulate flagellin levels in EPEC cells (Farris *et al*, 1998). The amounts of the two proteins expressed in an isogenic *bipA* null mutant of *S. typhimurium* were therefore compared with those in the wild type strain. However, no differences in phase-1 or phase-2 expression were seen (Figure 5.4a and f; Figure 5.6).

The DNA-binding protein, H-NS is required for the optimal expression of flagellin in *S. typhimurium* and *E. coli* (Hinton *et al*, 1992; Bertin *et al*, 1994). It regulates flagellar biosynthesis at the level of the master operon (Soutourina *et al*, 1999; Ko and Park, 2000a). H-NS levels were therefore examined in acid adapted exponential phase *S. typhimurium* cells. Immunoblots of whole cell lysates from bacteria grown under different pH conditions were probed with anti-H-NS antibodies. The expression levels of H-NS in cells grown at pH 7.2, pH 5.0 and in acid adapted cells grown at pH 3.0 were not detectably different (Figure 5.5.b). Therefore, H-NS expression is unaffected by low pH. This suggests that changes in H-NS concentration are not responsible for the observed decrease in flagellin synthesis during the ATR. However, H-NS can regulate gene expression without changes in cellular concentration. Instead, H-NS activity can be modulated by changes in oligomeric or heteroligomeric state (Smyth *et al*, 2000). Thus, further studies are required to identify any changes in H-NS activity during the ATR.

In summary, these results indicate that flagellin is negatively regulated by the PhoPQ two-component regulatory system and positively regulated by RpoS. In contrast, the BipA GTPase and H-NS do not appear to play a significant role in the regulation of flagellin levels in *S. typhimurium*.

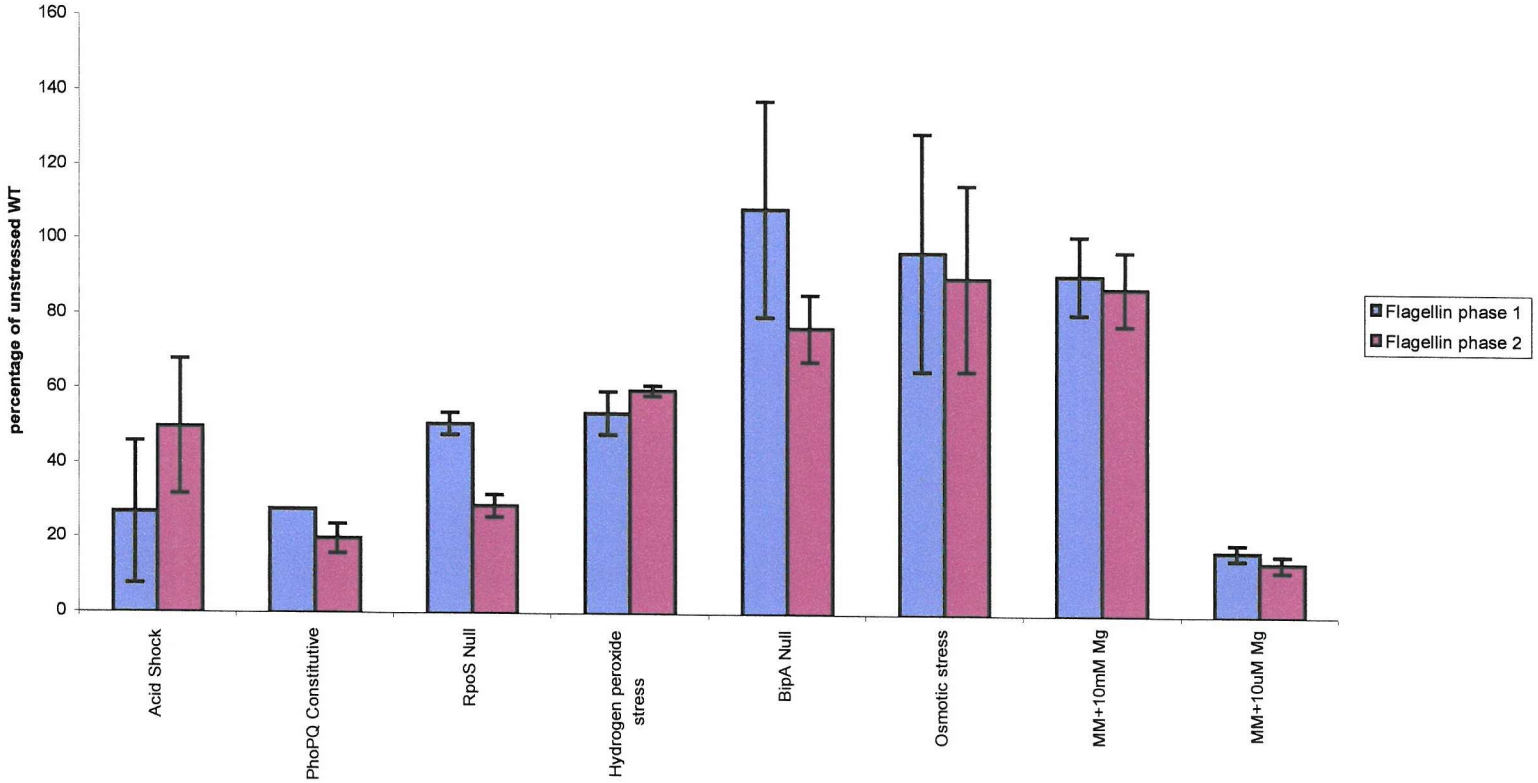


Figure 5.6. Relative amounts of phase-1 and phase-2 flagellin grown under the conditions shown in Figure 5.4. Expression level is depicted as a percentage of that in unstressed wild type cells, calculated using the Phoretix 2D Software v.5.1.

5.4. Oxidative Stress, but not Osmotic Stress Also Represses Flagellin.

As discussed in Chapter 1, a number of proteins are similarly regulated between the various stress responses. This is perhaps one of the mechanisms by which induction of acid tolerance can provoke cross protection to other stresses. The ATR cross protects against oxidative damage and high osmolarity (Leyer and Johnson, 1993). Therefore, the expression levels of flagellin in *Salmonella* cells treated with H_2O_2 or NaCl were investigated to determine if they were also affected by oxidative stress and high osmolarity. The levels of phase-1 and phase-2 flagellin in H_2O_2 adapted cells decreased to approximately 60% of that in uninduced cells (Figure 5.4a and e; Figure 5.6). In contrast, osmotic stress had a minimal effect on flagellin expression (Figure 5.4a and g; Figure 5.6).

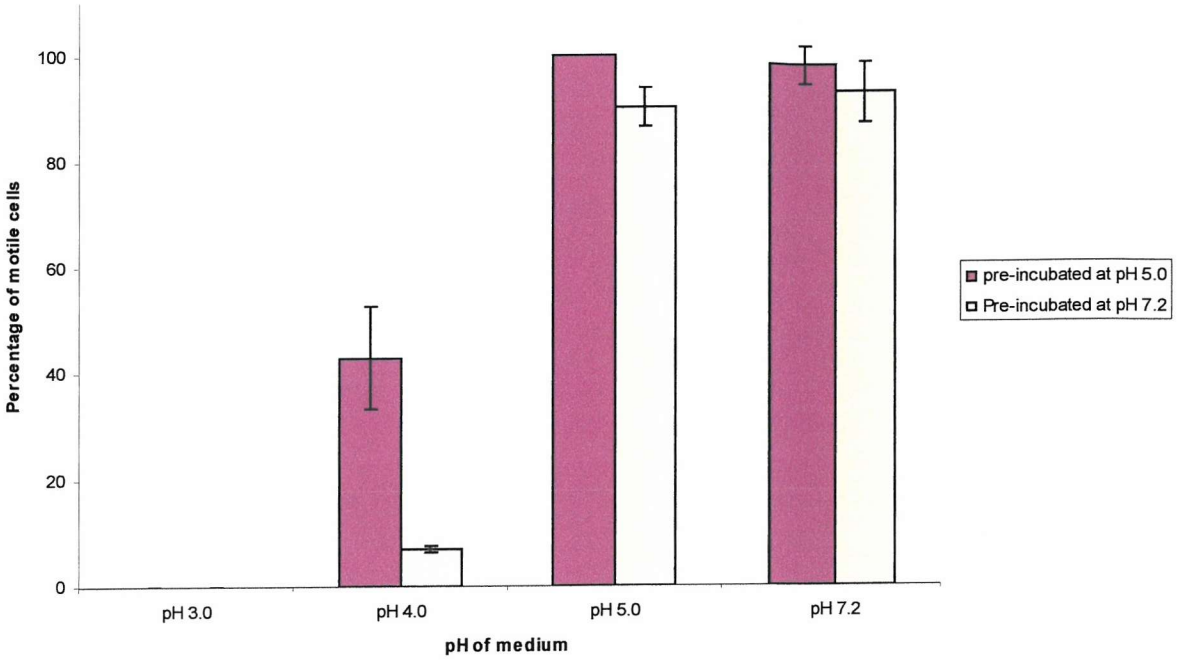
5.5. The ATR and the PhoPQ System Also Regulate Cell Motility.

A flagellin minus phenotype results in a loss of motility (Ikeda *et al*, 1983). Thus, it was predicted that cells undergoing an ATR should be markedly less motile. To examine this idea, the motility of *S. typhimurium* cells was measured at different pHs. As expected, *Salmonella* cells became progressively less motile as the pH was lowered and showed a non-motile phenotype at pH 3.0. This was true of cells with and without prior acid adaptation at pH 5.0. However, it is expected that non acid adapted cells exposed to pH 3.0 were non-motile due to a lack of viability. The degree of motility at pH 4.0 was dependent on whether an ATR had been induced. Thus, cells previously adapted to pH 5.0 were four-fold more motile than cells adapted to pH 7.2 medium (Figure 5.7a). Motility at the different pHs corresponds exactly with flagellin expression under the same conditions (figure 5.5a). This suggests that an ATR induced down-regulation of flagellin biosynthesis results in a lack of cell motility.

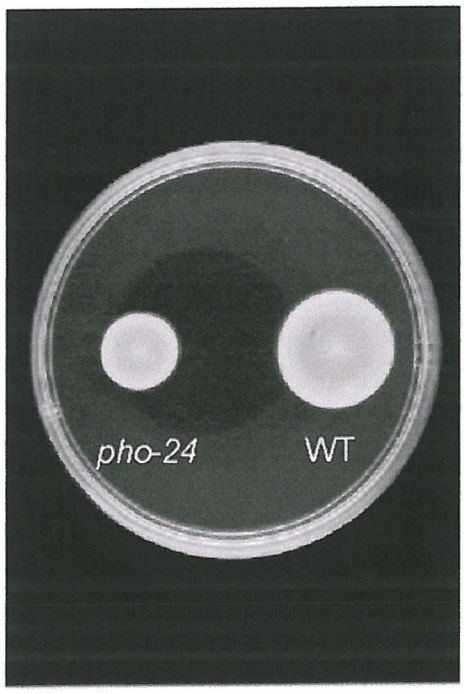
The reduced motility at low pH can be attributed to activation of the PhoPQ system, as the *S. typhimurium pho-24* mutant was significantly less motile than the wild type strain on swarm plates (Figure 5.7b).

Figure 5.7. (next page). PhoPQ mediated repression of cell motility. **a.** Effect of decreased pH on the motility of acid adapted and non-adapted *S. typhimurium* cells. SL1344 cells were grown to mid-exponential phase in pH 7.2 LB medium prior to pre-incubation for 60 minutes in fresh medium at either pH 7.2 or pH 5.0. The cells were then transferred to media adjusted to the pHs shown and cell motility determined after 5 minutes using phase contrast microscopy. **b.** Swarm plate assay to measure motility of SL1344 and its *pho-24* derivative. Swarm plates were prepared using N-minimal media containing 1mM Mg^{2+}.

a.


Percentage of motile cells
100
80
60
40
20
0
pH 3.0
pH 4.0
pH 5.0
pH 7.2
pH of medium
pre-incubated at pH 5.0
Pre-incubated at pH 7.2

b.


pho-24
WT

5.6. The ATR and PhoPQ Operate at the Level of the Class 1 Flagellar Genes, Whereas RpoS Regulates Class 2 and Class 3 genes.

The assembly of the bacterial flagellum is the consequence of a complex hierarchical expression of three classes of flagellar genes (see section 5.1.2.). The flagellin genes (FliC and FljB) are members of the third class. Their synthesis is thus dependent on the expression of both the class 1 and class 2 genes. Therefore, the ATR and PhoPQ may negatively regulate flagellin expression at any level in the flagellar biosynthetic pathway. Lac fusions to different classes of flagellar genes were therefore used to determine the point in the assembly pathway that is regulated by the Atr-PhoPQ system. Measurement of β-galactosidase activity showed the transcription of all classes of flagellar genes was decreased in ATR induced cells and also in the *pho-24* mutant relative to the parent strain (Figure 5.8a-c). Importantly, transcriptional repression was specific, as cells containing a lac fusion to a control promoter did not show a similar decrease in β-galactosidase activity under the same conditions (data not shown). These results suggest that activation of the PhoPQ system directly or indirectly represses the expression of the *flhCD* (class 1) master operon.

The optimal expression of flagellin was found to be dependent on RpoS (see section 5.3). Therefore, flagellar gene expression was also investigated in an *rpoS* null mutant. In corroboration with the proteomic analysis (Figure 5.4d; Figure 5.6), it was found that the class 1 gene *fliC* was repressed in the mutant. Transcription levels of the class 2 genes tested were also reduced, but expression of the class 1 gene, *flhD* was not significantly affected (Figure 5.8d and e). These results suggest that RpoS operates lower down the flagellar gene hierarchy than PhoPQ, although the precise location remains to be determined.

In summary, the PhoPQ system directly or indirectly represses flagellar gene expression at the level of the *flhCD* master operon. In contrast, RpoS stimulates expression of the class 2 and 3 flagellar genes and acts after transcription of the *flhCD* genes.

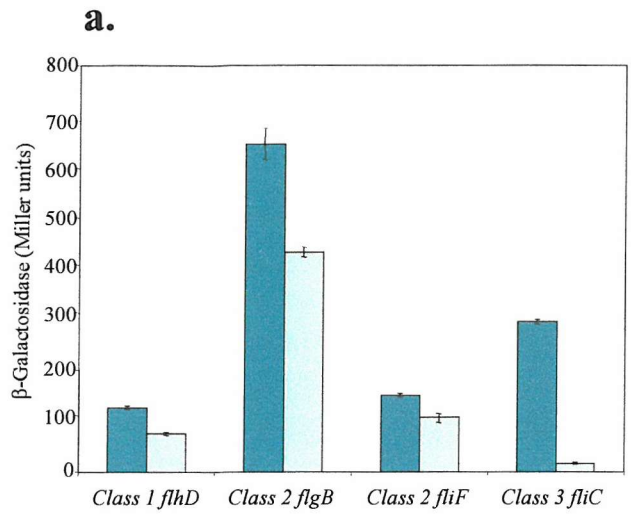
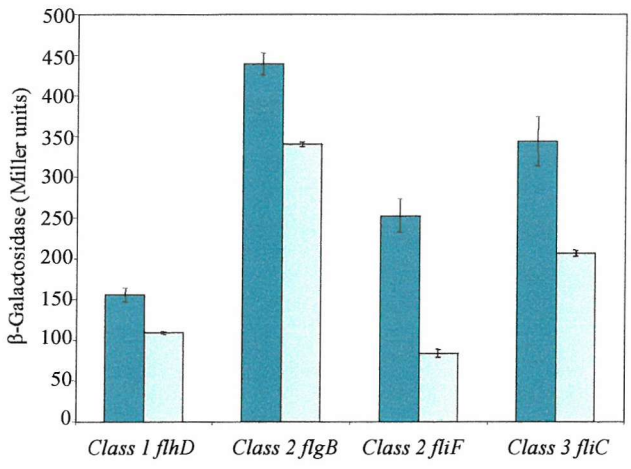
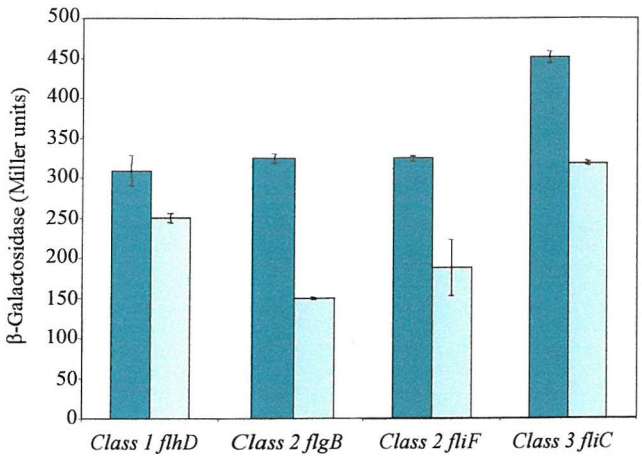
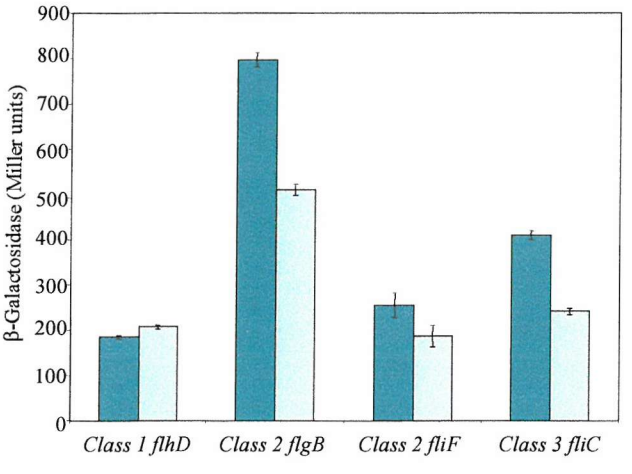
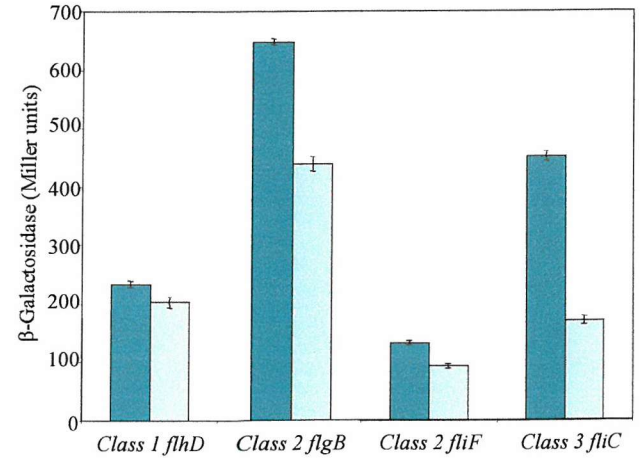


Figure 5.8. Transcription of the representative flagellar genes in wild type, pH 3.0 treated, *pho-24* and *rpoS::kan* mutants of *S. typhimurium* SL1344. In panel **a** the dark and lighter bars correspond to non-acid adapted and acid adapted cells respectively. In panels **b – e**, the dark and lighter bars correspond to wild type and mutant cells respectively. **a.** Lac fusion derivatives of mid-exponential phase SL1344 cells; **b.** Lac fusion derivatives of mid-exponential phase SL1344 *pho-24* cells; **c.** Lac fusion derivatives of stationary phase SL1344 *pho-24* cells; **d.** Lac fusion derivatives of mid-exponential phase SL1344 *rpoS::kan* cells; **e.** Lac fusion derivatives of stationary phase SL1344 *rpoS::kan* cells.









5.7. The ATR Survival Kinetics of a *S. typhimurium flhD* Mutant Strain are Identical to Those of the Wild Type Strain.

The ATR of *S. typhimurium* represses flagellar transcription at the level of the master operon. The absence of flagella synthesis in low pH conditions may thus confer a survival advantage on *Salmonella* cells. The ATR survival kinetics of a *S. typhimurium flhD* mutant were therefore investigated to identify any signs of constitutive acid tolerance. However, when compared with those of the wild type *Salmonella* strain (Figure 5.9), no differences in survival at pH 3.0 were seen. *flhD* minus cells previously adapted to pH 7.2 were, as wild type, unable to tolerate extreme acid pH after 15 minutes. Additionally, wild type and *flhD* mutant cells adapted to pH 5.0 were both capable of tolerating pH 3.0 conditions for at least 60 minutes.

Therefore, unlike for example pyruvate dehydrogenase (Foster and Hall, 1991), the repression of flagella synthesis does not impart constitutive acid tolerance upon *S. typhimurium*. It is more likely that flagella down-regulation constitutes a fraction of a larger acid tolerance mechanism. Hence, inducing only a small part of this mechanism would have very little effect. For example, it is probable that many proteins including the flagellar apparatus are differentially regulated during the ATR to reduce proton influx. The deletion of flagella alone would therefore have very little impact on this process.

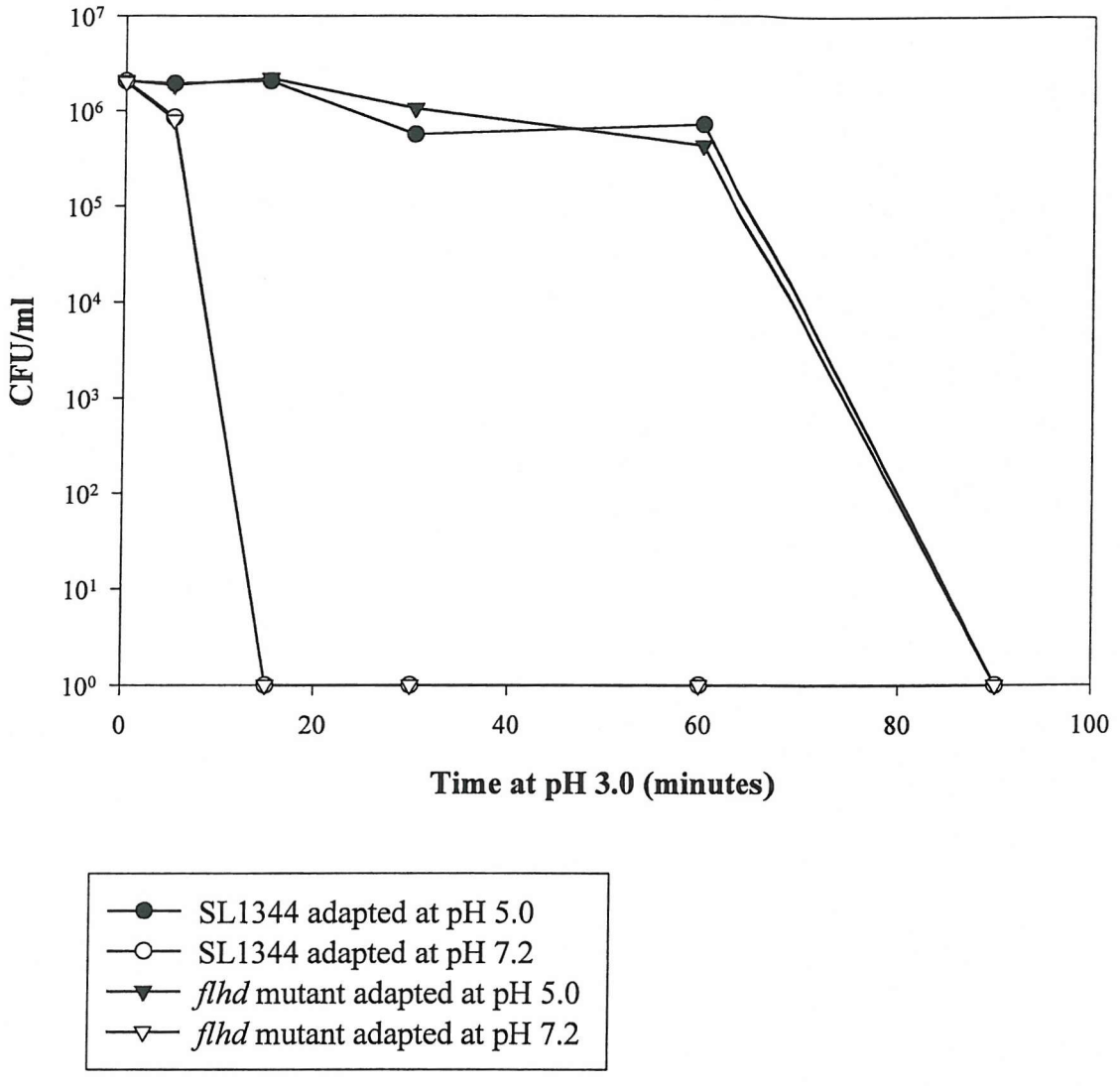


Figure 5.9. *S. typhimurium* SL1344 wild type and *flhD* mutant cells were adapted to inorganic acid at pH 5.0 or to pH 7.2 for 60 minutes prior to extreme inorganic acid exposure for various lengths of time at pH 3.0. Surviving culturable cells were counted after plating on LB agar (pH 7.2). Data for WT SL1344 are taken from Figure 3.5.

5.8 Discussion.

This chapter has described the negative-regulation of the *S. typhimurium* flagellar apparatus as a component of the ATR. The low pH associated repression of flagellar gene transcription is mediated via the PhoPQ two component regulatory system. This appears to be the first time that a two component signal transduction system has been linked with the expression of flagella. However, as described in section 5.1.4, this form of regulation is utilised in flagella mediated chemotaxis.

Induction of an ATR in *Salmonella* resulted in a significant repression of phase-1 and phase-2 flagellin synthesis. Further analysis indicated that flagellin expression was specifically down-regulated at pH 3.0 and not at pH 5.0 during the ATR. This range of pH regulation suggests flagellin constitutes an ASP rather than a component of inducible pH homeostasis. This is because the latter mechanism is operational at acidic pH ranges much closer to neutral.

As a known regulator of ASP expression (Bearson *et al*, 1998), the PhoPQ system was a possible candidate for the mediation of flagellin repression during the ATR. In keeping with this idea, activation of the PhoPQ system, either by use of a *pho-24* mutation or by growth of cells in Mg^{2+} depleted media, resulted in the repression of phase-1 and phase-2 flagellin synthesis. Further analysis also indicated that both the ATR and an activated PhoPQ system reduced flagella mediated cell motility.

The hierarchical regulation of flagellar biosynthesis and the non-motile phenotype suggested that the ATR and thus, PhoPQ, could be operating at the level of the *flhCD* master operon. Indeed, transcription of the class 1 genes was found to be significantly decreased in ATR positive and PhoPQ activated cells, resulting in the reduced expression of the class 2 and class 3 flagellar genes. As class 3 genes, *fliC* and *fljB* acted as indicators of this mechanism of flagellar gene repression. Collectively, these findings indicate that the PhoPQ system, which is activated on exposure to low pH, down-regulates flagellar gene expression during the ATR at the level of the master operon. However, it remains to be seen if repression is due to a direct interaction between the PhoP response regulator and the *flhCD* operon, or whether it operates indirectly. For example, Bearson *et al* (1998) have presented evidence to suggest that

PhoPQ may function to regulate ASP expression via a second two-component system, PmrAB.

Recent evidence has suggested that PhoQ can sense pH and Mg^{2+} ions independently (Bearson *et al*, 1998). Thus, it is possible that even greater repression of flagellar gene expression occurs when both low pH and sub-micromolar concentrations of Mg^{2+} ions are present. Such conditions may occur when *Salmonella* invades host cells as *in vivo* measurements suggest that *Salmonella* containing vacuoles are acidic and low in Mg^{2+} ions (Garcia del Portillo *et al*, 1992). An example of such an environment is present within the macrophage. As oxidative stress levels are also increased here, it is possible that a degree of co-regulation occurs between the ATR, the PhoPQ system and the oxidative stress response. In keeping with this hypothesis, phase-1 and phase-2 flagellin levels were also found to decrease significantly in *Salmonella* cells during the OSR. This important link between pathogenicity and the bacterial stress responses is also demonstrated by the co-regulation of glycerophosphodiester phosphodiesterase and D-galactose binding protein expression as detailed in Chapter 4.

Levels of the alternative sigma factor, RpoS, have been shown to increase during the exponential phase ATR of *S. typhimurium* (Lee *et al*, 1995). It was therefore of interest to examine its role (if any) in the repression of flagellar biosynthesis in acid conditions. Surprisingly, in contrast to the PhoPQ mediated repression effect, RpoS was found to stimulate flagella expression: reduced amounts of flagellin were seen in an *rpoS::kan* mutant. In addition, RpoS was found to stimulate transcription of both the class 2 and class 3 flagellar genes; class 1 gene expression was unaffected. A threshold amount of RpoS may be required for the optimal expression of class 2 and class 3 genes, whose transcription levels in mid-exponential phase and stationary phase were similar. The threshold RpoS concentration must be sufficiently low to allow the optimal expression of the flagellar genes in exponential phase cells, which contain relatively low levels of this sigma factor (Hengge-Aronis, 1996). The requirement for a threshold level of RpoS could explain why this global regulator does not counteract the effect of the PhoPQ system in acid conditions.

In contrast to the results described here, previous reports have indicated that RpoS does not affect the motility of *S. typhimurium* (Kutsukake *et al*, 1997). However, these

earlier studies used *Salmonella* strains derived from an LT2 parent. As described in Chapter 1, LT2 strains are known to contain a mutated *rpoS* allele (Wilmes-Riesenberg *et al*, 1997). The SL1344 strains used in the present study have a wild type *rpoS* gene (Coynault *et al*, 1996).

The DNA binding protein H-NS is required for optimal flagellar biosynthesis and acts at the level of the *flhCD* master operon (Soutourina *et al*, 1999; Ko and Park, 2000a). However, the results presented here indicate that a change in H-NS concentration is not involved in the repression of flagella during the ATR. However, H-NS activity could be modulated by changes in oligomeric or heteroligomeric state during acid stress (Smyth *et al*, 2000). Further work is therefore required to elucidate the role of H-NS in the repression of flagellar biosynthesis during the ATR. The fact that *fliC* expression is H-NS dependent (Soutourina *et al*, 1999), suggests the DNA-binding protein may have a role to play.

When a *Salmonella* cell encounters a mildly acidic environment, it can respond by moving back into more neutral pH conditions via negative chemotaxis (Kihara and Macnab, 1981), or it can initiate an acid tolerance response. It appears that in extremes of acid pH, a chemotactic response is not employed and instead, the induction of an ATR leads to down-regulation of flagella synthesis and a lack of cell motility. There are a number of possible explanations for this strategy. Firstly, motility would not confer an advantage to *Salmonella* in the specific areas in which it encounters extremely low pH. In the closed environments of both the macrophage and the stomach, movement would not propel the bacteria towards areas of increased pH. It is also of note that the bacteria would be moved through the stomach via host digestion mechanisms before the time at which acid adapted *Salmonella* are completely killed off by low pH (90 minutes – see Chapter 3). Secondly, flagella would serve no purpose in very low pH environments (pH $\leq$ 3.0), as depolymerisation of the filament would render bacterial cells immobile in these conditions (Macnab, 1996). Thirdly, flagella down-regulation may be employed to save or divert energy for use in temporarily more important survival mechanisms. The synthesis of a single flagellum consumes a significant amount of ATP: approximately 2% of the total biosynthetic energy expenditure of the cell in unstressed conditions (Macnab, 1996). This could increase

during acid stress. Therefore, the continued synthesis of flagella in extreme acid conditions may deplete ATP stocks that could otherwise be used to elicit acid tolerance.

A fourth consequence of reduced flagella expression would be a decrease in proton influx. The flagellum requires a flow of protons into the cell through the motor complex to power rotation. However, the number of hydrogen ions entering via this route is relatively small (a cell with eight flagella would require an influx of 5.7×10^7 protons per minute assuming roughly 1,200 ions enter per revolution at a rotational frequency of 100Hz (Macnab, 1996)). Therefore, if this method of acid tolerance is employed, it would probably be in conjunction with other mechanisms of reduced proton influx. For example, the increased activity (in reverse) of the Mg^{2+} dependent H^+ translocating ATPase during the ATR is thought to have this effect (Foster and Hall, 1991). Many mechanisms must be utilised to slow the inward current of hydrogen ions as studies have shown this process to be significantly reduced in ATR positive *Salmonella* cells (Foster and Hall, 1991). If flagellar gene regulation constitutes only a small part of this system, the fact that a reduction in flagellar transcription alone affords no survival advantage to cells in acid conditions (Figure 5.9) might be explained.

There is increasing evidence linking stress survival mechanisms, signal transduction systems and pathogenesis (see Chapter 1). Cell motility and flagellar biosynthesis is also essential for pathogenesis (Ottemann and Miller, 1997). The present proteomic studies have revealed a connection between these properties. A link between flagella mediated cell motility, the virulence associated PhoPQ regulatory system and the oxidative and acid stress responses has been identified in *Salmonella*. It is probable that these interlinked mechanisms have resulted from the bacterium's need to evolve processes enabling the induction of many survival responses in specific environmental niches.

CHAPTER 6.

THE ATR INDUCES PhoPQ DEPENDENT CROSS PROTECTION AGAINST OXIDATIVE STRESS IN EXPONENTIAL PHASE *S. typhimurium.*

CHAPTER 6. THE ATR INDUCES PhoPQ DEPENDENT CROSS PROTECTION AGAINST OXIDATIVE STRESS IN EXPONENTIAL PHASE *S. typhimurium.*

6.1. Introduction.

As an intracellular pathogen, *S. typhimurium* has to simultaneously contend with potentially harmful environmental conditions and regulate mechanisms required for host-bacterial interactions. Therefore, perhaps in the interests of conserving energy, the bacteria have evolved the ability to co-regulate stress responses with mechanisms of infection that are required in identical host environments. The PhoPQ system constitutes a global regulator required for pathogenesis (Groisman and Saier, 1990; Garcia Vescovi *et al*, 1994) that typifies this link.

The PhoPQ system is involved in the regulation of invasion of epithelial cells (Bajaj *et al*, 1996) and is required for intramacrophage survival (Fields *et al*, 1986; Ochman *et al*, 1996; Shea *et al*, 1996). It also controls the expression of a subset of ASPs as a regulon of the ATR (Bearson *et al*, 1998). As has been described in Chapter 1, the HilA protein is responsible for the positive regulation of SPI-1 (Bajaj *et al*, 1996), SPI-4 and SPI-5 (Ahmer *et al*, 1999), all of which are required for the intestinal stage of disease. *hilA* is repressed by the PhoPQ system (Bajaj *et al*, 1996), which detects and is activated by conditions of low pH and depleted Mg^{2+} found within the macrophage. Therefore, *Salmonella* avoids the wasteful expression of invasion genes once it has progressed to the systemic phase of infection. An activated PhoPQ system also results in the induction of SPI-2 (Ochman *et al*, 1996; Shea *et al*, 1996) and SPI-3 (Soncini *et al*, 1996) gene expression, which is essential for intramacrophage survival. In addition, PhoPQ simultaneously controls the expression of genes required for acid tolerance.

A second example of the co-ordinated expression of stress response and pathogenicity island genes occurs via the OmpR-EnvZ two-component regulatory system. The EnvZ sensor kinase detects changes in external osmolarity, resulting in the activation of the OmpR response regulator. This protein can subsequently activate the SsrAB system resulting in the transcription of SPI-2 genes (Lee *et al*, 2000). OmpR also enhances expression of *ompC* and inhibits transcription of *ompF*, aiding in osmotic tolerance

(Foster and Spector, 1995). Thus, the osmotic challenge that may be presented by the macrophage phagosome (Lee *et al*, 2000; Garcia-del Portillo *et al*, 1992) is tolerated by a system that also regulates a type III secretory apparatus required for intramacrophage survival.

In addition to the osmotic shock response, the OmpR response regulator has also been implicated in the ATR of *S. typhimurium* (Bang *et al*, 2000). Indeed, an additional example of bacterial co-ordinated regulation may involve the bacterial stress responses. As detailed in Chapter 1, many stress tolerance mechanisms can cross protect against additional potentially harmful environmental conditions (Leyer and Johnson, 1993). This is most likely attributable to the substantial number of global regulatory molecules and other proteins which are common to more than one tolerance mechanism. For example, the alternative sigma factor, RpoS and its associated regulon are components of the ATR (Lee *et al*, 1994), the OSR (Storz and Imlay, 1999), the osmotic shock response (Hengge-Aronis, 1996) and the starvation stress response (Spector, 1998). The existence of cross-protection is hardly surprising when the large number of different stresses encountered within single environmental niches is considered. For example, in the macrophage alone, *Salmonella* may have to contend with low pH, oxidative stress, cationic peptides, phosphate limitation, low osmolarity and a depletion of divalent cations (Garcia-del Portillo *et al*, 1992; Storz and Imlay, 1999; Lee *et al*, 2000). The bacteria thus have a distinct survival advantage if one stress response can induce additional tolerance mechanisms.

A number of similarities between the ATR and OSR mechanisms indicate that both acidic and oxidative stresses may induce a significant amount of cross protection against each other. For example, both tolerance mechanisms involve the increased activity of the RpoS and Fur global regulators (Foster, 1999; Storz and Imlay, 1999). The expression of genes that fall within these two regulons is therefore most likely to be altered in an equivalent manner during both stress responses. Indeed, the biosynthesis of the RpoS dependent α subunit of nitrate reductase II (NarZ) is increased in response to H_2O_2 and is required for acid tolerance induced by carbon starvation (Spector, 1998). Interestingly, *narZ* is regulated by a complex network of environmental stress signals including RpoS, C, N and P starvation, H_2O_2, ppGpp, CRP

and Fnr (Spector *et al*, 1999), suggesting that it may be heavily involved in cross protection. Other genes regulated in a similar manner by both the ATR and the OSR include those encoding the DNA binding protein Dps and DNA polymerase I which display increased expression and *ompF* which is repressed (Foster, 1999; Storz and Imlay, 1999).

The research presented in Chapters 4 and 5 also indicates a strong link between the acid and oxidative stress tolerance mechanisms. It has been shown that low pH and H_2O_2 stress results in the reduced expression of D-galactose binding protein, glycerophosphodiester phosphodiesterase and both phase-1 and phase-2 flagellin. Furthermore, in each case, the PhoPQ system has been identified as the mediator of the cross-regulation. Overall, the evidence suggests that acid induced H_2O_2 tolerance and H_2O_2 induced acid tolerance exists in exponential phase *S. typhimurium*. This cross protection raises the possibility of co-regulation by the PhoPQ system, which would also provide a link with pathogenicity. PhoPQ has not been previously linked with the oxidative stress response. However, as PhoPQ is essential for intramacrophage survival where oxidative stress is present, this connection is a distinct possibility. A linked PhoPQ-acid tolerance-oxidative stress response system would represent a potent form of resistance to macrophage induced stress.

This chapter presents research initiated to investigate the existence of acid induced H_2O_2 tolerance and vice versa in exponential phase *S. typhimurium*. A link with the PhoPQ regulatory system was also studied via the use of *pho-12* and *pho-24 S. typhimurium* mutant strains. Previous research has studied a connection between acid and oxidative stress tolerance in *Salmonella* (Foster and Hall, 1990; Leyer and Johnson, 1993). Data have however been inconsistent and have not covered cross protection survival kinetics in any detail. For example, Foster and Hall (1990) conclude that neither the ATR nor the OSR induce cross protection to H_2O_2 or low pH respectively. Leyer and Johnson (1993), however, suggest that exposure to low pH can induce tolerance to oxidative stress. This discrepancy may be attributable to differences in the oxidative stress challenge conditions used: Foster and Hall expose acid adapted cells directly to H_2O_2 at a concentration of 10mM, whereas, Leyer and Johnson expose similarly adapted cells to an activated lactoperoxidase system in the presence of unknown quantities of H_2O_2 generated by glucose oxidase. Additionally, Foster and

Hall measure cell survival at a single time point after H_2O_2 challenge, whereas Leyer and Johnson show cell survival under oxidative stress as a time course. It is possible that the time chosen by Foster and Hall to monitor cell survival may have missed evidence of cross protection that might have been prevalent at another time point. The fact that Leyer and Johnson show minimal cross protection after 20 minutes of oxidative stress challenge, rising to a maximum at 45 minutes re-enforces this idea. It is also evident that both studies used the *S. typhimurium* LT2 strain which has an altered *rpoS* allele. As this sigma factor has a broad role in stress tolerance, this strain may display a spurious cross protection phenotype. The research presented here utilises stress survival assays to monitor the effects of acid and H_2O_2 adaptation on subsequent tolerance, over time, of extremes of H_2O_2 and low pH, respectively, in SL1344.

This chapter presents data indicating that acid adaptation invokes cross protection against H_2O_2 stress in log phase *S. typhimurium*. However, the tolerance of peroxide stress is not as strong as that produced by an OSR inducing concentration of H_2O_2. In contrast, adaptation to oxidative stress did not provide any cross protection against extreme acid conditions. The results also indicate that the PhoPQ two component regulatory system plays a complex role in acid induced cross protection to oxidative stress and to heterogeneous stress challenges in general.

6.2. Adaptation to pH 5.0 Induces Cross Protection Against 30 μM H_2O_2.

The effect of acid adaptation at pH 5.0 on the induction of tolerance to 30 μM H_2O_2 was characterised in *S. typhimurium* SL1344. Exponential phase cells were fully acid adapted by exposure to inorganic acid at pH 5.0 for 60 minutes in LB medium. They were subsequently challenged with 30 μM H_2O_2 and monitored for survival over a time course of 90 minutes. As controls, parallel cell cultures were adapted to pH 7.2 in LB medium or 0.3 μM H_2O_2 (an OSR inducing concentration) in LB medium for 60 minutes prior to exposure to 30 μM H_2O_2. All assays were repeated at least three times. Figures show single representative experiments.

Figure 6.1 shows the effect of adaptation to acid or the control conditions on *Salmonella* survival in 30 μM H_2O_2. It is clear that an optimum tolerance is provided by adaptation to an OSR inducing concentration of H_2O_2. Survival levels here have only dropped by approximately one log order after 90 minutes of exposure to 30 μM H_2O_2. This is in contrast with cells previously adapted to pH 7.2 which were completely non-culturable after just 30 minutes of H_2O_2 challenge. Acid adaptation imparts an H_2O_2 tolerance on the bacteria that is equal to that of the OSR induced culture for the first 15 minutes of peroxide challenge. Thereafter, survival levels fall and a lack of detectable colony forming units is evident at 60 minutes. It is therefore concluded that acid adaptation induces cross protection to H_2O_2 stress. However, this tolerance is not as long lived as that conferred by prior adaptation to an inducing concentration of H_2O_2.

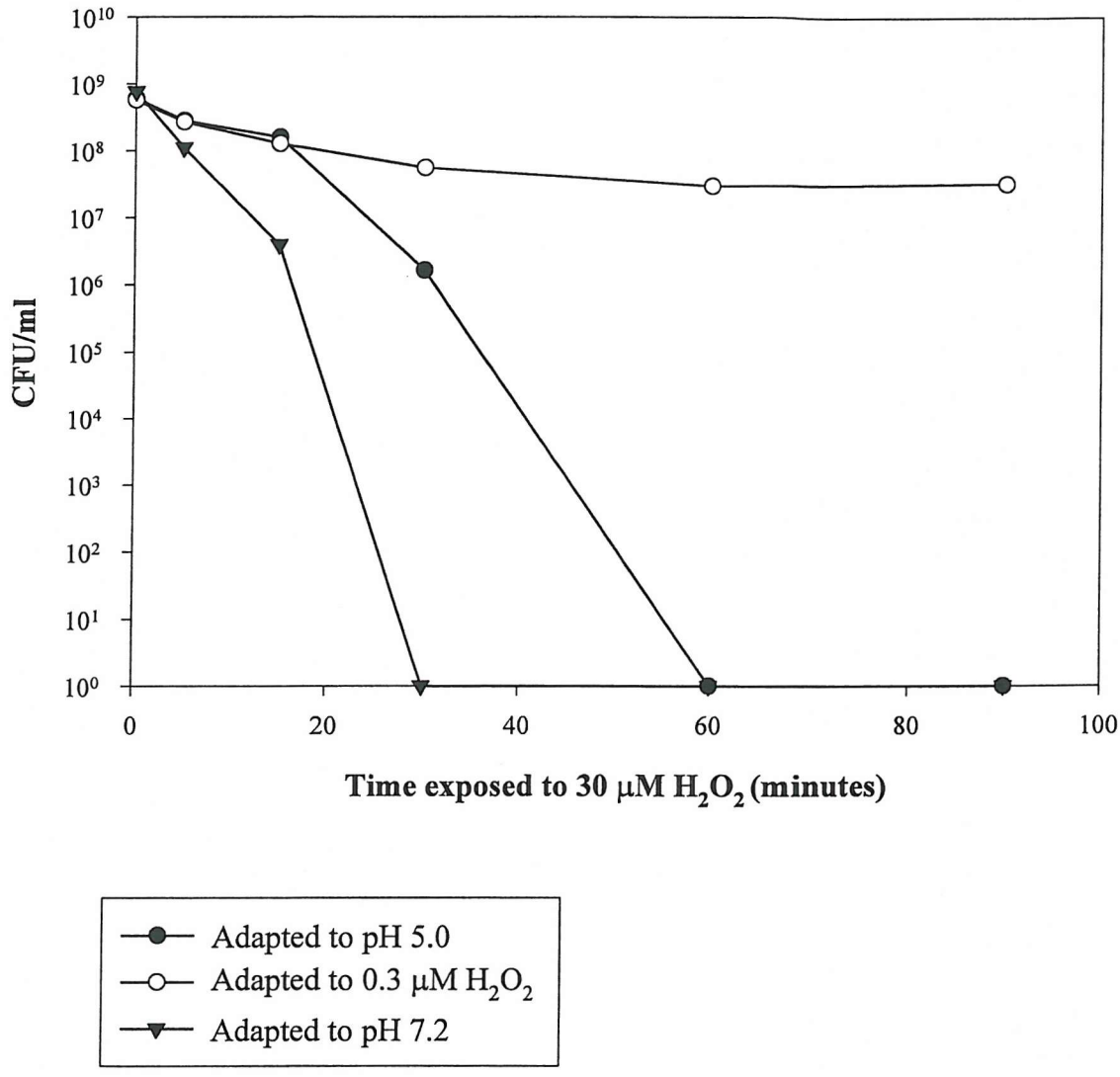


Figure 6.1. *Salmonella* cells adapted to pH 5.0 have increased survival against H_2O_2 oxidative stress. Exponential phase *S. typhimurium* SL1344 was adapted for 60 minutes to inorganic acid at pH 5.0, to 0.3 μM H_2O_2 or to pH 7.2 in LB medium. The cultures were then exposed to 30 μM H_2O_2 for up to 90 minutes in LB medium. Tolerance was monitored at the time points shown by determining the concentration of survivors (CFU/ml) after plating on LB agar at pH 7.2.

6.3. Adaptation to 0.3 μM H_2O_2 does not Induce Cross Protection Against Inorganic Acid Stress at pH 3.0.

Adaptation to 0.3 μM H_2O_2 for 60 minutes is required to induce an optimal OSR in exponential phase *S. typhimurium* SL1344 (G. Howell, personal communication). The effect of this OSR inducing adaptation on the generation of tolerance to extreme acid pH was therefore characterised. Exponential phase cells were exposed to 0.3 μM H_2O_2 for 60 minutes in LB medium and subsequently challenged with LB medium at pH 3.0 (inorganic acid). Survival was monitored over 90 minutes. As controls, parallel cell cultures were adapted to pH 7.2 or pH 5.0 LB medium for 60 minutes prior to pH 3.0 exposure. Assays were repeated at least three times. Figures show single representative experiments.

Figure 6.2 shows the consequences of adaptation to H_2O_2 and the control conditions on the survival of *Salmonella* when exposed to pH 3.0. An optimal tolerance of the acid challenge conditions was provided by prior adaptation to pH 5.0. *Salmonella* cells previously adapted to pH 7.2 fared less well at pH 3.0 and a rapid decline in culturability was seen. In these particular assay conditions, pH 7.2 adapted cells reached a zero survival level after 60 minutes of pH 3.0 exposure. A similar profile is discernible in the case of the H_2O_2 adapted cells. Therefore, H_2O_2 adaptation does not appear to confer any survival advantage to cells exposed to pH 3.0 conditions. It is concluded that an OSR inducing concentration of H_2O_2 does not significantly cross protect against extreme acid exposure in exponential phase *S. typhimurium*.

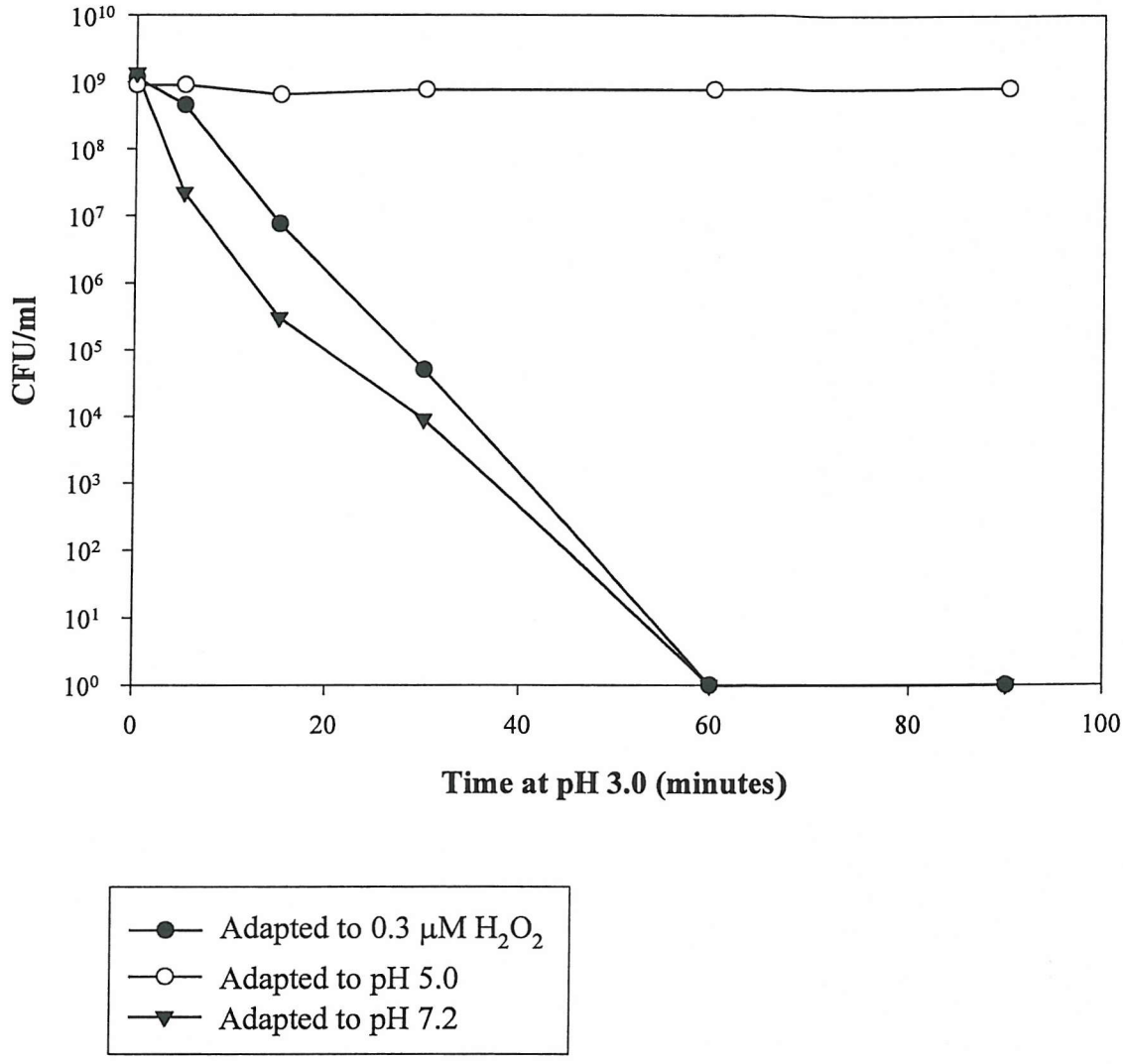


Figure 6.2. *Salmonella* cells with an induced OSR do not have enhanced survival at low pH. Exponential phase *S. typhimurium* SL1344 was adapted for 60 minutes to 0.3 μM H_2O_2, to inorganic acid at pH 5.0, or to pH 7.2 in LB medium. The cultures were then exposed to inorganic acid at pH 3.0 for up to 90 minutes in LB medium. Tolerance was monitored at the time points shown by determining the concentration of survivors (CFU/ml) after plating on LB agar at pH 7.2.

6.4. The PhoPQ Two Component Regulatory System Functions in Acid Induced Cross Protection Against Oxidative Stress.

The PhoPQ system constitutes a major regulator of the ATR in exponential phase *S. typhimurium* (Bearson *et al*, 1998). It has also been identified as a modulator of genes that are co-regulated by the acid tolerance and oxidative stress responses (see Chapter 4). Therefore, it is possible that the system might play a role in any cross protection identified between the two stress tolerance mechanisms. Thus, the effects of *S. typhimurium phoP* null (*pho-12*) and constitutive (*pho-24*) mutations on the cross protection survival assays were characterised. All assays were repeated at least three times. Figures show single representative experiments.

Figure 6.3 shows the effects of adaptation to pH 5.0, 0.3 μM H_2O_2 and pH 7.2 on the tolerance of a 30 μM H_2O_2 challenge in an exponential phase *S. typhimurium pho-12* mutant. Like the parent strain (Figure 6.1), adaptation to 0.3 μM H_2O_2 provided optimum tolerance to peroxide stress. At first glance, acid induced cross protection against H_2O_2 in the *pho-12* mutant also appears to follow the profile of the wild type strain. However, *pho-12* cells adapted to pH 7.2 survived a 30 μM H_2O_2 challenge for at least 90 minutes. Therefore, adaptation to pH 5.0 was actually detrimental in these circumstances. It is concluded that the inactivation of PhoPQ imparts a degree of constitutive H_2O_2 tolerance on *Salmonella* cells. This tolerance is (i) not as great as that conferred by prior exposure to OSR inducing concentrations of peroxide and (ii) reduced after prior exposure to pH 5.0.

The effect of acid adaptation on subsequent tolerance of a 30 μM H_2O_2 challenge in exponential phase *S. typhimurium pho-24* cells is depicted in Figure 6.4. Again, control cultures were adapted to pH 7.2 LB medium or 0.3 μM H_2O_2 in LB medium prior to exposure to 30 μM H_2O_2. It was found that *phoPQ* constitutive cells exposed to 0.3 μM H_2O_2 or pH 7.2 prior to peroxide challenge behaved similar to wild type cells. However, bacteria adapted to pH 5.0 lost the cross protection to oxidative stress seen in the parent strain (compare with Figure 6.1). This suggests that a constitutively active PhoPQ system has no effect on the OSR, but abolishes any cross protection to oxidative stress instigated by the ATR.

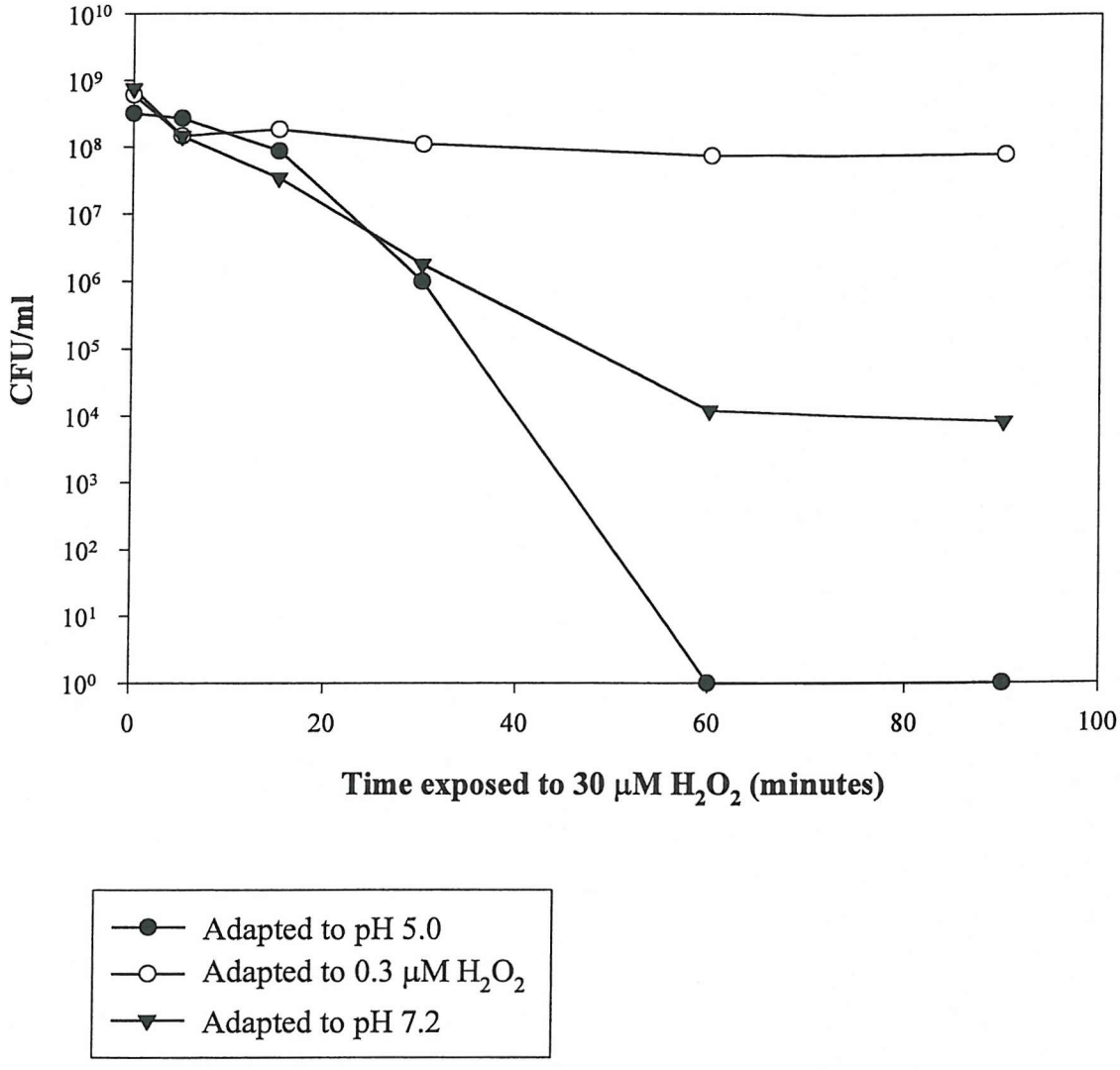


Figure 6.3. *Salmonella pho-12* cells adapted to pH 7.2, but not pH 5.0, show increased survival against H_2O_2 oxidative stress. Exponential phase *S. typhimurium* SL1344 *pho-12* was adapted for 60 minutes to inorganic acid at pH 5.0, to 0.3 μM H_2O_2 or to pH 7.2 in LB medium. The cultures were then exposed to 30 μM H_2O_2 for up to 90 minutes in LB medium. Tolerance was monitored at the time points shown by determining the concentration of survivors (CFU/ml) after plating on LB agar at pH 7.2.

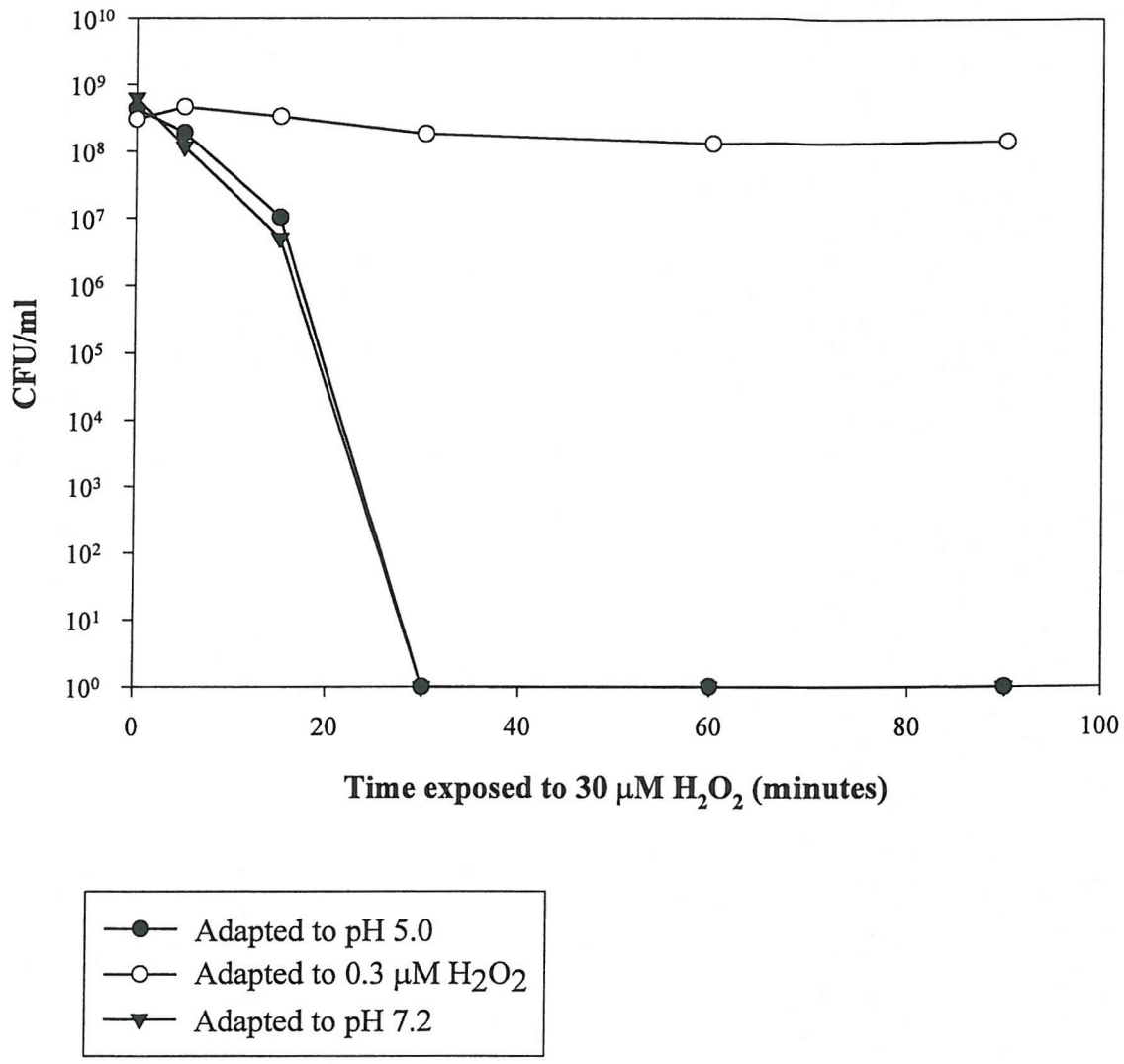


Figure 6.4. A *phoPQ* constitutive mutant, unlike the wild type, does not show increased survival against H_2O_2 oxidative stress following adaptation to pH 5.0. Exponential phase *S. typhimurium* SL1344 *pho-24* was adapted for 60 minutes to inorganic acid at pH 5.0, to 0.3μM H_2O_2 or to pH 7.2 in LB medium. The cultures were then exposed to 30μM H_2O_2 for up to 90 minutes in LB medium. Tolerance was monitored at the time points shown by determining the concentration of survivors (CFU/ml) after plating on LB agar at pH 7.2.

The effect of 0.3 μM H_2O_2 adaptation on the tolerance of pH 3.0 conditions was characterised in a *Salmonella pho-12 phoP* null mutant during log phase growth (Figure 6.5). Surprisingly, a lack of PhoPQ activity had no effect on the ATR as cells adapted to pH 5.0 prior to pH 3.0 exposure behaved as wild type. In addition, *pho-12* cells adapted to pH 7.2 displayed the same survival kinetics as similarly adapted wild type bacteria when exposed to pH 3.0 (compare with Figure 6.2). Interestingly, prior adaptation to 0.3 μM H_2O_2 was detrimental to the *phoP* null mutant's survival when challenged at pH 3.0. These cells survived for half the time of pH 7.2 adapted cells under extreme pH conditions. This could indicate that although PhoPQ is not involved in a cross protection effect here, it may be required for survival when both acid and oxidative stresses are present in the same environment.

The effect of H_2O_2 adaptation followed by acid pH challenge was characterised in a *S. typhimurium phoPQ* constitutive mutant (*pho-24*) during log phase growth (Figure 6.6). Control cells adapted to pH 5.0 prior to pH 3.0 challenge behaved as wild type and displayed a high level of acid tolerance. *pho-24* cells adapted to pH 7.2 before pH 3.0 exposure were able to tolerate extreme pH for 30 minutes longer than similarly treated wild type bacteria (compare with Figure 6.2). As the PhoPQ system is activated during the ATR, this result could indicate that the constitutive activation of PhoPQ imparts a degree of constitutive acid tolerance on exponential phase *S. typhimurium*. The *pho-24* cells adapted to 0.3 μM H_2O_2 and then exposed to pH 3.0 display a much less tolerant phenotype than wild type cells which survive for twice as long under the same conditions. It appears that aberrant continuous activation of the PhoPQ regulatory system may reduce the bacteria's ability to tolerate more than one stress condition in a particular environment.

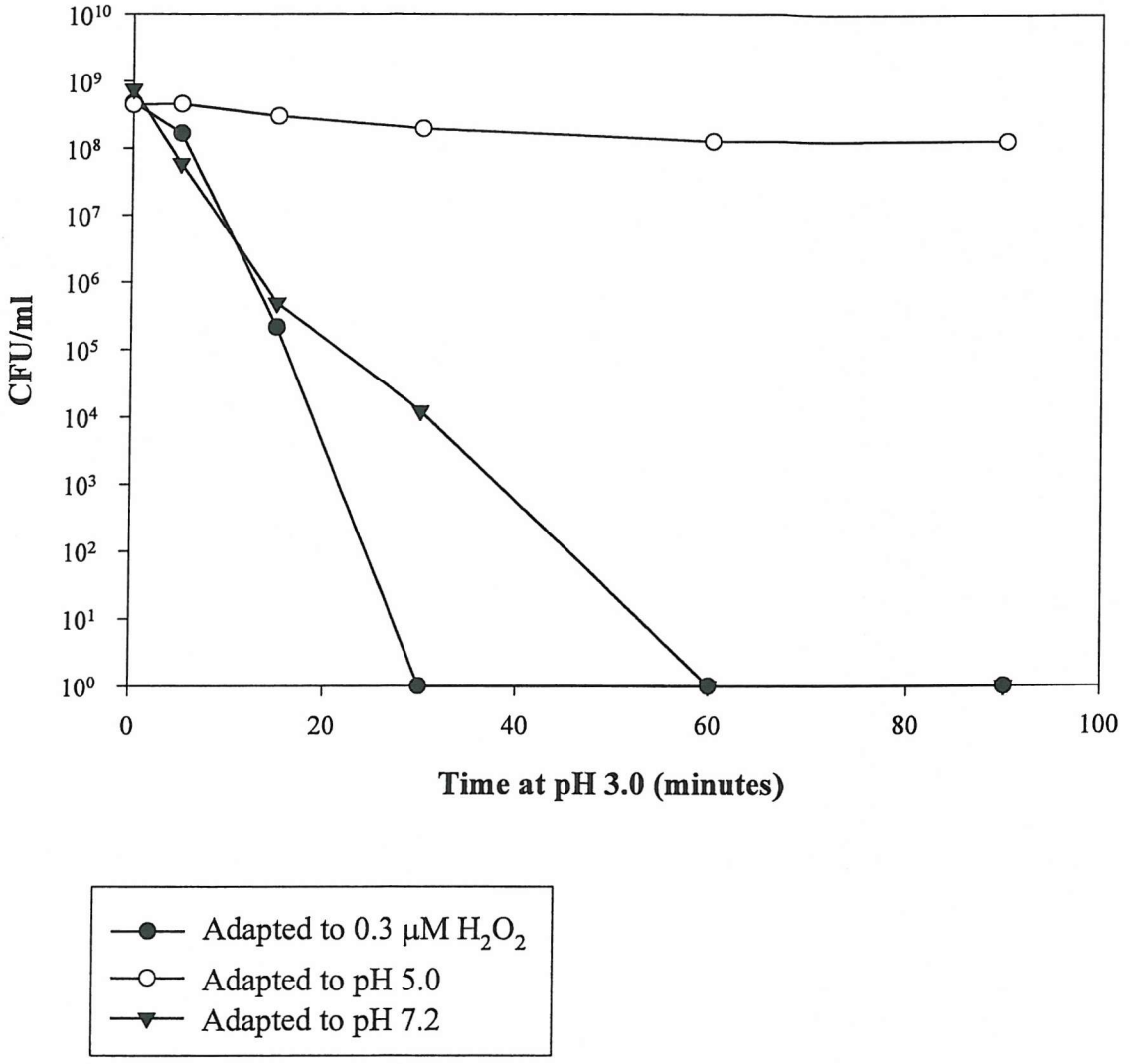


Figure 6.5. Decreased viability of *phoP*⁻ cells with an induced OSR, relative to pH 7.2 adapted cells, on exposure to low pH. Exponential phase *S. typhimurium* SL1344 *pho-12* was adapted for 60 minutes to 0.3 μM H_2O_2, to inorganic acid at pH 5.0, or to pH 7.2 in LB medium. The cultures were then exposed to inorganic acid at pH 3.0 for up to 90 minutes in LB medium. Tolerance was monitored at the time points shown by determining the concentration of survivors (CFU/ml) after plating on LB agar at pH 7.2.

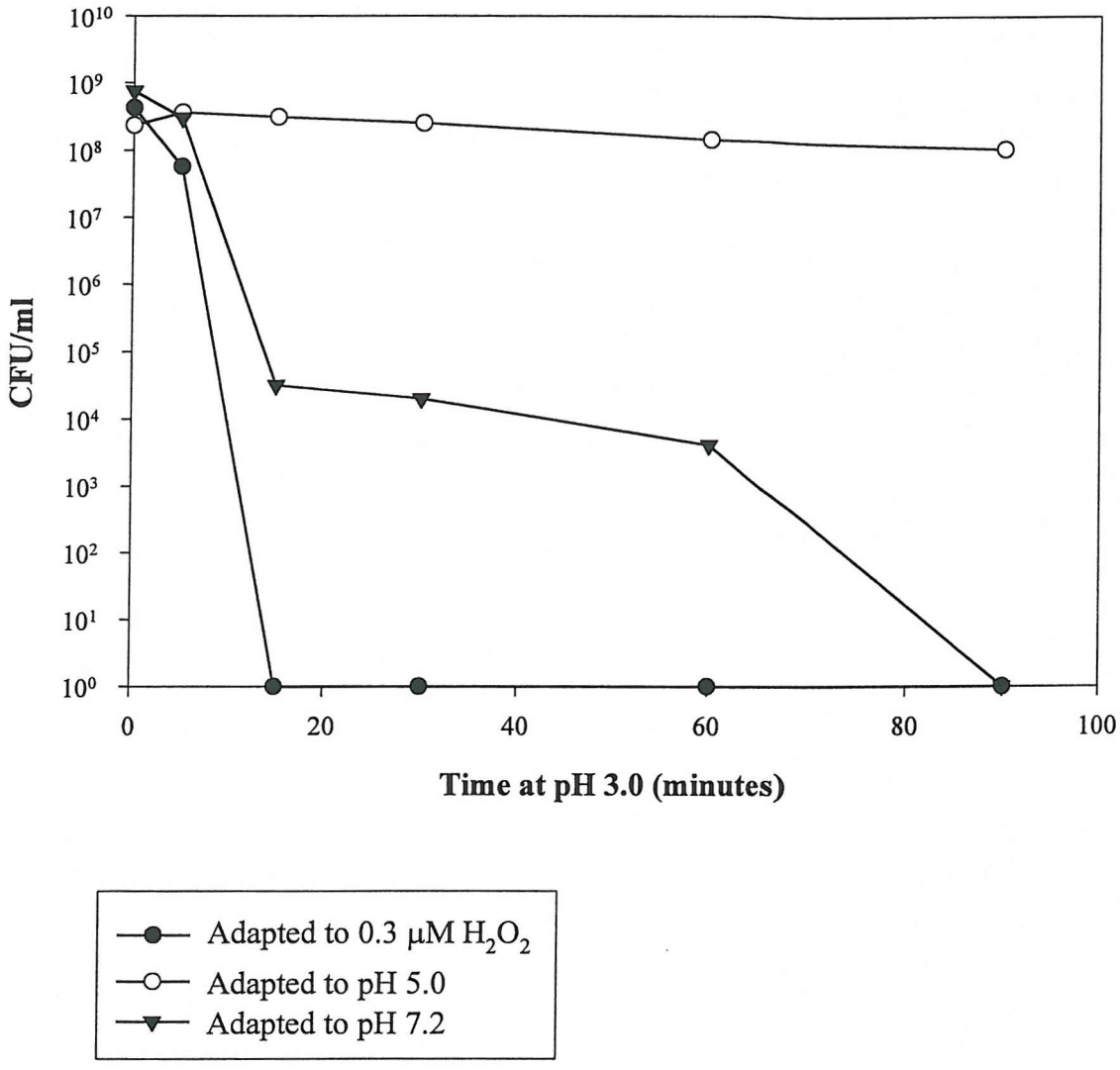


Figure 6.6. Decreased viability of a mutant with a constitutively active PhoPQ system and with an induced OSR, relative to pH 7.2 adapted cells, on exposure to low pH. Exponential phase *S. typhimurium* SL1344 *pho-24* was adapted for 60 minutes to 0.3 μM H_2O_2, to inorganic acid at pH 5.0, or to pH 7.2 in LB medium. The cultures were then exposed to inorganic acid at pH 3.0 for up to 90 minutes in LB medium. Tolerance was monitored at the time points shown by determining the concentration of survivors (CFU/ml) after plating on LB agar at pH 7.2.

6.5. Discussion.

This chapter reports the identification of a stress tolerance cross protection mechanism in exponential phase *S. typhimurium* that is dependent on the wild type expression of the PhoPQ system. A previously undetermined connection has been made between low pH induced tolerance of oxidative stress and a two-component regulatory system required for *Salmonella* virulence. This is indicative of a further mechanism by which this bacterial pathogen can inter-regulate virulence and stress responses that may be required in specific environmental niches.

The cross protection assays described here have shown that an optimal ATR inducing low pH stress can confer a significant degree of oxidative stress tolerance on log phase *Salmonella* cells. However, this acid induced tolerance of 30 μM H_2O_2 was not as sustained as that imparted by a weaker and sub-lethal 0.3 μM concentration of H_2O_2 (Figure 6.1). Therefore, although acid induced cross protection exists against H_2O_2, the tolerance levels induced are not optimal.

Similar assays have shown that the optimal OSR inducing 0.3 μM H_2O_2 stress is not able to confer a reciprocated tolerance of extreme acid stress in exponential phase *S. typhimurium* (Figure 6.2). 0.3 μM H_2O_2 induced bacteria behave as cells adapted to pH 7.2 when exposed to pH 3.0 conditions. These results suggest that the ATR constitutes a more general stress tolerance mechanism than the OSR. In this way, low pH could induce a number of processes that are appropriate to survival in other stress conditions, whereas H_2O_2 may mostly activate systems that are very specific to peroxide tolerance. After oral infection, the first real host mediated stress encountered by the pathogen is the acidic pH of the stomach. Therefore, the induction of a more general stress response to these conditions would benefit the cell, as it could prime the bacteria for the pathogenic process ahead.

The PhoPQ system has been implicated in pathogenicity (Garcia Vescovi *et al*, 1994) and the ATR (Bearson *et al*, 1998). It is also required for intramacrophage survival (Garcia Vescovi *et al*, 1994), an environment where both oxidative and acid stresses are probably encountered. A possible role for PhoPQ in acid induced cross protection

against oxidative stress was thus investigated. The cross protection assays were repeated using *S. typhimurium* strains with constitutively active PhoPQ systems (*pho-24*), or strains lacking PhoPQ activity (*pho-12*). Through the use of such assays, the role of PhoPQ in each of the two individual stress responses was also studied. These results will be discussed first.

Analysis of the survival kinetics of the *pho-12* strain indicates that a lack of PhoPQ activity has no effect on either acid induced acid tolerance (Figure 6.5) or H_2O_2 induced tolerance of peroxide (Figure 6.3) in the assay conditions used. However, the *pho-12* mutant does exhibit a degree of constitutive H_2O_2 tolerance which is lacking in the wild type strain (Figures 6.3 and 6.1). This tolerance is not as strong as that induced by prior exposure to 0.3 μM H_2O_2.

In comparison, examination of the survival kinetics of the *pho-24* strain suggests that a constitutively active PhoPQ system also has no effect on acid induced acid tolerance (Figure 6.6) or H_2O_2 induced H_2O_2 tolerance (Figure 6.4) under the same assay conditions. In contrast with the *pho-12* strain, no constitutive tolerance of H_2O_2 is seen in the *pho-24* mutant. However, an amount of constitutive acid tolerance is exhibited by this bacteria (Figure 6.6). Again, this tolerance is not as strong as that induced in the same strain in response to a weaker homologous stress.

Taken together, these results suggest that the PhoPQ system is involved in both the ATR and the OSR. Unlike previous reports (Bearson *et al*, 1998), the research described here indicates that a *phoP* null mutant does not impart acid sensitivity upon exponential phase *Salmonella*. However, in keeping with a positive role for PhoPQ in the ATR, constitutive expression imparts a degree of constitutive acid tolerance upon these cells. The PhoPQ induced acid tolerance is not as strong as that produced by an optimal ATR as many additional components are obviously required for a full response. Variations in the reported dependency of the ATR on the PhoPQ system may be explained by differences in assay conditions used. For example, PhoPQ may be required for an optimal ATR in minimal media as used by Bearson *et al* (1998), but not for acid tolerance in LB medium as utilised here: unlike minimal media, LB media contains high concentrations of Mg^{2+} that could reduce PhoPQ activity and possibly

result in wild type cells that behave as *phoP*⁻ mutants. This would account for the results described (Figure 6.5) where the removal of *phoP* is not detrimental to the ATR of wild type cells in LB medium. As an explanation for the role of PhoPQ in hydrogen peroxide tolerance, wild type expression of the two-component regulatory system may repress some OSR mechanisms (although this seems unlikely as the low pH, low Mg^{2+} environment of the phagosome induces PhoPQ, where both the ATR and OSR are required for survival). This repression may be overcome by 0.3 μM H_2O_2 adaptation. Therefore, no alteration in the OSR profile would be seen in a *phoP* null mutant, but a limited degree of constitutive H_2O_2 tolerance might be evident, as witnessed here. The hypothesised PhoPQ mediated repression of OSR mechanisms could still be overcome by H_2O_2 adaptation in a *phoP* constitutive mutant, explaining why no difference in the OSR profile is seen in Figure 6.4.

The function of PhoPQ in the acid induced cross protection against H_2O_2 stress and in heterogeneous stress challenges appears to be complex. Analysis of the survival kinetics of the *pho-12* and *pho-24* strains indicates that the acid induced H_2O_2 tolerance is lost when the PhoPQ system is aberrantly expressed (Figures 6.3 and 6.4). In fact, adaptation to pH 5.0 is actually detrimental to the constitutive H_2O_2 tolerance that is exhibited by the *phoP* null mutant (Figure 6.3). These results suggest that wild type levels of PhoPQ activity are required prior to any stress exposure for acid induced cross protection to function. In addition, these levels may also be required when the cell is exposed to acid and oxidative stress in quick succession, regardless of any cross protective mechanism. For example, PhoPQ is a component of the bacteria's defence system against acid stress. Therefore, exposure of a *phoP* null mutant to pH 5.0 could potentially weaken this strain, such that a subsequent exposure to H_2O_2 stress would be intolerable. This would explain the detrimental effect of pH 5.0 adaptation seen in the *pho-12* strain. Overall, low pH is probably inducing H_2O_2 tolerance via the regulation of systems that are common to both the ATR and the OSR. This regulation is most likely mediated by PhoPQ.

Further evidence suggests that wild type levels of PhoPQ appear to be beneficial for survival when acid and oxidative stresses are encountered in quick succession in any order. For example in relation to wild type cells, adaptation to 0.3 μM H_2O_2 in both the

pho-12 (Figure 6.5) and the *pho-24* (Figure 6.6) strains is deleterious to the subsequent survival of pH 3.0 conditions. Additionally, prior exposure to pH 5.0 is disadvantageous to the survival of both mutant strains in 30 μM H_2O_2 (Figures 6.3 and 6.4) when compared with wild type cells in the same conditions (Figure 6.1). The requirement for a properly expressed PhoPQ system in these conditions is quite perceivable. After all, both stress conditions are quite possibly present within the macrophage in which *Salmonella* cannot survive without a functioning PhoPQ system.

In conclusion, a form of low pH induced cross protection against a potentially lethal H_2O_2 stress has been identified in *S. typhimurium* SL1344. Wild type levels of PhoPQ activity have been found to be required for this cross protection, thereby indicating an additional link between virulence and stress tolerance in this micro-organism. In addition, it has been determined that the PhoPQ apparatus is required for a degree of tolerance when *Salmonella* are exposed in quick succession to more than one stress condition. Although not cross protection per se, this heterogeneous stress connection indicates that PhoPQ is probably involved in a general stress response mechanism. Indeed, the present research has corroborated previous work suggesting that PhoPQ is activated during the ATR and also provides a link to the regulation of H_2O_2 tolerance. The interconnected modulation of acid and oxidative stress responses via PhoPQ is perhaps expected as all three systems may be required for intramacrophage survival.

Although the mechanisms involved are complex, the data presented here suggest additional associations between the virulence properties and stress responses of *Salmonella* that may be beneficial to this pathogen.

CHAPTER 7.

THE BipA GTPase NEGATIVELY REGULATES THE ATR OF THE EPEC STRAIN, MAR001.

CHAPTER 7. THE BipA GTPase NEGATIVELY REGULATES THE ATR OF THE EPEC STRAIN, MAR001.

7.1. Introduction.

The BipA protein was first discovered in *S. typhimurium* when proteomic studies revealed that it was induced by more than seven fold in response to the cationic host defence peptide, BPI (Qi *et al*, 1995). BipA was first identified in *E. coli* as the novel phosphoprotein product of the *o591* gene (Freestone *et al*, 1995). Subsequent investigations utilising the EPEC MAR001 strain identified BipA as a novel tyrosine phosphorylated GTPase (Farris *et al*, 1998). Tyrosine phosphorylation was found to be markedly stimulated in the presence of a protein factor associated with the particulate fraction of MAR001. The phosphorylated form of the enzyme was also shown to exhibit a significantly increased nucleotide hydrolysing activity (Farris *et al*, 1998).

The functional properties of BipA in EPEC strains have been characterised in some detail. Results indicate that this protein is involved in the regulation of, or is required for, a number of important virulence associated processes in these bacteria. It is appropriate to summarise these properties here.

In line with the circumstances of its discovery in *Salmonella*, BipA also confers a degree of resistance to BPI in EPEC (Farris *et al*, 1998). *bipA::cat* mutants of MAR001 are significantly more susceptible to BPI than wild type cells. The protective effect of BipA may be fairly specific as a second cationic peptide, protamine, fails to elicit a marked decrease in the viability of *bipA::cat* cells (Farris *et al*, 1998). The mechanism behind the BipA induced BPI resistance is currently unclear. However, studies on a BPI derived peptide, P2, which retains antibacterial activity, indicate that this molecule may disrupt the bacterial respiratory chain in a manner that is circumnavigated via BipA (Barker *et al*, 2000). The addition of formate or succinate protects *Salmonella* cells from killing by P2, suggesting that these products act to bypass the point in the respiratory chain disrupted by the peptide. BipA was found to positively influence the degree of protection afforded by formate. Therefore, the GTPase may possibly protect against BPI by influencing the production of such protective organic acids during growth or their effects in the respiratory chain.

As described in Chapter 1, BipA is also known to regulate the ability of EPEC to induce A/E lesions on host cells. After localised adherence, *bipA::cat* mutants fail to trigger cytoskeletal rearrangements within host cells resulting in a lack of pedestal/pseudopod formation. A thickening of epithelial microvilli also occurs. In contrast, over-expression of *bipA* results in the formation of numerous pseudopods on the epithelial cell surface and an associated 40 percent increase in actin accumulation (Farris *et al*, 1998). BipA is therefore thought to contribute to the regulation of cytoskeletal reorganisation in host cells during EPEC pathogenesis.

A third virulence associated mechanism that appears to fall under the regulatory control of BipA is flagella mediated cell motility (Farris *et al*, 1998). EPEC *bipA::cat* mutants were found to over express flagellin, resulting in the increased secretion of this protein. BipA null mutants also displayed an increased motility in comparison with wild type strains (Farris *et al*, 1998). Thus, BipA negatively regulates flagella mediated cell motility in EPEC. In corroboration with these results, motility and flagellin expression were reduced in an EPEC strain over expressing *bipA* (Farris *et al*, 1998). The GTPase may also regulate chemotaxis in EPEC: BipA null nutants display an increase in smooth swimming, whereas, an elevated level of BipA results in a higher frequency of tumbling (Farris *et al*, 1998).

Proteomic studies comparing the protein expression profiles of an *E. coli* BipA null mutant with that of the wild type strain have identified additional BipA regulated loci that may be associated with virulence (Freestone *et al*, 1998). The carbon starvation inducible *csp15* gene displays increased expression levels in the null mutant, suggesting that BipA negatively regulates this locus. In addition, the universal stress protein, UspA requires BipA for its serine/threonine phosphorylation, as shown by reduced levels of the acidified isoform in the *bipA* mutant. These studies also indicate that the DNA binding protein, H-NS, may be negatively regulated by BipA in *E. coli*. The H-NS protein has been proposed to play a major role in signal transduction pathways involving virulence associated processes (Higgins *et al*, 1990; Atlung and Ingmer, 1997).

BipA has also been implicated in the regulation of group 2 capsule formation in *E. coli*. These virulence factors are known to be regulated by temperature, and are only

expressed above 20°C (Whitfield and Roberts, 1999). Their expression is controlled from two distinct promoters, from which the capsule genes are convergently transcribed (Whitfield and Roberts, 1999). BipA and the H-NS protein have recently been found to be required for maximal transcription from both promoters at 37°C. They are believed to function in the temperature regulation of these promoters as BipA and H-NS also repress transcription from both at 20°C (Rowe *et al*, 2000).

Cumulatively, the published evidence strongly suggests that the BipA GTPase is a key global regulator of a number of stress and virulence associated processes in enteropathogenic strains of *Salmonella* and *E. coli*. This prompted research designed to investigate a potential role for BipA in the regulation of an additional virulence-related mechanism in these bacteria, the ATR.

The abilities of wild type and *bipA* null mutant strains of enteropathogenic *E. coli* and *Salmonella* to tolerate extreme acid pH after adaptation to pH 5.0 conditions were compared. ATR survival assays identical to those described in Chapter 3 were used to characterise any potential effects of BipA on the acid tolerance of these strains. Figures 7.1 to 7.5 show single representative experiments. This chapter reports that an acid adapted EPEC MAR001 strain, in which the *bipA* gene had been disrupted by mutation, displayed a greater longevity in extreme acid conditions than the wild type parent. This was true if either inorganic or organic acid adaptation was used. Transcomplementation of *bipA* in the mutant strain resulted in a wild type acid survival phenotype. BipA is thus thought to negatively regulate the ATR of MAR001. In contrast, removal of the *bipA* gene had no effect on the acid survival kinetics of the EPEC E2348/69 and JPN15 strains, or the *S. typhimurium* SL1344 strain. Interestingly, the EAF plasmid cured JPN15 strain and its *bipA::cat* derivative both displayed an increased acid tolerance when compared with parental E2348/69 wild type and *bipA::cat* cells. It is concluded that a significant variation exists in the complex regulatory mechanisms of the ATRs of different enteropathogenic serovars, strains and species.

7.2. BipA Negatively Regulates the ATR of *E. coli* Strain MAR001 in Inorganic acid at Extreme pH.

The abilities of EPEC MAR001 wild type, *bipA::cat* and transcomplemented *bipA::cat* mutant strains to tolerate extreme inorganic acid challenge at pH 3.0 after adaptation to pH 5.0 or pH 7.2 conditions were compared. Tolerance of a pH 3.0 environment over time was characterised using defined ATR survival assay conditions, identical to those described in Chapter 3 (section 3.4). It was ensured that bacteria were fully adapted by exposing them to inorganic acid at pH 5.0 or to pH 7.2 LB medium for 60 minutes prior to pH 3.0 challenge. As before, ATR assays were carried out in duplicate on mid-exponential phase cells. They were repeated at least twice.

As previously described, acid adapted MAR001 wild type cells were able to tolerate pH 3.0 conditions for a relatively short length of time. Viable cells were undetectable after 30 minutes of pH 3.0 exposure (Figure 7.1). In contrast, pH 5.0 adapted MAR001 *bipA::cat* mutants displayed a minimal decrease in survival after 30 minutes at pH 3.0. In fact, a total loss of viability was not seen until 60 minutes of exposure to extreme acid conditions (Figure 7.1). To ensure that this effect could be attributed to the BipA protein, ATR assays were performed on a MAR001 *bipA::cat* mutant into which the wild type *bipA* gene had been reintroduced. The transcomplemented strain displayed an ATR survival profile similar to that of the wild type strain (Figure 7.1). The ATR "super response" of the *bipA* null mutant indicates that the BipA GTPase negatively regulates one or more unknown functions of the inducible inorganic ATR in EPEC MAR001. In all three strains of MAR001 used (wild type, *bipA::cat* and transcomplemented *bipA::cat*), pH 7.2 adapted cells were unable to tolerate exposure to pH 3.0, even for only 5 minutes (Figure 7.1). This suggests that BipA is not responsible for imparting any constitutive acid sensitivity or tolerance on the MAR001 strain.

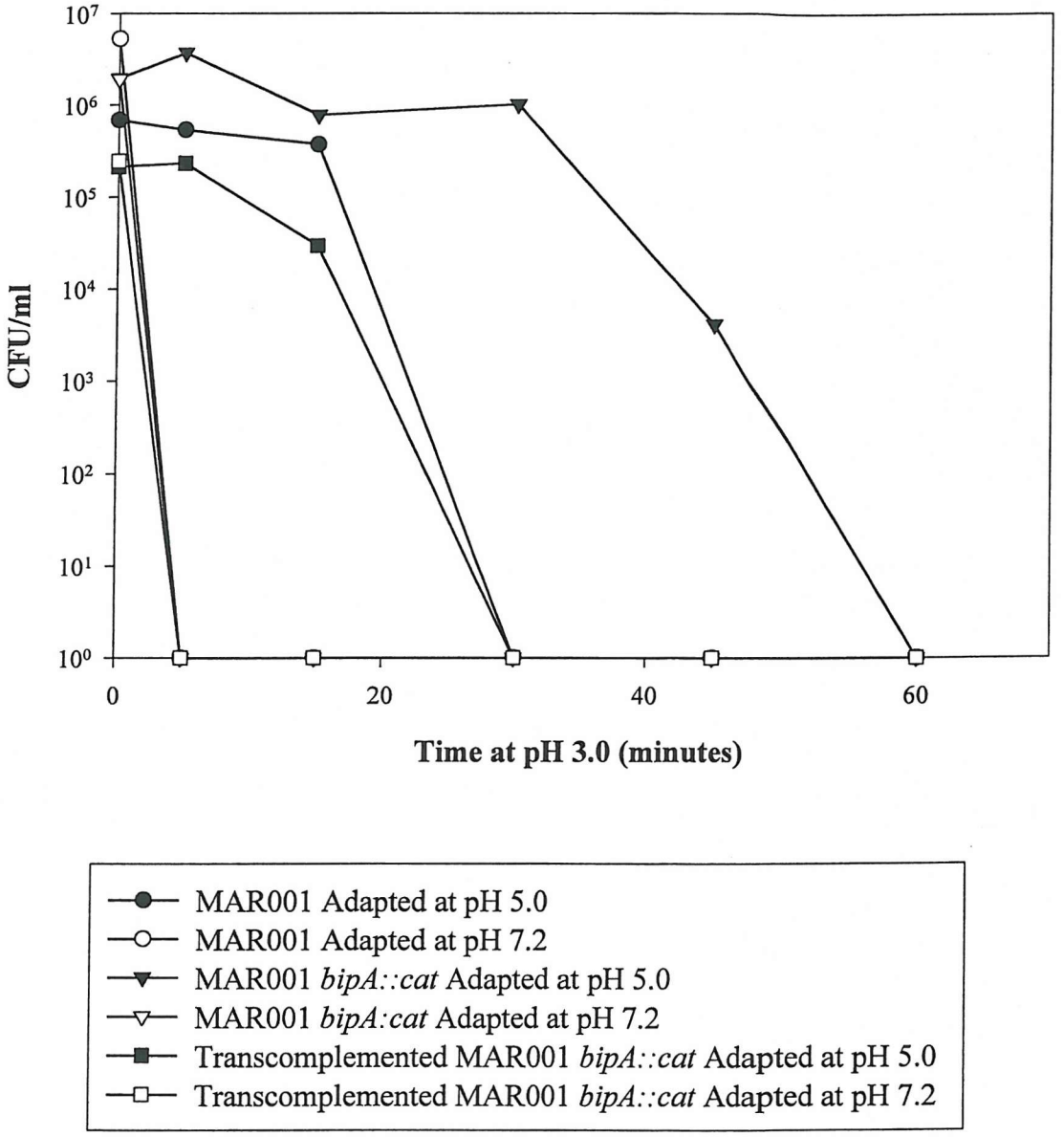


Figure 7.1. Increased survival of a *bipA* null mutant of MAR001 at pH 3.0 following adaptation at pH 5.0. *E. coli* MAR001 wild type, *bipA::cat* and transcomplemented *bipA::cat* strains were adapted to inorganic acid at pH 5.0 or to pH 7.2 for 60 minutes prior to extreme acid exposure for various lengths of time at pH 3.0. Surviving culturable cells were counted after plating on LB agar (pH 7.2).

7.3. BipA Negatively Regulates the ATR of *E. coli* Strain MAR001 at Extreme pH in the Presence of Organic acid.

To determine if the negative effect of BipA on the ATR of MAR001 was specific to inorganic acid, the survival assays described in section 7.2 were repeated, using the organic acid sodium benzoate, as a component of the acid stress. The ATR assays used were identical to those described in Chapter 3 (section 3.5) to investigate the effect of organic acid at low pH. That is, after adaptation to pH 5.0 or pH 7.2 LB medium, bacteria were exposed to pH 3.0 in the presence of 0.5 mM sodium benzoate and their survival was monitored after various lengths of time. Experiments were performed in duplicate and repeated at least twice.

As was previously described, wild type MAR001 cells adapted to pH 5.0 were able to tolerate extreme acid pH in the presence of 0.5 mM sodium benzoate for a shorter length of time than when exposed to pH 3.0 conditions alone (Figure 7.2. See also Figure 3.8). Here, a lack of cell viability was detectable after only 15 minutes. In a similar manner, the pH 5.0 adapted MAR001 *bipA::cat* strain displays a reduced tolerance of extreme acid when sodium benzoate is present. However, the viability of this strain becomes undetectable after a longer acid stress exposure time of 30 minutes (Figure 7.2). Again, the increased survival of *bipA::cat* cells was lost when transcomplemented with p*bipA*, resulting in an ATR profile similar to that of the wild type strain (Figure 7.2). In all three MAR001 strains used, pH 7.2 adapted bacteria were unable to tolerate exposure to pH 3.0 plus sodium benzoate, even for 5 minutes (Figure 7.2). This again suggests that BipA is not responsible for imparting any constitutive acid sensitivity or tolerance on the MAR001 strain.

The data indicate that the BipA protein negatively regulates the inducible organic and inorganic acid tolerance responses of exponential phase MAR001 cells.

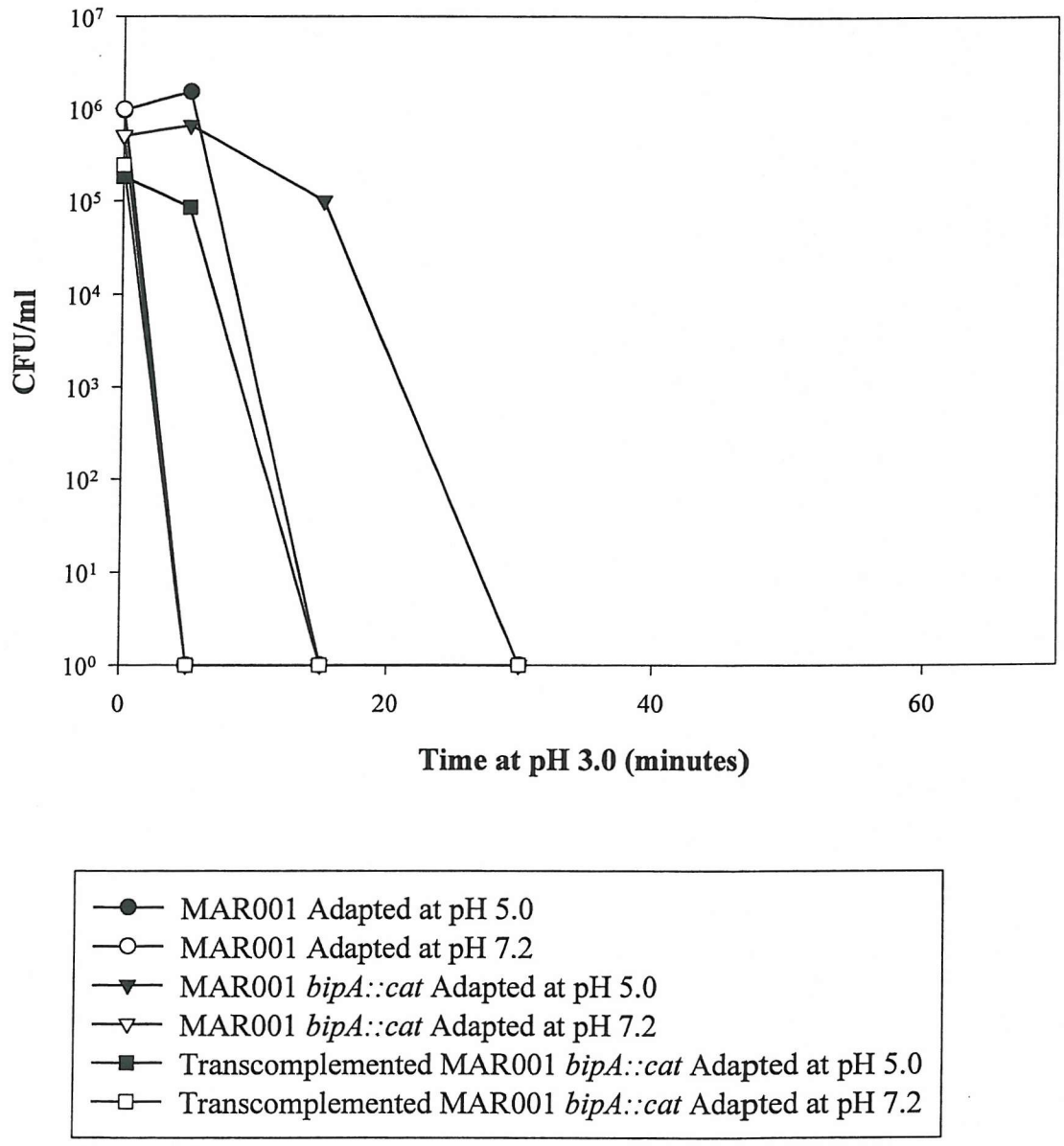


Figure 7.2. Increased survival of a *bipA* null mutant of MAR001 at pH 3.0 in the presence of 0.5 mM sodium benzoate following adaptation at pH 5.0. *E. coli* MAR001 wild type, *bipA::cat* and transcomplemented *bipA::cat* strains were adapted to inorganic acid at pH 5.0 or to pH 7.2 for 60 minutes prior to extreme acid exposure for various lengths of time at pH 3.0 in the presence of 0.5 mM sodium benzoate. Surviving culturable cells were counted after plating on LB agar (pH 7.2). Data for WT MAR001 are taken from Figure 3.8.

7.4. BipA has no Effect on the Survival Kinetics of EPEC Strain E2348/69 During the Exponential Phase ATR.

To determine if BipA was involved in the regulation of the ATR of other *E. coli* serovars, the survival kinetics of the E2348/69 wild type and *bipA::cat* strains were compared in acid. Inorganic ATR survival assays, identical to those described in Chapter 3 (section 3.4) and section 7.2 of this chapter were used. As before, ATR assays were carried out in duplicate on exponential phase cells. They were repeated at least twice.

As described previously, the pH 5.0 adapted E2348/69 wild type strain displayed a similar pH 3.0 tolerance to MAR001. Cell viability was undetectable after 30 minutes of exposure to pH 3.0 (Figure 7.3). However, unlike the MAR001 *bipA::cat* strain, E2348/69 *bipA::cat* cells displayed a wild type phenotype in extreme acid conditions. It is concluded that the BipA protein has no effect on the exponential phase ATR of *E. coli* E2348/69. This suggests that the regulation of the ATR is variable among EPEC isolates.

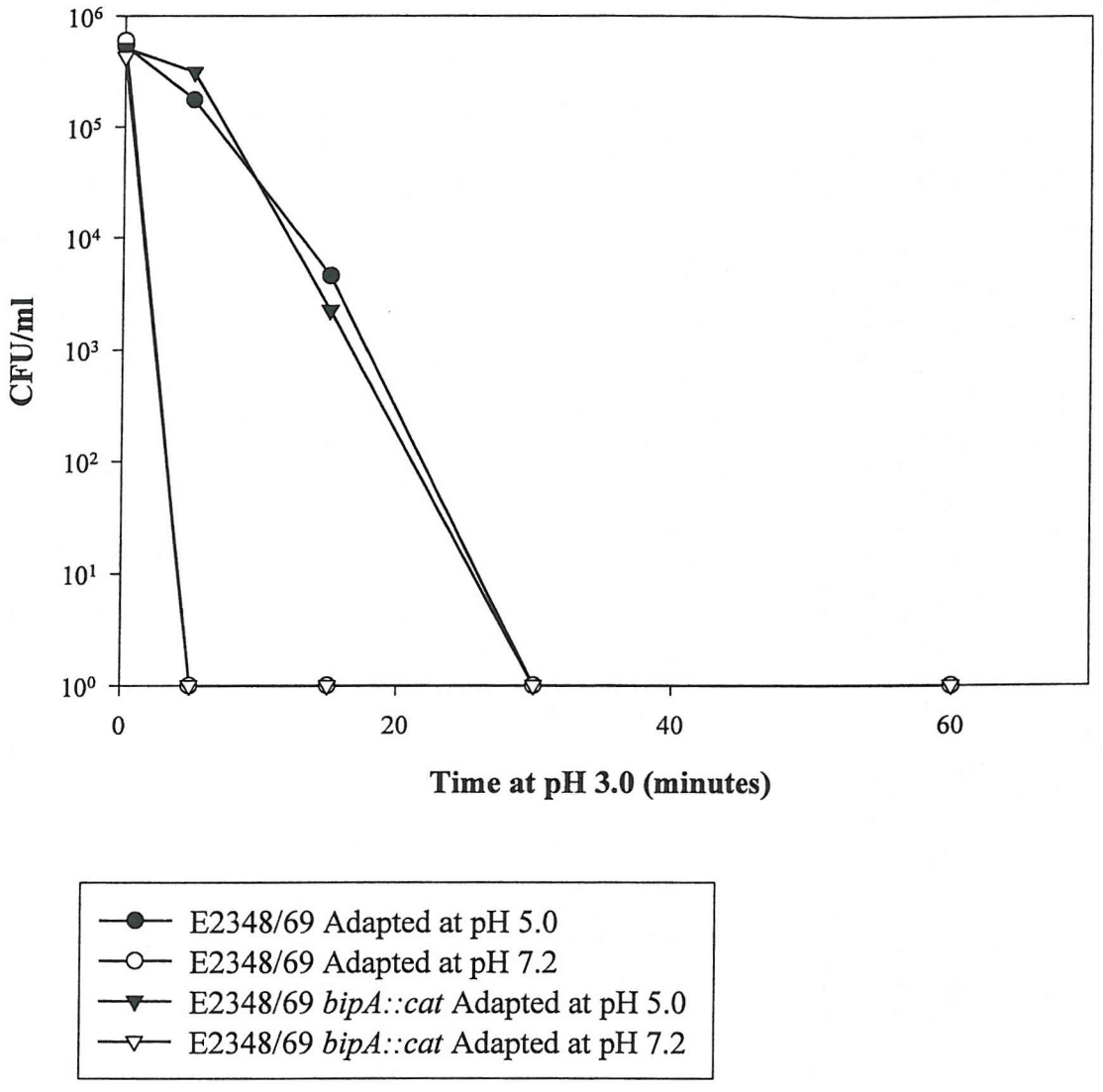


Figure 7.3. Lack of increased survival of a *bipA* null mutant of E2348/69 at pH 3.0 following adaptation at pH 5.0. *E. coli* E2348/69 wild type and *bipA::cat* strains were adapted to inorganic acid at pH 5.0 or to pH 7.2 for 60 minutes prior to extreme acid exposure for various lengths of time at pH 3.0. Surviving culturable cells were counted after plating on LB agar (pH 7.2).

7.5. BipA has no Effect on the Survival Kinetics of EPEC Strain JPN15 During the Exponential Phase ATR.

The *E. coli* MAR001 strain lacks the EAF plasmid (pMAR2) (Baldini *et al*, 1983), which is required for the full virulence of the E2348/69 strain (Jerse and Kaper, 1991). As described in Chapter 1, the EAF plasmid encodes factors such as the bundle forming pili, essential for EPEC localised adherence to the host epithelia (Girón *et al*, 1993). The *per* locus is also situated on the EAF plasmid and is required for transcriptional activation of the *eae* gene (Gomez-Duarte and Kaper, 1995), essential for intimate adherence and A/E lesion formation (Jerse *et al*, 1991). As BipA has also been linked with the regulation of A/E lesion formation (Farris *et al*, 1998), it was possible that the absence of pMAR2 in MAR001 could be responsible for the BipA induced ATR effects displayed by this strain.

To determine if the lack of an EAF plasmid allows BipA to exert a negative regulatory effect on the ATR, the acid survival assays were repeated using *E. coli* JPN15, an EAF cured derivative of E2348/69 (Jerse and Kaper, 1991). Again, assays were carried out in duplicate on exponential phase cells. They were repeated at least twice.

Surprisingly, JPN15 showed an increased survival time at pH 3.0 relative to the parental E2348/69 cells (compare Figure 7.4 and Figure 7.3). This was true of both pH 5.0 and pH 7.2 adapted bacteria. After 30 minutes of pH 3.0 exposure, the concentration of viable acid adapted JPN15 cells was four log orders above that of similarly treated E2348/69 cultures. The viability of pH 5.0 adapted JPN15 cells did not reach zero until the bacteria had been exposed to pH 3.0 for 60 minutes (Figure 7.4). Likewise, JPN15 bacteria adapted to pH 7.2 were able to tolerate extreme acid conditions for longer than their E2348/69 counterparts. Here, JPN15 cell viability was undetectable after 15 minutes (Figure 7.4), as opposed to 5 minutes for the E2348/69 strain (Figure 7.3). Interestingly, *bipA* appears to have no effect on the acid tolerance response of JPN15, as the *bipA::cat* derivative showed ATR survival kinetics similar to those of the wild type strain (Figure 7.4).

These results suggest that removal of the EAF plasmid might confer a degree of constitutive acid tolerance on E2348/69 cells. This, more hardy acid tolerant phenotype

results in increases in survival time when exposed to pH 3.0 conditions in ATR induced and non-induced bacteria.

The pH 3.0 survival profile of pH 5.0 and pH 7.2 adapted JPN15 *bipA::cat* cells was found to be similar to that of the wild type strain (Figure 7.4). It is therefore concluded that removal of the EAF plasmid from EPEC strains does not influence the ability of BipA to regulate the ATR.

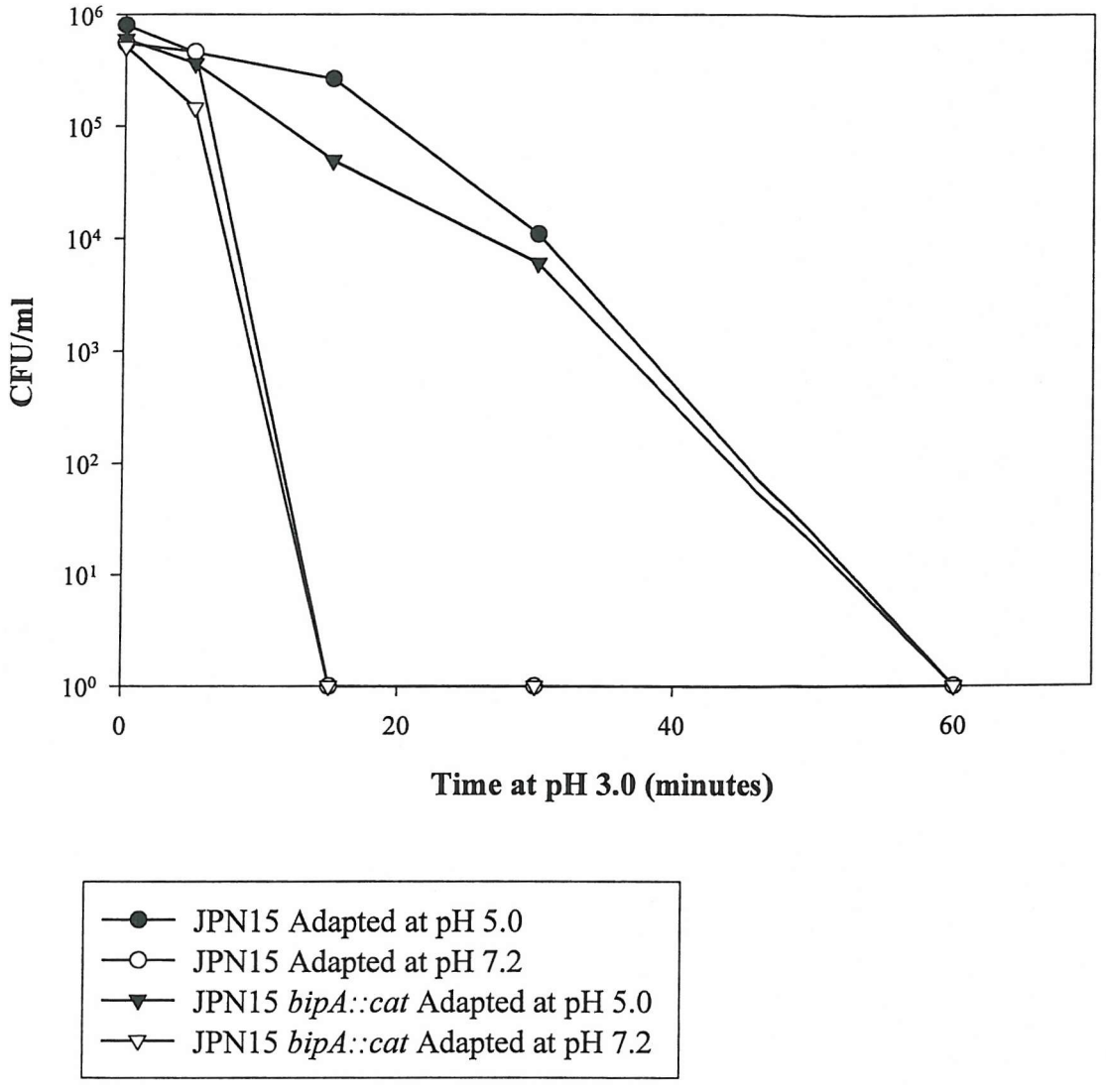


Figure 7.4. Increased survival of an EAF plasmid cured derivative (JPN15) of E2348/69 at pH 3.0 following acid adaptation at pH 5.0 (see Figure 7.3). The JPN15 *bipA* null mutant shows no increased survival relative to the JPN15 parent under these conditions. *E. coli* JPN15 wild type and *bipA::cat* strains were adapted to inorganic acid at pH 5.0 or to pH 7.2 for 60 minutes prior to extreme acid exposure for various lengths of time at pH 3.0. Surviving culturable cells were counted after plating on LB agar (pH 7.2).

7.6. BipA has no Effect on the Survival Kinetics of *S. typhimurium* SL1344 During the Exponential Phase ATR.

To determine if the BipA protein was involved in the exponential phase ATR of *S. typhimurium*, the survival kinetics of the SL1344 strain and its *ΔbipA* derivative were compared in acid. ATR survival assays identical to those described in Chapter 3 (section 3.4) and section 7.2 above were used. As before, ATR assays were carried out in duplicate on exponential phase cells. They were repeated at least twice.

As described previously, pH 5.0 adapted wild type SL1344 cells were able to tolerate pH 3.0 conditions with no significant fall in survival for up to 60 minutes. Cell viability was undetectable after 90 minutes (Figure 7.5). An essentially identical survival profile was displayed by the acid adapted SL1344 *ΔbipA* strain when exposed to pH 3.0 (Figure 7.5). There were also no comparable differences between the extreme acid survival profiles of the pH 7.2 adapted SL1344 and *ΔbipA* cells (Figure 7.5).

These data indicate that the BipA protein does not regulate the ATR in exponential phase *S. typhimurium*. Moreover, it is clear that the regulatory systems behind the ATR mechanisms of enteropathogenic bacteria appear to vary between serovars and species.

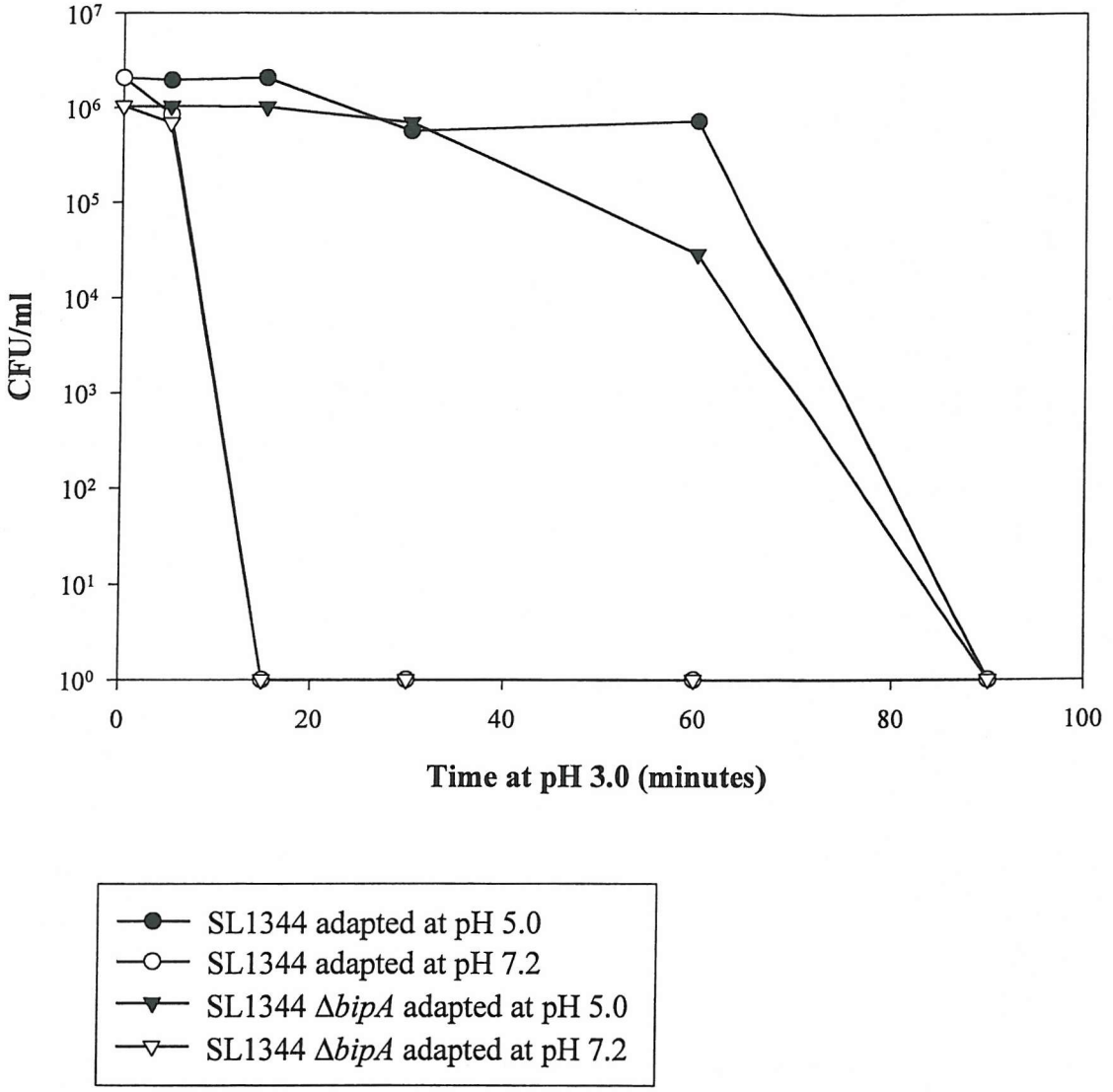


Figure 7.5. Lack of increased survival of a *bipA* null mutant of SL1344 at pH 3.0 following adaptation at pH 5.0. *S. typhimurium* SL1344 wild type and *ΔbipA* strains were adapted to inorganic acid at pH 5.0 or to pH 7.2 for 60 minutes prior to extreme acid exposure for various lengths of time at pH 3.0. Surviving culturable cells were counted after plating on LB agar (pH 7.2). Data for WT SL1344 are taken from Figure 3.5.

7.7. The Lipopolysaccharide (LPS) Profile of Neither *E. coli* MAR001 nor *S. typhimurium* SL1344 is Altered by BipA.

The BipA GTPase is up regulated in response to, and is required for protection against the host defence peptide, BPI in both *E. coli* MAR001 and *S. typhimurium* SL1344 (Qi *et al*, 1995; Farris *et al*, 1998; Barker *et al*, 2000). BPI has a potent bactericidal activity against a wide variety of gram negative bacteria (Weiss *et al*, 1978). The specificity of this cationic peptide is due to its high affinity for the lipid A region of LPS to which it binds with an apparent Kd of approximately 2 to 5 nM (Gazzano-Santoro *et al*, 1992). Therefore, it was hypothesised here that BipA may protect against BPI by altering the LPS profile of the bacterial outer membrane, producing a concomitant decrease in the binding affinity of the host defence protein.

The PhoPQ regulatory system has already been shown to protect against host cationic antimicrobial peptides (CAMP) in *S. typhimurium* in a similar manner to that suggested here for BipA (Guo *et al*, 1997). PhoPQ regulates structural modifications of lipid A, including the addition of aminoarabinose and 2-hydroxymyristate. The fact that mutants lacking the PhoP activated *pagP* gene demonstrate an increased outer membrane permeability in response to CAMP supports the theory that increased lipid A acetylation represents a CAMP resistance mechanism (Guo *et al*, 1998). In conjunction with PhoPQ, the PmrAB system also regulates the modification of LPS (Gunn *et al*, 1998). PmrAB modifies the LPS core and lipid A regions with ethanolamine and adds aminoarabinose to the 4' phosphate of lipid A.

As both the PhoPQ and PmrAB systems are activated during the ATR of *S. typhimurium* (Bearson *et al*, 1998), structural modifications of LPS may be a key requirement of acid tolerance. It was therefore hypothesised that the BipA protein may alter the LPS structure in such a way as to be detrimental to acid tolerance, but beneficial to protection against BPI. This could explain the negative effect of BipA seen on the ATR in the *E. coli* MAR001 strain.

To determine if BipA had any effect on LPS structure, the LPS composition of wild type and *bipA* null mutant strains was compared. The LPS was isolated from the MAR001 and SL1344 strains by proteinase K digestion of whole cell extracts. It was

then visualised by silver staining after SDS-PAGE (Hitchcock and Brown, 1983). The morphological heterogeneity of SDS-PAGE LPS profiles can be attributed to variations in the O-specific polysaccharides, core oligosaccharides and lipid A content of the LPS. The SDS-PAGE LPS profiles appear as characteristic low molecular weight (less than 14 kDa) single or doublet bands of varying colouration.

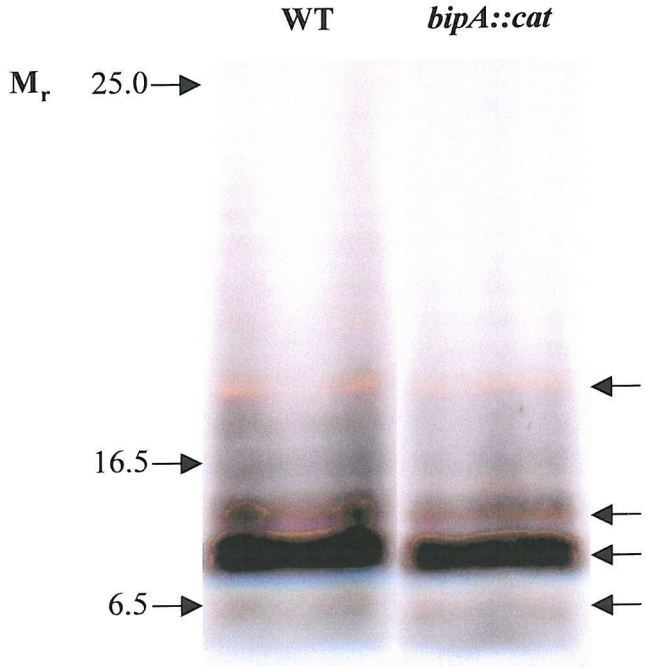
The LPS profiles of the wild type and *bipA::cat* strains of *E. coli* MAR001 were identical (Figure 7.6a). Characteristic grey and red doublets and single yellow bands (arrowed) were observed in both strains. Changes in the LPS migration pattern due to alterations in the LPS profile of the *bipA::cat* mutant were therefore not seen.

The LPS profile of the wild type and *ΔbipA* strains of *S. typhimurium* SL1344 were also identical (Figure 7.6b). Characteristic red doublets (arrowed) and grey and yellow single bands were seen (arrowed). Changes in the LPS migration pattern due to the aforementioned alterations in LPS structure were thus not observed in the *ΔbipA* mutant.

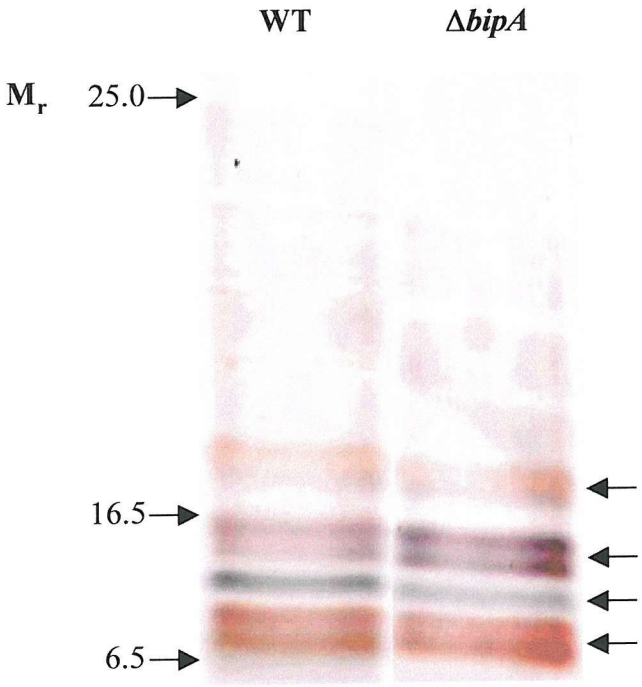
These results suggest that BipA does not impose morphological changes in LPS structure in *E. coli* and *S. typhimurium*. It is therefore concluded that the negative regulation of the ATR in the *E. coli* MAR001 strain is not mediated by alterations in LPS constituents.

Figure 7.6. (next page). LPS was isolated from *E. coli* MAR001 and *S. typhimurium* SL1344 strains by proteinase K digestion. It was then visualised by silver staining after SDS-PAGE. **a.** The LPS profiles of the MAR001 wild type and *bipA::cat* strains. Equal sample loadings were used. Characteristic low molecular weight grey and red doublets and single yellow bands were viaualised (arrowed). **b.** The LPS profiles of the SL1344 wild type and *bipA::cat* strains. Equal sample loadings were used. Characteristic low molecular weight red doublets (arrowed) and grey and yellow single bands (arrowed) were visualised.

a. MAR001



b. SL1344



7.8. Discussion.

Previous studies have shown that the BipA GTPase is required for, or regulates a number of virulence-related processes including (i) the ability of EPEC to induce A/E lesions on mammalian host cells (Farris *et al*, 1998); (ii) the resistance of *Salmonella* and *E. coli* strains to certain antimicrobial peptides (Farris *et al*, 1998; Barker *et al*, 2000); (iii) flagella mediated cell motility and chemotaxis (Farris *et al*, 1998); (iv) the H-NS, UspA and Csp15 proteins (Freestone *et al*, 1998) and (v) group 2 capsule formation in *E. coli* (Rowe *et al*, 2000).

This chapter reports for the first time that in both inorganic and organic acid environments, BipA negatively regulates the ATR of the EPEC strain, MAR001. Targeted disruption of the *bipA* gene prior to acid adaptation resulted in cells that survived twice as long at pH 3.0 than the similarly adapted parental strain (Figures 7.1 and 7.2). Transcomplementation of the *bipA* null mutant with a plasmid carrying the wild type *bipA* gene restored normal levels of acid tolerance (Figures 7.1 and 7.2). This result confirmed that the increased survival time observed at pH 3.0 was due to the absence of the BipA protein. Disruption of the *bipA* gene had no effect on the acid tolerance of MAR001 cells that had been adapted at pH 7.2 (Figures 7.1 and 7.2). This indicates that removal of the BipA protein does not introduce a degree of constitutive acid tolerance, but actually enhances the protection afforded by the induction of the ATR mechanism itself. This is the first time that the disruption of a gene has been found to induce a stronger ATR, rather than increase the overall level of inherent acid resistance of a particular strain. For example, previous work has identified that pyruvate dehydrogenase (Foster and Hall, 1991) and phosphoglucoisomerase (Foster and Bearson, 1994; Bearson *et al*, 1998) null mutants display an increased acid tolerance, but this is at the constitutive level: these mutants survive extreme acid conditions, even if taken directly from a neutral pH environment. Therefore, BipA is likely to restrict the optimum performance of ATR mechanisms in the *E. coli* MAR001 strain.

The precise mechanistic effect of BipA on the ATR of MAR001 remains to be identified. One possibility investigated here was the ability of BipA to modify the morphology of the bacterial LPS structure. The PhoPQ system, which like BipA, is

induced by host defence peptides, uses a similar mechanism to reduce the binding affinity of these antimicrobials to the bacterial outer membrane. As PhoPQ is also activated by low pH, it was thought that an altered LPS structure may participate in acid tolerance. Thus, if BipA altered the LPS structure to prevent peptide binding, but in a different manner to that of PhoPQ, this could lead to a reduction in the acid tolerance of BipA positive strains. BipA negative strains would be more acid tolerant as they would have a 'more PhoPQ like' LPS morphology. This hypothesis, however, was not borne out. The LPS structures of MAR001 wild type and *bipA::cat* strains visualised on SDS-PAGE gels were found to be equivalent in composition (Figure 7.6a). It is possible that any BipA dependent structural alterations to LPS are subtle and hence not detectable using gel analysis. However, it will be necessary to carry out detailed mass spectrometry experiments on purified LPS to test this idea.

Another possible explanation is that BipA could regulate gene expression in an antagonistic fashion to that of the ATR. Exposure of *S. typhimurium* SL1344 to BPI resulted in the up regulation of BipA expression together with changes in the profiles of a number of other proteins visualised on 2D gels (Qi *et al*, 1995). It is probable that this altered expression profile is essential for BPI resistance, and at least some of the proteins may be regulated by BipA. Interestingly, the proton-translocating ATPase (Atp) was down regulated and the pyruvate dehydrogenase E3 subunit was up regulated in response to BPI. It has been previously shown that *atp* expression increases during the ATR of *S. typhimurium* (and *atp* mutants are acid sensitive); in addition a lack of pyruvate dehydrogenase expression in the same bacteria results in constitutive acid tolerance (Foster and Hall, 1991). The present study also identifies the pyruvate dehydrogenase E1 subunit as a down regulated component of the *Salmonella* ATR. These results suggest that BPI resistance may require a protein expression profile which is deleterious to acid tolerance. This may be regulated by BipA. However, when the 2D gel profiles of BipA mutants are compared with those produced during the ATR, no antagonistic protein regulation is seen (S. Payot, personal communication). In fact, in MAR001, BipA negatively regulates flagellin synthesis (Farris *et al*, 1998), a process that the present study has shown to be true of the ATR. These results suggest that BipA may influence the ATR via an as yet undefined novel regulatory mechanism.

In contrast to the EPEC strain, MAR001, the BipA GTPase had no effect on the ATR of another EPEC serovar, E2348/69, or on the ATR of a different species, *S. typhimurium* SL1344. Targeted disruption or deletion of the *bipA* genes of E2348/69 and SL1344 respectively did not result in any differences in extreme acid survival time when compared with the parental strains. This was true of both pH 5.0 and pH 7.2 adapted bacteria (Figures 7.3 and 7.5).

These results suggest that the BipA GTPase has different regulatory properties concerning the ATR in specific EPEC serovars and in distinct species of enteropathogenic bacteria. Two of the most prominent explanations for the intraspecies differences are (i) variations in the pathogenic properties of *E. coli* and *Salmonella* and (ii) dissimilarities in the ATR mechanisms employed by these two bacteria. As an intracellular pathogen, *S. typhimurium* does not form the A/E lesions and pseudopods on epithelial cells that are characteristic of EPEC infections. It is therefore clear that BipA cannot function in the positive regulation of A/E lesion formation in *Salmonella.* The negative regulation of the ATR in MAR001 by BipA may therefore be connected in some way with this role in the intestinal phase of disease. For example, acid tolerance is not required in the neutral pH of the small intestine. Therefore, in *E. coli*, it is advantageous to inhibit the ATR (which could be an unnecessary drain on resources) via a mechanism required for long term intestinal survival. As *Salmonella* does not form A/E lesions, and is exposed to the luminal side of the intestinal epithelia for a relatively short time, it may not require this function. The primary role of BipA in *Salmonella* may be as a defence against host antimicrobial peptides such as BPI. Due to the nature of the pathogenic mechanism of this bacterium, it is likely to be in a more acidic environment than *E. coli* when CAMP exposure occurs (for example, in the macrophage "phagosome"). It would thus be self defeating for BipA to have a negative effect on the ATR of this micro-organism under such circumstances.

As detailed in Chapter 1, a number of differences in the ATR mechanisms of *E. coli* K-12 and *S. typhimurium* exist. For example, components of the *E. coli* K-12 ATR such as increased CFA synthesis (Brown *et al*, 1997; Chang and Cronan, 1999), acid shock RNA (Suziedeliene *et al*, 1999), extracellular induction components (Rowbury and Goodson, 1999a) and the oxidative system of acid tolerance (Castaine-Cornet *et al*, 1999) have, as yet, not been reported in *S. typhimurium*. BipA could thus be regulating

an ATR mechanism specific to *E. coli*, explaining an absence of any effect in *S. typhimurium*.

The differential regulation of the ATR by BipA in different serovars of *E. coli* is more difficult to explain. It may stem from the presence of variations in the ATR mechanisms employed by specific strains. This is difficult to ascertain as published reports tend to specialise in particular organisms. It is almost impossible to compare research on the ATRs of *E. coli* serovars between reports as, invariably, different ATR assay conditions are used. At least, from the studies reported here (Chapter 3), the EPEC MAR001 and E2348/69 strains appear to have very similar ATR profiles in inorganic and organic acids under identical assay conditions.

It was thought possible that the absence of an EAF plasmid in MAR001 might explain the differential regulatory properties of BipA in this bacteria compared with the EAF positive E2348/69. However, the ATR profile of *E. coli* JPN15 (an EAF minus derivative of E2348/69) was not altered by disruption of the *bipA* gene (Figure 7.4), suggesting that this hypothesis was wrong. Interestingly though, removal of the EAF plasmid from E2348/69 had a positive effect on the acid tolerance of this strain: survival time at pH 3.0 was increased in both pH 5.0 and pH 7.2 adapted cells (Figure 7.4). This suggests that the EAF plasmid, like BipA, negatively regulates the ATR. Here, though, a small degree of constitutive acid tolerance is seen. BipA and the EAF plasmid share an additional similarity in that they both promote A/E lesion formation. The *per* locus on EAF is required for transcriptional activation of the *eae* gene within the LEE pathogenicity island. This is essential for intimate adhesion and A/E lesion formation (Gomez-Duarte and Kaper, 1995). As the intestinal phase of the disease process takes place near neutral pH, the EAF plasmid and BipA may act together in *E. coli* to negatively regulate the energetically wasteful ATR, which would probably have previously been induced upon passage through the stomach.

In summary, the research reported here identifies additional regulatory links between mechanisms required for pathogenic properties of a micro-organism and a stress tolerance response, also essential for virulence. It is becoming increasingly clear that bacteria are able to co-regulate a number of processes to optimise their survival

potential in particular host environments. The complexity of these systems and their variability between different bacterial strains and species cannot be underestimated.

CHAPTER 8.

GENERAL DISCUSSION.

CHAPTER 8. GENERAL DISCUSSION.

Enteropathogenic strains of *E. coli* and *Salmonella* are responsible for some of the most common foodborne diseases in man. Potentially fatal bacterial infections attributable to food affect approximately 8 million people and cause 9000 deaths per year, even in the most sanitised of cultures (Altekruse *et al*, 1997). These disease-causing bacteria utilise specific virulence mechanisms to co-ordinate the pathogenic process within the host. Furthermore, in this environment the micro-organisms are frequently exposed to potentially life threatening stress conditions. They thus also have to employ particular tolerance mechanisms if they are to survive the extreme environments encountered and precipitate disease. The detailed characterisation of such virulence and stress tolerance processes obviously has great relevance towards the future treatment of foodborne illnesses. Research could potentially uncover novel drug targets of importance to both the pharmaceutical and food preservation industries.

This thesis has presented detailed studies of the exponential phase acid tolerance responses of enteropathogenic strains of *E. coli* and *S. typhimurium*. The complete elucidation of these mechanisms alone will be of immense benefit to future strategies designed to combat these bacteria. For example, the removal of acid tolerance should result in the eradication of the micro-organism's ability to survive the extreme pH conditions found within the stomach. This could abolish the oral route of infection completely. An ATR negative bacteria would also be unable to survive in acidic foodstuffs, perhaps allowing the development of more comprehensive, organic acid based food preservation techniques. The inhibition of the ATR via food additives would seem a more appropriate way to combat food poisoning than the administration of drugs. As has been so clearly demonstrated with the prevalent use of previous antibiotics, the use of drugs may only result in the evolution of more drug-resistant strains. The implications of the results obtained in this study will be discussed here.

Studies presented in this thesis have established a protocol using a defined set of assay conditions that enables the ATR survival kinetics over time to be easily compared between bacterial species and strains in identical growth phases. In this way, new detailed profiles pertaining to the survival of exponential phase enteropathogenic *Salmonella* and *E. coli* strains in defined acidic environments have been characterised.

The effects of acid adaptation time and extreme acid exposure time on the survival of these bacteria have been identified in both organic and inorganic acid environments. These data enabled the exact conditions required for the induction of optimal ATRs in the strains examined to be deduced.

Differences in the ATR survival profiles between the *Salmonella* and EPEC strains were thought to be attributable to differences in the pathogenic habits of the two species. For example, *Salmonella* was more tolerant of inorganic acid conditions at low pH, as perhaps found within the macrophage. The EPEC strains, were relatively more capable of enduring exposure to organic acid, a phenotype that might be advantageous when exposed to the fermentation by-products of the gut for extended periods. The ATR differences were also described in relation to known variations in the ATR mechanisms employed by each species. To further investigate these theories, the ATR survival assay protocols could be applied to monitor the acid tolerance of the bacteria in conditions closer to those found in their natural environments. For example, the pH adjusted LB medium could be replaced by synthetic gastric fluid or a solution representing the intestinal lumen. The effects of acid adaptation could also be studied *in vivo*, perhaps by monitoring intramacrophage survival or faecal counts of orally administered bacteria. The ability of the EPEC strains to endure organic acid environments implies that the use of these chemicals in food preservation may be insufficient. This hypothesis could be tested by using the ATR assays to monitor survival in foodstuffs preserved in this way.

Characterisation of the conditions required to induce an optimal ATR in exponential phase *S. typhimurium* enabled the initiation of proteomic studies to identify changes in protein expression attributable to this tolerance mechanism. The exponential phase ATR stimulon of the LT2 positive *S. typhimurium* SL1344 strain was identified using whole cell lysates with an IPG of pH 4-7. The majority of the ATR associated changes in protein synthesis involved repression effects. This was plausible as the literature allies the ATR with a more hardy, less metabolically active, stationary phase phenotype that includes the up-regulation of RpoS levels (see for example, Lee *et al*, 1994) and the possible induction of the stringent response (Rallu *et al*, 2000).

Through the use of tryptic mass fingerprinting in conjunction with nanoelectrospray mass spectrometry and/or N-terminal sequencing, the majority of proteins falling within the ATR stimulon of SL1344 and detectable in whole cell lysates have been identified. Most of these proteins could be divided into one of three groups according to possible acid tolerance functions: i) those whose altered expression would reduce proton influx and conserve energy in the form of ATP; ii) those whose altered expression would result in a hardier, less metabolically active cell and iii) those which are co-regulated by oxygen availability. A further group of identified proteins were hitherto hypothetical gene products of unknown function resulting from genome sequencing projects. Future investigations could focus on identifying or clarifying the roles that the above proteins have to play in the ATR.

Knock-out or constitutive mutants could be constructed in order to study the effect of particular gene products on the ATR survival kinetics of SL1344. However, the fact that the altered expression of one protein alone may not have any effect on acid tolerance has to be considered. For example, the decreased expression of a whole group of proteins may be required to produce enough of a reduction in proton influx to be beneficial in acidic conditions. Therefore, the effect of multiple mutations in ATR associated genes may have to be studied, although these could render the cells nonviable. Any gene products producing significant alterations in the bacteria's ability to tolerate acidic conditions could represent potential drug targets.

The predicted functions of the ATR associated proteins could be investigated. For example, to identify whether a particular protein has an effect on proton influx, the pHi of strains lacking the relevant gene could be compared with that of wild type bacteria. Bacterial pHi values have been deduced in the past by measuring the distribution of radio-labelled weak acids or bases across the cellular membrane (Foster and Hall, 1991).

It would be of benefit to investigate whether a similar ATR stimulon is present in other *Salmonella* strains or bacterial species when exposed to comparable environmental conditions. It would also be useful to compare the ATR stimulon expressed *in vivo* with that reported in the lab. Proteomic studies could be used for these purposes. Any

acid tolerance associated gene products found to be common to all scenarios could represent future drug targets.

These studies have highlighted the power of the proteomic technique in identifying components of the ATR in a relatively short time period and that may have taken years to come to light (or may not have been discovered at all) using more traditional, genetically based procedures, such as transposon mutagenesis. It should be noted, however, that the detection of low abundance proteins is still a major problem without enrichment procedures. It is likely that further development of proteomics will ameliorate this problem. Also highlighted, is the ability of the proteomic approach to make good use of the data provided by genome sequencing projects. It is predicted that as more genomes are sequenced, proteomic and associated bioinformatic procedures will become more widely used. Genome sequences themselves cannot tell us much about the conditions, or the specific cell type (in the case of multicellular organisms) in which genes are expressed. Proteomics will be the ideal tool for this purpose.

Proteomic studies identified flagellin as a protein that was repressed during the *S. typhimurium* SL1344 ATR. The subsequent detailed investigation of the regulatory mechanisms behind this effect indicated that the flagellar apparatus was repressed at the level of the class one master operon during the ATR. It was discovered that this effect was mediated, either directly or indirectly via the PhoPQ two component regulatory system. These results revealed the existence of intimate connections between the regulation of the ATR, flagellar mediated cell motility and other virulence associated processes required for pathogenicity. One reason why the flagellar apparatus is down regulated at low pH could be to reduce proton influx and conserve energy in the form of ATP. Additionally, it would be disadvantageous for the cell to synthesise flagella under these conditions, as depolymerisation of the flagellin filament occurs at low pH rendering the bacteria immobile (Macnab, 1996). Again, the hypothesised function of flagella repression in acid tolerance could be investigated by comparing pHi measurements between mutant and wild type strains. Studies could also be initiated to investigate whether the flagellar apparatus is regulated in a similar manner in other bacterial species and strains.

The reported repression of flagellar biosynthesis by the PhoPQ system is only one of several results described within this thesis which have identified a close connection between the regulation of virulence associated mechanisms required for pathogenicity and the ATR. Proteomic studies using mutant strains have enabled a number of additional ATR associated proteins in *S. typhimurium* to be assigned to the PhoPQ regulon. Interestingly, these gene products were found to be regulated in a similar manner during the oxidative stress response. This suggested that the connection between virulence properties and the ATR could also be extended to other stress responses. Indeed, a number of ATR associated proteins were found to be regulated by RpoS, a sigma factor that is known to regulate a number of different stress response mechanisms (Foster and Spector, 1995; Hengge-Aronis, 1996). On the basis of these results, it was suggested that the ability to co-regulate stress response mechanisms and virulence associated processes would impart a significant survival advantage on the bacteria during pathogenesis. For example, *Salmonella* may be able to regulate pathogenicity islands and stress responses required for intramacrophage survival via the same regulatory molecules. In this context, it would be interesting to use proteomic studies to ascertain if additional global regulatory molecules could control the expression of the ATR associated proteins identified here.

The co-regulation of elements of the acid and oxidative stress responses by PhoPQ prompted an investigation of any cross-protection afforded by these two tolerance mechanisms. It was found that the ATR could cross protect against oxidative stress and that this was in some way mediated by PhoPQ. The fact that the cross tolerance was unreciprocated suggested that the ATR may be a more general stress response than the OSR. Therefore, it was suggested that when induced within the stomach, the ATR may be used to prime the bacteria for defence against subsequent stressful environments within the host.

The link between the regulation of pathogenesis and stress response mechanisms was potentiated further when ATR assays of mutant strains indicated that BipA and the EAF plasmid also affected the ATR. Interestingly, both BipA and the EAF plasmid are required for A/E lesion formation in EPEC and have been found here to negatively regulate the ATR in certain strains of these bacteria. BipA was found to have no effect on the ATR of *S. typhimurium*. Therefore, it was concluded that the regulation of the

ATR is variable between different species and strains. It was suggested that this may be linked to the specific pathogenic properties of particular bacteria. For example, *Salmonella* spends proportionately more time than *E. coli* in acidic conditions during pathogenesis. It was thus thought that *Salmonella* would be at a disadvantage if BipA inhibited acid tolerance. When bound to the external, mucus coated face of epithelial cells, *E. coli* may not have to contend with low pH. Thus, this bacterium would be at an advantage if the ATR, recently induced by passage through the stomach, was inhibited by BipA at this stage of infection.

Under defined assay conditions, the ATR survival kinetics of enteropathogenic strains of *E. coli* and *S. typhimurium* have been characterised. Subsequent proteomic studies have identified the exponential phase ATR stimulon of *S. typhimurium*. This research has also elucidated a number of potential ATR regulatory mechanisms. In particular, intimate connections have been revealed between the regulation of the ATR and that of other stress responses and virulence mechanisms required for pathogenesis. One of the most prominent of these discoveries was the link between motility, acid tolerance and the PhoPQ system. The results have important implications regarding the design of future strategies required to combat these bacterial pathogens. Global regulators that serve to co-regulate these systems may prove to be ideal drug targets for future treatments of 'food poisoning'. For example, in the invention of new food preservation techniques. It is potentially to its advantage that the bacteria employs a mechanism of regulation whereby one global regulator can control a number of different processes in a particular environment. However, this could turn out to become the micro-organisms' Achilles heel.

APPENDIX A.

A number of the proteins that were found to be differentially regulated during the exponential phase ATR of *S. typhimurium* SL1344 were not identified (see Chapter 4), even though tryptic mass fingerprinting followed by nanoelectrospray mass spectrometry produced good peptide mass data. It is thus possible that these proteins are novel and currently do not exist in the databases searched. Therefore, for future reference, the 2D gel spot numbers for these proteins (corresponding to those in Figure 4.2) are listed below alongside their respective peptide mass data.

Spot Number	Peptide Masses Obtained (Da)
1.	676.993; 735.166; 790.376; 795.333; 1097.579; 1172.576; 1247.615; 1381.708; 1617.763; 1694.861; 1947.976; 2028.064; 2163.071; 3389.967.
7.	625.364; 636.406; 773.367; 837.354; 1461.769; 1476.845; 1937.122; 2163.101; 2273.222; 2550.287; 2659.378; 3212.552.
11.	790.399; 795.347; 1029.679; 1181.607; 2185.122; 2201.097; 2301.251; 2549.303; 2659.397; 4143.562.
17.	790.382; 1440.723; 1539.761; 2028.978; 2185.084; 3320.746; 3676.858; 4150.184.
13.	613.239; 671.340; 693.316; 735.319; 837.363; 901.347; 1223.513; 1261.508; 1837.601; 2550.329.

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