UNIVERSITY OF SOUTHAMPTON

FACULTY OF MEDICINE, HEALTH AND LIFE SCIENCES

School of Biological Sciences

A Proteomic Analysis of the Osmotic Shock Response in *Salmonella enterica* Serovar Typhimurium.

by

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ABSTRACT

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A PROTEOMIC ANALYSIS OF THE OSMOTIC SHOCK RESPONSE IN *SALMONELLA ENTERICA* SEROVAR TYPHIMURIUM.

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Bacteria inhabit diverse and rapidly changing environments, and one of the most common stresses encountered is a change in osmotic conditions. Enteric bacteria in particular, such as *Salmonella enterica* serovar Typhimurium provide good examples of the problems faced as they need to survive for long periods of time in low osmolarity conditions (e.g. pond water) as well as in the high osmolarity conditions of regions of the gastrointestinal tracts of their hosts. Osmotically regulated components may be involved in virulence and study of these may shed light on genes involved in the disease process as well as on mechanisms of adaptation to hyper-osmotic stress. The aims of this study were to investigate the osmotic responses of *Salmonella* and in particular to identify changes that occur at the protein level when *Salmonella* encounters hyper-osmotic conditions.

A reliable 2-dimensional gel electrophoresis methodology for the analysis of the *Salmonella* Typhimurium proteome was established and combined with mass spectrometry to detect and identify proteins that were differentially expressed when *Salmonella* was exposed to hyper-osmotic stress. In wild type *S.* Typhimurium 24 proteins were differentially expressed, 13 were up-regulated and 11 down-regulated. The proteins identified were involved in a range of bacterial systems including protein handling, protein synthesis, detoxification, metabolism and cell morphogenesis. In addition, an *ompR*$^-$ mutant was used to determine which of these differentially expressed proteins were regulated by or independent of the OmpR/EnvZ regulatory system. Generally, a similar set of components were differentially expressed in the *ompR*$^-$ mutant. However, the degree of induction or repression tended to be exaggerated. Additional analyses using the non-gel LC/MS based Protein Expression System™ strongly supported these results, further highlighting the up-regulation of proteins involved in cell morphogenesis, and also suggesting a subtle down-regulation of TCA cycle components.

Two of the proteins that were highly induced by hyper-osmotic stress, and implicated in determining cell shape, were MreB and YgaU (the latter was the most strongly induced protein, as detected by 2-D gel analyses, in both *Salmonella* strains). Further mRNA analyses indicated that YgaU is transcriptionally up-regulated under hyper-osmotic stress, whereas MreB appears to be post-transcriptionally controlled. Mutational analysis also confirmed that MreB is involved in cell morphology in *Salmonella.*

These current findings suggest that investigation of cell wall remodelling processes may be important to further the understanding of the osmoadaptative processes of *S.* Typhimurium.

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ABBREVIATIONS

ATP	Adenosine Triphosphate
ATR	Acid Tolerance Response
BCA	Bicinchoninic Acid
BSA	Bovine Serum Albumin
CHAPS	3-[(3-Colamidopropyl)dimethylammonio]-1-Propanesulfonate
CRP	C-Reactive Protein
CT	Threshold Cycle
DEPC	Diethylpyrocarbonate
DNA	Deoxyribonucleic Acid
dNTP	Deoxyribonucleotide Triphosphate (nucleotides)
DTT	Dithiothreitol
E. coli	*Escherichia coli*
EDTA	Ethylenediaminotetraacetic Acid
FLP	Flippase
FRT	FLP Recognition Target
HCL	Hydrochloric Acid
HEPES	N-(2-hydroxyethyl)-piperazine-N'-2-ethanesulfonicacid
HIV	Human Immunodeficiency Virus
H-NS	Histone-like Nucleoid Structuring Protein
HSP	Heat Shock Protein
ICAT	Isotope-Coded Affinity Tagging
IEF	Isoelectric Focusing
IPG	Immobilised pH Gradient
kDa	KiloDalton
LB	Luria Bertani
LC	Liquid Chromatography
MALDI-TOF	Matrix Assisted Laser Desorption /Ionization- Time Of Flight
MOPS	3-(N-morpholino)propanesulfonic acid
mOsm	Milliosmol
MS	Mass Spectrometry
NCBI	National Centre for Biotechnology Information
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PEEC	Pathogen-Elicited Epithelial Chemoattractant
pI	Isoelectric Point
PMN	Polymorphonuclearleukocyte
PMSF	Polymethylsulphonylflouride
Q-TOF	Quadrupole Time-of-Flight
RNA	Ribonucleic Acid
ROS	Reactive Oxygen Species
SDS	Sodium Dodecyl Sulphate
SDS-PAGE	SDS-Polyacrylamide Gel Electrophoresis
SIP	*Salmonella* Invasion Protein
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

TAE	Tris-acetate EDTA Buffer
TTSS	Type-Three Secretion System

CHAPTER ONE

INTRODUCTION

CHAPTER 1. INTRODUCTION

1.1 General Introduction

It has been known since the dawn of biochemistry that enzymes and other bio-molecules function best at particular ionic strengths and that deviation from the optimal salt concentration can have potentially disastrous consequences. As a consequence of these strict requirements living organisms have evolved multiple, complex mechanisms to control intracellular osmolarity.

Maintenance of intracellular osmolarity is particularly critical for free-living unicellular organisms such as bacteria, as they often face dramatic swings in external osmotic conditions. Enteric bacteria, such as *Escherichia coli* and *Salmonella enterica* provide good examples of the types of problems faced as they need to survive for substantial periods of time in low osmolarity conditions (e.g. pond water) as well as in the high osmolarity conditions of the gastrointestinal tract of their hosts.

This thesis focuses on the osmotic responses of *Salmonella* and in particular on the identification of changes that occur at the protein level when *Salmonella* encounters hyper-osmotic conditions. In addition, it is also concerned with an analysis of the adaptive changes that occur when the bacterial pathogen *Salmonella enterica* serovar Typhimurium (hereafter referred to as *S.* Typhimurium) is exposed to hyper-osmotic stress induced by increasing the external NaCl concentration. Although many previous studies have been conducted in this area using *S.* Typhimurium and especially the related bacterium *Escherichia coli*, most have focused on the transcriptional responses using genetic approaches and direct analyses of changes at the protein level, and it is a poorly researched area. Accordingly, the present studies focus on a proteomic analysis of the osmotic stress responses of *S.* Typhimurium. Before describing the results of the previous work on this topic, however, it is pertinent to describe the general features of *S.* Typhimurium and why it has been the subject of investigation for over a century.

1.2 *Salmonella*

1.2.1 General information

Salmonella cause a wide range of disease syndromes ranging from a normally mild short-lived gastroenteritis to a severe systemic infection of the reticuloendothelial system as caused by the bacterium *Salmonella* Typhi. It has been estimated that in 1994 there were 16.6 million cases of typhoid fever globally with approximately 600,000 deaths (Pang et al., 1995; Bernard Ivanoff and Klaus Stohr, 2005).

The symptoms of *Salmonella* infection can vary greatly. For example *S.* Typhimurium often causes acute gastroenteritis, which usually starts 24 – 48 hours after the ingestion of the infectious dose. The symptoms of this gastroenteritis are diarrhoea, abdominal cramps, fever, nausea, vomiting and chills. All or few of these symptoms may be present as this also depends on the actual strain of infections and the person infected. The gastroenteritis normally lasts for approximately 3 days. Non-typhoidal salmonellosis typically results in a fatality rate of less than 1% globally but it is important to note that despite a reported reduction in typhoid fever cases there is actually an increase in non-typhoidal salmonelloses world-wide.

Typhoid fever often caused by *S.* Typhi or *S.* Paratyphi is much more serious. Onset is approximately 7 to 21 days after infection, during which a number of symptoms present including: loss of appetite, headache and high fever. Typhoid fever can generally be treated intensively with a wide range of antibiotics (ampicillin and gentamycin are common) for about 4 weeks post infection. However, in recent years the problem of antibiotic resistant *Salmonella* (amongst other bacteria) has arisen. This kind of infection has a very high fatality rate without treatment. The elderly and immunocompromised people such as HIV-infected patients are particularly susceptible to this kind of infection. *S.* Typhi is host specific and infects only humans whereas *S.* Typhimurium can infect a range of hosts.

People with diarrhoeal *Salmonella* infections normally recover completely although bowel habits may take several months to return to normal. Some patients however can go on to develop further S*almonella* related complications such as Reiter's Syndrome. Reiter's syndrome consists of painful joints, irritation of the eyes and

painful urination. This may last for months and may lead to chronic arthritis. Others may become carriers of *Salmonella* if they colonise an immunopriviledged site such as the gall bladder. Some of these carriers, e.g. the notorious 'Typhoid Mary', may shed *Salmonella* for years. It is thought that she may have directly infected up to 1300 people with *Salmonella* and may be responsible for the death of a young girl.

1.2.2 Importance of *Salmonella* as a pathogen

Salmonella food poisoning costs the UK economy a minimum of £60 million annually (Roberts and Sockett, 1994) for the estimated 50,000 cases that occur each year (Wheeler et al., 1999) and more than 150,000 cases were reported from the 14 European Union member states in 2001 (European Commission, 2001). *Salmonella* spp. are also the most important food-borne pathogens in terms of deaths caused in the UK; 119 deaths in England and Wales were attributed to *Salmonella* spp. in 2000, which was 29% of the total of food-borne disease-associated deaths (Adak et al., 2002). Moreover, non-typhoidal *Salmonella* spp. were the most common cause of hospital admissions among the food-borne pathogens and 1516 people in England and Wales were admitted to hospital with *Salmonella* infections in 2002, with a total bed occupancy of 8793 days (Adak et al., 2002) . Globally, *Salmonella* spp. cause an estimated 1.6 billion cases of infection each year worldwide and 3 million deaths (Pang et al., 1995). Startling recent data from Denmark (Helms et al., 2003) suggested that 3% of people with a *Salmonella* infection will die within a year and that infection with *S.* Typhimurium doubles relative mortality. While opinion is mixed over the interpretation of these data, they emphasise the impact and public perception of this zoonosis even in developed countries.

1.2.3 Classification

The genus *Salmonella* is a member of the family Enterobacteriaceae. Other members of this family include *Escherichia coli*, *Shigella, Citrobacter, Klebsiella* and *Yersinia* all of which are known pathogens and are the causative agents of a range of diseases. *Salmonella* are Gram-negative, non-spore forming, generally motile (although *S.* Gallinarum and *S.* Pullorum are always non-motile) facultative anaerobic bacilli.

Salmonella is generally considered to comprise of 2 species: *S. enterica* and *S. bongori*. Of these, *S. enterica* is further subdivided into 6 sub species: *enterica,*

salamae, *arizonae, diarizonae, houtenae*, and *indica*. In addition, *S. enterica* subspecies contains about 60% of the *Salmonella* serotypes, including *S.* Enteritidis, *S.* Typhimurium and the vast majority of mammalian and avian pathogens.

Below the species level, to date, approximately 2501 different serovars of *Salmonella* have been described. It should be noted, however, that fewer than 2% are responsible for *Salmonella* infections in animals and humans. (Davies, 2001). Different serovars cause different types of disease with a range of symptoms. *S.* Typhi, *S.* Paratyphi A, *S.* Paratyphi C and *S.* Sendai are host adapted to man and usually cause systemic disease, a septicaemia-typhoidal syndrome. Other serovars, however, never spread beyond the gastrointestinal tract so the infection is limited to gastroenteritis. *S.* Typhimurium is limited to the gastrointestinal tract in humans, but in the mouse causes systemic disease and therefore is a useful model for human typhoid fever. *S.* Typhimurium in conjunction with *S.* Enteritidis causes the majority of zoonotic salmonellosis in most countries. Because of this predominance of a few serovars to cause disease it is important to differentiate between the various serotypes. Differing strains of *Salmonella* as well as other species are distinguished from each other by serotyping. Using serological tests, closely related bacteria can be distinguished from each other by slight chemical differences in their (O) antigen that occur in their O-specific side chains of lipopolysaccharide, which constitute the outermost layer of the cell and H antigens which indicate flagellin variants. The antigenic formula of *S.* Typhimurium is 1, 4, [5], 12:i:1,2 . This antigenic formula indicates the following: the presence of O antigens 1, 4, 5 and 12. The 1 is underlined to indicate that this antigen is present as a result of phage conversion, [5] indicates the variable presence of this antigen. i: 1, 2 indicates phase 1H antigen and phase 2 H antigens 1 and 2. (Singleton, 1999). This method of strain differentiation is called the Kauffmann-White scheme.

There is however still a degree of controversy regarding the 'correct' method of bacterial nomenclature, so much so that it was suggested that *Salmonella* and *E. coli* should be re-classified into a single genus, which is not unsurprising as *Salmonella* and *E. coli* share 85% DNA-DNA homology (Sharp, 1991).

1.2.4 Epidemiology

The primary mode of transmission of non-typhoidal salmonellosis remains that of *Salmonella* contaminated food and water (Pang et al., 1995). To actually cause a food-borne illness an infectious dose has to be reached or exceeded. This is where a sufficient number of microbial cells are consumed to cause disease. This infectious dose varies greatly between pathogens and between people. For example the infectious dose for the Gram-negative microaerophilic bacteria, *Campylobacter jejuni* is approximately 500 organisms whereas the infectious dose of non-typhoidal *Salmonella* is 10^5-10^7 organisms. This number varies greatly depending on *Salmonella* serovar and also depending on the food source, in which the bacteria are present. For example *S.* Eastbourne present in chocolate, only requires 10 – 25 organisms to cause illness (Craven et al., 1975; Adams and Motarjemi, 2005; Adams and Motarjemi, 1994).

Despite great improvements to the monitoring and reporting of food-borne illnesses the socio-economic cost for food-borne illness is still vast and it should be noted that for each reported case of S*almonella* infection it is thought that there are at least 3 further cases that go unreported in the UK (Roberts, 2000) and this figure is undoubtedly far higher in developing countries. It has been estimated that the cost on the economy for intestinal infections in England in 1999 was £750 million. (Roberts, 2000) and *Salmonella* related infections cost the US economy $2.3 billion dollars a year according to data from the US Centres for Disease Control (http://.www.ncbi.nlm.nih.gov/coffeebreak/CB8_SipA/pahe/html)

1.2.5 *Salmonella* as a model organism

Salmonella is one of the most intensely studied pathogens. This is because: (i) there are several features that are remarkably conserved between many other bacterial pathogens including; resistance to antimicrobial peptides, adherence and invasion of mammalian cells and survival with phagocytic cells, (ii) it is very amenable to genetic manipulation and analysis, (iii) it has well defined interactions with host cells, (iv) subgroup I *Salmonella* serovars, although essentially identical, have very different host specificity, therefore the genetic basis of host-specificity can be studied (http://mcb.berkeley.edu/courses/mcb103/1031ec19.htm).

1.2.6 The pathogenesis of salmonella-induced enteritis

The method and severity of the *Salmonella* infection depends greatly on the *Salmonella* serotype and host species involved. During the pathway by which *Salmonella* reach the intestine of the host, the bacteria experience a wide variety of different conditions. The process from ingestion to excretion is summarised as follows.

1. Oral ingestion of *Salmonella.*
2. Passage into the stomach, exposing the cells to a mean pH of 2.0.
3. After the stomach, any surviving pathogens pass into the lumen of the small intestine, where they invade the intestinal mucosa. Infected epithelial cells are extruded into the intestinal lumen with subsequent villus blunting and loss of absorptive surfaces. The cause of this is the invasion of enterocytes and M cells. *Salmonella* also induce a polymorphonuclear leukocyte (PMN) influx into infected mucosa and induce watery diarrhoea, which may contain blood (Wallis and Galyov, 2000). A schematic diagram of the pathogenesis of *Salmonella*-induced enteritis is shown overleaf in Figure 1.1.

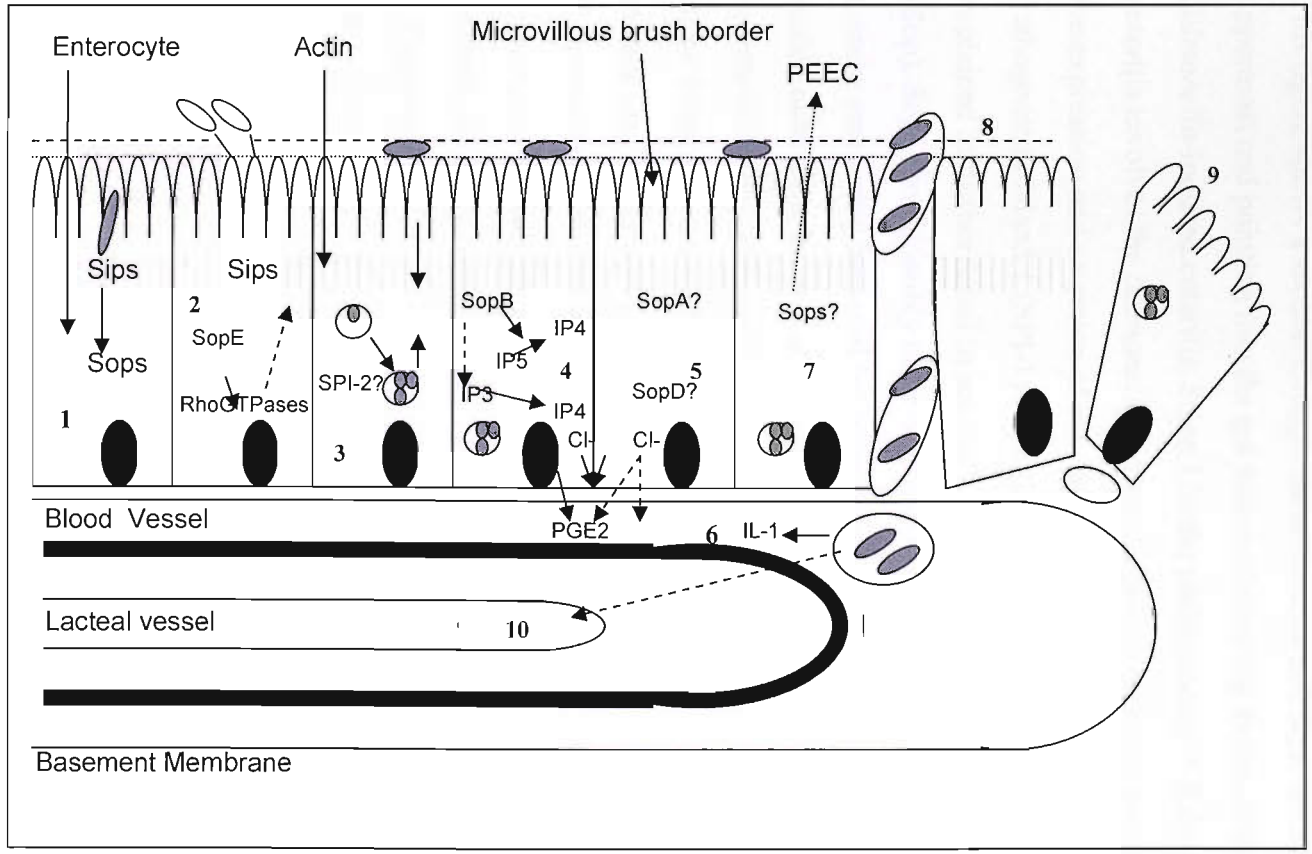


Figure 1.1 For figure information see next page.

Figure 1.1 A schematic representation of the ten stages of pathogenesis of *Salmonella*-induced enteritis (adapted from Wallis and Galyov, 2000).

This figure shows a section through the enterocyte cell layer with the microvilli uppermost and pointing into the gut lumen illustrating the ten stages of pathogenesis of *Salmonella*-induced enteritis. Stage 1 in the pathogenesis of *Salmonella*-induced enteritis involves the *Salmonella*-mediated penetration of the enterocyte, which requires the expression and secretion of effector proteins that are translocated by the *Salmonella* Pathogenicity Island 1 (SPI-1) Type III secretion system (TTSS). SPIs and the TTSS are explained in further detail in section 1.3. Stage 2 involves the *Salmonella* outer protein (Sop), SopE, and possibly other Sops inducing ruffling of the enterocyte membranes, thereby promoting bacterial invasion. Stage 3 is where the intracellular bacteria reside within membrane bound vesicles and possibly continue translocation of TTSS-1 secreted effectors and replication of bacteria within the vesicles is promoted by TTSS-2. Stage 4 involves intracellular SopB protein affecting inositol phosphate signalling events. One such event can antagonise the closure of chloride channels, which in turn influence net electrolyte transport and thus fluid secretion. In Stage 5 epithelial cells infected by the bacteria secrete chemokines and prostaglandins that recruit inflammatory cells to the site of infection, so that in Stage 6, *Salmonella* interact with inflammatory cells and stimulate the release of proinflammatory cytokines that enhance the inflammatory response. Stage 7 sees *Salmonella*-infected epithelial cells release pathogen-elicited epithelial chemoattractant (PEEC) across the apical membrane. This substance acts to stimulate polymorphonuclear leukocyte (PMN) transepithelial migration between the enterocytes and in Stage 8, infiltrating inflammatory cells phagocytose *Salmonella* (the fate of these cells is unclear). In Stage 9 *Salmonella*-infected enterocytes become extruded from the villus surface, leading to the shedding of the infected cells into the intestinal lumen and results in villus blunting and loss of absorptive surfaces. Finally, in Stage 10 some of the infected cells migrate to the draining lymphatics, carrying *Salmonella* to systemic sites.

It is in the small intestine that *Salmonella* cells are most likely to experience a change in osmolarity (the stress with which this thesis is primarily concerned). During stage 3 of pathogenesis, as described previously, once the bacteria enter into the epithelial cells, they reside within membrane bound vesicles within the cells. Entry into these vacuoles (also known as phagosomes) is thought to expose *Salmonella* cells to a shift in osmolarity, in addition to changes in pH, reactive oxygen species and bactericidal cation peptides.

4. Invasive strains e.g. *S.* Typhi penetrate through the intestinal barrier pass into the lymphatic system and are engulfed by phagocytes within which they multiply. Later these bacteria re-enter the bloodstream, causing septicemia.
5. Finally *Salmonella* are excreted into the external environment.

Each of the different conditions such as acidity, osmolarity, temperature or exposure to bactericidal cations, that *Salmonella* encounter during the infection process causes the utilisation of specific virulence factors that have been proposed to aid virulence. Some of the key ones are summarised in Table 1.1 (Lucas and Lee, 2000). The *Salmonella* pathogenicity islands SPI-1 and 2, are discussed in further detail in the following section 1.3. Other SPI pathogenicity islands have also been described, however, their roles in virulence and/or sites of induction *in vivo* are currently unclear.

Table 1.1 Regulation of *S.* Typhimurium factors linked to virulence (adapted from Lucas and Lee, 2000).

Proposed site in host	**Virulence associated genes**	**Proposed functions**	**Environmental conditions**	**Regulatory factors**	***In vivo* induction**
Stomach	*atr* genes	Log phase acid tolerance response	Acid pH	Fur, RpoS	
Intestine	SPI-1	Invasion, chemokine production, PMN migration, apoptosis	Oxygen, osmolarity, pH, growth phase, cations(?), P_i levels (?)	EnvZ/OmpR, PhoPQ, PhoRB, CsrAB, SirA/BarA, HilA, HilC, HilD, InvF	
Macrophages	SPI-2	Replication in macrophages	Cation levels, P_i levels, osmolarity (?)	PhoPQ, SsrAB, EnvZ/OmpR	Mouse spleens, macrophage line
Liver/spleen	PmrA-dependent PhoP activated genes	Resistance to cationic peptides and killing by macrophages and PMNs	Cation levels, pH	PhoPQ, PmrAB	Macrophage line

1.3 *Salmonella* Pathogenicity Islands and Type III Secretion Systems

In order for bacterial pathogens to invade and infect their hosts, multiple factors are required (Groisman and Ochman, 1997). It has been stated by Bowe *et al* that approximately 4% of the entire *S.* Typhimurium genome is required for the fatal infection of mice, which translates into over 200 genes. (Bowe et al., 1998) *Salmonella's* interaction with the host is known to be very complex and it is thought that this complexity reflects the large number of virulence determinants expressed by *Salmonella* (Jones and Falkow, 1996). Not only are there a large number of genes required in *Salmonella* virulence but their gene products must act at the correct time and the correct location. (Bowe et al., 1998). These genes are found on plasmids or on the chromosome as units of one, or a few or in large virulence cassettes composed of a series of genes and operons. These are known as *Salmonella* Pathogenicity Islands (SPI) (Marcus et al., 2000) of which 5 have been identified to date (Table 1.2).

Table 1.2 The 5 SPIs that have so far been identified (Marcus et al., 2000).

SPI	Function
1	Primarily required for the bacterial penetration of the epithelial cells of the intestine (invasion) and linked to enteritis in cattle
2, 3, 4	Required for the growth and survival of the cells within the host, manifested in the systemic phase of the disease. SPI-1 and 2 also encode type III secretion systems, which mediate the respective virulence phenotype by translocating bacterially-encoded proteins into various compartments of the host cell .
5	Mediates the inflammation and chloride secretion which characterize the enteric phase of the disease

1.3.1 Type III secretion systems (TTSS)

Many Gram-negative animal pathogenic bacteria contain highly homologous protein secretion systems. These are known as type III secretion systems (TTSS) (Hueck, 1998). The primary role of TTSS is to co-ordinately translocate and secrete a set of effector proteins into eukaryotic target cells. *Salmonella* has at least two TTSS

secreting virulence-associated effector proteins, TTSS-1 and TTSS-2 (Wallis and Galyov, 2000; Hueck, 1998). *Salmonella* pathogenicity island I (see 1.3.2) encodes more than 30 proteins which includes regulatory proteins, structural components of TTSS-1, and several TTSS-1 secreted proteins and their chaperones. The SPI-1 TTSS and the invasive phenotype are expressed during growth conditions that mimic those found in the lumen of the intestine, these include, low oxygen tension, and high osmolarity (Ernst et al., 1990; Galan and Curtiss, III, 1990; Lee and Falkow, 1990; McBeth and Lee, 1993)

1.3.2 *Salmonella* pathogenicity island - 1

This pathogenicity island is 40kb in size and is located at 63 centisomes on the *S.* Typhimurium chromosome. SPI-1 contains at least 29 genes encoding various components of a TTSS, its regulators and its secreted effectors (Collazo and Galan, 1997). SPI-1 enables *S.* Typhimurium to efficiently penetrate the intestinal epithelium. Evidence suggests that SPI-1 mutants are attenuated for virulence when inoculated orally but not systemically. SPI-1 expression appears to be limited to when *Salmonella* invade nonphagocytic cells of the gut epithelium whereas other virulence determinants were later acquired to allow the establishment of systemic disease. Translocation of effectors via the SPI-1 encoded TTSS allows *Salmonella* spp. to enter non-phagocytic cells such as the M cells of the intestinal epithelium (Lee and Schneewind, 1999). The invasive process involves massive ruffling of the host cells plasma membrane at the site of interaction with the bacterium and bacterial uptake into large vesicles resembling micropinosomes (Francis et al., 1993; Garcia-Del Portillo and Finlay, 1994). Crucial for invasion are rearrangements of the host cell actin cytoskeleton, witnessed by the ability of cytochalasins to inhibit *S.* Typhimurium uptake (Finlay et al., 1991). Importantly, invasion is mediated by the delivery of effectors that directly engage host cell signalling pathways (Marcus et al., 2000). There are numerous regulators of SPI-1, including internal genes and external two-component systems, cascades of cellular events and also environmental signals. The primary example of the internal regulation is through HilA. HilA is a member of the ToxR/OmpR regulon that activates the *sip* operon, which encodes secreted proteins, and the *inv/spa* and *prg* operons, encoding components of the secretion apparatus (Bajaj et al., 1995). A regulator outside of SPI-1 known to control cellular invasion is the two-component regulator BarA/SirA. BarA is a sensor kinase of the

phospho-relay type and SirA is its cognate response regulator (Altier et al., 2000; Johnston et al., 1996). It has been shown that SPI-1 genes are maximally expressed through HilA under low oxygen conditions (Jones and Falkow, 1994). An increase in osmolarity (> 300 mOsm) (Fordtran et al., 1968) in the small intestine may also be a probable signal for invasion and SPI-1 gene expression. The elevated osmolarity induces *hilA* expression and also causes changes in DNA supercoiling that affect invasion gene transcription (Galan and Curtiss, III, 1990; Bajaj et al., 1996; Bajaj et al., 1995).

1.3.3 *Salmonella* pathogenicity island - 2

The SPI-2 locus was initially defined by signature-tagged mutagenesis as a chromosomal region present in *S. enterica*, but absent in the related non-pathogenic species *Escherichia coli*, K-12. It is 40kb in size and consists of 42 open reading frames. Further analysis revealed that only a subset of these genes is required for systemic infections in the mouse model of salmonellosis, i.e. genes encoding a TTSS (Shea et al., 1996).

Several of the predicted SPI-2 genes show sequence similarity to protein families of the TTSS subunits and a nomenclature for SPI-2 virulence genes was proposed that reflected the relationship to these protein families (Hensel et al., 1997b). Genes encoding components of the type III secretion apparatus were designated *ssa* (secretion system apparatus), genes encoding type III substrate proteins and their specific chaperones were designated *sse* and *ssc* respectively. Genes encoding regulatory proteins of SPI-2 virulence genes were termed *ssr*.

It is interesting to point out that several genes on SPI-2 do not appear to code for proteins associated with virulence (Hensel et al., 1999). It has been shown that the conditions within all eukaryotic cells tested induce SPI-2 gene expression. However to date SPI-2 virulence genes have not been identified in bacteria species other than *Salmonella* spp. DNA-hybridisation analysis has revealed that the genes are present in *S. enterica* but absent in *S. bongori* and it is interesting to note that *S. bongori* species is supposed to be phylogenetically older than *S. enterica* and only very rarely associated with human disease (Hensel et al., 1997a).

Analysis of SPI-2 expression using single-copy *luc* reporter gene fusions to SPI-2 genes, has demonstrated that SPI-2 gene expression is induced by Ca^{2+} and Mg^{2+} deprivation, and phosphate starvation (Deiwick et al., 1999). Interestingly, the effects of low Ca^{2+} and Mg^{2+} correlate with genes under the control of the PhoPQ system, a global regulator of *Salmonella* virulence (Miller, 1991). Furthermore, SPI-2 gene expression is reduced in a *phoP*$^-$ background.

The two component system SsrA-SsrB has been shown to be critical for the expression of SPI-2 genes (Shea et al., 1996). SsrA-SsrB encodes the sensor and regulator of the two component system and mutations in either gene cause the same level of attenuation as mutations in the TTSS. Recent work by Lee *et al* revealed that the expression itself is regulated by OmpR/EnvZ, another 2 component system with a global regulatory function. In *S.* Typhimurium, OmpR/EnvZ may act as a sensor for low osmolarity and low pH inside of the phagosome (Lee et al., 2000). This is summarised overleaf in Figure 1.2 and OmpR/EnvZ is discussed in further detail in section 1.5.3.1.

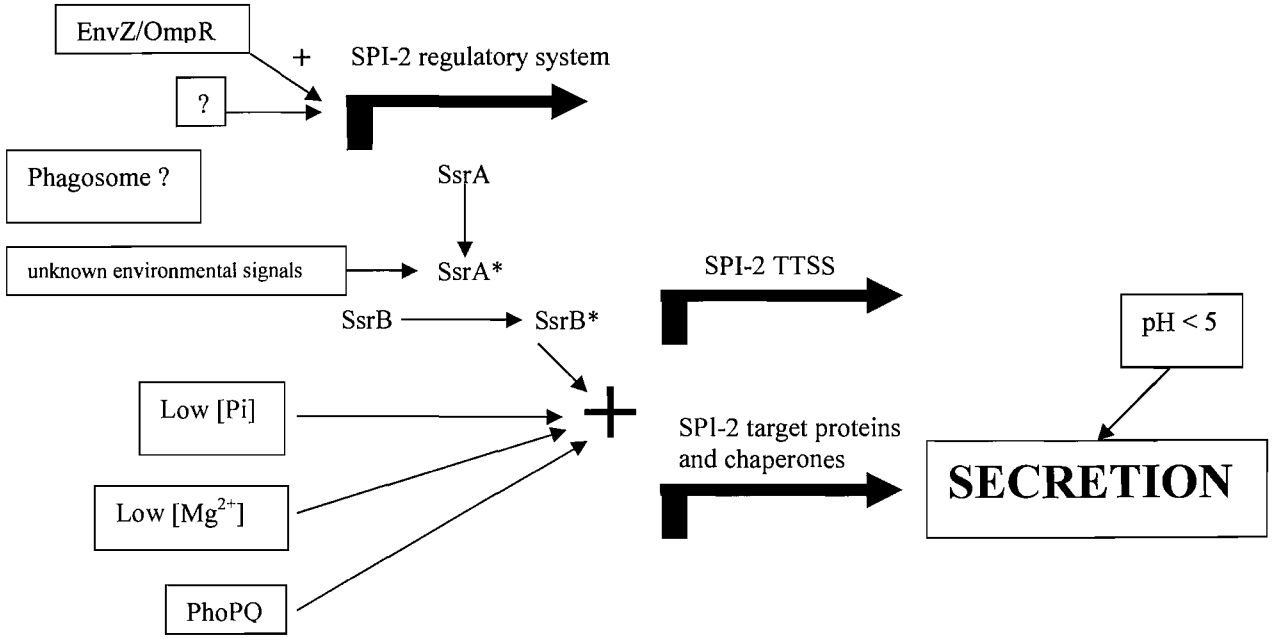


Figure 1.2 Proposed model for the regulation of the SPI-2 virulence gene expression (adapted from (Hensel, 2000))

*SsrA, a sensor kinase, becomes autophosphorylated in response to several stimuli, some of which remain to be characterised. In turn, SsrB becomes phosphorylated, leading to the induction of SPI-2 genes.

It can be seen from the above considerations that *Salmonella* encounters a very wide range of stresses during its life cycle, including changes in pH, temperature, oxygen, growth phase, iron concentration and osmolarity. It is likely, therefore, that its ability to cope with these stresses is a critical determinant of its survival and capacity to cause disease. Moreover, some of the external stress factors may also serve as sensory cues to alert *Salmonella* to major changes such as ingestion by a host. As such, these stresses may also trigger the expression of components required to cause disease. It is therefore relevant to consider *Salmonella* stress responses in more detail, with particular emphasis on its responses to hyperosmotic stress.

1.4 Environmental Adaptation: *Salmonella* Stress Responses

The mechanisms that bacteria employ to adapt to their immediate surroundings are complex and diverse. Stress responses may involve adaptive responses such as physiological adjustments or simple evasive measures such as those employed by motile bacteria. An overview of some of the stress responses mounted by *Salmonella* follows, before a detailed coverage of the osmotic stress response.

1.4.1 The acid tolerance response

In the case of enteric bacteria such as *Salmonella*, one of the commonest and potentially harmful stresses encountered is a shift in pH. At low pH levels *Salmonella* cells may display negative chemotaxis with further decreases in pH resulting in expression changes of at least 50 proteins (acid shock proteins) leading to acid tolerance (Foster, 1999; Foster, 1995). Acid shock proteins include the heat shock proteins DnaK and GroEL, however the identity of many has yet to be determined (Foster, 1991).

Control of the acid tolerance response (ATR) in the exponential phase is complex and involves a number of regulatory proteins, including the alternative sigma factor $\sigma^{S,}$ encoded by *rpoS*, *fur* and the *phoPQ* signal transduction system (Bearson et al., 1998; Lee et al., 1995). *Salmonella* also has an acid-inducible

stationary phase ATR that is σ^S – independent, but appears to involve OmpR (Foster, 1999), a transcription factor associated with the control of osmolarity and explained in further detail in section 1.5.3.1. This is perhaps unsurprising, as there appears to be a co-regulation of different stress responses in many bacteria.

Waterman and Small have published data that indicates there is a relationship between the ability of different bacterial species to resist acid stress and their infectious dose (Waterman and Small, 1998). This also appears true for *Salmonella*, with mutants lacking key ATR regulators, having reduced virulence in both mice and man (Fang et al., 1992; Fields et al., 1986; Hohmann et al., 1996; Miller et al., 1989)

1.4.2 The oxidative stress response

Reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2) are extremely damaging to proteins and nucleic acids. By-products of aerobic respiration are a source of ROS, and perhaps more importantly so, is the oxidative stress that enteric bacteria such as *Salmonella* may encounter in the phagosome of host macrophages. In response to exposure to reactive oxygen species bacteria can elicit an oxidative stress response (OSR) involving the induction or repression of specific genes.

The OSR of *S.* Typhimurium primarily involves two regulons. One regulon is controlled by the SoxR-SoxS transcription factors, the other by OxyR (Demple, 1999). Proteins induced by oxidative stress include manganese superoxide dismutase, resistant fumerase and aconitase (Storz and Imlay, 1999). A common theme amongst the OSR induced proteins is that they improve cellular repair activities and anti-oxidant capability.

1.4.3 The starvation stress response (SSR)

When *Salmonella* cultures are deprived of a carbon source (and less importantly deprivation of phosphate and nitrogen) the starvation stress

response is induced. This induction of the SSR also offers the cell a degree of protection against other stresses that it may encounter such as: high temperature, low pH, exposure to H_2O_2, and the antimicrobial peptide polymixin B (McLeod and Spector, 1996; Seymour et al., 1996; Spector et al., 1999). Unlike the stationary-phase response, the SSR is elicited in response to a single specific parameter, carbon-energy source starvation, and at cell densities 1 to 2 logs lower than those measured under typical stationary-phase conditions. Eventually the SSR results in a more resistant and energy efficient cell.

The two known major SSR regulators are σ^s and cAMP:CRP (the cAMP bound cyclic-AMP receptor protein). RpoS positively regulates genes under SSR, whereas the cAMP:CRP complex is involved in both positive and negative regulation of SSR loci .

One of the σ^s dependent systems involved in the SSR in *S.* Typhimurium is the *narZYWV* operon. This operon encodes part of the nitrate reductase system (Bonnefoy and Demoss, 1994) and includes the *narZ* gene (formally known as *stiA* (starvation inducible A). The *narZ* gene is also involved in carbon starvation induced heat tolerance and acid tolerance (Spector et al., 1999). The KatE protein (peroxide degradative hydroperoxidase II) induced by oxidative stress is also induced by carbon starvation and appears to be involved in cross resistance of these stresses with the *stiC* gene which is also regulated by σ^s. The *dadA* (*stiB*) gene is required for utilisation of L-alanine and other D-amino acids and is induced by carbon or phosphate starvation (Spector, 1998). These findings highlight the overlapping nature of many of the responses mounted by pathogens such as *Salmonella* in response to apparently diverse stresses.

1.4.4 The heat shock response

The major adaptive response to a rise in temperature (such as might be encountered during entry into a warm blooded host) is the heat shock response. The heat shock response involves the induction of heat shock proteins (HSPs) such as molecular chaperones, ATP-dependent proteases and folding catalysts

involved in protein folding, assembly and repair both under normal and stress conditions (some HSPs are found only in cells responding to stressors, but most are also present at much lower concentrations under normal conditions i.e. are constitutively expressed and play essential roles in cellular protein homeostasis). A primary mechanism of effect of many stressors is protein denaturation and/or aggregation. It is thought that under stressful conditions HSPs take on additional but related functions to molecular chaperoning, helping to repair denatured proteins and protecting others from damage. This protects cells allowing them to recover and survive the stress. The heat shock response is also induced in bacteria by other stressors including: osmotic shock, oxidative stress and pH shifts, further highlighting the co-regulation of different stress responses in many bacteria.

In Gram-negative bacteria the HSPs are under common transcriptional control of σ^{32}, the transcriptional activator of the heat shock regulon (Bukau, 1993; Erickson et al., 1987). The main bacterial HSPs include the chaperones GroEL, GroES, DnaK (Hsp70), DnaJ and GrpE. Other HSPs include the proteases such as Lon and ClpB (Hsp104). DnaK forms a chaperone machinery with DnaJ and GrpE and is involved in many cellular processes, such as DNA replication of the bacterial chromosome, RNA synthesis and protein transport (Gamer et al., 1996; Hendrick and Hartl, 1993). Furthermore, this chaperone machinery interacts with many unfolded and misfolded proteins and assists in proper folding, helps in refolding, prevents aggregation, and mediates the degradation of denatured proteins. Recently Takaya *et al* discovered a novel small heat shock protein (sHSP) named AgsA (aggregation-suppressing protein) in the thermally aggregated fraction from an *S.* Typhimurium *dnaK*-null strain (Tomoyasu et al., 2003). AgsA appears to be a chaperone that prevents aggregation of denatured proteins and maintains them for refolding in *S.* Typhimurium at high temperatures.

Heat shock proteins have been shown to be directly involved in the pathogenesis of *S.* Typhimurium, e.g. a 66 kDa HSP appears to be responsible for *S.* Typhimurium binding to mucosal cell walls (Ensgraber and Loos, 1992). It has also been reported that a mutation of the protease gene, *htrA* (a

periplasmic serine protease involved in the degradation of abnormally folded proteins) results in attenuation of the bacteria in BALB/c mice (Johnson et al., 1991). More recently it has been shown that the ATP-dependent proteases ClpXP (Yamamoto et al., 2001) and Lon (Takaya et al., 2003), which are also HSPs, are essential for systemic infection with *S.* Typhimurium in BALB/c mice. ClpXP and Lon are required for the survival and growth of *S.* Typhimurium within macrophages and cope with the accumulation of damaged proteins within phagosomes. The DnaK/DnaJ chaperone machinery has also been shown to be involved in bacterial invasion of epithelial cells (Takaya et al., 2004).

In view of the topic of the present studies it is appropriate to consider osmotic stress responses of Gram-negative bacteria, in particular *S.* Typhimurium in greater detail. Many of the relevant previous studies have been conducted using the related Gram-negative bacterium *E. coli* rather than *Salmonella.* Where appropriate, therefore, relevant work with this organism will also be discussed.

1.5 The Osmotic Shock Response

The availability of water is one of the most significant environmental parameters affecting the growth and survival of microbes. Changes in external osmolarity can trigger fluxes of water along osmotic gradients that could results in cells bursting (hypotonic environment) or plasmolysis (hypertonic environment). In considering the effects of osmotic shock upon Gram-negative bacteria such as *Salmonella*, it is important to consider the Gram-negative cellular architecture, which differs to that of Gram-positive organisms. The important aspect of this differing cellular architecture is that of the cell wall. The Gram-positive cell wall consists of a single 20 to 80nm thick peptidoglycan or murein layer lying outside the plasma membrane. In contrast to this, the Gram-negative cell wall is quite complex and consists of a 1 to 3 nm peptidoglycan layer surrounded by a 7 to 8 nm thick outer membrane. The structures outside the plasma membrane are often referred to as the envelope.

The cell wall is very important in the protection of the cell from either lysis or plasmolysis due to any osmotic pressure applied to it. Within the cell, solutes are much more concentrated than in the external environment which is normally hypotonic. During osmosis, water moves across selectively permeable membranes such as the plasma membrane from dilute solutions (high water concentration) to more concentrated solutions (lower water concentration). Therefore water normally enters bacterial cells and the osmotic pressure may reach 300 psi (Prescott et al., 1993). Understandably, the plasma membrane can not withstand such high pressures and the cell will eventually lyse. The cell wall is important in resisting this cell swelling. The reverse is also true i.e. in hypertonic environments the solutes are more concentrated in the environment as opposed to within the cell and as a consequence water flows outwards and the cytoplasm pulls away from the cell wall (plasmolysis)(Prescott et al., 1993). Plasmolysis can be detected as an increase in the turbidity of the cultures upon introduction of the cells into media of an increased osmolarity (Csonka, 1989). Assuming that the hyper-osmotic shock is not too severe the plasmolysis is transient and after a lag period the volume will increase due to the osmotic adaptation of the cell. Sudden plasmolysis results in the inhibition of a variety of physiological processes, ranging from nutrient uptake (Roth et al., 1985b; Roth et al., 1985a; Walter et al., 1987) to DNA replication (Meury, 1988). Bacteria can avoid these disastrous outcomes by taking counter measures, such as controlling intracellular solute concentrations. As Gram-positive and Gram-negative bacteria are affected differently by osmotic stress and use different strategies to respond to changes in osmolarity (Poolman and Glaasker, 1998) the following section considers Gram-negative bacteria only.

1.5.1 Responses to hyper-osmotic shock

During hyper-osmotic stress, the primary focus of this study, exposure of cells to high external osmotic pressure results in an efflux of water from the interior. The decrease in the internal water content brings about a reduction in the turgor pressure and a fall in cytoplasmic volume. It is this reduction in turgor and

consequent alteration of membrane shape and turgor that may be a part of the osmotic protection machinery of the cell, with this 'sensory mechanism' able to initiate the osmotic protection cascade. The cascade of adaptive responses that *Salmonella* and related bacteria implement has been described to occur in the following three phases (Wood, 1999).

In phase one, which occurs in the first 1 to 2 minutes, the cell is dehydrating and shrinking and most transport and respiration ceases. At the same time there is an increase in levels of potassium and glycine, betaine and proline (the latter three solutes via ProP). Potassium is the principle osmolyte and it is accumulated in order to restore turgor and hence an increase in potassium uptake is the first response to counter osmotic stress. Two potassium uptake mechanisms exist in *E. coli*, a low affinity, constitutive system (Trk) and an osmotically induced system Kdp. Activity of the Kdp and Trk transport proteins are modulated to ensure that there is no excess in potassium uptake, this ensures that the optimum K^+ concentration is maintained. Despite K^+ being a very useful osmolyte due to its abundance in the environment it is not without flaws. If the internal concentration of K^+ increases too much the increased cytoplasmic ionic strength will inhibit normal enzyme activity. Although potassium and glycine, betaine and proline uptake may occur simultaneously, potassium accumulation by Trk is faster and more vital in this first phase.

In phase two, which lasts 20-60 minutes, nucleic counter-ions (nucleic acids have many charged phosphate groups, and would have a very large net anionic charge in the absence of counter-ions. An imbalance in these counter-ions could also lead to conformational changes) are being replaced and rehydration begins. Putrescine is extruded, bacteria accumulate compatible solutes and respiration resumes, though at a reduced rate. Compatible solutes are those that can be accumulated to high concentrations with minimal damage to normal cell metabolism or enzyme function. They are generally inert and have no net charge. Some of the most universally used are N-substituted amino acids, betaine and proline. Although many bacteria use the strategy of accumulating compatible solutes, the types accumulated and accumulation mechanisms differ among species. The compatible solute of choice for *E. coli* and *S.*

Typhimurium appears to be betaine although trehalose is also important (Higgins et al., 1987).

In phase three, which can take over an hour, DNA and protein synthesis resume, cell growth and division resumes. This involves the expression of osmo-responsive genes e.g. *proP, proU* and *betT*. The *proU* gene has been shown to encode a high affinity transport system for the osmoprotectant betaine, which is accumulated to high concentrations in response to osmotic stress (Cairney et al., 1985; Csonka, 1981; Le Rudulier and Bouillard, 1983; Perroud and Le Rudulier, 1985).

These 3 phases are summarised in Table 1.3.

Table 1.3 A summary of the phases of the hyper-osmotic stress response for *E. coli* K-12 (adapted from Wood, 1999).

Phase	Approximate Duration	Structural Change	Physiological Change
I	1 - 2 minutes	Cell dehydrates and shrinks Cytoplasmic water activity decreases Cytoplasmic crowding increases Wall/membrane turgor altered	Respiration and most transportation ceases Trk/ProP activated ΔpH increased transiently ATP level increased transiently
II	20 – 60 minutes	Nucleic acid counterions replaced and rehydration begins	Putrescine extruded K^+ glutamate and compatible solutes accumulate Respiration is resumed (albeit at a reduced rate)
III	60+ minutes	Cell wall and nucleoid changes DNA/protein synthesis resume Cell growth and division resume Co-solvent composition adjusted	Osmoresponsive genes expressed (e.g. *proP*, *proU*, *kdpFABC*, *betT*) Compatible solute uptake/efflux cycle established

1.5.2 Responses to hypo-osmotic shock

Responses to osmotic down-shifts are poorly characterised, but are also thought likely to occur in three similar phases to those against salt up-shift, as summarised in the following table (Table 1.4).

Table 1.4. A summary of the phases of the hypo-osmotic stress response for *E. coli* K-12 (adapted from Wood, 1999).

Phase	Approximate Duration	Structural Change	Physiological Change
I	< 1 minute	Cell hydrates and swells Cytoplasmic water activity increases Cytoplasmic crowding decreases Wall/membrane turgor altered	Channels open
II	1-2 minutes	Cell shrinks & cytoplasmic crowding increases	Co-solvents and water extruded
III	10-20 minutes	Currently unknown	Channel close

Upon hypo-osmotic shock *E. coli* has been reported to rapidly release potassium, glutamate and trehalose, whilst retaining alanine, lysine, arginine and sucrose (Schleyer et al., 1993).

The mechanisms by which bacteria sense changes in osmolarity and the regulation of the aforementioned osmotic stress responses are now discussed.

1.5.3 Osmo-sensing and regulators of the osmotic shock response

Responses to hyper-osmolarity are governed by; two-component systems (e.g. EnvZ/OmpR and KdpD/KdpE), other protein osmosensors (e.g. the previously discussed ProP, ProU and BetT) and sigma factor σ^S (RpoS).

Theoretically, changes in osmolarity could be detected directly as changes in extracellular water activity or indirectly as changes in the cell wall/ plasma membrane structure or in the composition of the cytoplasm.

One of the best studied osmosensors in *E. coli* is KdpD - the induction of the *kdp* operon is mediated by the two-component system KdpD/KdpE. Sutherland *et al* have shown that the expression of Kdp and ProU, though both under osmotic control, are regulated in response to entirely different signals. Rather than responding to changes in turgor as appears to be the case with Kdp, ProU expression is principally determined by the intracellular accumulation of potassium ions (Sutherland et al., 1986). Recently it has been found that amphipathic compounds such as chlorpromazine effect KdpD phosphorylation in the same way as high-medium osmolarity, suggesting that the osmotic signal that is sensed by KdpD is a membrane stretch (Poolman and Glaasker, 1998). There is still debate over the activation mechanism of *proU* (the transcription of which is stimulated over 100-fold in response to increases in external osmolarity). However, as activation occurs in the latter stages of the osmotic shock response, this suggests that neither raised external osmotic pressure nor reduced turgor pressure activates this pathway.

An osmosensor that is a mechanosensitive channel has been described in the cytoplasmic membrane of *E. coli*. The osmosensor known as MscL is sensitive to pressure changes in the lipid bilayer and is voltage dependent (Blount et al., 1997b; Blount et al., 1997a).

In addition to the osmotic stress response observed in *E. coli* due to EnvZ/OmpR and RpoS, evidence exists which illustrates that an increase in intracellular concentrations of K^+ (in an initial response to elevated osmolarity)

causes the induction of a periplasmic protein strongly induced by hyper-osmotic stress called OsmY. This product of the *osmY* gene was first described by Barron *et al* (1986). It was initially thought that the *proU* and *osmY* may respond to the same regulatory signal, that being the signal molecule, potassium glutamate. Induction of ProU expression has been shown to correlate strongly with the intracellular accumulation of potassium glutamate (Sutherland et al., 1986). It has now been shown that there are regulatory differences between *proU* and *osmY* and that they differ in how long the induced state is maintained following osmotic stress (Yim and Villarejo, 1992). OsmY is also transcriptionally regulated and its expression may involve direct interaction between the *osmY* promoter and RpoS, the putative stationary-phase sigma factor (Yim et al., 1994; Vijayakumar et al., 2004).

The direct function of OsmY is still not clear although it is thought that it could be a binding protein for the transport of an alternative osmolyte. Since there is only a slight growth disadvantage to cells lacking wild-type *osmY*, the osmolyte is not likely to be an effective osmoprotectant (Yim and Villarejo, 1992). There are a number of such osmolytes in *E. coli* and these include γ-aminobutyric acid (Measures, 1975), 3-(N-morpholine) propane-sulphonate (Cayley et al., 1989), and taurine (McLaggan and Epstein, 1991).

The two-component system, EnvZ/OmpR, is of particular interest for this study and therefore is discussed in greater detail.

1.5.3.1 The EnvZ/OmpR two-component system.

The EnvZ/OmpR two-component system is an important regulator of the osmotic shock response in *E. coli* and *Salmonella*. EnvZ, an integral inner membrane sensor kinase, regulates the concentration of phosphorylated OmpR in response to osmolarity. Phosphorylated OmpR (OmpR-P) is a transcription factor that binds to the porin genes *ompF* and *ompC* to regulate their expression. At high osmolarity, an increase in the kinase activity of EnvZ results in an increase in the concentration of OmpR-P, and OmpR-P binds to

the low affinity sites on *ompF*, this represses OmpF expression. OmpR-P binds also to low affinity sites on *ompC*, activating *ompC* at high osmolarity. This is summarized in Figure 1.3.

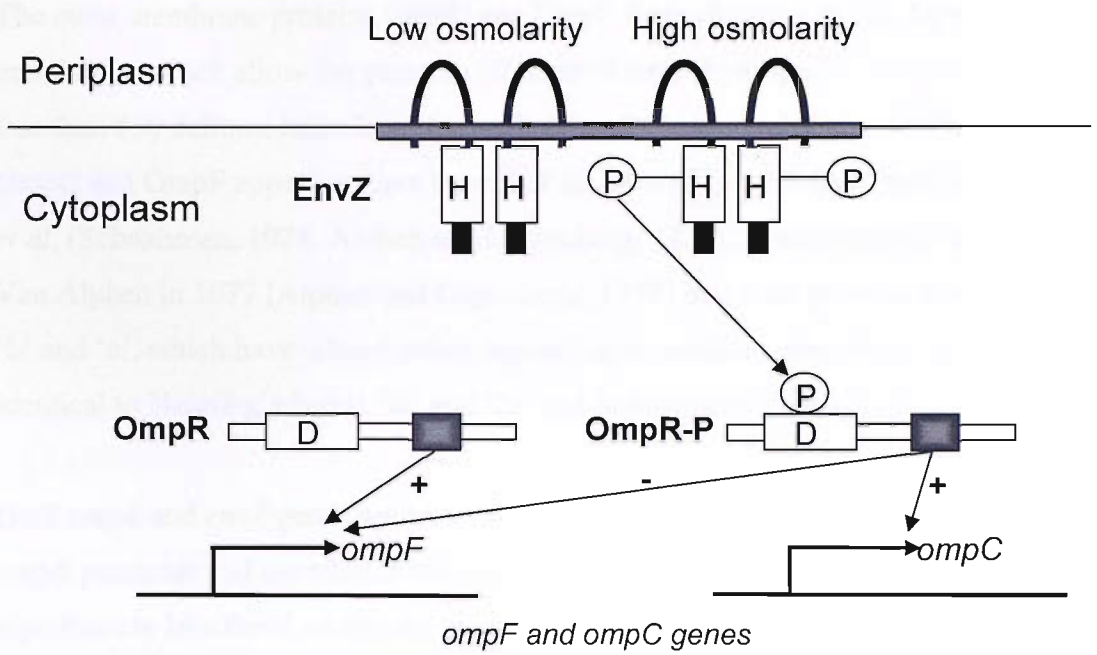


Figure 1.3 A schematic diagram illustrating the mechanism of regulation of the outer membrane proteins OmpC and OmpF by the two-component system EnvZ/OmpR.

The outer membrane proteins OmpC and OmpF form channels in the outer membrane, which allow the passive diffusion of small hydrophilic molecules of less than 650 daltons, including glycine-betaine (Nikaido and Vaara, 1985). OmpC and OmpF appear to have been first discovered in 1974 by Schnaitman *et al*, (Schnaitman, 1974; Alphen and Lugtenberg, 1977). It was determined by Van Alphen in 1977 (Alphen and Lugtenberg, 1977) that their proteins labelled 'b' and 'c', which have altered ratios depending on medium osmolarity, are identical to Henning's bands 'Ia' and 'Ib' and Schnaitman's 'protein 1'.

Both *ompR* and *envZ* genes are located at the *ompB* locus under control of the *ompB* promoter and are transcribed as a single polycistronic mRNA. However significantly less EnvZ molecules are produced than OmpR. During exponential growth of *E. coli* there were ~3500 molecules of OmpR and only 100 molecules of EnvZ per cell (Cai and Inouye, 2002).

As discussed previously, EnvZ/OmpR regulates expression of the SsrA-SsrB two-component system that controls expression of genes encoding components of the SPI-2. Recent work by Garmendia *et al* suggests the effects on SP1-2 gene expression activated by *low* osmolarity are transmitted predominately through the SsrA-SsrB system, with EnvZ-OmpR required for full expression of the SPI-2 genes (Garmendia et al., 2003).

EnvZ/OmpR is also implicated in the regulation of curli production in *E. coli*. Curli fibres are proteinaceous fibres produced by *E. coli* and *Salmonella* spp. and involved in colonisation of inert surfaces, biofilm formation, binding to host proteins and internalisation of *E. coli* by eukaryotic cells (Collinson et al., 1991; Olsen et al., 1998; Olsen et al., 1989; Vidal et al., 1998; Jubelin et al., 2005).

1.5.4 Osmotic shock and cross protection

There are distinct differences in the regulation of the bacterial stress responses, between cells which are in the exponential growth phase and those that are in the stationary phase of growth. The major difference between these two phases in terms of master regulators is the secondary sigma factor, RpoS (Lange and Hengge-Aronis, 1991). The RpoS sigma factor is hardly present in exponentially growing cells, however, it is essential to cells that are entering the stationary phase of growth or that are exposed to a range of other stresses (Lange and Hengge-Aronis, 1991; McCann et al., 1991). As RpoS is known to be involved in regulating, hyper-osmotic responses (Hengge-Aronis et al., 1993), care was taken to ensure all cells used for this study were from mid-exponential phase cultures.

In *E. coli* and *S.* Typhimurium, and other Gram-negative species, RpoS is the master regulator of a general stress response (as mentioned previously there appears to be a co-regulation of different stress responses in many bacteria), with RpoS mediated gene expression controlled by a complicated interaction between global regulatory proteins such as H-NS, Lrp and CRP (Hengge-Aronis, 1999). In *E. coli* the σ^S regulon contains over 80 genes, conferring resistance to a wide range of stresses including: osmotic stress, oxidative stress, starvation and pH shift. Genome-wide expression profiling data indicates that up to 10% of *E. coli* genes are under direct or indirect control of σ^S, impacting not only on stress tolerance but the entire cell physiology under sub-optimal conditions (Weber et al., 2005). The study by Weber *et al* also suggests that σ^S dependent genes represent a complex network with differentially controlled modules and links to other global regulons.

An important phenomena that has been shown to be a regulator of the osmotic shock response in bacteria is that of DNA supercoiling. DNA supercoiling has been most characterised in *E. coli* however it has also been described in various other bacterial species (Rohde et al., 1994; Jordi et al., 1995; Ali et al., 2002). The expression of global transcriptional regulators such as Fis (*fis*), cyclic AMP receptor protein (*crp*), and stress induced factor RpoS are all dependent

on the supercoiling state of the cell (Finkel and Johnson, 1992; Schneider et al., 1999). Supercoiling regulates the transcriptional effects of these regulators directly by affecting the efficiency of protein binding to their DNA targets, or indirectly by altering the transcriptional expression of the regulators themselves. These observations have raised the possibility that DNA supercoiling may play a functional role in coupling stress detection to transcriptional activity (Dorman, 1996).

Elevated extra-cellular osmotic conditions cause a rapid increase in negative supercoiling, with subsequent relaxation to preinduction values as the cell recovers. Increased negative supercoiling is reported to stimulate the transcription of several osmoregulated genes *in vitro* and *in vivo*, including the primary active transporter for the osmoprotectants proline and glycine-betaine (*proU*) (Higgins et al., 1988; Jordi and Higgins, 2000), and the lipoprotein OsmE (Conter et al., 1997), as well as to repress the transcription of the outer membrane β-barrel porin OmpF (Graeme-Cook et al., 1989). Through a series of genome wide expression profiling experiments under conditions of high salt and perturbation with drug antibiotics a regulatory role for DNA supercoiling in the osmotic stress response of *E. coli* has been identified (Cheung et al., 2003). DNA supercoiling also appears to have a potential role in co-ordinating the regulation of overlapping gene expression in response to both osmotic and anaerobic stress (Bhriain et al., 1989) and the heat shock protein, DnaK has been reported to maintain negative supercoiling of DNA against heat shock (Ogata et al., 1996).

It can be seen from the above discussion that osmotic stress responses and their integration with other adaptive processes is a complex matter. Moreover, to date most studies have necessarily focused on specific aspects rather than on a direct analysis of the components involved at the protein level. Recently, however, it has become possible to embark on a systematic and relatively large-scale analysis of protein changes involved in such responses. Such analyses, termed proteomics, are still in their infancy. However, they have the potential to reveal components of hitherto unknown function, as well as

uncovering components known to be involved in other processes that had not been suspected of involvement in the stress response under investigation. The following section briefly describes proteomics as it has been described for the study of *Salmonella* stress responses.

1.6 Proteomics

The focus of this thesis is the investigation of protein level changes in *Salmonella* under hyper-osmotic shock and for this purpose, a proteomic approach was chosen for the study.

As the genomes of a number of organisms have been defined it has become clear that it is almost impossible to assign functions to many of the genes. Although genomics provides information on the ways in which an organism may express its genes, it does not provide any insight into how an organism can modify the pattern of gene expression in responses to various conditions. This can only be done by investigating gene expression directly at the mRNA or protein level. However there are problems associated with studies of mRNA (Anderson and Anderson, 1998; Haynes et al., 1998). For example there is often a poor correlation between mRNA abundance and amount of corresponding protein in a cell. In addition mRNA studies provide no information on the processes of co- and post-translational modification, which can have a profound effect on the functional properties of proteins. These obstacles can in part be overcome by proteomics i.e. analysis of the proteome, 'the protein complement expressed by the genome of an organism, or in multi-cellular organisms, as the protein complement expressed by a tissue or differentiated cell' (Wilkins et al., 1996).

The first requirement of proteome analysis is to separate the complex mixture of proteins obtained from an organism or cell line etc. Currently this is most commonly done using two-dimensional gel electrophoresis (studies of the proteome began in the late 1970s following the development of 2-D electrophoresis in 1975 (O'Farrell, 1975)) when proteins are first separated by

isoelectric focusing (IEF) and then by SDS-PAGE i.e. proteins are first separated by molecular charge using a pH gradient and then by mass. For effective analysis 2-D electrophoresis must be capable of reproducible, high-resolution separation. This was initially problematic when carrier ampholytes were used to generate pH gradients for IEF. However, better resolution and reproducibility has been achieved with use of immobilised pH gradients (IPGs) developed by Amersham Pharmacia Biotech (Bjellqvist et al., 1993). Separated proteins are then visualised by high sensitivity staining or autoradiography, producing two-dimensional arrays of proteins.

The next step of proteomics is the identification of the separated proteins. In addition to the information on apparent molecular weight, pI and relative abundances from the gel, traditionally individual proteins from gels have been identified by N-terminal sequencing, internal peptide sequencing, immuno-blotting or co-migration with known proteins. However, with the growth of protein databases there was a shift in the way proteins were identified, with sequences now frequently determined by correlating mass spectrometry data of peptides derived from proteins with information in databases. This approach, known as peptide mass mapping, involves the generation of residue specific enzyme digests (most often a trypsin digest) of separated proteins, that are analysed by mass spectrometry to accurately determine the masses of the resulting peptides and produce a peptide mass profile or 'fingerprint'. This experimental profile or 'fingerprint' can then be compared against theoretical peptide profiles generated from protein sequence databases to create a list of likely protein identifications.

Challenges remain with abundant spots obscuring minor ones and poor resolution of very hydrophobic, very basic or very large proteins in current 2-D systems (Gygi et al., 2000). However, new technologies are constantly arising to address these problems e.g. the use of isotope-coded affinity tags (ICATs)(Gygi et al., 1999) to label and extract peptides for analysis and sequencing. One ICAT can be used to tag proteins from control cells and another (containing a fixed number of ^{2}H or ^{13}C atoms) is used to tag corresponding proteins from cell grown under a selected condition. Using this

approach, the relative abundance of each sequenced peptide can be determined and as a result differentially expressed proteins identified. It is also possible in many cases to cultivate cells in media containing specific isotopically-labelled amino acids for similar types of quantitative analyses

1.6.1 Proteomic analysis of *Salmonella* stress responses

Microbes are ideal organisms for proteomic analysis. They are relatively simple to manipulate in the laboratory, have small genomes and mutants can generally be more easily constructed in microbes than in many other experimental systems. In addition, many microbes are well characterised in terms of genetics, biochemistry and physiology (O'Connor et al., 2000).

With regards to *Salmonella*, proteomics has been used to study a number of disparate questions. For example, to investigate the repertoire of proteins synthesised by *S.* Typhimurium during growth in host cells. Abshire and Neidhart (1993) compared the protein patterns expressed by *S.* Typhimurium growing within macrophage-like U937 cells and an extracellular control culture. Levels of approximately 40 proteins were observed to increase during growth within the cells, while approximately 100 were repressed. Furthermore, the pattern of proteins expressed by *S.* Typhimurium growing within the U937 cells was compared to patterns produced when *S.* Typhimurium was exposed to various environmental conditions in the laboratory. This revealed that the intracellular environment imposes many stresses on *S.* Typhimurium, but that the macrophage-induced response was not a sum of individual stress responses displayed during extracellular growth (Abshire and Neidhardt, 1993). More recently, Burns-Keliher *et al* (1997) improved on the traditional technique of using chemicals to stop host cell synthesis to allow labelling of bacterial proteins only. Instead, they used a labelled lysine precursor that mammalian cells cannot use, to enable specific labelling of bacterial proteins without interfering with host cell metabolism. This enabled the detection of 57 proteins synthesised by *S.* Typhimurium SL1344 (Δasd mutant) during growth in a

human intestinal cell line, 34 appearing to be unique to the intracellular environment. (Burns-Keliher et al., 1997).

Few proteins maps have been published for *Salmonella*, one notable exception being a 2-D reference map of proteins forming the cell envelope fraction of *S.* Typhimurium SL1344. (Qi et al., 1996).

The usefulness of a proteomic approach for investigating the stress responses of *Salmonella* has been clearly illustrated by Adams *et al* (2001). Using proteomics, Adams *et al* investigated the influence of low pH stress on the *Salmonella* proteome, revealing that phase 1 and 2 flagellin were down-regulated when acid-adapted *Salmonella* were exposed to pH 3.0, and that this was dependent upon activation of the PhoPQ pathway, an unanticipated link (Adams et al., 2001).

To date, however, there has been no global proteomic analysis of *Salmonella* Typhimurium responses to osmotic stress, the primary focus of this study. As mutants that are defective in osmotic adaptation are non-virulent, further identification of proteins involved in osmotic stress responses could provide new avenues to investigate strategies to reduce *Salmonella* viability/virulence.

1.7 Aims of the Study

The aims of this study were:

1. To compare protein expression profiles of *Salmonella* cells grown under standard laboratory conditions and under conditions of hyper-osmotic stress.
2. To detect and identify proteins which are up- or down-regulated when *Salmonella* has adapted to hyper-osmotic stress.
3. To determine if proteins that have altered abundance under conditions of hyper-osmotic stress are regulated by the relatively well-characterised, OmpR/EnvZ two-component system or if they are regulated by some other means.
4. To determine the contributions of specific *Salmonella* proteins to the adaptation process.

CHAPTER TWO

MATERIALS AND METHODS

CHAPTER 2. MATERIALS AND METHODS

2.1 Reagents

In alphabetical order, the following specialist materials and reagents were used (with source):

Bacto Agar N°3 (Oxoid)

Broad range molecular weight markers (New England BioLabs)

DNA ladder: 1Kb (Gibco BRL)

Ethidium Bromide (Sigma)

EZ mix LB broth base (Sigma)

Glass cover slips (BDH)

Premium glass microscope slides (BDH)

Trypsin, sequencing grade (Roche)

2-D gel equipment and reagents (Amersham Pharmacia Biotech.)

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

2.2 Bacterial Strains Used, Culture and Storage Conditions

The following strains of *S.* Typhimurium were used in this study:

- SL1344 (histidine auxotroph) (Wray and Sojka, 1978)
- SL1344 *ompR*$^-$ (CJD359 *ompR1009*::Tn*10*) (Dorman et al., 1989)
- SL1344 *mreB*$^-$ (PRA118, Peter Alefounder, University of Southampton)

Luria-Bertani (LB) medium was used for all microbiological growth unless stated otherwise. The LB medium contained 20.6 g of LB EZ mix per litre of deionised water. LB culture plates were made by supplementing LB with 1.5% (w/v) agar (Bacto Agar N° 3, Oxoid). Media was sterilised by autoclaving at 121°C at 15 psi for 15 minutes. Once cooled to below 50°C the appropriate antibiotics were added if necessary (Table 2.1).

Table 2.1 Antibiotics used in this work

Antibiotic	Concentration	Final Concentration	Sterilised by:	Storage
Ampicillin (sodium salt)	25 mg/ml in water	25 µg/ml	Filtration 0.2 µm	-20°C
Chloramphenicol	25 mg/ml in 100% ethanol	25 µg/ml	Not required	-20°C
Kanamycin sulphate	50 mg/ml in water	50 µg/ml	Filtration 0.2 µm	-20°C
Tetracycline hydrochloride	12.5 mg/ml in 50% ethanol	12.5µg/ml	Filtration 0.2 µm	-20°C

Strains were generally cultured aerobically at 37°C in LB containing any necessary antibiotic. To set up a culture, 2-4 average size colonies were inoculated into an appropriate volume of broth and allowed to grow overnight so that a stationary phase culture was produced. An appropriate amount of the overnight culture was then added to fresh broth to produce a mid-exponential phase culture for experimental use. Cell growth was measured by monitoring the change in turbidity using a spectrophotometer at 600 nm, and with a blank of LB medium. To prepare strains for long-term storage, 0.5 ml of overnight culture was dispensed into a pre-sterilised cryo-tube containing 0.5 ml of a 50% glycerol solution. After mixing, the tubes were flash-frozen and stored at -70°C until required.

2.3 Induction of An Osmotic Stress Response

Overnight cultures of *S.* Typhimurium SL1344 or its *ompR*⁻ derivative were diluted 1:40 into fresh pre-warmed LB media (typically 400 ml in a 2 litre conical flask) and grown with vigorous aeration at 37°C. The cell density of the culture was monitored by measuring the absorbance at 600nm (A_{600}) with a spectrophotometer. Immediately after dilution of the overnight culture into fresh media, the A_{600} was 0.05. From a stock solution of 4.5M sodium chloride in LB, the required volume was added to give a final molarity of 0.5M and at such a time that 60 minutes after addition of the sodium chloride, the cells reached an A_{600} of 0.5, which equates to a mid-exponential growth phase. The corresponding control sample treated with a corresponding volume of pre-warmed LB media.

2.4 Preparation of Whole Cell Extracts of *Salmonella* for 2-D PAGE

The following procedure was performed as quickly as possible to reduce the severity of any proteolytic degradation.

Cultures of cells that were ready for harvesting were cooled by immersion in ice water for 20 minutes prior to transfer of the culture to pre-cooled centrifuge pots. Cells were then collected by centrifugation in a pre-cooled rotor for 10 minutes at 6000 rpm at 4°C using a JA21 rotor in a Beckman Avanti centrifuge. The cells were washed with pre-cooled 0.9% w/v sodium chloride solution (~250 mls) and re-pelleted before resuspension in 1.6 ml lysis buffer (2.5% IPG Phor buffer (Amersham Pharmacia) pH 4-7, 2.5% Triton X-100, 2.5% β-mercaptoethanol, in 8 ml of analytical grade water).

Phenylmethylsulphonylfluoride (5 mg) was added to the re-suspended pellet, which was then sonicated to complete cell lysis the (a total of 6 cycles using a MSE Soniprep 150: 15 sec burst of an amplitude of 15 microns, followed by 1 min with no sonication). The sonication was performed with the sample cooled in ice water to reduce the probability of unwanted spontaneous post-translational modifications such as deamidation and carbamylation. To assist further protein denaturation and sample clarification a further 1.2 g of analytical grade urea was added, for a final urea concentration of 12M, and allowed to dissolve at room temperature with gentle agitation.

2.5 Preparation of Cell Envelope Extracts of *Salmonella* for 2-D PAGE

S. Typhimurium SL1344 (or other strains) were grown up in LB medium (where necessary, supplemented with the appropriate antibiotic) overnight at 37°C with vigorous agitation. The overnight culture was diluted 1:25 into fresh pre-warmed LB medium (400mls) and was grown with vigorous shaking at 37°C until an A_{600} of 0.5. The cells were then harvested by centrifugation (all centrifugation was performed in pre-cooled centrifuges at 4°C) at 4000g and re-suspended in 40 ml of phosphate buffered saline (PBS). The suspension was re-centrifuged again for a further 10 minutes at 4000g and re-suspended in 16 ml of distilled water. The suspension was sonicated at 15 microns for 6 cycles. Each cycle consisted of 30 seconds on and 60 seconds off, whilst constantly being cooled on ice water. To remove unlysed cells, the sonicate was re-centrifuged at 3000*g* for 5 minutes. The supernatant was carefully decanted into a

new sterile oakridge tube and centrifuged at 23,000*g* for 40 minutes at 4°C. The supernatant was then discarded and the tubes were allowed to drain for several minutes to ensure the removal of all the supernatant. The pellet was then re-suspended in 5 ml of PBS and re-centrifuged at 23,000*g* for 40 minutes at 4°C. This cell pellet constituted the extracted bacterial cell envelope. Outer membrane proteins were also extracted from the sample by re-suspending the pellet in 4 ml of 2% Triton X-100 solution. After centrifugation at 23,000*g* for 40 minutes at 4°C the supernatant consisted of predominantly cytoplasmic membrane proteins whereas the pellet consisted of outer membrane proteins.

2.6 Protein Determination Analysis

To establish the optimum concentration of protein to be used in the two-dimensional protein separations, two techniques were generally used. Due to interference by some of the components of the lysis buffer, a number of commercially available kits were unsuitable for this application. The protein concentration of the sample was estimated by diluting 1:100 with analytical grade water and then determining the absorbance at 280nm. An A_{280} of 1 was assumed to be equivalent to 1 mg/ml of protein. In conjunction with this method, the sample was also separated on a one dimensional Novex™ gel (8-16% Bis-Tris, MOPS running buffer). This was to ensure that despite the inadequacies of the absorbance at 280nm method any control and test samples were equally loaded in order for a direct comparison of protein expression to take place.

To determine the protein concentration of the secreted protein samples the BCA protein assay (BioRad) was used, following the manufacturer's instructions. Lyophilised bovine serum albumin (Sigma P7656) was used to produce protein standard curves.

Unless otherwise stated, the protein molecular weight markers used on SDS-PAGE gels were those described in Table 2.2.

Table 2.2 Protein molecular weight markers used in SDS-PAGE

Protein (Broad range, New England Biolabs.)	Molecular Weight (kDa)
Myosin	212
MBP-β-galactosidase	158.194
β-galactosidase	116.351
Phosphorylase B	97.184
Serum albumin	66.409
Glutamate dehydrogenase	55.561
Maltose binding protein-2	42.710
Lactose dehydrogenase	36.487
Triose phosphate isomerase	26.625
Trypsin inhibitor	20.040-20.167
Lysozyme	14.313
Aprotinin	6.517
Insulin A, B chain	2.34-3.40

2.7 Two-Dimensional Polyacrylamide Gel Electrophoresis

2.7.1 First dimension isoelectric focussing

Proteins were separated in the first dimension according to their isoelectric point using the IPGPhor^{TM} isoelectric focussing system (Amersham Pharmacia Biotech) as per the manufacturer's instructions. 18cm immobilised pH gradient strips (IPGs) pH 4-7 were used unless otherwise stated.

The sample was diluted with sample rehydration solution (8M urea, 33mM CHAPS, 2% v/v IPG buffer pH 4-7, 20mM DTT, a few grains of Orange G) to a final protein concentration of 4 mg in 400 µl. The sample-buffer solution was then added to the base of an 18cm IPG strip holder ensuring even coverage with no air bubbles. Using fine forceps, the plastic covering of the IPG strip was removed and then the strip was lowered into the IPG strip holder, gel side down into the solution and overlaid with 1.5 ml of dry strip cover fluid to prevent drying out and urea crystallisation. A lid was placed on the strip holder before transfer to the IPG Phor unit. Ensuring that both electrodes were in contact with the appropriate termini of the holder, the unit was set to rehydrate the strips at 20°C for 12 hours followed by an isoelectric focussing step for a total of 75kV hours. Next IPG strips were frozen at –70°C prior to the second dimension electrophoresis.

2.7.2 Second dimension polyacrylamide gel electrophoresis (PAGE)

The second dimension, which separates proteins according to molecular weight, used pre-cast 12-14% polyacrylamide gels (ExcelGel XL, Amersham Pharmacia Biotech.).

IPG strips were equilibrated with 2 x 10 ml of equilibration solution (50 mM Tris-HCl pH 6.8, 6 M urea, 30% v/v glycerol, 1% SDS) using sealed 10 ml pipettes, with 10 minutes agitation. The first 10 ml volume contained 16 mM DTT to reduce disulphide bonds. The second volume contained 0.2M iodoacetamide to counteract gel streaking caused by DTT. A few grains of bromophenol blue were included to aid the monitoring of electrophoresis. After equilibration, the dry strips were drained on filter paper, on their sides. Next, a Multitemp II thermostatic circulator and cooling plate were set up (as per manufacturer's instructions) to run at 15°C. An ExcelGel XL was placed onto a

cooling plate coated with paraffin oil and equilibrated to 15°C. Anodic and cathodic ExcelGel SDS buffer strips were placed at opposite ends of the gel. The equilibrated IPG dry strip was then positioned gel side down, onto the ExcelGel XL, approximately 1mm from the cathodic buffer strip. Small pieces of filter paper were inserted at either end of the IPG strip, touching the gel to prevent capillary action from transporting buffer around the edges of the gel. Good contact was ensured between the IPG strip and ExcelGel by gently running a pair of forceps over the plastic backing of the IPG strip. Electrodes were then positioned to make contact with the appropriate buffer strips. The second dimension was then run using the following steps (all at constant current):

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

B. The IPG strip and filter papers pieces were removed at this stage

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

D. The cathodic buffer strip and corresponding electrode were then moved to cover the position previously occupied by the IPG strip on the ExcelGel

E. 1000 V, 40 mA, 40 W for 145 minutes

F. Once the dye front entered the anodic buffer strip, electrophoresis was stopped.

Gels were then stained for 20 minutes with a modified Coomassie Brilliant Blue stain (0.2% Coomassie Brilliant Blue R-250 dissolved in 0.5% v/v glacial acetic acid and 20% v/v/ methanol) and destained overnight with 30% methanol.

2.7.3 Image analysis

Gels were scanned into a computer at 600dpi using an Epson GT scanner using standard parameters. The protein expression patterns were then analysed using Phoretix 2-D Advanced v5.1 Software, following the process recommended by the manufacturer that is summarised overleaf in Figure 2.1.

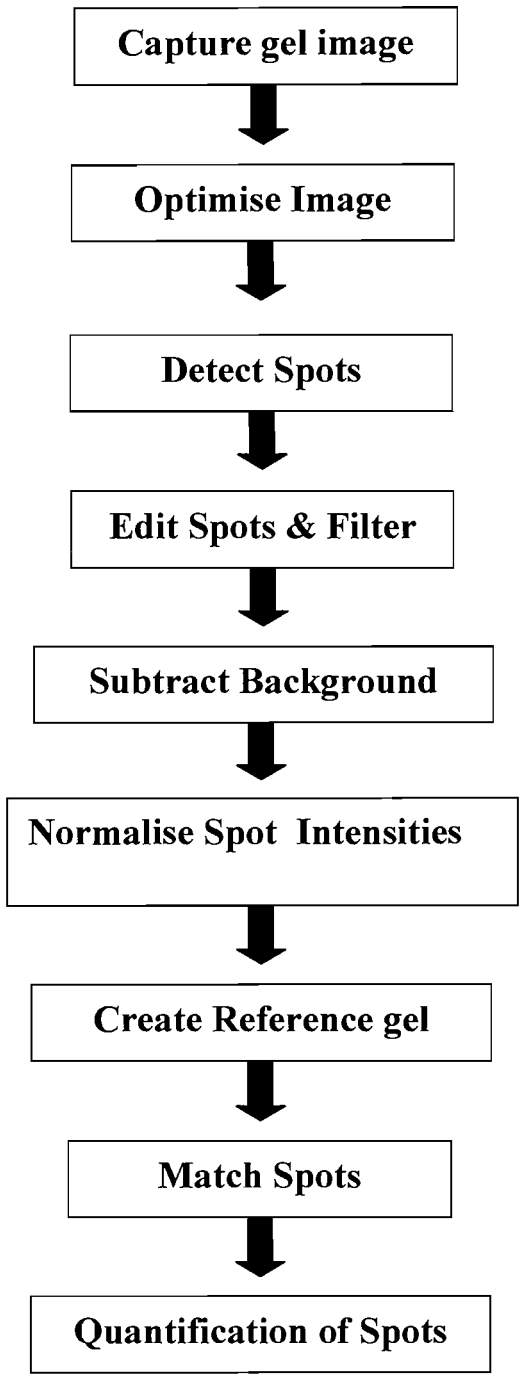


Figure 2.1 A flow diagram summarising the steps involved in 2-D gel image processing for spot quantification.

2.8 In-Gel Digestion of Proteins Separated by 2-D-PAGE and Identification by Mass Spectrometry and Database Mining

2.8.1 In-gel digestion

Two-dimensional gels were rinsed with sterile, analytical grade water and protein spots of interest excised into separate 0.6 ml tubes. Then excised protein spots were cut into 1mm x 1mm pieces in the tubes with a sterile scalpel blade. Tubes were checked regularly to ensure they were free from contamination. The gel particles were then rinsed with sterile, analytical grade water (100 – 150 µl), before being dehydrated with 100% acetonitrile (3-4 times the volume of the gel pieces). Next, the gel pieces were immersed in sterile 10 mM DTT in 0.1 M NH_4HCO_3 (enough liquid to cover the gel pieces) for 30 minutes at 56°C to reduce proteins. Gel particles were then dehydrated again with 100% acetonitrile before alkylation with 55mM iodoacetamide in 0.1 M NH_4HCO_3 (40 µl) for 20 minutes, room temperature in the dark. Following a wash with 0.1M NH_4HCO_3 for 15 minutes, the samples were dehydrated in 100% acetonitrile. If gel particles retained Coomassie blue stain at this point, they were incubated overnight in 1:1 v/v 0.1 M NH_4HCO_3:acetonitrile before dehydrating once more with 100% acetonitrile. Protein digestion was started by rehydrating the gel pieces in 50 mM NH_4HCO_3, 5 mM $CaCl_2$, 1.5 ng/µl trypsin at 4°C for 45 minutes. Finally the supernatant was removed and 50 mM NH_4HCO_3, 5 mM $CaCl_2$ added for overnight digestion at 37°C

2.8.2 Peptide extraction

After overnight digestion (as above), the gel pieces were incubated in 25 mM NH_4HCO_3 at 37°C for 15 minutes with shaking. Two volumes (volume of the gel pieces) of acetonitrile were then added to the gel pieces before incubating at 37°C for another 15 minutes with shaking. The gel pieces were then centrifuged in a microcentrifuge, 8000 rpm for 1 minute. The supernatant was collected into fresh 0.6 ml microtubes. The gel particles were then vortexed in 5% v/v formic acid at 37°C for 15 minutes. Another two volumes (volume of the gel pieces) of acetonitrile were then added to the gel pieces before incubating at 37°C for another 15 minutes with shaking. The gel pieces were then centrifuged again in a microcentrifuge, 8000 rpm for 1 minute. The supernatants were then pooled.

2.8.3 Mass spectrometry

Peptide extracts were analysed by Matrix-Assisted Laser Desorption Ionisation Time Of Flight (MALDI-TOF) mass spectrometry using a MALDI-TOF instrument (Waters). If required, tandem MS mass spectrometry was also used. In this case, individual peptides were separated using a CapLC system and eluted on-line into a Q-TOF Global Ultimate MS instrument (Waters). Fragmentation data was collected after collision induced dissociation. This analysis was performed by Mr Paul Skipp at the Centre for Proteomic Research in the University of Southampton.

2.8.4 Database searching and protein identification

The peptide masses experimentally derived using mass spectrometry were used to search the NCBI nr database using the peptide mass fingerprinting programme MS-FIT (part of the Protein Prospector suite, http://prospector.ucsf.edu/). Fragmentation data from individual peptides was used to search the same database (which was updated monthly) using ProteinLynx Global Server 2 software (Waters Ltd).

2.9 Preparation of *S.* Typhimurium Cells for Analysis by the Waters® Protein Expression System

Samples were prepared, in this case wild type and *ompR*⁻ *S.* Typhimuirum cell extracts from both non-stressed and salt-stressed cultures, as follows:

1. An overnight culture of SL1344 or *ompR*⁻ *S.* Typhimurium was prepared by inoculating 2-3 colonies into 50 ml LB broth in a 500 ml flask. This was incubated overnight at 37°C with shaking.
2. Following the 14 hour incubation 10 ml of the overnight culture was inoculated into 390 ml of fresh pre-warmed media in a 2 litre flask. Similarly, inoculants were prepared in 7 other 2 litre flasks. All cultures were incubated at 37°C with shaking.
3. At A600 = 0.5 50 ml of pre-warmed NaCl/LB was added to flasks 1-4 and 50 ml of pre-warmed LB to flasks 5-8.
4. The incubation was continued for 60 minutes at 37°C.
5. The cultures were chilled in ice-water for 10 minutes then centrifuged at 6000rpm for 10 minutes at 4°C.

6. Each cell pellet was re-suspended in 250 ml of ice-cold 10mM HEPES buffer and re-centrifuged at 6000rpm for 10 minutes at 4°C.
7. Each pellet was re-suspended in 1.6 ml of HEPES and sonicated (6 cycles of 15 micron; 15 seconds on, 1 min off on ice).
8. Samples were transferred to well labelled tubes and snap frozen in liquid nitrogen before storage at -80°C.

The subsequent processing and analyses were carried out by Drs Mark Ritchie and Therese McKenna at Waters Corporation and Mr Paul Skipp at the University of Southampton's Centre of Proteomic Research. In brief, 150µg (total protein) of the protein extracts were diluted and solubilised by incubation at 80°C for 15 minutes in 0.1% RapiGest™ SF before reduction with 5mM DTT and alkylation with 10mM iodoacetamide. The proteins were then digested with 1% (w/w) sequencing grade trypsin overnight (16 hours). RapiGest™ SF was cleaved by addition of HCl, followed by removal via centrifugation. Samples were diluted with 0.1% formic acid to a final concentration of 1µg/µl prior to analysis using the Waters CapLC™ Q-Tof LC-MS system. RapiGest™ SF is a mild denaturant that solubilises and unfolds proteins, making them more amenable to cleavage, without modifying them, suppressing endoproteases, or interfering with HPLC, MALDI-TOF MS or HPLC/MS techniques. This approach enables the analysis of all proteins in the lysate including membrane related proteins.

2.10 Analysis of Secreted Protein Profiles

Overnight cultures of the relevant bacteria were diluted 1:40 into fresh, pre-warmed LB and grown aerobically at 37°C until the cells had reached mid-exponential growth. At this point, the cells were treated with either LB (control) or LB + salt (to a molarity of 0.5 M). Cell growth was then allowed to continue until an A_{600} of 0.5 had been achieved. Bacterial cells were removed by centrifugation at 18000g for 10 minutes and the supernatant was filtered through a 0.45 µm filter to remove further cells. Trichloroacetic acid (10% w/v) was then added to precipitate proteins present in the supernatant. Following thorough mixing and incubation on ice for 60 minutes, the samples were centrifuged at 18000g for 30 minutes. The resultant pellet was then washed twice with acetone with centrifugation as before. The pellet was allowed to air dry for 5 minutes before it was re-suspended in 150 µl of sterile distilled water. The

secreted protein pellet was snap frozen and then stored at -70°C prior to analysis. Secreted protein profiles were then analysed by separating proteins on 4-12% NuPage Bis-Tris gels (Invitrogen) using MOPS buffer and visualising with SYPRO Ruby stain (Molecular Probes) as per manufacturer's instructions.

2.11 Sample Preparation for Phase Contrast Microscopy and Imaging for Cell Size Analysis

Cultures of *S.* Typhimurium SL1344 or its *ompR*⁻ derivative were grown as described in Section 2.3 and, following salt addition, were permitted to grow aerobically at 37°C for 60 minutes, with samples taken after 1 min or 60 min of treatment. All cultures were at mid-exponential growth (A_{600} range: 0.473 – 0.529) at the end of treatment, i.e. 60 minutes after the addition of LB or LB + NaCl. To immobilise cells, sodium azide was added to a final concentration of 5 mM. The length and width dimensions of at least 100 well-oriented bacteria were then recorded using a X60 objective on a Leica DM RBE Microscope fitted with a digital camera (Hamamatsu, Orca CCD). Length and width measurements were collected using ImageJ version 1.29X software, a public domain image analysis program developed at the National Institutes of Health (www.//rsb.info.nih.gov/ij/). Images of cells were taken at a standard size and resolution, that being 1280 x 1024, 8 bit and 72 dpi. Images of at least 100 cells were taken and also an image of a calibration slide at the same size and resolution as the cells.

2.12 Transcriptional Analysis Techniques

2.12.1 RNA isolation from bacterial cells grown in suspension. (Trizol Reagent Method)

Bacterial cells were grown aerobically in LB until an A_{600} = 0.5. 1 ml samples of cells were collected (10,000 rpm in a microcentrifuge for 10 minutes at 4°C), prior to the addition of 1 ml of the Trizol reagent (Invitrogen) to each pellet and homogenisation by repeated pipetting for 30s followed by vortexing for 1 minute. Finally 3 cycles of freeze/thawing was performed by flash freezing in liquid nitrogen and thawing in 50°C water (i.e. until thawed but not permitted to heat up). The homogenate was then incubated at 20°C for 5 minutes, prior to the addition of 0.2 ml of chloroform. Tubes were vigorously shaken for 15 seconds by hand and incubated for 3 minutes at 20°C. To

separate the three distinct phases, the sample was centrifuged at 12,800 rpm in a microcentrifuge for 15 minutes at 4°C. The top, colourless, aqueous phase was then carefully removed and transferred to a clean microcentrifuge tube; the remaining phases containing DNA and protein were discarded. To ensure the absence of DNA from the RNA preparation, 1 µl of RNase-free DNase I (Promega) was added prior to incubation at 37°C for 30 min. RNA was precipitated from the aqueous phase by the addition of 0.5 ml of isopropyl alcohol and incubated for 10 minutes at 20°C. The sample was then centrifuged at 12,800 rpm in a microcentrifuge for 10 minutes at 4°C. Next, the supernatant was removed and the pellet was washed by addition of 1ml of 75% ethanol for every ml of Trizol used. The sample was vortexed and then centrifuged at 10,150 rpm in a microcentrifuge for 5 minutes at 4°C. The ethanol was removed and the pellet air dried for 5-10 minutes before resuspending it in 20µl of DEPC water by drawing through a pipette tip. The resuspended pellet was then incubated at 57°C for 10 minutes before freezing at –70°C.

2.12.2 cDNA synthesis

1 µg of RNA was heated at 65°C for 2-3 minutes then chilled on ice for 5 minutes. Next, 12.5 µl of master mix was added (4 µl of 5 x RT PCR buffer, 0.5 µl RNasin® ribonuclease inhibitor (Promega), 2µl dNTPs (10 mM), 1µl random hexameric primers, 1 µl reverse transcriptase and 4 µl $MgCl_2$ (25 mM)). This reaction mixture was then incubated at 37°C for 60 minutes. Negative control samples were also required and this involved the following. 2 µl of RNAse buffer I (Promega) was added to 10 µl of RNA and RNAse I and 7 µl of sterile water. This mixture was incubated at 37°C for 90 minutes. On completion of incubation the volume was made up to 50 µl by the addition of 30 µl of sterile distilled water.

Ethanol precipitation of RNA was achieved by following standard procedures, as described in Promega's 'protocols and applications guide' (1996). Negative control sample cDNA was also prepared, as described above.

2.12.3 RT-PCR protocol (using the primers described in Table 2.3)

A reaction mix was prepared consisting of 5 µl of 10X reaction buffer, 4µl of 25 mM $MgCl_2$ (5 µl when using the *mreB* primers), 1 µl of dNTPs, 0.5 µl each of *ygaU* or *mglB* or *mreB* primers, 1 µl of cDNA or negative control RNA, 0.5 µl hotstart Taq and 37.5 µl of DEPC water (36.5 µl when using *mreB* primers). A negative control reaction was also set up omitting the cDNA or negative control RNA and a positive control reaction with the *gapA* primers in place of the *ygaU* or *mglB* or *mreB* primers.

The amplification conditions used for the *mreB* and *ygaU* reactions were as follows:

1 cycle	95°C 10 minutes
30 cycles	95°C 1 minute (denaturation)
	55°C 45 seconds (annealing)
	72°C 50 seconds (extension)
	72°C 10 minutes (final extension)

For *mglB* a 60°C annealing temperature replaced the 55°C indicated above.

Resulting RT-PCR products were analysed by agarose gel electrophoresis (2.5% gels run for 40 minutes in 1 x TAE at 100v/200mA and visualised using ethidium bromide 0.006% w/v in the 1 x TAE).

Table 2.3 Primers used in RT-PCR experiments. (Supplied by Qiagen Operon GmbH)

Target Gene	Sequence (5' – 3')
mreB (forward)	GACGGCGTTATCGCTGAC
mreB (reverse)	CACTGTTCCAGCGCCACC
ygaU (forward)	GATAAGGACGATCAGGCGAA
ygaU (reverse)	GCTTTGAAATAGCGCTCAGG
mglB (forward)	CGCGCCGGATGTTCAGCT
mglB (reverse)	TTCAGCGCCTCTACCGCG
gapA (forward)	CTGACGAAACCGCGCGTAAA
gapA (reverse)	CGCGGAACGCCATACCAGTC

These primers were developed *ab initio,* specifically for this study.

2.12.4 Analysis of gene expression using quantitative PCR (using the primers described in Table 2.4)

A reaction mix was prepared consisting of 10.4 µl of a 2X master mix (consisting of 2X reaction buffer, 5 mM $MgCl_2$, 500 mM dNTP solution, 2.5U/ µl of Hot GoldStar enzyme Hotstart Taq, 1.25 µl of SYBR green (from a 1:100 in DMSO stock)), 1.6 µl of primers, 1.6 µl of cDNA or negative control RNA and 6.4 µl of DEPC water. A negative control reaction was also set up omitting the cDNA or negative control RNA and a positive control reaction with the *gapA* primers in place of the *ygaU* or *mglB* or *mreB* primers. The master mix components were those supplied as part of the Eurogentec qPCR™ Core Kit.

Each reaction was run on a DNA Engine Opticon 2 system (MJ Research) in 96 well plates using the following cycling conditions:

- 95°C 10 minutes
- 94°C 10s
- 58°C 25s
- 72°C 10s
- Repeat for 40 cycles
- 72°C 10 minutes

A melt curve analysis was carried out for each reaction, to check that the selected primers only amplified a single template, did not interact to form primer dimers and that the assay was stringent enough to allow the use of SYBR green. The melt curve was run using:

- 55°C ramped to 95°C in 0.1°C per second increments

Differences in the CT values between assays can only be compared when it is known that both assays have a similar amplification efficiency. PCR efficiencies were calculated using Opticon 2.01 software.

Table 2.4 Quantitative PCR primers (Supplied by Invitrogen Life Technologies)

Target Gene	**Sequence (5'-3')**
*mreB*q(forward)	GGCGTGGTTTACTCTTCTTCTGT
*mreB*q(reverse)	CGTTCTGCGGTGGCTTCA
*ygaU*q(forward)	GAATACCCGACGCTGACAAAGT
*ygaU*q(reverse)	CGATATTCCCCACGGCAACC
*mglB*q(forward)	TCCGTGGTGCGTAAGGCTAT
*mglB*q(reverse)	CGTTTTGCGAGTCATTCATCAGT
*gapA*q (forward)	CATCTCAGAACATCATCCCGTCCT
*gapA*q (reverse)	GCCATACCAGTCAGTTTGCCATT

These primers were developed *ab initio*, specifically for this study.

2.13 DNA Techniques

2.13.1 Agarose gel electrophoresis

Analytical gel electrophoresis was performed as previously described by (Sharp et al., 1973). 0.9% (w/v) agarose was made up in TAE buffer (0.04M Tris-acetate; 1 mM EDTA) containing 0.6 μg/ml of ethidium bromide. DNA was mixed with equal volumes of gel loading buffer (10% v/v glycerol; 0.4% orange G made up in TE buffer (10 mM Tris-HCl pH 7.5; 5 mM Na_2EDTA) and loaded onto the gel. A 5 μl volume of 1 kb DNA ladder (Invitrogen 1 kb DNA ladder) was used as a size marker (see Table 2.3). Electrophoresis was performed at a constant 120 V for approximately 45 minutes in TAE buffer containing 0.6 μg/ml ethidium bromide. Gels were visualised and photographed using a UV transilluminator camera system.

Table 2.5 DNA markers used in agarose gel electrophoresis

DNA Band	Size (kb)
1	12.216
2	11.198
3	10.180
4	9.162
5	8.144
6	7.126
7	6.108
8	5.090
9	4.072
10	3.054
11	2.036
12	1.636
13	1.018
14	0.506, 0.517
15	0.396
16	0.344
17	0.298
18	0.220
19	0.201
20	0.154
21	0.134
22	0.075

2.13.2 Preparation of chromosomal (genomic) DNA

From a 10 ml overnight culture initially inoculated with 2 or 3 colonies a cell pellet was collected by spinning the culture at 4000 rpm in a Beckman JA-20 rotor for 10 minutes at 4°C. The supernatant was discarded and the pellet was resuspended in a mixture of TE and proteinase K (110 mM Tris-HCl (pH 8.0), 1 mM EDTA containing 1 mg/ml proteinase K). The pellet was resuspended by continuous pipetting and then incubated in a waterbath at 65°C for 30 minutes. SDS (10%) was added and the solution incubated at 65°C for a further hour. Phenol/chloroform (2 ml) was added to the solution in a phaselock tube (Eppendorf) and the gently agitated and spun at 4000rpm in a Megafuge 1.0 Heraus 7570E. The supernatant was decanted in to another phaselock tube and the phenol extraction was performed twice more. The resulting supernatant was then transferred to a Falcon tube containing 3 ml of 100% ethanol and then inverted until the precipitated DNA took on a fluffy appearance. The DNA was extracted from the Falcon tube using a hook made from a Pasteur pipette and gently swirled in -20°C, 70% ethanol. Residual fluid from the DNA was permitted to drip away from the DNA before the pellet was allowed to air dry for 30 minutes. Finally, the pellet was resuspended in TE buffer and stored at –20°C.

2.13.3 Ethanol precipitation

DNA was recovered by the addition of 0.1 volumes of 3 M NaAc (pH 5.6) and 2.5 volumes of absolute ethanol. The DNA was incubated in methanol dry-ice for 30 minutes. This sample was then allowed to thaw and spun at 15000rpm for 10 minutes in a micro-centrifuge to pellet the DNA. Ethanol was removed and the pellet washed in 300 µl of -20°C 75% ethanol to remove any residual salts. The pellet was then air dried for 5 minutes before being resuspended in sterile analytical grade water.

2.13.4 Estimation of DNA quantity and purity

DNA estimations were made by recording the absorbance readings at 260 nm and 280 nm (using quartz cuvettes) against a suitable blank. DNA was estimated using the assumption that an A_{260} of 1 was the equivalent to a DNA concentration of 50 µg/ml. The resulting DNA was only used if the 260 nm to 280 nm ratio was above 1.6. Where necessary, samples were further de-proteinised, e.g. by phenol/$CHCl_3$ extraction.

2.13.5 DNA extraction from agarose gels

DNA fragments visible on agarose gels were excised using a clean scalpel blade. The DNA from these bands was then extracted using a Qiagen Gel-Extraction kit following the manufacturer's instructions. The procedure was based on the principle that the DNA binds to the silica-membrane columns and any contaminant are washed through. DNA was eluted from the column either with sterile analytical grade water or alternatively with Elution Buffer (10 mM Tris-HCl, pH 8.5).

2.13.6 One step inactivation of chromosomal *ygaU* in *S.* Typhimurium using PCR products

The method of chromosomal gene disruption as described by Datsenko & Wanner for *E. coli* was utilised in order to try and produce a mutant of *S.* Typhimurium in which chromosomal *ygaU* was inactivated, to enable a study of the impact of *ygaU* loss upon *S.* Typhimurium responses to osmotic shock and other parameters (Datsenko and Wanner, 2000).

In brief, the approach involves replacing a chromosomal sequence (in this case *ygaU*) with a selectable antibiotic resistance gene that is generated by PCR. After selection, the resistance gene is then eliminated using an FLP (flippase) expression plasmid.

The first step of the process was the amplification of an FRT (FLP recognition target)-flanked resistance gene by PCR. In this case this was either chloramphenicol or kanamycin resistance from either a pKD3 or pKD4 plasmid. The method of PCR utilised was as described by Sambrook *et al.* (1989). All PCR reactions were performed in 50 µl reaction volumes in 0.5 ml eppendorf tubes using either a Techne thermal cycler or a Techne gradient thermal cycler. The DNA polymerase used was either Taq polymerase (Promega) or Pfu DNA polymerase (Stratagene) depending upon application. The PCR reaction buffer contained a final concentration of 1 x Promega buffer, 1.5 mM, 0.2 nM of each dNTP, 20 pmols of each primer and 2.5U of DNA polymerase. The PCR components were then heated to 94°C for approximately 30s before the addition of the DNA polymerase. This temperature was then maintained for a further 2 minutes 30 seconds. The thermal cycler conditions typically consisted of 94°C

for 1 minute, 50°C for 40 seconds and 72°C for 2 minutes, all experiments used either 30 or 35 cycles and a final extension time at 72°C for 10 minutes. The oligonucleotides used in this study were synthesized by Invitrogen, Sigma, or MWG biotech and are detailed below in Table 2.6.

Table 2.6. Oligonucleotides used in the PCR reaction to amplify an FRT-flanked resistance gene for use in the one step inactivation of chromosomal *ygaU* in *S.* Typhimurium.

Primer Name	Sequence
YgaU-KO-H1-alt	GACAAGCGAGGTGAATATGGTGTAGGCTGGAGCTGCTTC
YgaU-KO-H2-alt	GCGACCTGATAGCCATTACATATGAATATCCTCCTTAG
YgaU-KO-H1	TCATTCCCTCTTGCTACACTTTCTTTGTGTGTTATTTTTGACAAGCGAGGTGAATATGGTGTAGGCTGGAGCTGCTTC
YgaU-KO-H2	CCATTCTTCGCCAGGCGATGGCAGGGAAAATGTAGCCTGGCGCGACCTGATAGCCATTACATATGAATATCCTCCTTAG

These primers were developed *ab initio*, specifically for this study.

Once the FRT-flanked resistance gene had been amplified the PCR fragment was digested with *Dpn*I and re-purified using a Qiagen kit as described in 2.13.7 and transformed into electrocompetent *S.* Typhimurium SL1344 (pKD46) as described in 2.13.8. One-half of the 1 ml sample was plated onto agar containing chloramphenicol or kanamycin and if no growth occurred within 24h the rest of the sample was plated after standing overnight at room temperature. Any colonies that grew were re-streaked onto LB agar without antibiotic and incubated at 37°C. The colonies were also tested for ampicillin sensitivity to verify loss of the pKD46 plasmid and if it was lost re-streaked and incubated at 43°C before re-testing. Finally the wild type gene replacement by the mutant was verified using PCR.

2.13.7 Restriction enzyme digests

Restriction enzyme digestion of DNA was based on the procedure as described by (Sambrook et al., 1989). Specifically, the PCR fragment consisting of the kan^R or Cm^R antibiotic resistance cassette including FRT modules from either pKD4 or pKD3 respectively was digested using *dpn1* in buffer B (60 mM Tris-HCl, 500 mM NaCl, 60 mM $MgCl_2$ and 10 mM dithiothreitol (DTT)) at 37°C for 2 hours. DNA digests were purified using the Qiagen QIAquick gel extraction kit protocol. The DNA was eluted in 40 µl of sterile analytical grade water.

2.13.8 Transformations into electrocompetent cells

Electrocompetent cells for transformations were prepared by growing cultures under vigorous aeration to an A_{600} of approximately 0.6 optical density units, harvesting (6000rpm, Beckman JLA 10500 rotor for 10 minutes at 4°C) and washing the cells twice with sterile, ice cold, analytical water. The cells were then resuspended in sterile, ice cold, 10% v/v glycerol and pelleted (6000rpm, Beckman JLA 10500 rotor, for 10 minutes at 4°C). The cells were resuspended for a final time in 10% v/v glycerol and then aliquoted into 40 µl batches snap frozen and stored at -70°C. The DNA to be transformed was added to 40 µl of electrocompetent cells in either 0.1 or 0.2 ml electroporation cuvettes. This was mixed thoroughly and incubated on ice for no longer than 1 minute. The cells were exposed to electric current at either 1.25mV or 2.5mV for either the 0.1 ml or 0.2 ml electrocuvettes respectively, for between 4.5-5.0 milliseconds. At this point the cells were resuspended immediately in 1 ml of sterile SOC medium (10mM NaCl; 2.5 mM KCl; 10 mM $MgCl_2$; 10 mM $MgSO_4$; 20 mM Glucose; 2% Bacto-tryptone; 0.5% w/v Bacto-yeast extract) and incubated at 37°C for 1-2 hours prior to plating on suitable media.

2.13.9 Cloning the *ygaU* gene codon into pAJG38

The following method was used to try and produce a clone of *S.* Typhimurium SL1344 that expressed YgaU under the control of an arabinose inducible promoter.

The first step of the process was the amplification of the *ygaU* gene by PCR using SL1344 chromosomal DNA (prepared as described in section 2.12.2) as a template. The PCR reaction was performed in 5 0µl reaction volumes in 0.5 ml thin-walled Eppendorf tubes using either a Techne thermal cycler or a Techne gradient thermal cycler. The PCR reaction mixture contained 5 µl of 1 x Promega buffer, 4mM magnesium sulphate, 0.5 µl of each primer, 0.2 mM dNTPs, 0.5 µl of template, 1.25U of Pfu DNA polymerase (Stratagene) and 40.5µl of sterile distilled water. The PCR conditions consisted of 95°C for 1 minute, 95°C for 1 minute, 60°C for 30 seconds and 72°C for 2 minutes, repeated for 30 cycles and followed by 72°C for 5 minutes. The oligonucleotides used in this study were synthesised by Invitrogen Life Technologies and are detailed in Table 2.7.

Table 2.7. Oligonucleotides used in the PCR reaction to amplify chromosomal *ygaU* from *S.* Typhimurium.

Primer Name	Sequence
ygaU-clone-forward	CCCCCCCCCCTCGACAAGCGAGGTGAATATGGG
ygaU-clone-reverse	AAAAAAAAACTGCAGTTATTCTTCAGGAATACGCAACAC

These primers were developed *ab initio*, specifically for this study.

The PCR product was purified on and extracted from an agarose gel as described in section 2.13.5.

Next, both the PCR product and a *bipA*$^{+}$ plasmid pAJG38 (Figure 2.2: an L-arabinose-inducible *bipA* generated by removal of a 1.7 kb *Sal*I-*Pst*I fragment from pAJG31, and replacement with a 1.6kb *Sal*I-*Pst*I fragment PCR amplified from E2348/69 chromosomal DNA Ap^{r} and described in (Grant et al., 2003)) were digested with *Sal*I-*Pst*I (Promega).

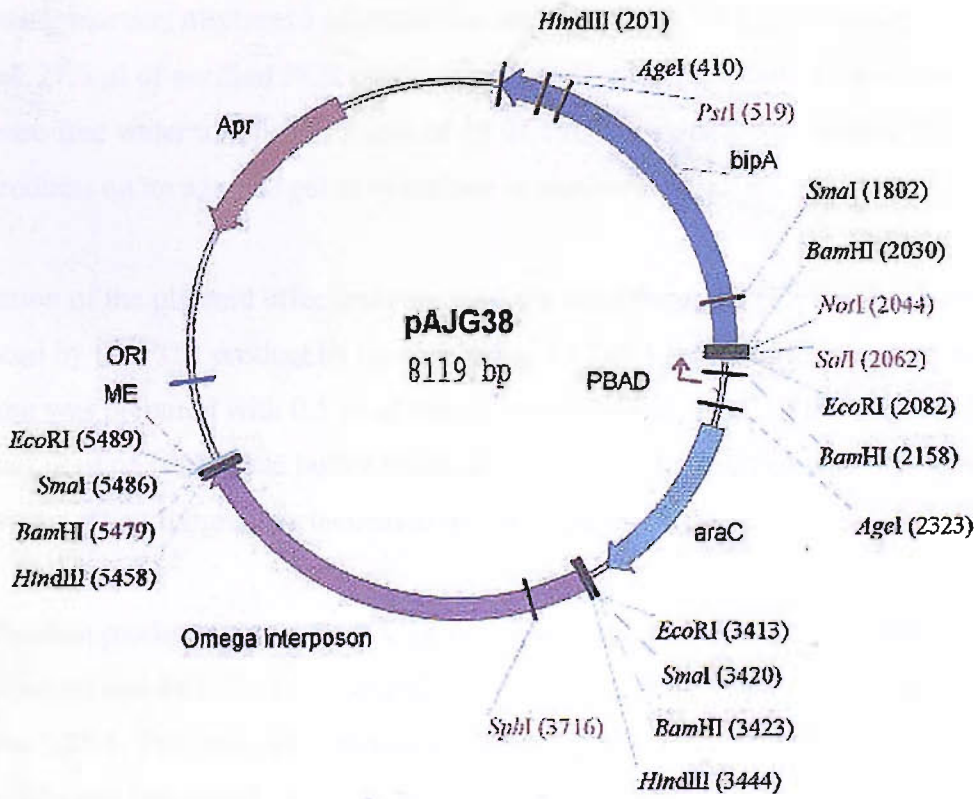


Figure 2.2 Plasmid pAJG38

The PCR product and plasmid were digested separately at 30°C overnight in the following reaction mixture: 5 µl restriction enzyme buffer D, 5 µl acetylated BSA 1 mg/ml, 27.5 µl of purified PCR product or plasmid, 60U *Sal*I, 60U *Pst*I and sterile nuclease-free water to a final volume of 50 µl. Product sizes were checked by running the products on an agarose gel as described in section 2.13.1.

Digestion of the plasmid effectively excised the *bipA* fragment (Figure 2.2) to be replaced by the PCR product by ligation using T4 DNA ligase as follows: a reaction mixture was prepared with 0.5 µl of the digested plasmid, 10 µl of the digested PCR product, 2 µl of 10X ligase buffer made up to a final volume 20 µl with sterile nuclease-free water. The mixture was incubated for 14 hours at 17.5°C.

The ligation product (plasmid pAJG38 with *bipA* replaced by *ygaU*) was then transformed into MACH-1 *E. coli* cells (Invitrogen Life Technologies) as described in section 2.13.8. The cells were then plated onto LB plates containing 100 µg/ml ampicillin and incubated overnight at 37°C. Any ampicillin resistant colonies were grown in ampicillin containing broth, and a plasmid preparation produced from the culture using a Qiagen MiniPrep kit. The plasmid preparation was then sent for sequencing to confirm it was the *ygaU* containing plasmid present in the MACH-1 cells. The sequencing was performed by Lark Technologies.

Next, electrocompetent *S.* Typhimurium were prepared and transformed using the plasmid preparation from the ampicillin resistant MACH-1 cells, confirmed by sequencing to contain the *ygaU* gene. The transformed *S.* Typhimurium cells were selected by growth on ampicillin containing media.

Finally, cultures of the ampicillin resistant (*ygaU* plasmid containing) MACH-1 *E. coli* or *S.* Typhimurium were grown to various times points in 1% arabinose or glucose in LB broth containing 100 µg/ml ampicillin and protein extracts were prepared for analysis by 1-D SDS-PAGE (12% Bis-Tris Novex gels and MES buffer (Invitrogen Life Technologies)).

CHAPTER THREE

BASELINE STUDIES: STRAIN VERIFICATION AND OPTIMISATION OF SAMPLE ANALYSIS METHODS

CHAPTER 3. BASELINE STUDIES: STRAIN VERIFICATION AND OPTIMISATION OF SAMPLE ANALYSIS METHODS

3.1 Baseline Studies to Determine the Effect of an Osmotic Up-shift on *Salmonella* Growth.

Before undertaking proteomic analyses of *Salmonella* grown under hyper-osmotic conditions it was critical to determine the growth responses of *S.* Typhimurium SL1344 and its *ompR*⁻ derivative to 0.5M NaCl, not least to ensure that these strains displayed the relevant phenotypes. Figures 3.1 A and B show typical growth curves of SL1344 and *ompR*⁻ SL1344 cells before and after the addition of 0.5M NaCl. It can be seen that both strains were capable of recommencing growth after salt addition. However, both strains displayed a marked phase of growth retardation of similar magnitude prior to recommencing growth. It was concluded that SL1344 and its *ompR*⁻ derivative both display an adaptive response to osmotic up-shift. These results imply that the OmpR transcription factor is not essential for adaptation to hyper-osmotic stress even though its involvement in osmoregulation is well documented.

3.2 Optimisation of *Salmonella* Protein Preparation and Separation by 2-D Gel Electrophoresis

When these studies commenced, the most viable route for the proteomic analysis of *Salmonella* stress responses was the comparative analysis of protein profiles obtained by two-dimensional gel electrophoresis. Previous studies had shown that a range of *Salmonella* protein could be fractionated in this way (O'Connor et al., 1997; Qi et al., 1996; Qi et al., 1995). However, the approach had not been optimised in terms of the amount of protein that could be loaded onto such gels. This was important as a key objective in the present study was the identification of proteins of interest rather than just their detection. Additionally, it was not known whether single pH range gels offered a significant advantage over broader range gels or if pre-fractionation of the cell envelope would be useful for routine analyses. These parameters were also investigated as part of the base-line studies.

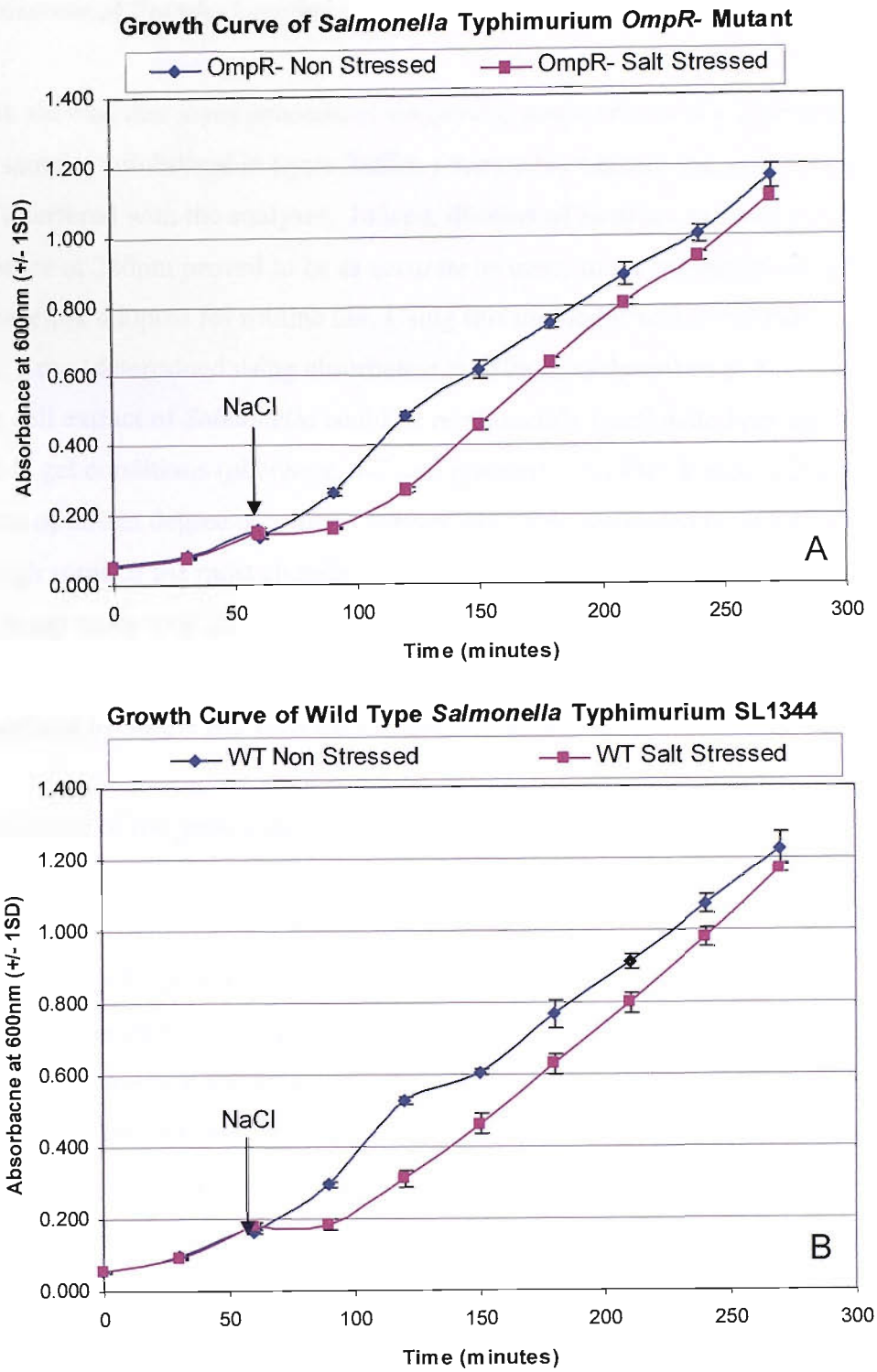


Figure 3.1 Osmoadaptation characteristics of the two main *Salmonella* strains used in this study. Cultures were grown in LB medium as described in section 2.3 and salt was added to a final concentration of 0.5 M at an A_{600} of approximately 0.2.

A. Growth of SL1344 *ompR*⁻ before and after osmotic up-shift.

B. Growth of SL1344 before and after osmotic up-shift.

3.3 Optimisation of Protein Loading

Initial work showed that many procedures for protein quantitation were inaccurate when used with samples solubilised in Lysis Buffer, presumably because the urea contained in the buffer interfered with the analyses. Indeed, dilution of samples and measurement of the absorbance at 280nm proved to be as accurate as many more complicated methods and was therefore adopted for routine use. Using this method it was found that, nominally, 5 mg (determined using absorbance at 280nm, as described in Section 2.6) of a whole cell extract of *Salmonella* could be reproducibly fractionated per gel using standard 2-D gel conditions (pH range: 4-7; gel gradient: 12-14%). Panels 3.2 A and B illustrate the optimum degree of protein loading obtained, compared to an under-loaded gel. Although some of the most abundant proteins exhibit minor amounts of vertical streaking, many more spot features can be detected.

3.4 Comparison of Single pH Unit and Broad pH Range Gels

During the course of the present study, single pH unit gels became commercially available. It was therefore of interest to determine if they represented a significant advantage over broader pH gels for the fractionation of *Salmonella* proteins. As illustrated in Figure 3.3, pH 4.5 – 5.5 gels gave reasonable results and had a significantly greater protein loading capacity than broader pH range gels. However, the number of additional protein spots observed relative to the equivalent region of a pH 4-7 range gel was not significantly greater. Further, the other single pH unit gels tested did not produced satisfactory results with *Salmonella* lysates. The pH 5.5-6.7 dry strips proved to be problematic in terms of poor reproducibility and focussing despite attempts to alter the first dimension protocol and solubility criteria (data not shown). Some possible reasons for the apparently poor quality of gels in the 5.5-6.7 pH region could be that the proteins that are being separated are less abundant than in the 4.5-5.5 range. It was therefore concluded that the inclusion of single pH unit gels in the routine analysis of *Salmonella* protein expression profiles was not justified in terms of the overall benefits and the increased cost and analysis time.

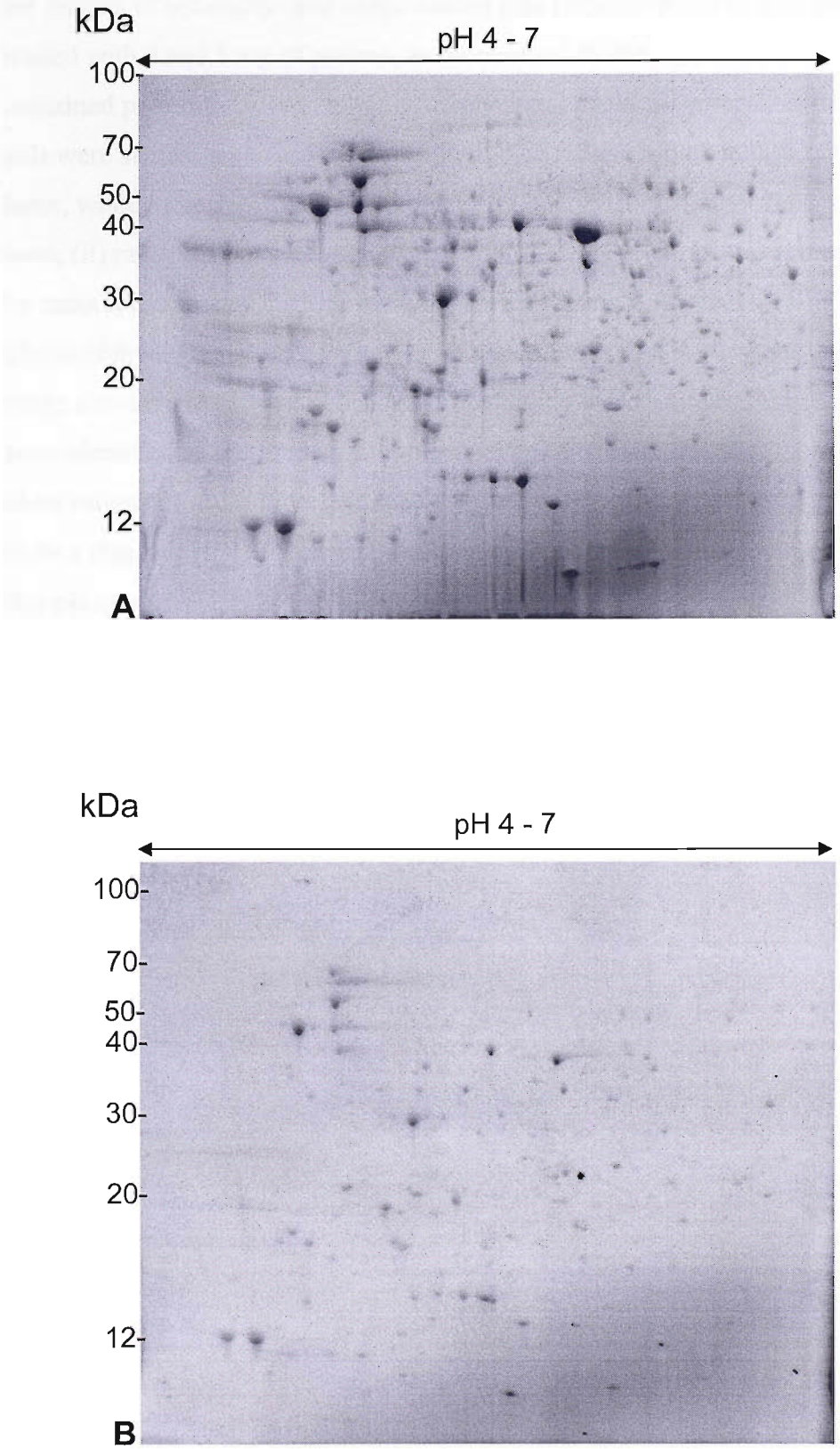


Figure 3.2 See following page for description

Figure 3.2 (previous page). Optimisation of protein loading conditions. Panels A and B are images of optimally- and under-loaded gels respectively. The gels were nominally loaded with 5 and 1 mg of protein, as determined by the A_{280} measurements. Each gel contained proteins derived from mid-exponential phase *Salmonella* cells. In each case, gels were stained with Coomassie brilliant blue rather than by silver staining as the latter, while more sensitive, (i) has a very limited dynamic range when densitometry is used; (ii) produces much higher backgrounds and (iii) complicates protein identification by mass spectrometry, especially if a procedure using a protein cross-linking agent (e.g. glutaraldehyde) is used. Experiments using a pH range of 3-10 rather than the pH 4-7 range shown here showed some additional highly basic proteins. These have previously been identified as predominantly ribosomal components (David O'Connor, unpublished observations). Furthermore, the relatively crowded nature of pH 3-10 gels was deemed to be a disadvantage relative to the few additional proteins that could be visualised with this pH range.

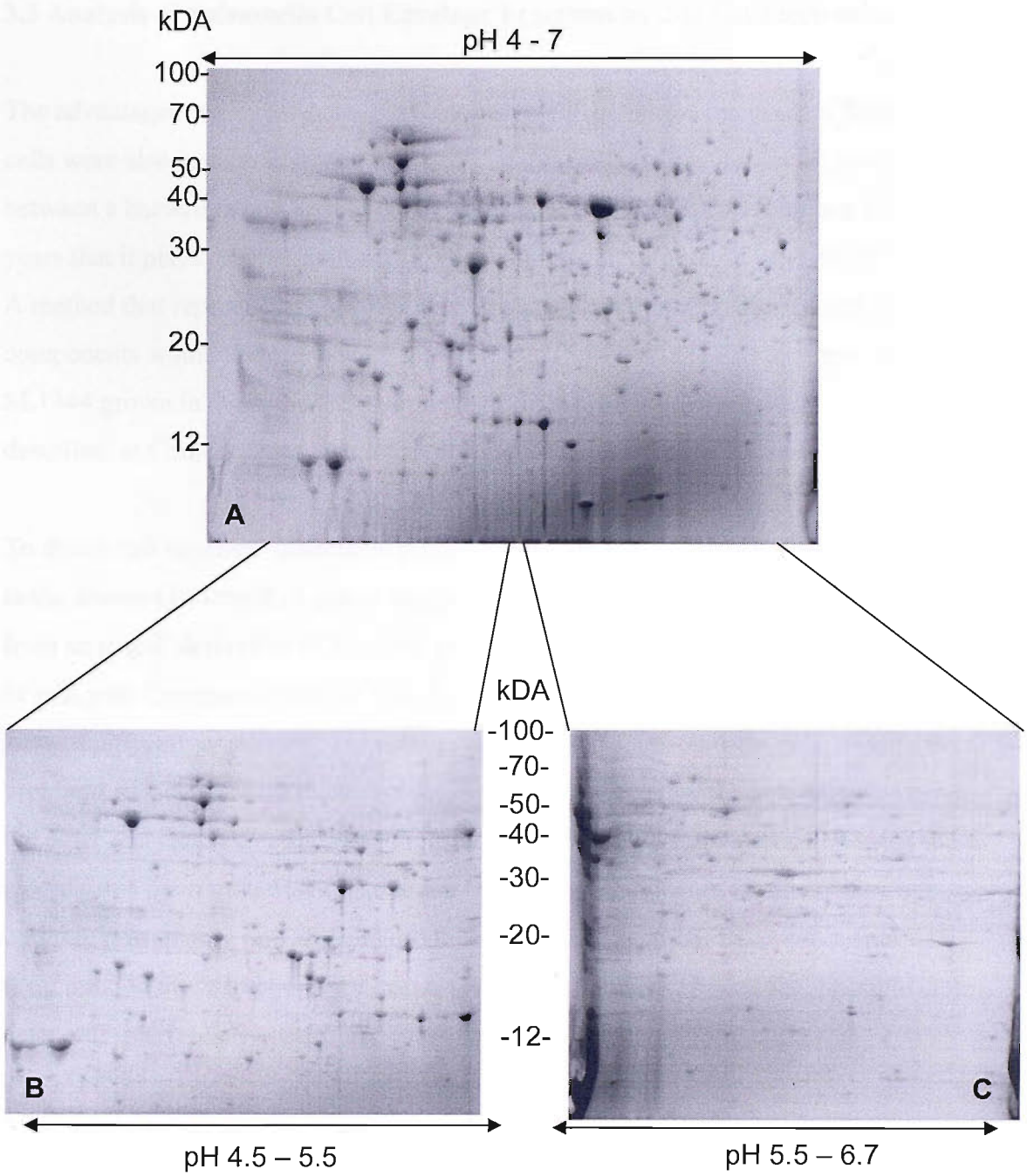


Figure 3.3 Comparison of single pH unit and broad pH range gels for *Salmonella* proteome analysis. Note the increased resolution of spots achieved using narrow range IPG strips. Gel A shows the pattern for whole cell lysate prepared from wild type *S.* Typhimurium SL1344 separated by 2-D-PAGE using pH 4-7 IPG strip. Gels B and C shows the equivalent patterns obtained with pH4.5-5.5 and 5.5-6.7 IPG strips, respectively.

3.5 Analysis of *Salmonella* Cell Envelope Fractions by 2-D Gel Electrophoresis.

The advantages and disadvantages of analysing cell envelope fractions of *Salmonella* cells were also assessed. It is recognised that the cell envelope forms the interface between a bacterium and its immediate environment and it has been known for many years that it plays an important role in maintaining cell turgor and integrity (Chapter 1). A method that reproducibly allowed the high-resolution fractionation of cell envelope components would therefore be of some utility. Cell envelope extracts from cells of SL1344 grown in the presence or absence of 0.5 M NaCl were prepared for analysis as described in Chapter 2 and fractionated using 2-D gels with a pH range of 4-7.

To detect cell envelope-associated proteins that responded to hyper-osmotic stress, even in the absence of OmpR, 3 paired batches of cell envelope fractions were also prepared from an *ompR*$^-$ derivative of SL1344, grown under identical conditions. Initial staining of gels with Coomassie brilliant blue failed to reveal many protein spots (~20). Accordingly, silver staining was used to visualise the spots on these gels, despite the problems associated with the technique. Figures 3.4-3.6 show the gels prepared using the outer membrane fractions and Figures 3.7 – 3.9 show the gels prepared using the cytoplasmic membrane fractions. It can be seen that the images obtained were sub-optimal, presumably due to the hydrophobic nature of many of the components containing in these fractions and consequent poor solubility. It was also possible that detergent interference contributed to the poor resolution.

The outer membrane fraction gels (Figures 3.4 – 3.6) had good reproducibility but a highly abundant component of approximately 40 kDa displayed horizontal streaking and a distorted pattern. The cytoplasmic membrane fraction gels (Figures 3.7 – 3.9) were far less reproducible with many components displaying horizontal streaking. In view of the disappointing results obtained with these sub-cellular fractions, detailed image analysis was not performed nor were any spots taken for protein identification. It was possible that the use of alternative detergents in the solubilisation buffer system that was employed might have improved matters (Santoni et al., 2000). However, it was judged that further efforts in this direction would outweigh the potential benefits as alternative approaches for measuring cell envelope components were becoming available.

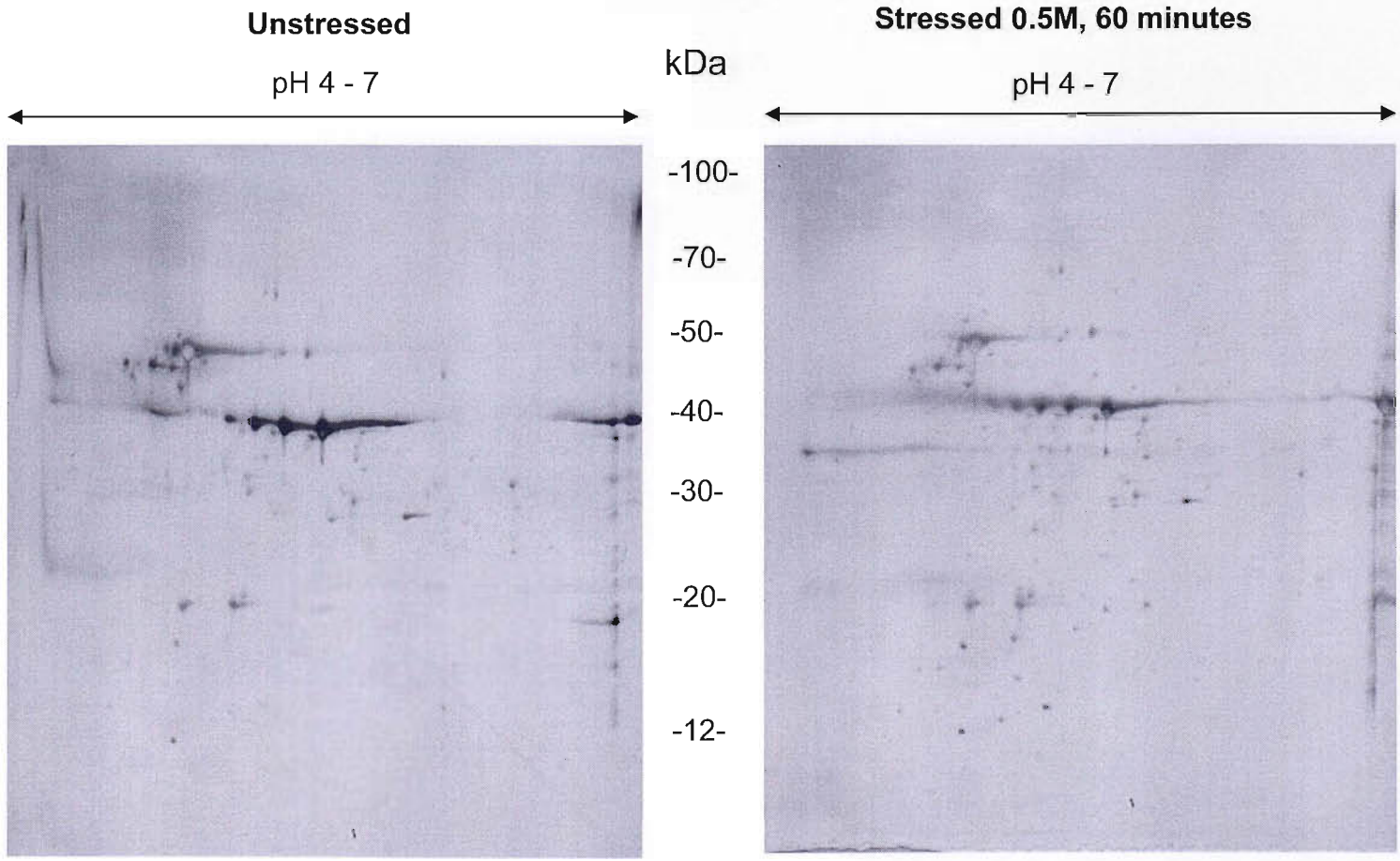


Figure 3.4 Comparison of gels separating outer membrane protein fractions from salt shocked (stressed) and non-salt shocked (unstressed) *ompR*⁻ *S.* Typhimurium SL1344. Salt shock was induced as described in Chapter 2, Section 2.3. (Replicate 1).

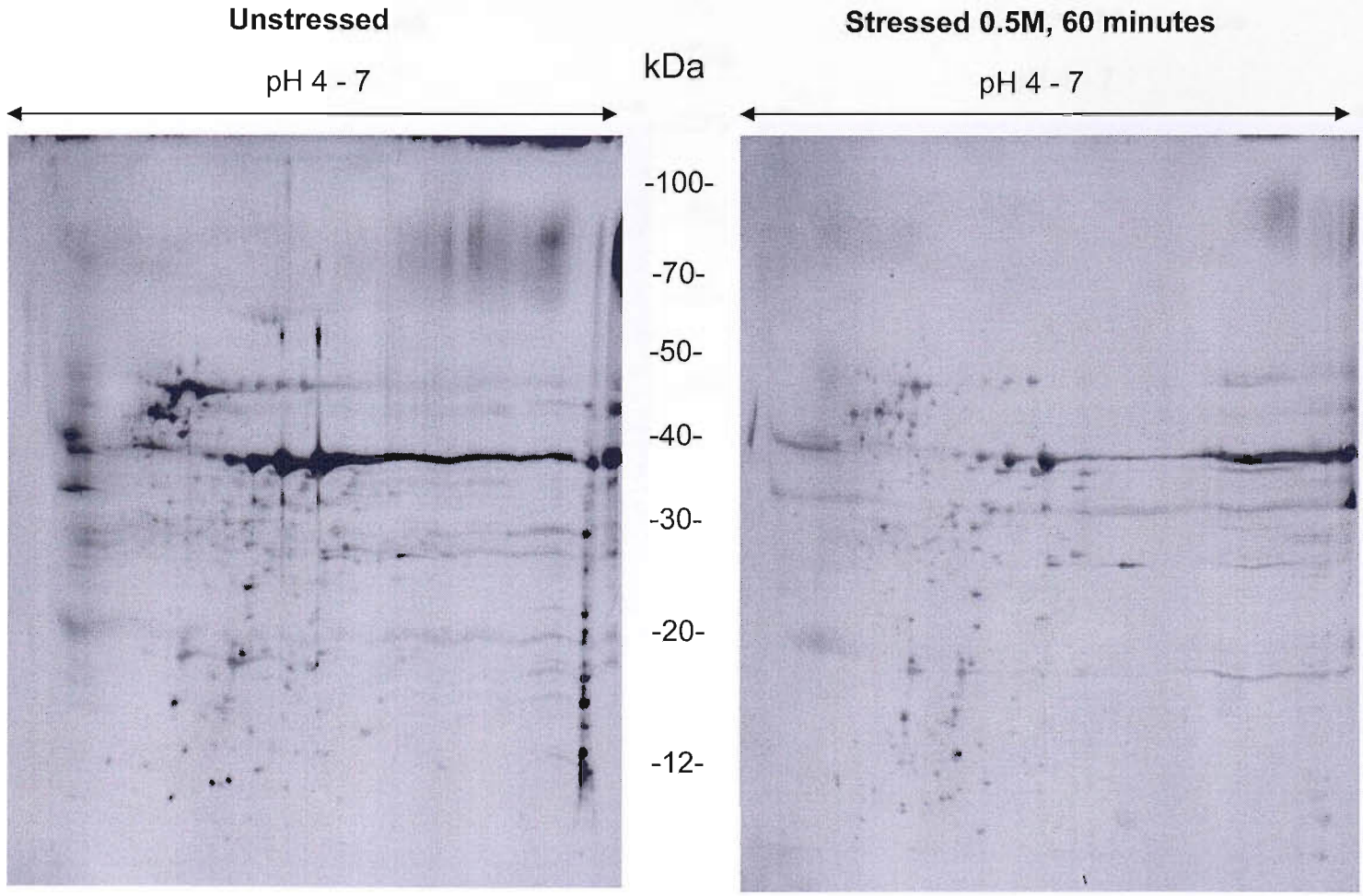


Figure 3.5 Comparison of gels separating outer membrane protein fractions from salt shocked (stressed) and non-salt shocked (unstressed) *ompR*⁻ *S.* Typhimurium SL1344. Salt shock was induced as described in Chapter 2, Section 2.3. (Replicate 2).

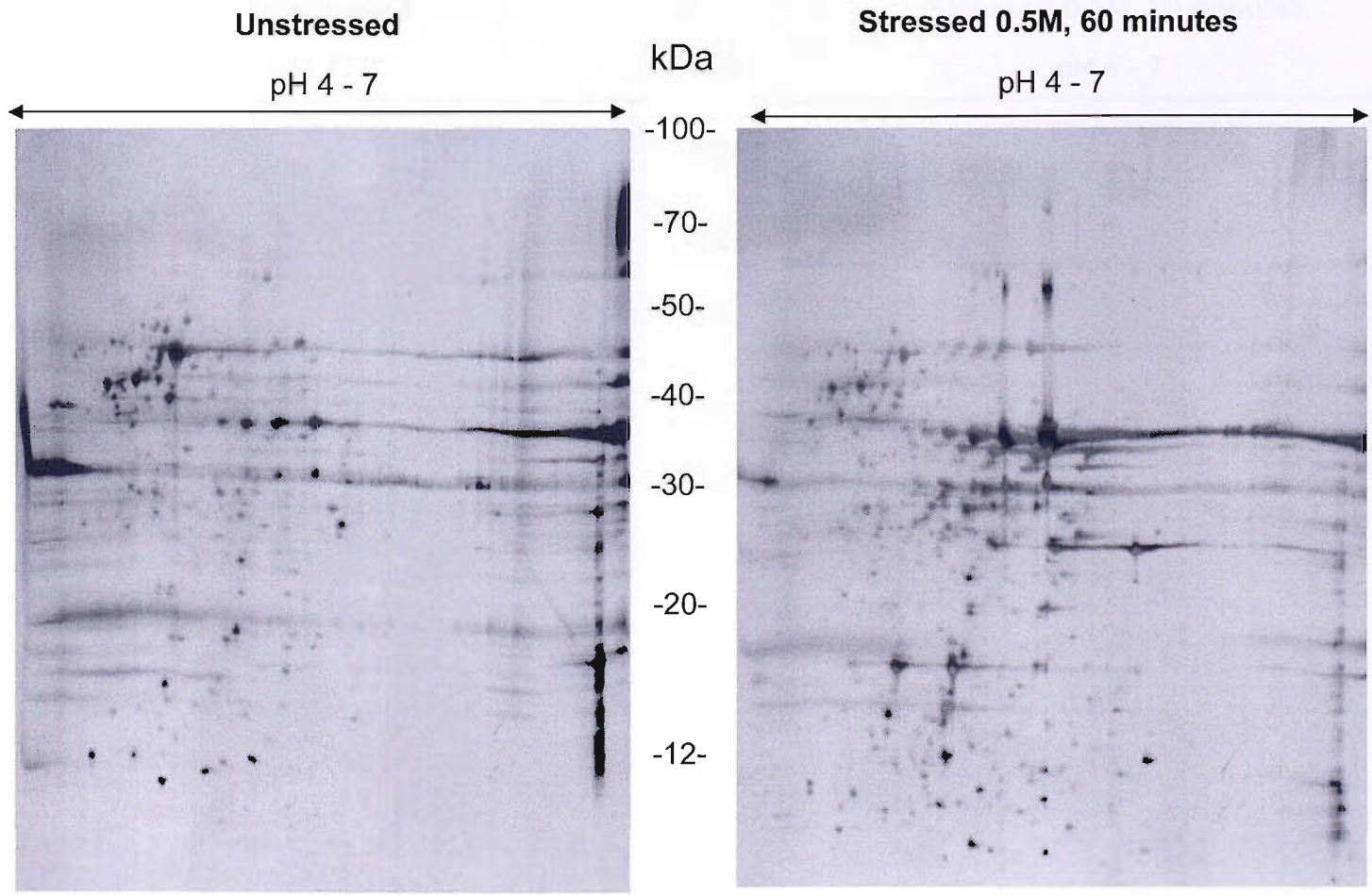


Figure 3.6 Comparison of gels separating outer membrane protein fractions from salt shocked (stressed) and non-salt shocked (unstressed) *ompR*⁻ *S.* Typhimurium SL1344. Salt shock was induced as described in Chapter 2, Section 2.3. (Replicate 3).

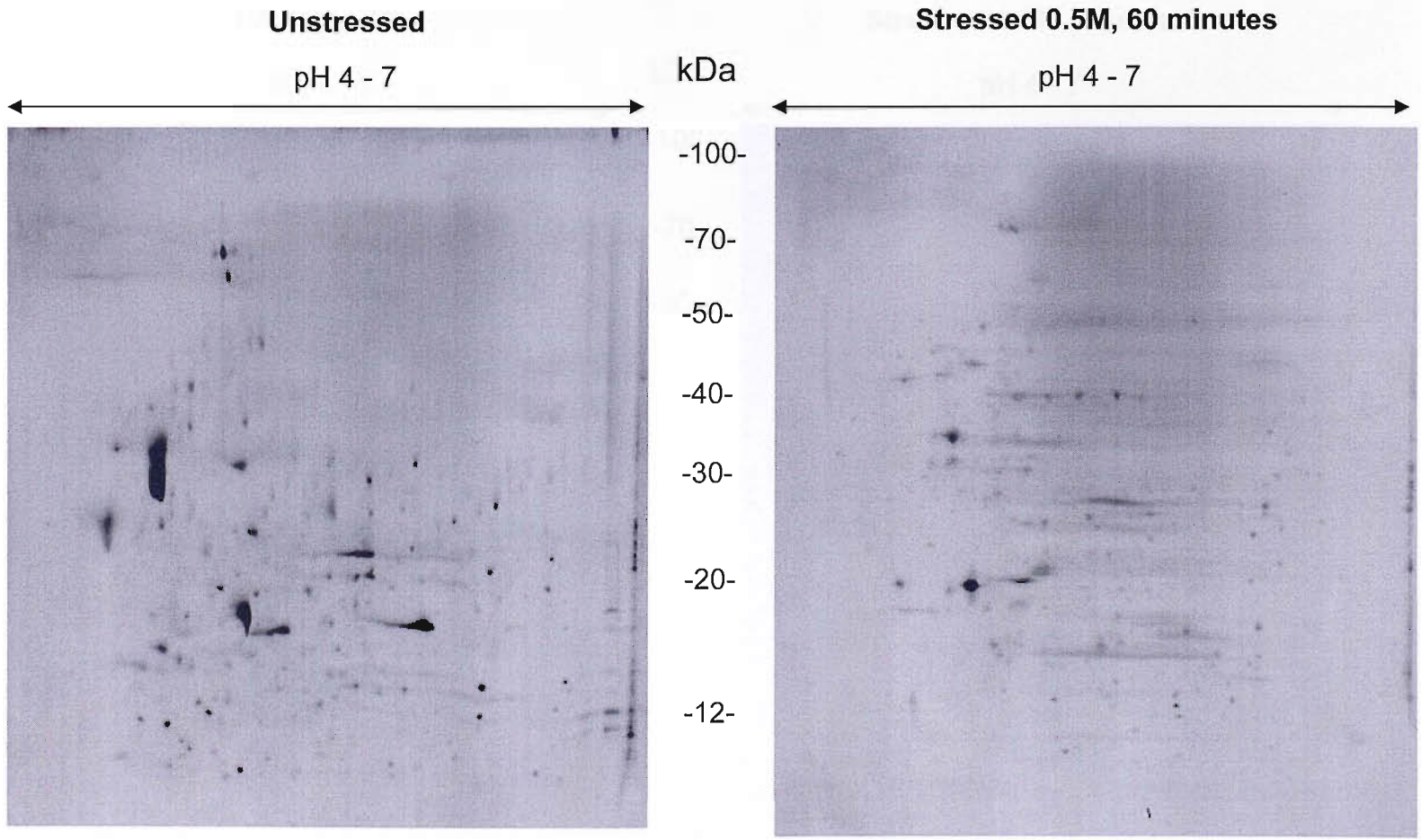


Figure 3.7 Comparison of gels separating cytoplasmic membrane protein fractions from salt shocked (stressed) and non-salt shocked (unstressed) *ompR*⁻ *S.* Typhimurium SL1344. Salt shock was induced as described in Chapter 2, Section 2.3. (Replicate 1).

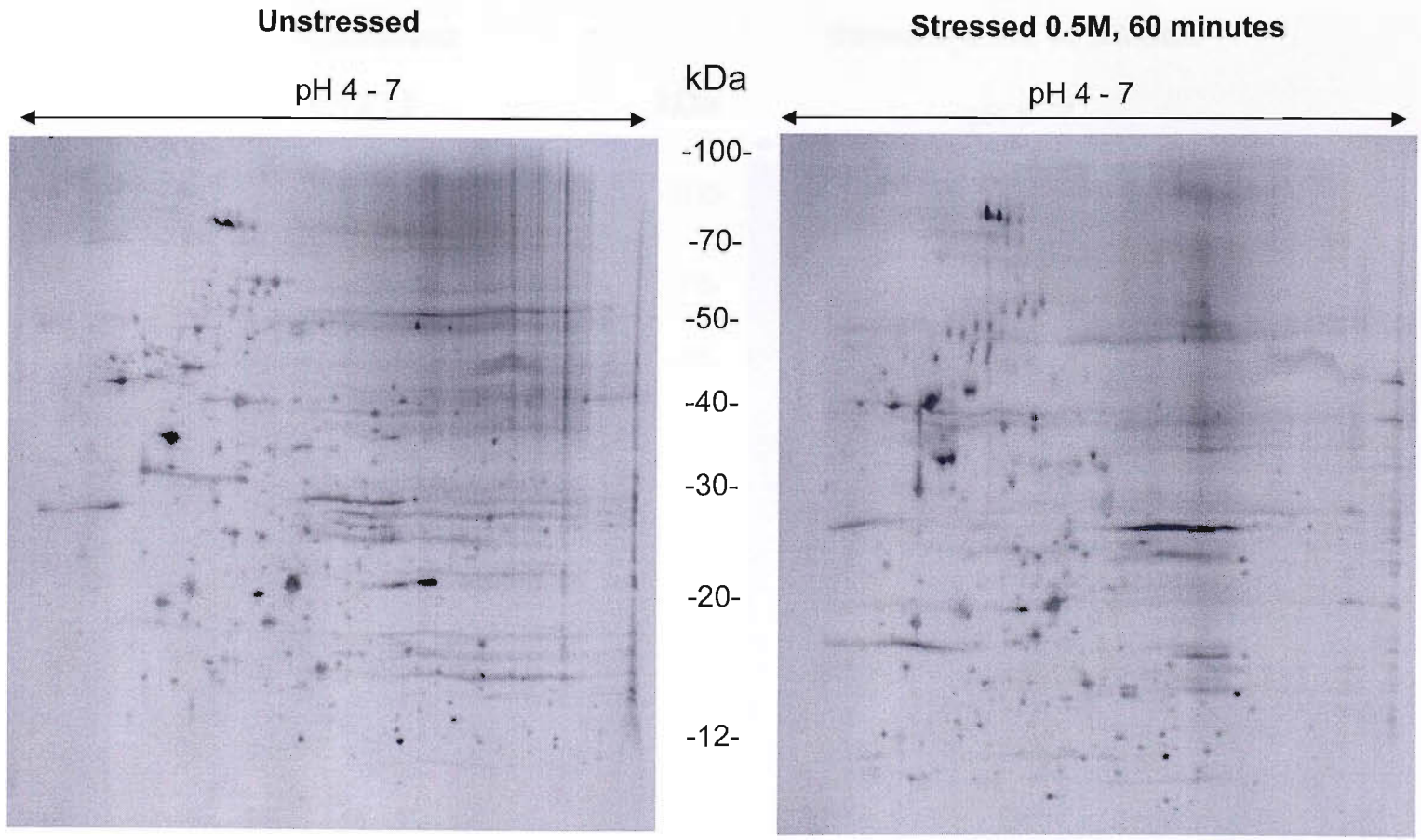


Figure 3.8 Comparison of gels separating cytoplasmic membrane protein fractions from salt shocked (stressed) and non-salt shocked (unstressed) *ompR*⁻ *S.* Typhimurium SL1344. Salt shock was induced as described in Chapter 2, Section 2.3. (Replicate 2).

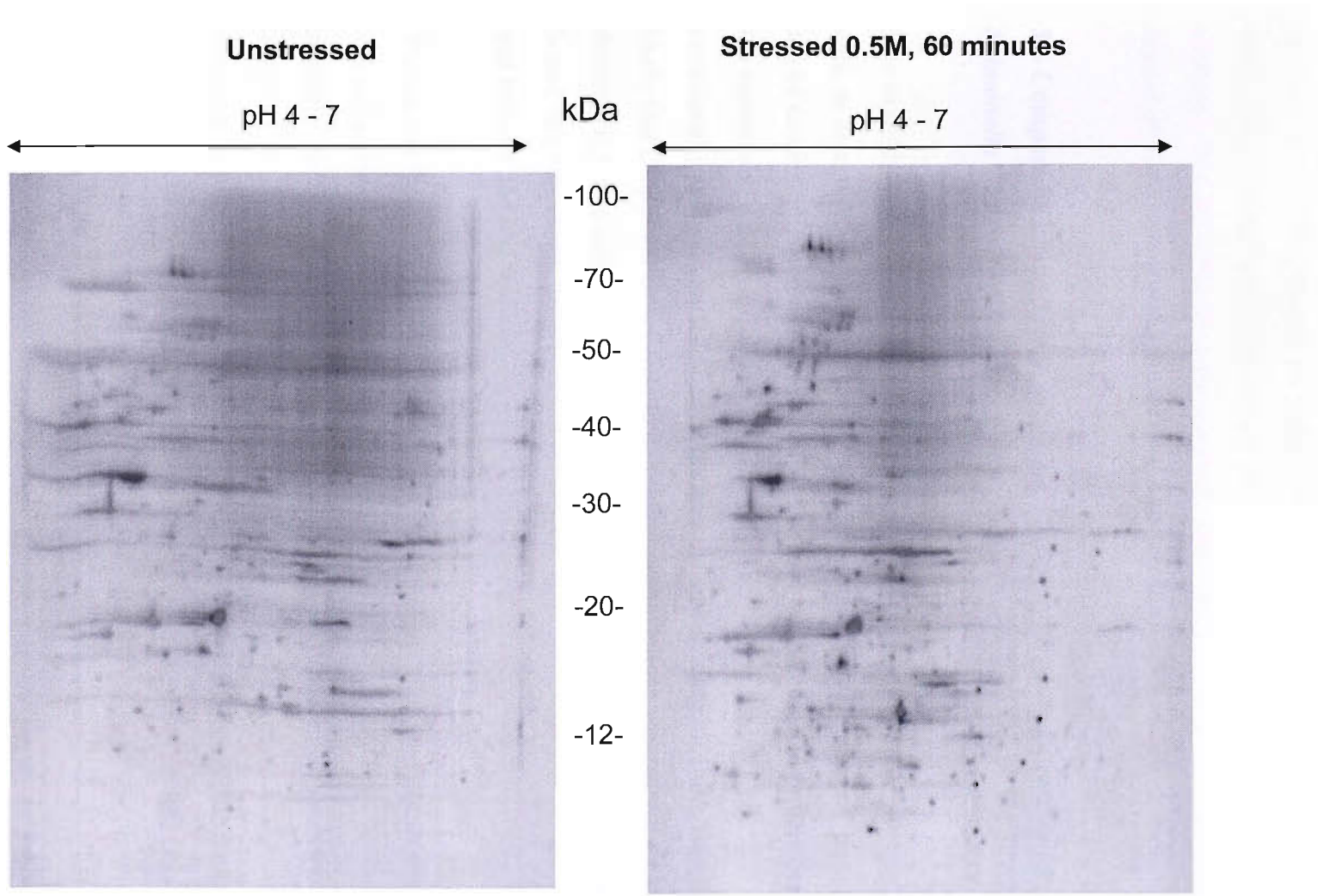


Figure 3.9 Comparison of gels separating cytoplasmic membrane protein fractions from salt shocked (stressed) and non-salt shocked (unstressed) *ompR*⁻ *S.* Typhimurium SL1344. Salt shock was induced as described in Chapter 2, Section 2.3. (Replicate 3).

Because of the difficulties of working with cell envelope fractions and sub-fractions, efforts were subsequently focussed on obtaining good quality gels using whole cell extracts. This proved to be more successful and Figure 3.2 A shows a gel image that is typical of those that can be routinely obtained.

3.6 Comparison of the Secreted Protein Profiles of Wild Type and *ompR*⁻ *Salmonella* Under Non-Stress and Salt-Stress Conditions

Due to difficulties of resolving the outer membrane proteins OmpF and OmpC on 2-D gels, as described in this chapter, the loss of these proteins in the *ompR*⁻ mutant could not be confirmed. Therefore it was decided to analyse the secreted protein profiles of the mutant and wild type *Salmonella*. The rationale behind this idea was that the cells occasionally shed cell surface fragments into the surrounding medium. Hence it was likely that OmpF and OmpC would be present in the culture supernatant and would be detected if it was concentrated prior to analysis by SDS-PAGE. In addition, it was hoped this may reveal differences in other secreted proteins under hyper-osmotic stress and between the two strains.

The secreted proteins of wild type and *ompR*⁻ mutant *S.* Typhimurium under non-stress and salt stress conditions were prepared and analysed as described in Chapter 2. Four replicate pairs of stressed and unstressed cultures were prepared for each strain and analysed. Although resolution was poor due to the high salt content of the samples, the resulting protein profiles for the strains (Figure 3.10 A & B) shows that a band of ~37-38kDa (most likely a combination of both OmpF (38.3 kDa) and OmpC (37.1kDa)) was induced in the wild type cells on addition of salt. In contrast, the same protein(s) were present at comparable levels, irrespective of salt addition in *ompR*⁻ cells. Subsequently, the protein band for the salt stressed sample was identified as OmpC by mass spectrometric analysis.

It can thus clearly be seen that OmpC is up-regulated in the wild type *Salmonella* under salt stress, but has unchanged expression in the *ompR*⁻ mutant. OmpF and OmpC are only 1kDa different in molecular weight and therefore the band identified is probably a combination of both proteins. That it was identified (by mass spectrometry) as OmpC in the salt-stressed band, is not surprising, as under conditions of increased osmolarity, expression of OmpC is raised, whereas OmpF is repressed to very low levels.

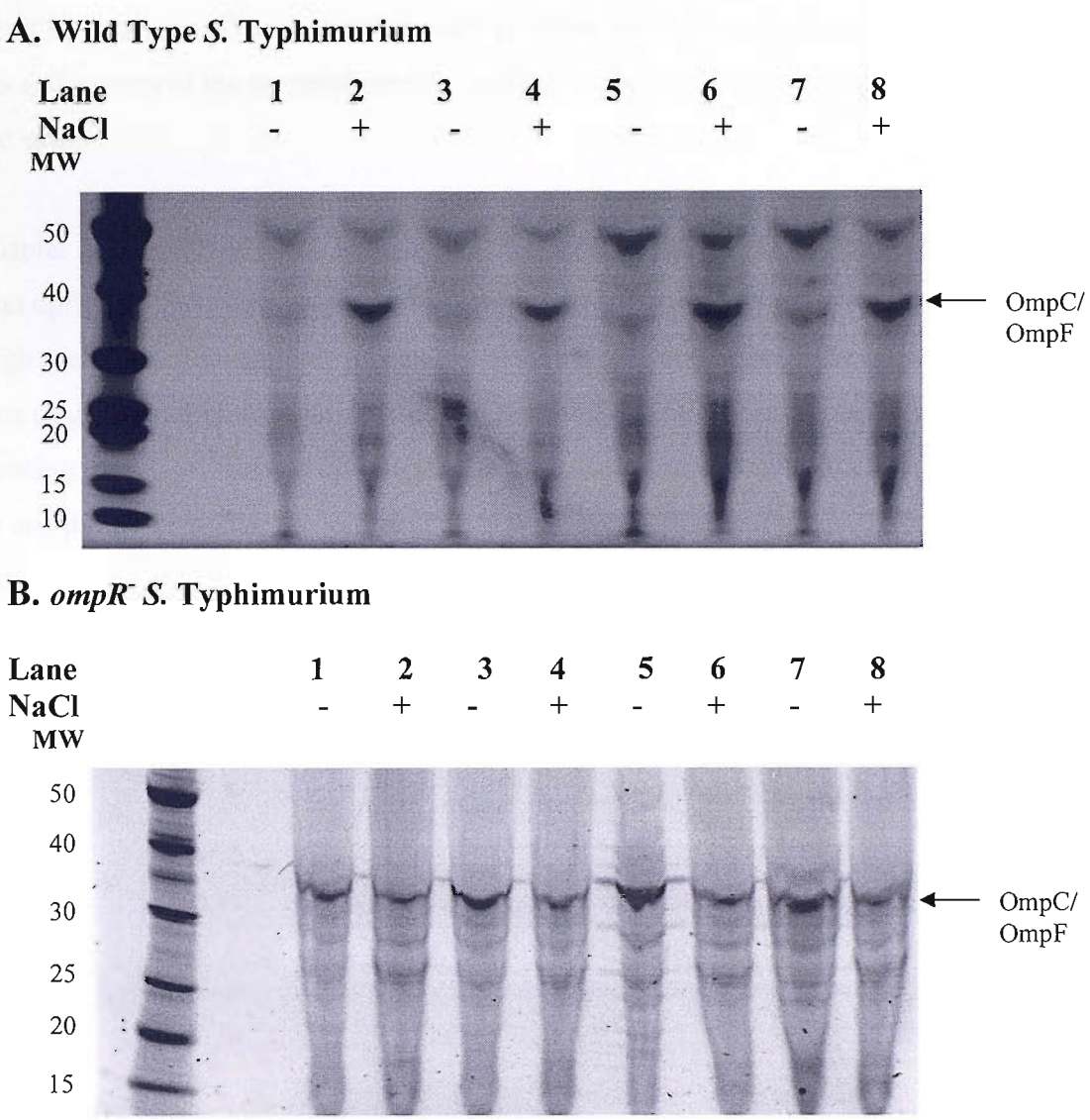


Figure 3.10 A & B. Sypro Ruby stained, 4-12% NuPage Bis-Tris gels separating secreted protein preparations from wild type (A) and *ompR*⁻ (B) *S.* Typhimurium cultured under non-stress (Lanes 1, 3, 5, and 7) and salt-stress (Lanes 2, 4, 6 and 8) conditions.

The lack of ability to control OmpC/OmpF expression in the *ompR*⁻ mutant was confirmed by analysis of the secreted protein profiles, however there were no other obvious differences in the secreted protein profiles of the two strains under hyper-osmotic stress.

This chapter has described some base-line studies to validate the major strains being used and optimise the fractionation of *Salmonella* proteins via 2-D gel electrophoresis. Although preliminary attempts to use single pH units gels and to produce sub-cellular fractions of good quality were disappointing, the final protocols for preparing and fractionating whole cell extracts proved to be sufficiently robust to produce good quality and highly reproducible gels and were used for work described in the following chapters.

CHAPTER FOUR

PROTEINS THAT ARE DIFFERENTIALLY EXPRESSED IN *S.* TYPHIMURIUM SL1344 CELLS FOLLOWING OSMOTIC UP-SHIFT

CHAPTER 4. PROTEINS THAT ARE DIFFERENTIALLY EXPRESSED IN *S.* TYPHIMURIUM SL1344 CELLS FOLLOWING OSMOTIC UP- SHIFT

4.1 Introduction

Studies described in the previous chapter established suitable conditions for the 2-D gel analysis of proteins from *S.* Typhimurium SL1344. In contrast to the more widely studied LT2 strain, SL1344 was isolated relatively recently and, more importantly, retains full virulence in mice. In part, the increased virulence of the latter strain may be due to the presence of some 350 genes that are not found in the LT2 genome (M. Pallen and J. Parkhill personal communication). However, it is also known that some genes common to both strains differ in their functionality. Thus, *rpoS*, a key regulator of the general stress response, has been reported to be altered in strain LT2 (Ibanez-Ruiz et al., 2000). Accordingly, it was decided to restrict the proteomic analyses to SL1344 to increase the relevance to studies on *Salmonella* pathogenesis being conducted elsewhere. This chapter describes the detection and identification of proteins of *S.* Typhimurium SL1344 that are differentially expressed when the strain is shifted to a high salt concentration.

4.2 Detection and Quantification of *Salmonella* Typhimurium Proteins That Are Differentially Expressed Under Hyper-Osmotic Conditions

Whole cell lysates were prepared from 4 paired batches of salt-stressed and unstressed cells, proteins were then separated using 2-D electrophoresis and stained with Coomassie Brilliant Blue and the gel images digitally captured and analysed as previously described (Chapter 2) to detect proteins that were significantly up- or down-regulated in response to hyper-osmotic stress. For the purposes of this study, a protein was deemed to be significantly up- or down- regulated if the following criteria were met: consistently up- or down- regulated on all sets of gels, greater than or equal to 2 fold increase or decrease in expression *and* for each protein that had altered expression the normalised spot intensities on the control sample gels were statistically compared to the intensities on the gels separating salt treated samples (using SPSS v11 software, a Mann-Whitney comparison of medians was performed due to non-normally distributed

data) for significant differences. In order to ensure rigorous quantification, it was crucial that particular care was taken at the image analysis stage.

From these analyses, it was concluded that at least 24 protein spots were altered in a hyper-osmotic environment. Inspection of their distribution showed that the differentially expressed proteins had a wide range of pIs and molecular masses, indicating that there was unlikely to be any significant bias in the analytical methods that were employed to detect them. Figure 4.1 is a representative 2-D gel illustrating the effects of hyper-osmotic stress on protein expression in wild type cells, showing 13 up-regulated (including those spots not detectable on the control gel) and 11 down-regulated proteins. Further, more detailed 2-D gel images illustrating the differentially expressed proteins are depicted in Figure 4.2.1-4.2.6. Analyses of the magnitude of the changes showed that the most highly up- and down-regulated proteins were spots 6 and 16, respectively (Figure 4.3), which showed ~10 fold and 0.5 fold alterations respectively.

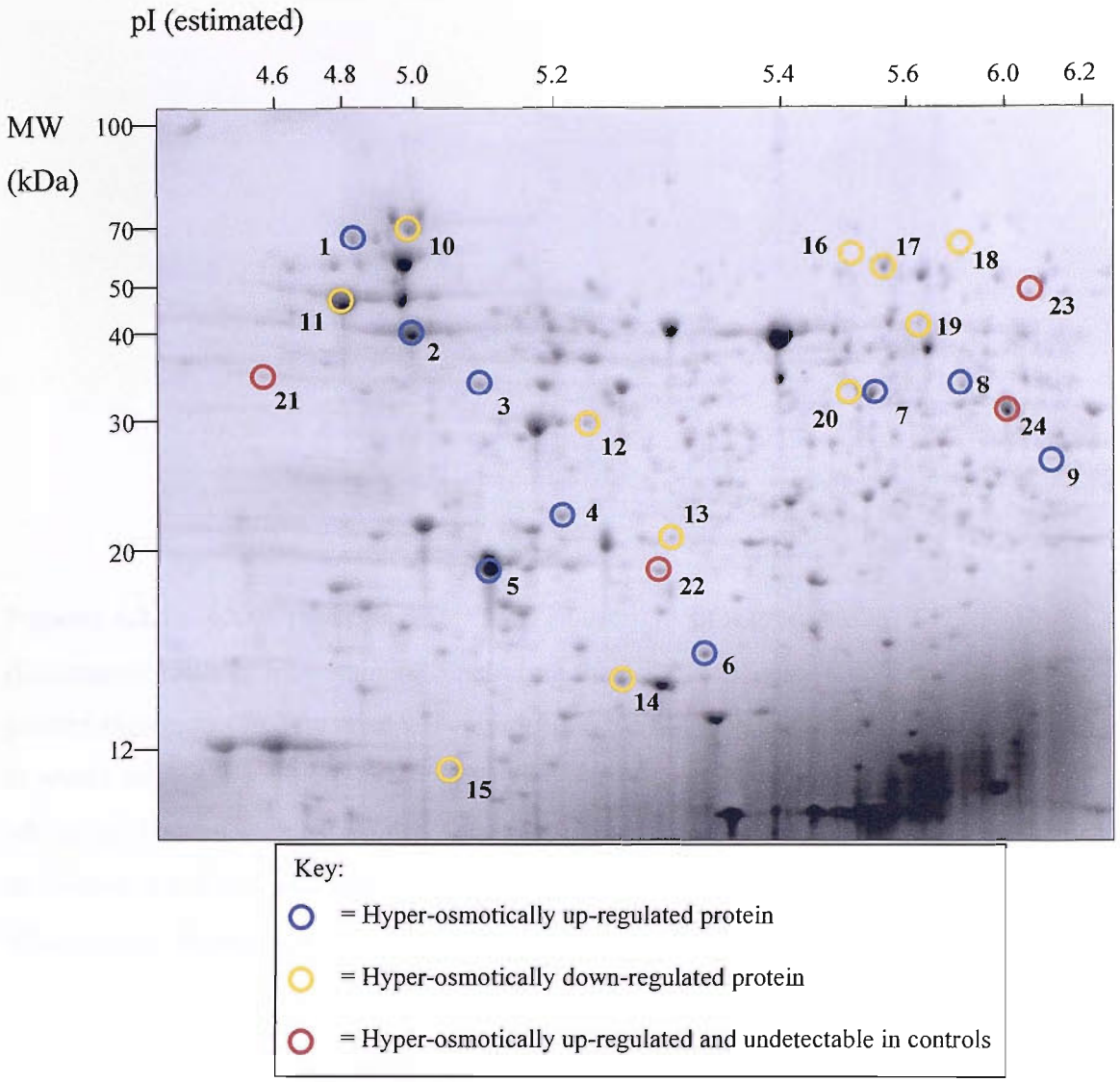
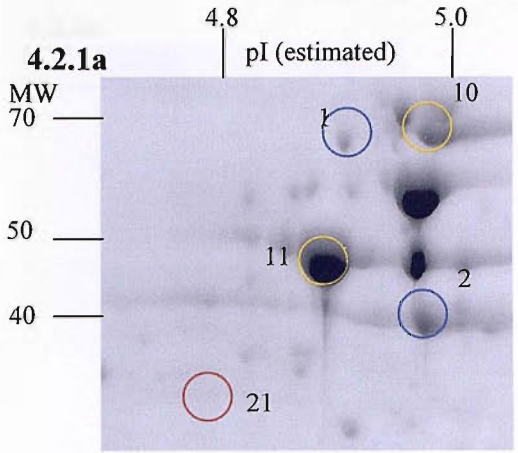


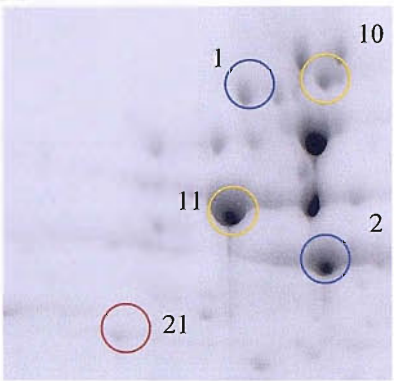
Figure 4.1 A representative 2-D gel (Coomassie brilliant blue stained) illustrating the effects of hyper-osmotic stress on protein expression in wild type *S.* Typhimurium SL1344. The pI and MW (kDa) scales are on the top and left hand sides of the gel image respectively. Proteins that were up – or down- regulated, when cells are hyper-osmotically stressed are ringed in blue and yellow respectively. Proteins that were up-regulated under the same conditions but were undetectable in cells grown in control conditions are ringed in red.

Figures 4.2.1a-4.2.6b (next pages). Enlarged sections of representative 2-D gels (Coomassie brilliant blue stained) illustrating the effects of hyper-osmotic stress on protein expression in wild type *S.* Typhimurium SL1344. In each case 'a' corresponds to whole cell lysates from unstressed *S.* Typhimurium SL1344 and 'b' corresponds to whole cell lysates from osmotically-stressed cells. Protein spot numbers relate to those on Figures 4.1 & 4.3 and Table 4.1. Additionally, the colour coding for the rings around spots of interest is the same as in Figure 4.1.

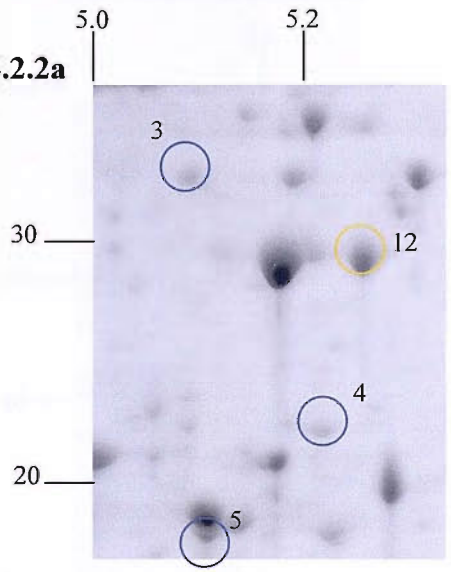
4.2.1a



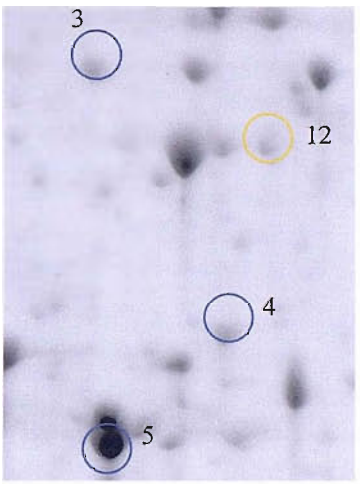
4.2.1b



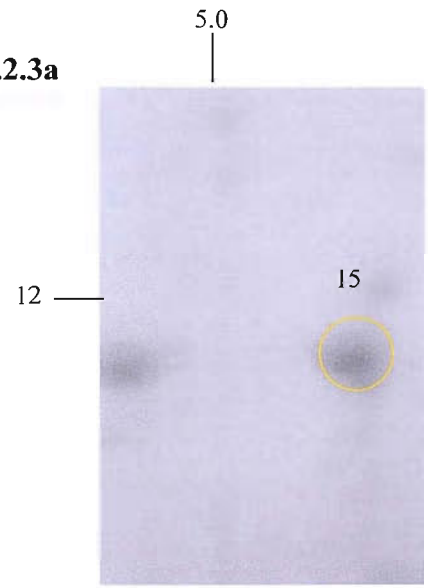
4.2.2a



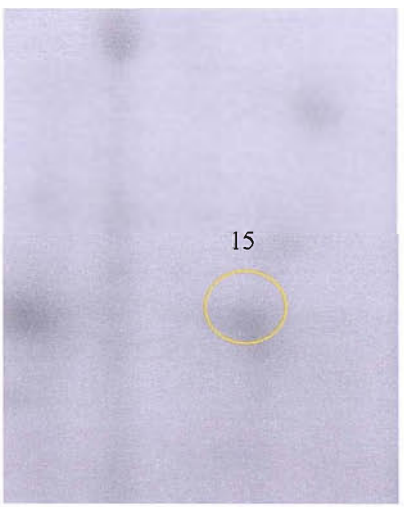
4.2.2b



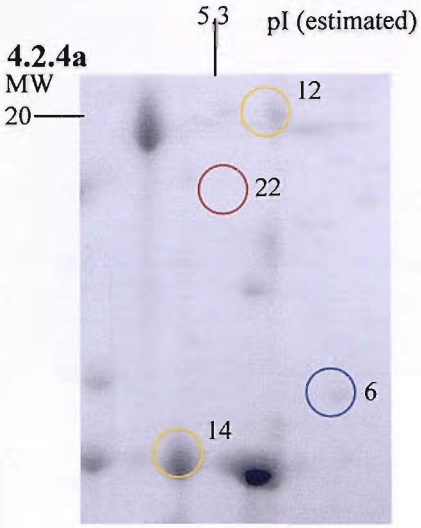
4.2.3a



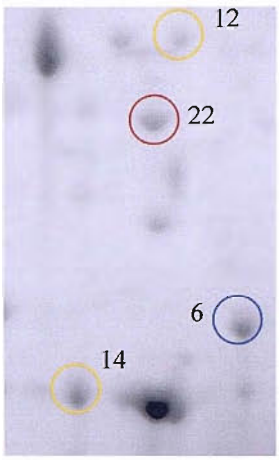
4.2.3b



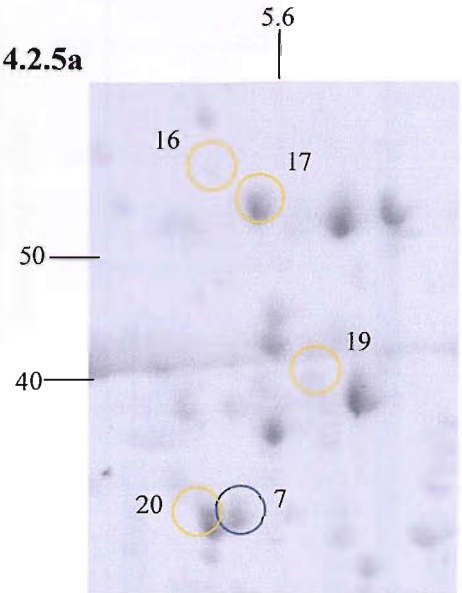
4.2.4a



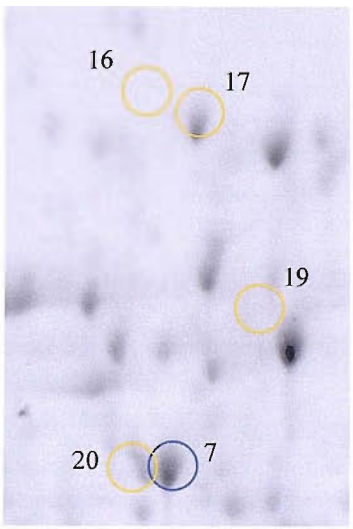
4.2.4b



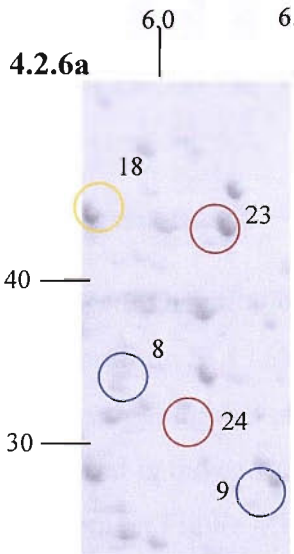
4.2.5a



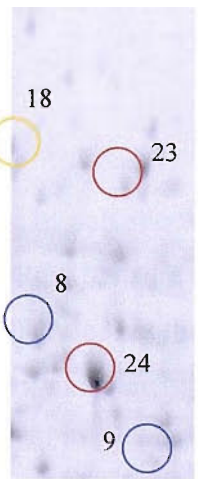
4.2.5b



4.2.6a



4.2.6b



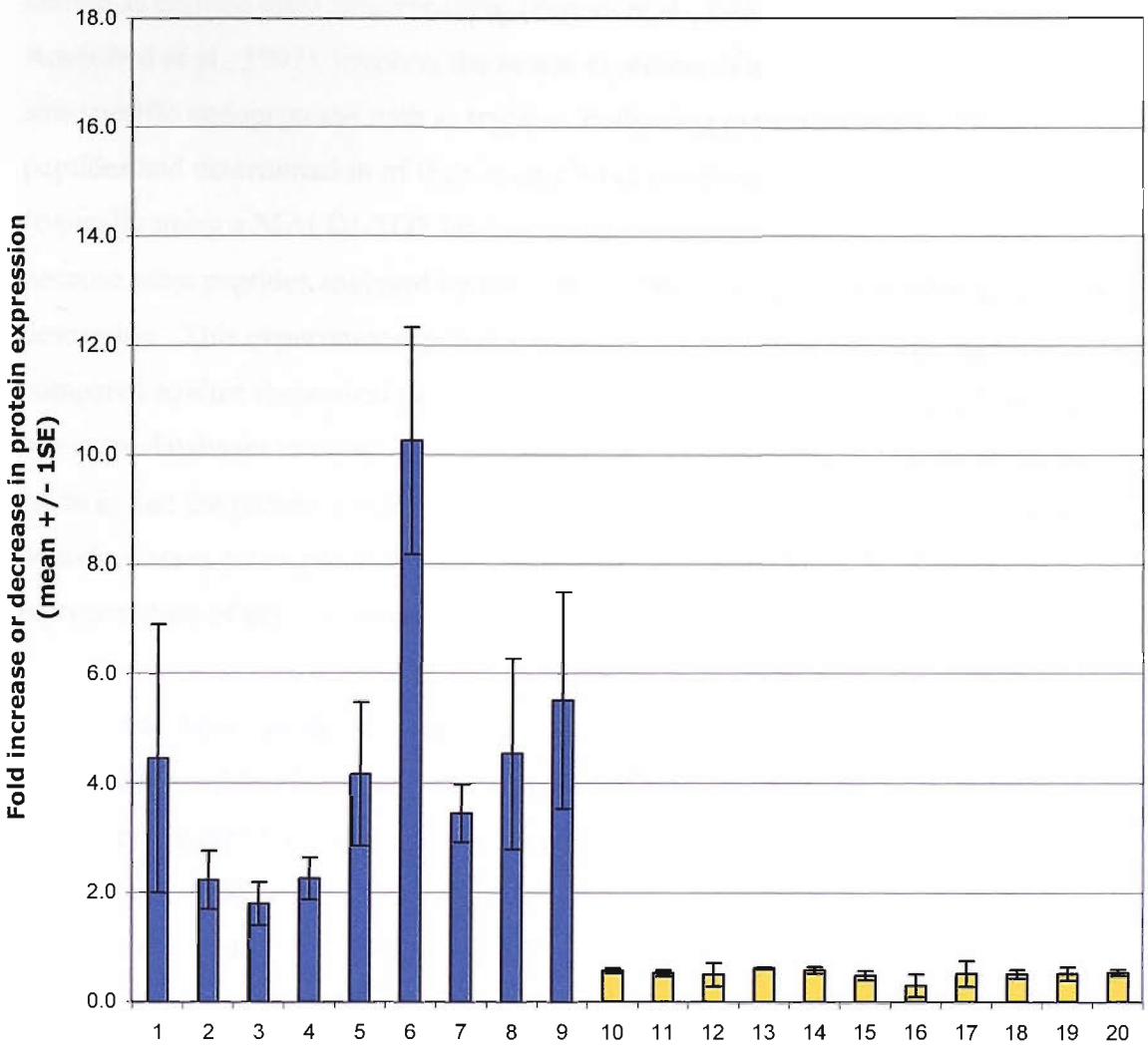


Figure 4.3 Quantitation of protein expression induced by salt shock in wild type *S.* Typhimurium SL1344 (blue bars are up-regulated, yellow bars are down-regulated). Proteins highlighted in red in Figure 4.1 cannot be illustrated, as these were not expressed in the control gels. The numbers along the x-axis correspond to the protein numbers on Figure 4.1 and in Table 4.1. Please note that the y-axis is scaled in order to facilitate comparison with Figure 5.4.

4.3 Identification of *Salmonella* Typhimurium Proteins That Are Differentially Expressed Under Hyper-Osmotic Conditions.

To determine the identities of the differentially expressed spots uncovered following the analysis of whole cell extracts, two mass spectrometric approaches were used. The first, known as peptide mass fingerprinting (Pappin et al., 1993; Henzel et al., 1993; Rosenfeld et al., 1992), involves the *in situ* digestion of an excised protein spot with a site-specific endoprotease such as trypsin. Following extraction of the resulting peptides and determination of their mass:charge (m/z) values via mass spectrometry (typically using a MALDI-TOF instrument), their masses can be readily derived. This is because most peptides analysed by this method produce singly charged ions upon laser desorption. This experimental peptide mass profile or 'fingerprint' can then be compared against theoretical peptide profiles ('virtual digests') generated from protein sequence databases to create a list of likely protein identifications. The assumption made is that the protein candidate with the highest number of matching predicted peptide masses corresponds to the protein in the original sample. A schematic representation of peptide mass fingerprinting is shown in Figure 4.4.

The second mass spectrometry approach used in these studies was tandem MS. Peptides derived from a protein spot of interest were pre-fractionated via a nano-scale reverse phase HPLC column and injected via an electrospray inlet into a tandem mass spectrometer (a hybrid quadrupole-Time-of-flight instrument known as a Q-TOF). In this way, a peptide of a specific m/z value could be gated into a collision chamber where it was bombarded with inert gas atoms so as to fragment it. The masses of the resulting fragments were then determined using the "MaxEnt" software package. The information thus obtained was sufficient to allow the searching of a protein sequence database using the ProteinLynx Global Server 2 search engine. Briefly, this first produces a list of candidate peptides, from sequences in the database, which have intact molecular masses that closely match that of the parent ion. The theoretical fragment ion patterns for each of these candidate peptides is then compared to the experimental fragmentation data to eliminate spurious matches and to assign a score to the match. Finally the software predicts which "y" ions should be present in the highest-ranking candidate peptide and checks to see if these can be found in the experimentally derived fragmentation data. In order to assign the candidate peptide as a good match, at least three consecutive "y" ions must match between the candidate and experimentally derived data. Figure 4.5 shows a

schematic representation of peptide identification using tandem mass spectrometry. In this way, several proteins that could not be identified by the first method were successfully assigned.

Table 4.1 gives a listing of the identified proteins, together with some relevant ancillary information. It can be seen that there are some discrepancies between the observed and theoretical parameters for the identified proteins in Table 4.1, for example, for oligopeptide transport ATP-binding domain OppD and 30S ribosomal protein S1 (Protein 2 in Table 4.1, but not Protein 10). This could be for a number of reasons including: the inherent inaccuracy of molecular weight determination by electrophoresis and the fact that many database entries for protein molecular weights and pIs are based on translations of nucleic acid sequences. Parameters predicted from nucleic acid sequences do not take into account events such as the loss of signal peptides or post-translational modifications that may be significantly alter the predicted molecular weight and pI of a protein (Cottrell, 1994).

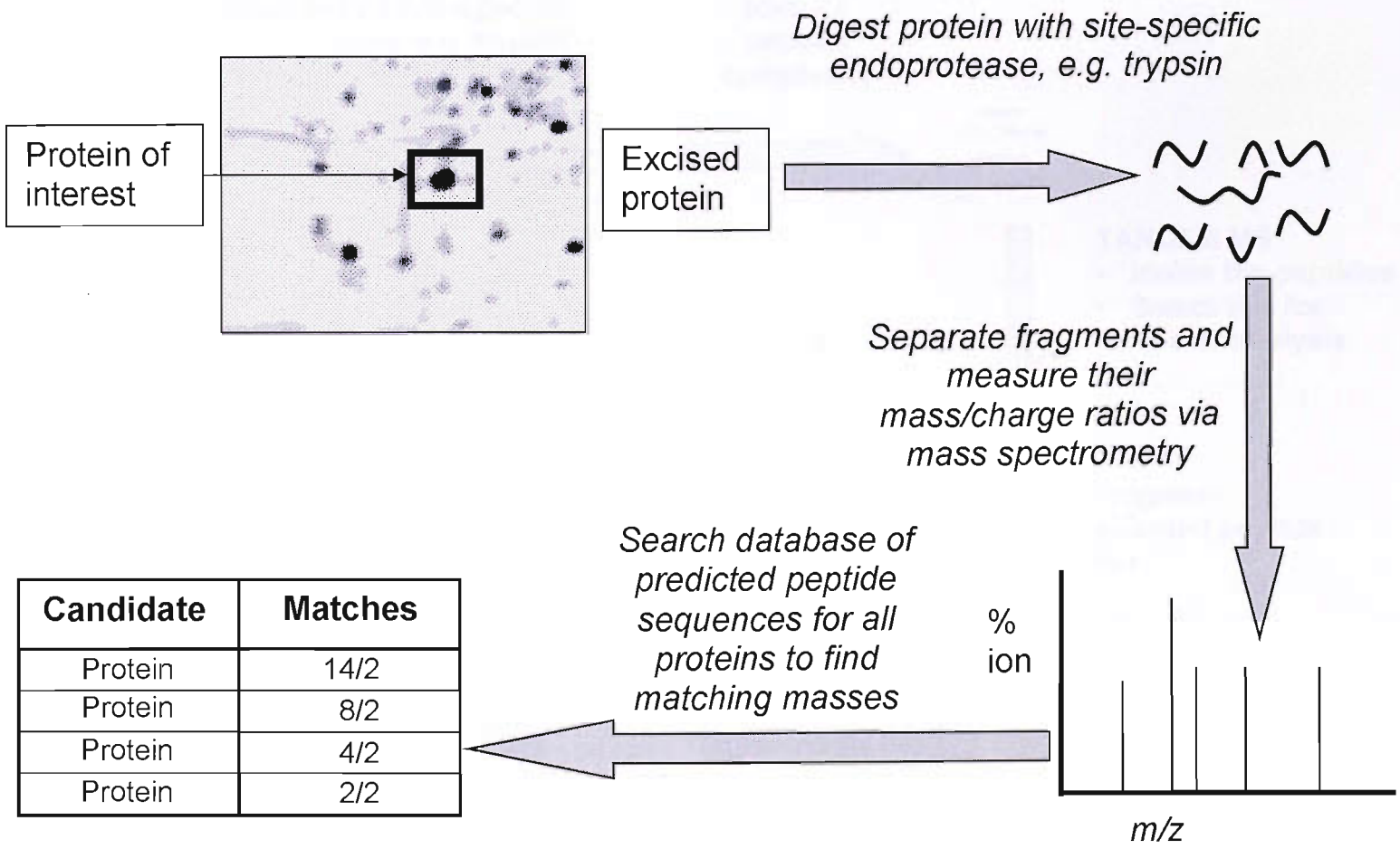


Figure 4.4 An overview of the processes involved during peptide mass fingerprinting.

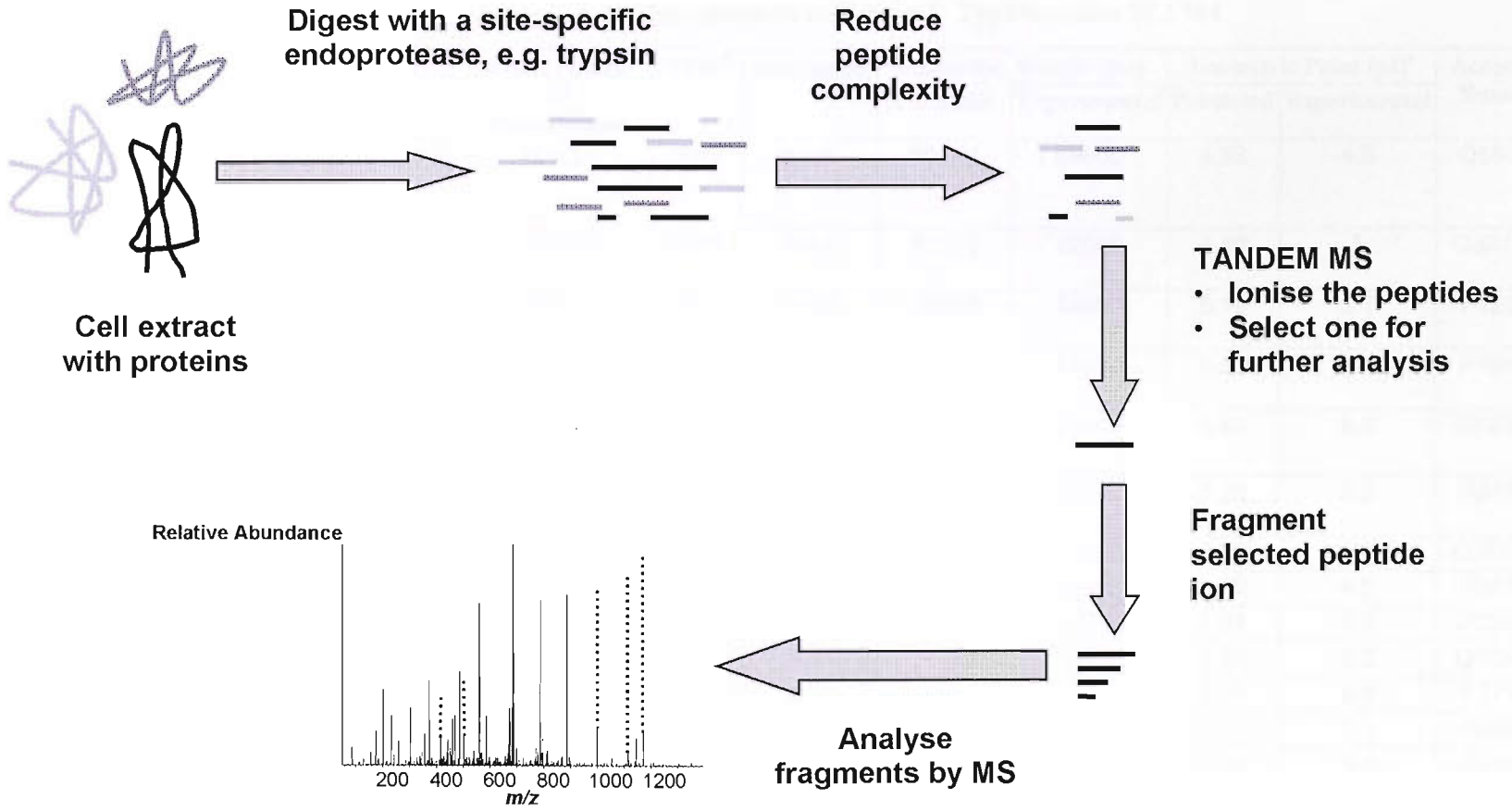


Figure 4.5 An overview of the processes involved in protein sequencing by tandem mass spectrometry (sequencing several proteins in the same experiment).

Table 4.1 Identified proteins with altered expression under hyper-osmotic stress in wild type *S.* Typhimurium SL1344.

Protein No[a]	Protein Name	% Peptide Coverage[b]	Score[c]	Main Method of Identification	STM[d]	Gene name	Molecular Weight (Da)		Isoelectric Point (pI)[e]		Accession Number
							Predicted	Experimental	Predicted	Experimental	
1	Chaperone Hsp70 in DNA biosynthesis/cell division (DnaK)	27	178	MALDI	0012	*dnaK*	69127	69000	4.83	4.8	Q56073
2	30s ribosomal protein S1 (cleavage fragment)	26	162	MALDI	0981	*rpsA*	61173	42000	4.89	5	Q8XGK9
3	Rod shaped determining protein	53	54.5	MALDI	3374	*mreB*	36952	34000	5.19	5.1	P13519
5	Alkyl hydroperoxide reductase c22 protein	42	79	MALDI	0608	*ahpC*	20616	19000	5.03	5.1	P19479
6	Unknown protein from 2-D gel (Putative LysM domain)	70	118	MALDI	2795	*ygaU*	16121	15000	5.44	5.3	Q8ZML9
7	Glyceraldehyde-3-phosphate dehydrogenase	96	132	MALDI	1290	*gapA*	35455	32000	6.32	5.6	P24165
10	30s ribosomal protein S1	32	242	MALDI	0981	*rpsA*	61173	70000	4.89	5	Q7CQT9
11	Flagellin	33	120	MALDI	1959	*fliC*	51481	47000	4.79	4.8	P06179
12	D-galactose binding protein	65	29	MALDI	2190	*mglB*	35814	30000	5.81	5.2	P23905
14	Heat shock protein A	50	69	MALDI	3809	*ibpA*	15750	14000	5.23	5.2	Q7CPF1
17	Pyruvate kinase (Kpy1)	80	177	MALDI	1378	*pykF*	50661	60000	5.66	5.6	P77983
20	Phosphofructokinase	-	-	MALDI	4062	*pfkA*	34915	33000	5.57	5.5	P65692
22	NADH dehydrogenase I chain E	50	70	MALDI	2325	*nuoE*	18602	18000	5.29	5.3	P33903
23	Oligopeptide transport ATP-binding protein OppD	40	50.4	MALDI	1743	*oppD*	36846	50000	5.84	6.1	P04285

[a] Protein numbers refer to the spots highlighted in Figure 4.1

[b] % Peptide coverage indicates what % of the full-length sequence is accounted for by the tryptic peptides that were assigned to the protein.

[c] Mowse score from Mascot search results for proteins 1,2,5,10,11,14 & 22. Score from ProteinProbe search results for all other proteins.

[d] STM indicates the ordered locus name. A name used to represent an open reading frame in a completely sequenced genome, generally based on a prefix representing the organisms, e.g. *Salmonella* Typhimurium, and a number representing the sequential ordering of the genes on the chromosome.

[e] The pI values calculated using the amino acid sequence data and the pI/Mw tool on the ExPASy website (http://au.expasy.org/cgi-bin/pi_tool?).

4.4 Discussion

This chapter has described the detection and identification of a number of proteins that are differentially expressed when SL1344 cells are exposed to osmotic up shift by addition of 0.5 M salt for 60 min.

Because of the stringent criteria used for defining differentially expressed proteins, the final number (24) is relatively small. However, it should be noted that the most highly induced protein corresponds to the product of hitherto hypothetical gene, while others have never previously been reported to respond to hyper-osmotic stress. Thus, these investigations have produced novel data that, due to the rigorous parameters that were applied, are likely to retain significance. It is therefore appropriate to discuss the putative roles of the components that have been uncovered with respect to what is already known about salt stress.

4.4.1 The functions of proteins identified in this study and their potential roles in salt stress

4.4.1.1 DnaK and Heat Shock Protein A (HspA)

Two heat shock proteins were found to be differentially regulated under salt stress in this study. As discussed in the general introduction to this thesis, when bacteria are exposed to heat shock and other insults that lead to protein misfolding, there is an increase in levels of heat shock proteins (Hsps) such as the chaperone DnaK. Chaperones interact with other cellular proteins to prevent aggregation and to catalyse renaturation. DnaK was found to be up-regulated approximately 4.5 fold by osmotic shock in wild type *S.* Typhimurium in this study, which concurs with what has been previously reported in *E. coli* (Bianchi and Baneyx, 1999; Meury and Kohiyama, 1991). Muery and Kohiyama (1991) provided the first evidence that Hsps are involved in osmotic stress responses by demonstrating that the cellular content of DnaK rises 2-3 fold after treatment with 0.6 M NaCl. Bianchi and Baneyx (1999) also saw an increase in DnaK when mid-exponential cells were treated with 0.464 M sucrose. A reason for an increase in Hsps during osmotic shock could be the need to chaperone newly synthesised proteins and/or refolding of proteins damaged due to water efflux. Another heat shock protein, the small heat shock protein HspA was down-regulated,

approximately 0.5 fold, under salt stress in this study. This protein is far less well understood. It appears to be a minor stress protein that is reported to be dispensable in *E. coli* (Thomas and Baneyx, 1998).

4.4.1.2 Ribosomal protein S1

Protein synthesis takes place on ribosomes, which in prokaryotes are formed by the 50S and 30S subunits. Ribosomal protein S1 is a constituent of the 30S subunit and was present in lower levels under salt stress, but with a corresponding increase in levels of a cleavage fragment. This suggests that the protein is being degraded under salt stress rather than down regulated *per se*. If this proposal were true then it would be useful to search for an up-regulated (or activated) protease.

4.4.1.3 Alkyl hydroperoxide reductase c22 (AhpC subunit)

Alkyl hydroperoxide reductase c22 (AhpC) protein was up-regulated under osmotic stress. A homologue of this protein has been previously reported as induced by osmotic shock in *Staphylococcus aureus* (Armstrong-Buisseret et al., 1995). AhpC, which has peroxidase activity, is a component of Ahp and helps to protect cells against organic peroxides. The two proteins making up Ahp act together; AhpF uses NADH or NADPH as electron donor to AhpC, which reduces physiological lipid peroxides, such as linoleic acid hydroperoxide, as well as thymine hydroperoxide and nonphysiological alkyl hydroperoxides to their respective non-toxic alcohol forms. AhpC has been demonstrated to act as specific alkyl hydroperoxide-scavenging enzyme for protection against oxygen radical damage (Jacobson et al., 1989). The induction of AhpC under salt stress may be an indication that the cells are experiencing more oxidative stress or may simply be the result of the switching on of general stress responses.

4.4.1.4 Flagellin

Flagellin was down-regulated under salt-stress, which concurs with much of the literature. It has been know for some time that flagellar synthesis is inhibited when bacteria are grown under stressful conditions, including osmotic stress (Adler and Templeton, 1967; Shi et al., 1993; Li et al., 1993). It has been shown that the level of acetyl phosphate modulates flagellar expression in the *flh*DC master operon and that the effect is mediated by the osmoregulator OmpR (Shin and Park, 1995). This role of OmpR in flagellar regulation is further demonstrated in this thesis, by the fact that when the effect of hyper-osmotic stress on an *ompR*$^-$ mutant was analysed there was *no* change in flagellar expression (Chapter 5).

4.4.1.5 D-galactose binding protein

D-galactose binding protein (MglB) was found to be down-regulated (approximately 0.5 fold) under salt stress. MglB is a component of the ATP-binding cassette (ABC) importer galactose permease, which involved in the uptake of galactose and glucose. (Harayama et al., 1983; Muller et al., 1985). MglB also has a role in chemotaxis (Manson et al., 1998). Under conditions of hyper-osmotic stress the import of galactose or glucose may be reduced and/or chemotaxis may be turned off (also indicated by the repression of flagellin), leading to a reduction in levels of MglB.

4.4.1.6 Phosphofructokinase, glyceraldehyde-3-phosphate dehydrogenase, NADH dehydrogenase I and pyruvate kinase.

Levels of phosphofructokinase, which controls the entry of substrate into glycolytic pathways (Roehl and Vinopal, 1976) and pyruvate kinase, a catalyst involved in ATP-regenerating reactions in carbohydrate metabolism were slightly reduced under salt stress. In contrast, there was an increase in levels of glyceraldehyde-3-phosphate dehydrogenase and the E subunit of NADH dehydrogenase I, key metabolic components. The apparent different effects on the various components of the glycolytic pathways may indicate that only the critical components are up-regulated during hyper-osmotic stress. However it is worth noting, that although it is generally thought that osmo-adaptation is an energy-consuming situation (Oren, 1999), a study using *E.* coli to

investigate energy requirements in relation to osmotic stress, concluded that a low water activity did not impose a "large energetic burden" (Krist et al., 1998).

4.4.1.7 Unknown protein from 2-D gel (YgaU), rod shaped determining protein (MreB) and oligopeptide transport ATP binding protein (OppD)

Three of the proteins with altered expression under salt stress appear to be involved with *Salmonella* cell shape. The most highly induced protein - YgaU protein (149 amino acid residues) - currently has no known function, but does contain two conserved domains: a BON (bacterial OsmY and nodulation) domain (at residues 28-91) and LysM motif (at residues 99-147). A BON domain typically has an α/β predicted fold, conserved glycine residue and several hydrophobic regions, suggestive of a binding or structural function rather than a catalytic function. Most proteobacteria seem to possess one or two BON-containing proteins, typically of the OsmY-type proteins. As mentioned in the general introduction to this thesis, the OsmY protein is a 20kDa outer membrane or periplasmic *E. coli* protein that is expressed in response to a variety of stress conditions, in particular, helping to provide protection against osmotic shock. One hypothesis is that OsmY prevents shrinkage of the cytoplasmic compartment by contacting the phospholipid interfaces surrounding the periplasmic space (Oh et al., 2000; Liechty et al., 2000). The domain architecture of two BON domains alone suggests that these domains contact the surfaces of phospholipids, with each domain contacting a membrane (Yeats and Bateman, 2003). The LysM motif is present in enzymes that degrade cell walls (Joris et al., 1992; Bateman and Bycroft, 2000) and also forms part of proteins such as intimin which is involved in bacterial pathogenesis (Bateman and Bycroft, 2000). Interestingly, through leader sequence analysis, it appears that YgaU lacks a signal peptide. This may indicate that it is cytoplasmically located rather than periplasmically, where it exerts its affect. In Gram-negative bacteria, most periplasmic and outer membrane proteins have a signal sequence (also called a leader peptide) in the N-terminus, which is cleaved off after the translocation of the cytoplasmic membrane. Some of the cytoplasmic membrane proteins also have cleavable signal sequences but some N-terminal signal sequences in the cytoplasmic membrane proteins are not cleaved off, remaining as transmembrane segments. PSORT software was utilised to predict the presence or absence of any leader sequence using McGeoch's method modified by Nakai and Kanehisa (McGeoch, 1985; Nakai and Kanehisa, 1991). In brief it considers the N-terminal basically charged region (CR) and

the central hydrophobic region (UR) of signal sequences. A score was calculated from the: length of UR, peak of UR and net charge of CR. A large positive score meaning a high possibility of possessing a signal sequence. Upon analysis of the YgaU amino acid sequence a McGeoch's score of −14.97 was returned. Next PSORT applies von Heijne's method of signal sequence recognition (von Heijne, 1986). A large positive output means a high possibility that the protein has a cleavable signal sequence. A von Heijne's score of −6.8 was returned for YgaU, which indicates that the sequence has no N-terminal signal sequence.

MreB is a homologue of actin that forms a bacterial cytoskeleton (Jones et al., 2001; van den Ent et al., 2001) and is discussed in detail in Chapter 6. OppD is a component of OppABCDF, an ATP-dependent oligopeptide transporter and member of the ATP-Binding Cassette (ABC) superfamily of transporters. OppABCDF transports oligopeptides two to five amino acids in length and in addition functioning in oligopeptide uptake and also has a role in recycling cell wall peptides (Hiles et al., 1987).

Under conditions of hyper-osmotic shock, the cell wall of *Salmonella* is under stress as water leaves the cell. The up-regulation of these three proteins (MreB, YgaU and OppD) suggests that systems exist to remodel cell walls and/or to adapt cell shape, as required in response to osmotic stress. Rapid degradation of cell wall peptides may be occurring to increase cell wall flexibility, helping prevent the cytoplasmic membrane 'tearing' away under conditions of hyper-osmotic stress.

4.5 Phylogenetic Analysis

Sequence similarity searches were carried out to gain some insight into the phylogenetic distribution of the proteins identified. An initial search for sequence similarity was done using the UniProt NREF (Non-redundant REFerence) database to identify clusters of proteins with >90% homology. This was followed by other searches including BLASTP analysis and the Interpro database from EMBL-EBI. The wider phylogenetic links revealed by the latter database are detailed below in Figures 4.6 – 4.18. The figures are Taxonomy Displays giving an 'at a glance' view of the taxonomic range of the sequences associated with each protein. The number of sequences associated with a particular lineage is indicated by the numbers on a Taxonomic Display, which has the taxonomy-tree root as its centre and the model organisms selected populate the outer most circle. The lineages were carefully selected to provide a view of the major groups of organisms. Nodes of the taxonomy-tree are placed on the inner circles and radial lines lead to the description for each node. No significance is attached to the position of the node on a particular inner-circle, other than convenience, though some attempt has been made to group nodes. Nodes are either true taxonomy nodes (with an NCBI taxonomy number) or are artificial nodes created for this display; of which there are three: 'Unclassified', 'Other Eukaryota (Non-Metazoa)' and the 'Plastid Group'.

For the majority of identified proteins, the closest homologous proteins were - not surprisingly - identified in other members of the Enterobacteriaceae, primarily the generas *Salmonella*, *Escherichia*, *Shigella* and *Erwinia*. From the UniProt NREF and BLASTP analyses two interesting bacteria to appear on the searches were *Buchnera aphidicola* (with >90% homologous DnaK) and *Wigglesworthia glossinidia brevipalpis* (with >90% homologous MreB). These are both endosymbiotic bacteria (of aphids and tsetse flies) that are descendents of free-living gamma-Proteobacteria and recent analyses suggest that they form a sister group with *E. coli*, *Salmonella* spp., and *Yersinia pestis* (Canback et al., 2004). It would be interesting to know if these homologous proteins retain the capacity to respond to osmotic stress. In addition, *Shewanella oneidensis*, which belongs to the Gram-negative gamma subgroup that includes the Enterobacteriaceae, was also listed as having highly homologous MreB (>90%). *Shewanella* spp. are interesting as they inhabit diverse environments, with the majority inhabiting marine environments and therefore tolerant of sodium salt stress. Interestingly, a recent study of *Shewanella oneidensis* MR-1 in response to elevated salt

conditions has reported that the bacteria requires relatively high salt concentrations for optimal growth despite being isolated from a freshwater environment (Liu et al., 2005).

DnaK as a member of the Hsp70 family is shown to have a wide number of related sequences distributed across all lineages on the Interpro Taxonomy Display (Figure 4.6). This is not surprising due to the highly conserved nature of the Hsp70 proteins. The 30S ribosomal protein subunit S1 was also broadly distributed across the lineages (Figure 4.7). This is most likely due to the S1 domain appearing in a wide range of RNA associated proteins within nature. It should be noted however that S1 is not found in Gram-positive bacteria. In contrast to these widely distributed proteins, MreB only revealed related sequences in the bacteria and to a limited degree in the cyanobacteria and archaea (Figure 4.8). Some of the related sequences reported were due to homology with the N-terminal half of Hsp70. However as discussed in Chapter 6, MreB has the closest homology with another member of the Hsp70 super family which is actin. This is illustrated in Figure 4.19, showing the matches for MreB from the Interpro database for bacteria numbered 151-175 (of the total 188 bacterial sequence matches reported), which has alignment data for MreB with both Hsp70 and actin. It is also interesting to note that non-spheroidal bacteria have at least 1 mreB gene (Jones et al., 2001).

Alkyl hydroperoxide reductase had a wide taxonomic distribution, as would be expected for a member of a broad, ubiquitous group of antioxidant enzymes (Figure 4.9). Whereas YgaU has two Taxonomy Displays to cover the distribution of the LysM and BON domains, which are very different. The LysM motif is found in all lineages except the Archaea (Figure 4.10a), whereas the BON domain is limited to the bacteria and a select few Cyanobacteria (Figure 4.10b).

Glyceraldehyde-3-phosphate dehydrogenase is yet another protein with sequence similarity to proteins across the taxonomic lineages (Figure 4.11) reflecting the variety of functions of this protein including an involvement in glycolysis. Flagellin, however, is unique to the bacterial lineage (Figure 4.12) illustrating its very specific function in polymerising to form bacterial flagella filaments. D-galactose binding protein (Figure 4.13) was similar to YgaU in that it was primarily limited to the bacteria and a select few Cyanobacteria. Surprisingly a phylogenetic link also appears for D-galactose binding protein to another organism, the African malaria mosquito *Anopheles Gambiae*, which is shown by a number 1 next to the Eukaryota, Metazoa and Arthropoda.

HspA, pyruvate kinase, phosphofructokinase and NADH-quinone oxidoreductase returned sequences across all lineages (Figures 4.14, 4.15, 4.16 and 4.17). HspA is a heat shock protein and as such is conserved, as are pyruvate kinase and phosphofructokinase being key proteins in the glycolytic pathway, and NADH-quinone oxidoreductase as a key respiratory chain enzyme found in mitochondria and chloroplasts. The final protein analysed, OppD, was however limited in sequence similarity to proteins only within the bacteria, archaea and cynanobacteria lineages (Figure 4.18).

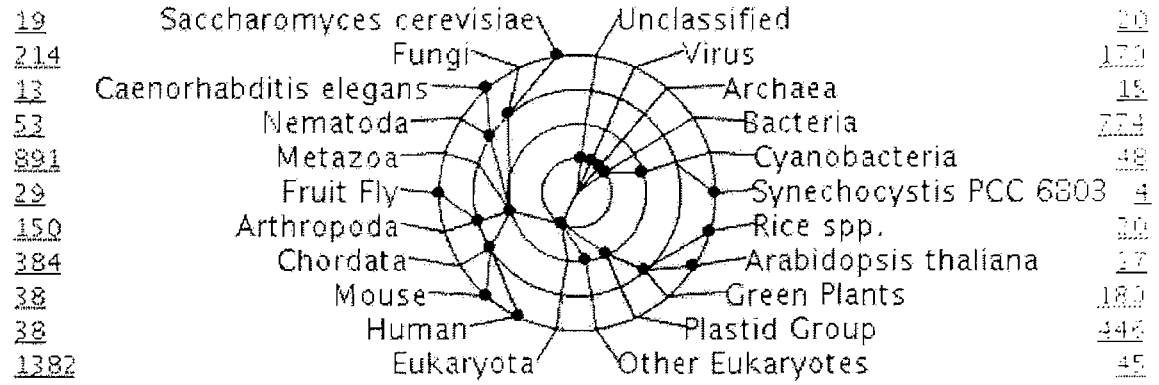


Figure 4.6 The Interpro Taxonomy Display for DnaK (Protein No. 1).

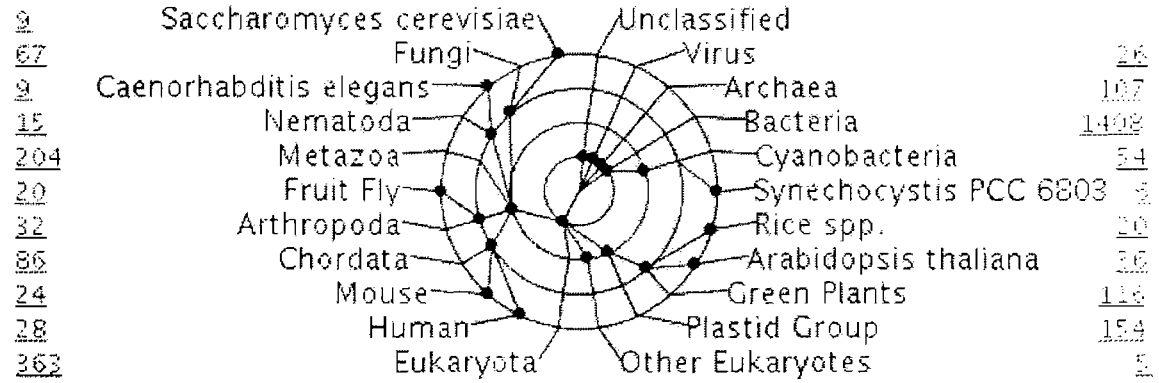


Figure 4.7 The Interpro Taxonomy Display for 30S ribosomal protein S1 (Protein No. 2).

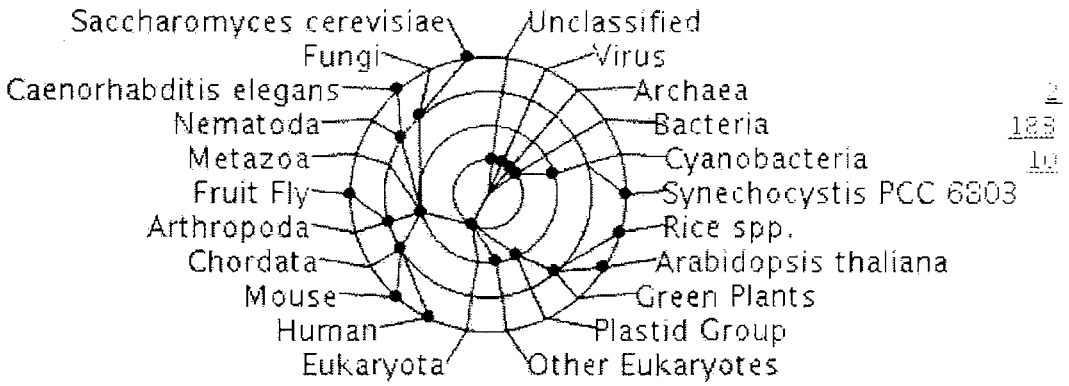


Figure 4.8 The Interpro Taxonomy Display for rod shape-determining protein MreB (Protein No. 3).

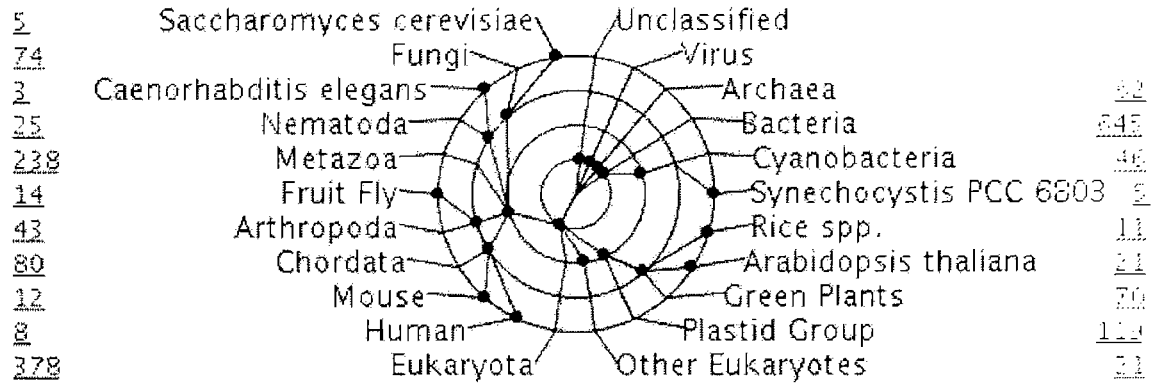


Figure 4.9 The Interpro Taxonomy Display for alkyl hydroperoxide reductase (Protein No. 4).

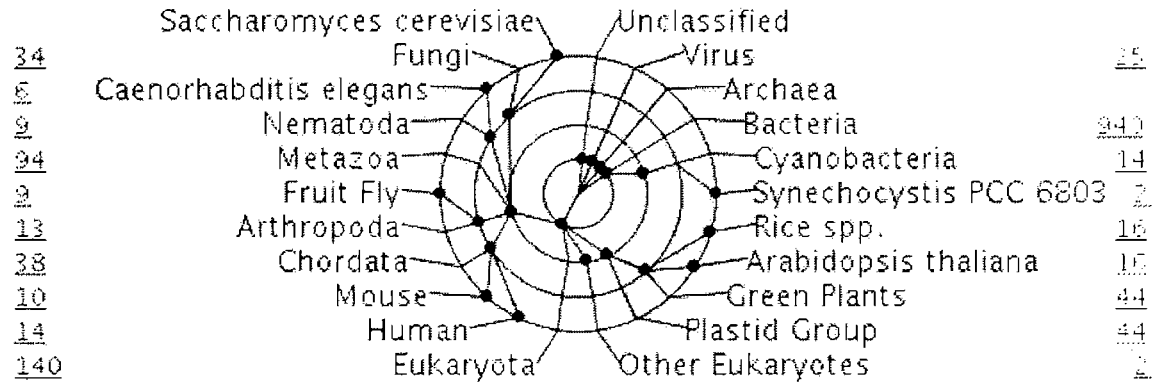


Figure 4.10a The Interpro Taxonomy Display for peptidoglycan-binding LysM (A match with YgaU due to the LysM domain. (Protein No. 6)).

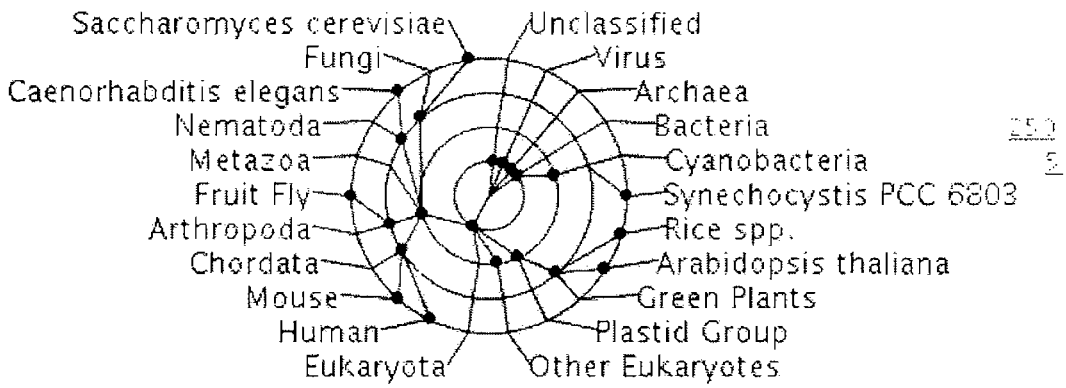


Figure 4.10b The Interpro Taxonomy Display for transport-associated protein (A match with YgaU due to the BON domain. (Protein No. 6))

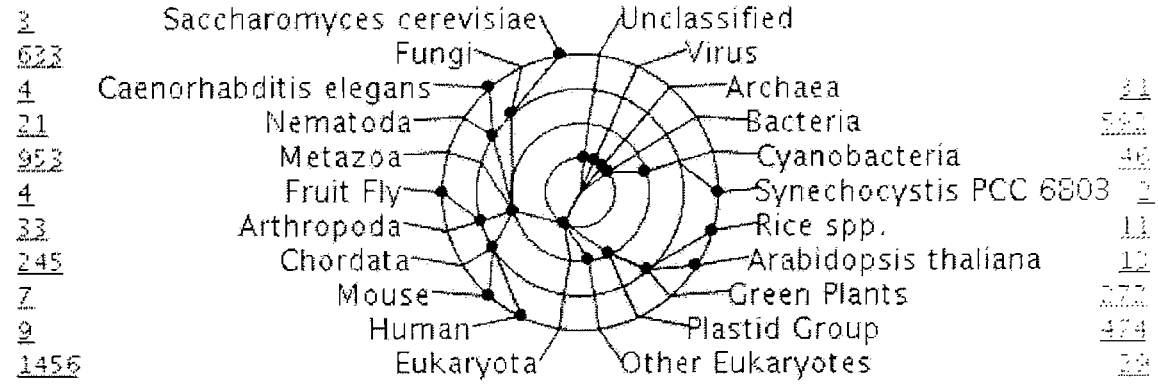


Figure 4.11 The Interpro Taxonomy Display for glyceraldehyde-3-phosphate dehydrogenase (Protein No. 7).

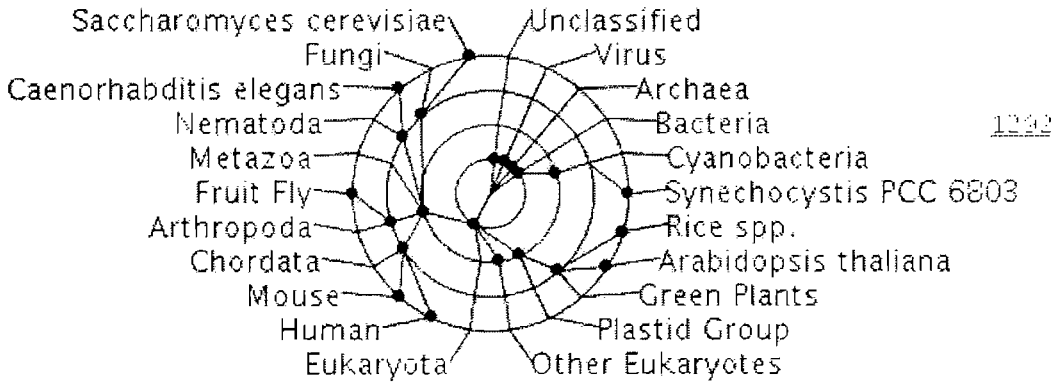


Figure 4.12 The Interpro Taxonomy Display for flagellin (Protein No. 11).

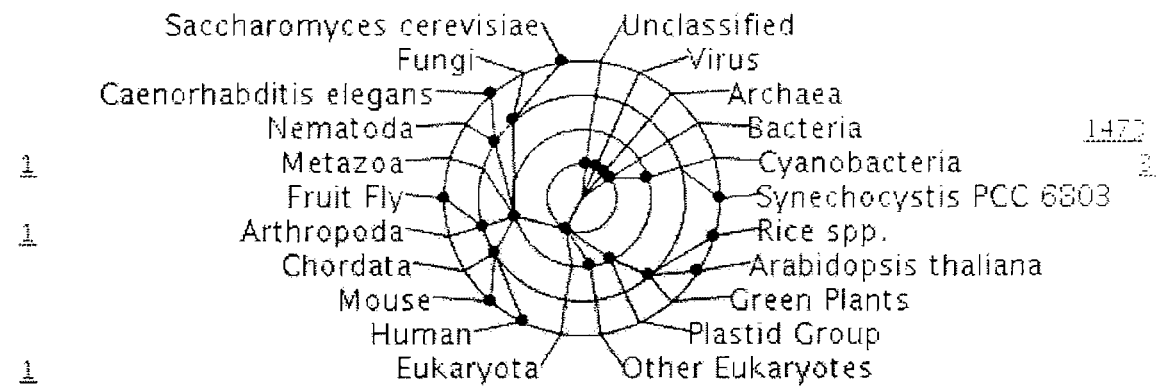


Figure 4.13 The Interpro Taxonomy Display for D-galactose binding periplasmic protein (Protein No. 12).

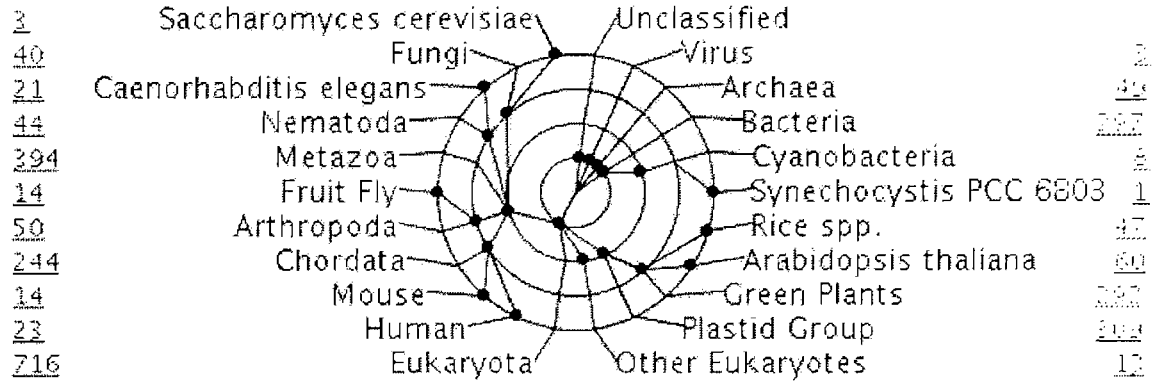


Figure 4.14. The Interpro Taxonomy Display for heat shock protein A (Protein No. 14).

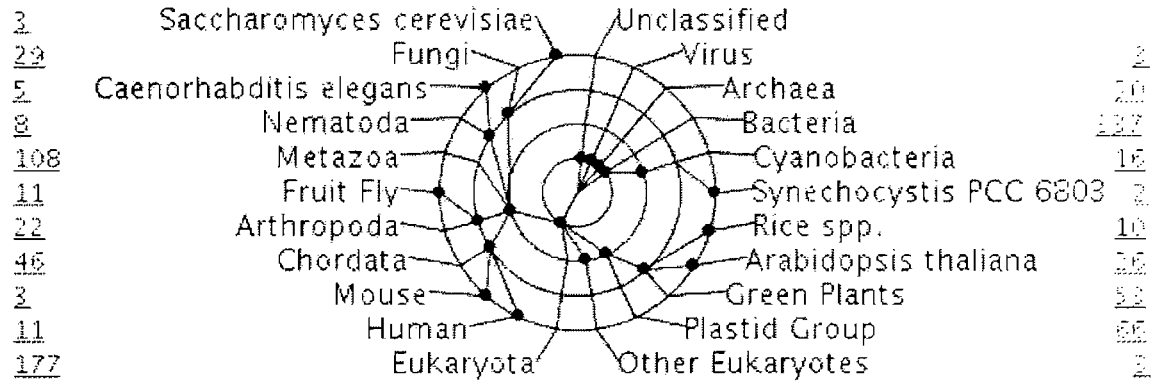


Figure 4.15. The Interpro Taxonomy Display for pyruvate kinase (Protein No. 17).

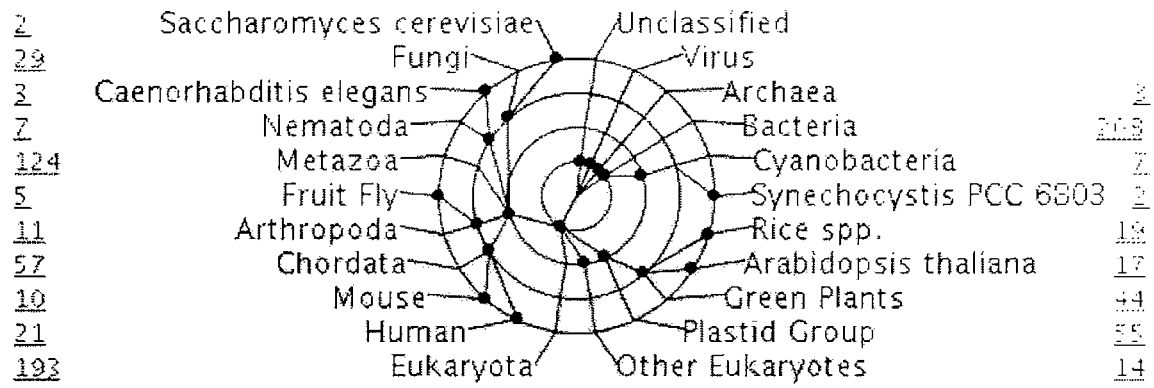


Figure 4.16. The Interpro Taxonomy Display for phosphofructokinase (Protein No. 20).

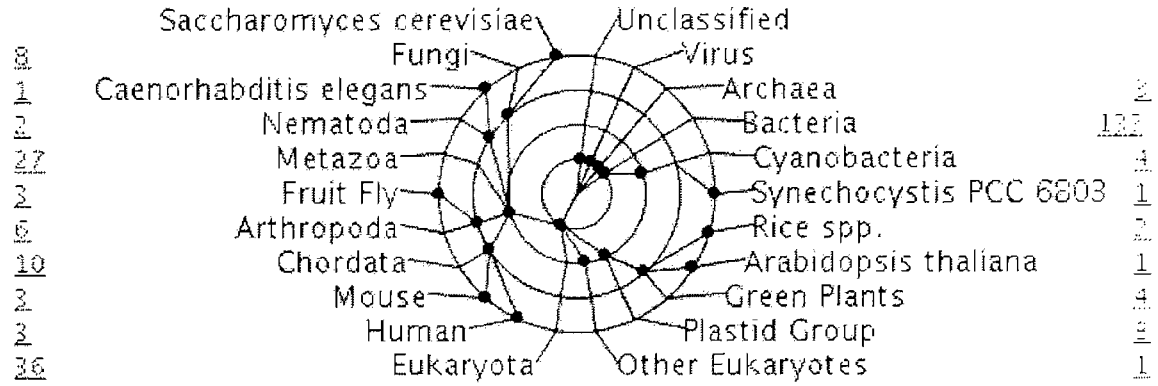


Figure 4.17. The Interpro Taxonomy Display for NADH-quinone oxidoreductase chain E (Protein No. 22).

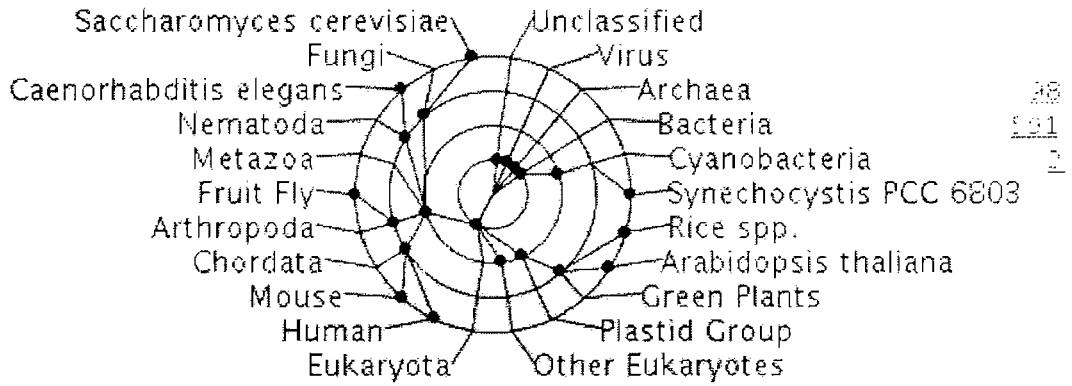


Figure 4.18. The Interpro Taxonomy Display for oligopeptide transport ATP binding protein OppD (Protein No. 23).

Bacteria

Proteobacteria

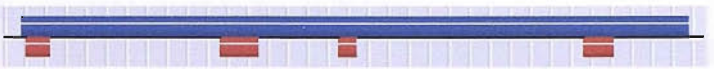
Rickettsia conorii Tax id: 781

UniProt/TrEMBL Q92GC5_RICCN (Q92GC5) 10aa 

Rickettsia prowazekii Tax id: 782

UniProt/TrEMBL Q9ZCH4_RICPR (Q9ZCH4) 10aa 

Rickettsia sibirica Tax id: 35793

UniProt/TrEMBL Q7P9U3_RICSI (Q7P9U3) 10aa 

Rickettsia typhi Tax id: 785

UniProt/TrEMBL Q68VY0_RICTY (Q68VY0) 10aa 

Salmonella paratyphi-a Tax id: 54388

UniProt/TrEMBL Q5PJU7_SALPA (Q5PJU7) 10aa 

Salmonella typhi Tax id: 601

UniProt/Swiss-Prot MREB_ECOLI (P13519) 10aa 

Salmonella typhimurium Tax id: 602

UniProt/Swiss-Prot MREB_ECOLI (P13519) 10aa 

Shewanella oneidensis Tax id: 70863

UniProt/TrEMBL Q8EA11_SHEON (Q8EA11) 10aa 

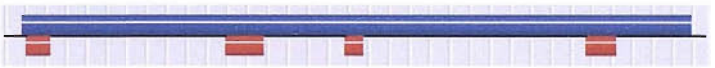
Shigella flexneri Tax id: 623

UniProt/Swiss-Prot MREB_ECOLI (P13519) 10aa 

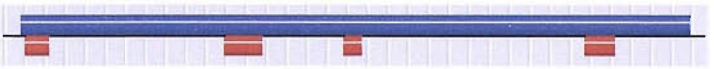
Silicibacter pomeroyi Tax id: 89184

UniProt/TrEMBL Q5LWC2_SILPO (Q5LWC2) 10aa 

Vibrio cholerae Tax id: 666

UniProt/TrEMBL Q9ZA92_VIBCH (Q9ZA92) 10aa 

Vibrio fischeri (strain ATCC 700601 / ES114) Tax id: 312309

UniProt/TrEMBL Q5E7X9_VIBF1 (Q5E7X9) 10aa 

Vibrio parahaemolyticus Tax id: 670

UniProt/TrEMBL Q87LC1_VIBPA (Q87LC1) 10aa 

Vibrio vulnificus (strain YJ016) Tax id: 196600

UniProt/TrEMBL Q7MHD7_VIBVY (Q7MHD7) 10aa 

Vibrio vulnificus Tax id: 672

UniProt/TrEMBL Q8DCH1_VIBVU (Q8DCH1) 10aa 

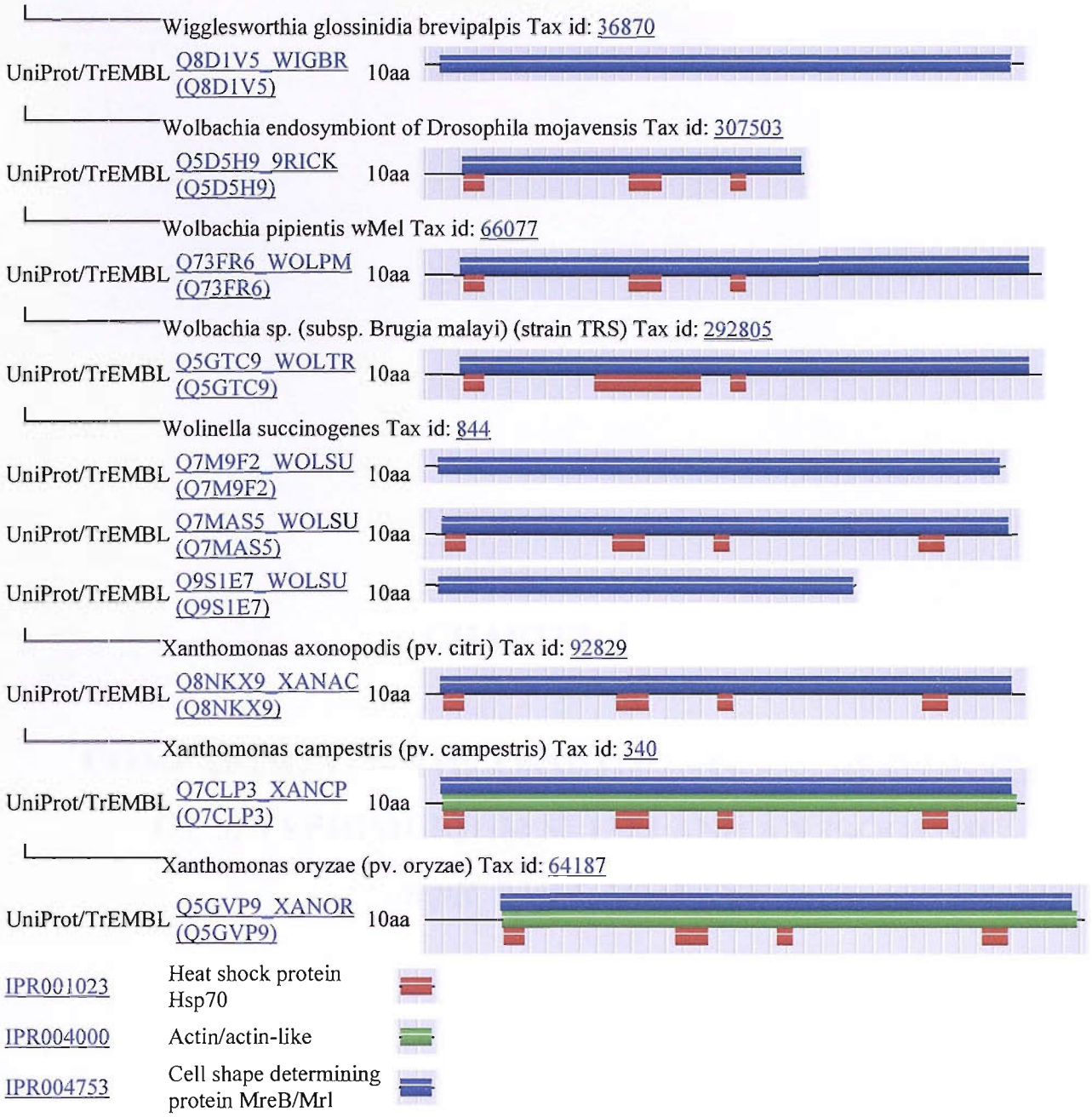


Figure 4.19 MreB results from the Interpro database for bacteria numbered 151-175. The blue bar represents the MreB sequence, with each segment of the light blue background covering 10 amino acids. Hsp70 alignment is represented in red and actin alignment in green.

CHAPTER FIVE

COMPARISON OF THE OSMOTIC STRESS RESPONSES OF *S.* TYPHIMURIUM SL1344 AND AN ISOGENIC *ompR*$^-$ NULL MUTANT

CHAPTER 5. COMPARISON OF THE OSMOTIC STRESS RESPONSES OF *S.* TYPHIMURIUM SL1344 AND AN ISOGENIC *ompR*⁻ NULL MUTANT

5.1 Introduction

As stated in the General Introduction (Chapter One), the EnvZ/OmpR two-component signal transduction system plays an integral part in bacterial adaptation to osmotic stress. Several targets of this regulatory system have previously been identified, including: the SsrA/B two component regulatory system, the OmpC and OmpF porins and *csgD* ; a transcriptional regulator involved in curli fibre formation. However, a comparison with the numbers of targets controlled by other two-component systems, and consideration of the limitations of the methods used to identify the targets, suggests that many remain to be identified.

The studies described in the previous chapter identified several proteins that were differentially expressed when wild type cells of *S.* Typhimurium SL1344 were subjected to hyper-osmotic stress. While this has uncovered several components that had not previously been implicated in the stress response, it was possible that other components were not revealed. Possible reasons for their lack of detection would include their low abundance or poor solubility, lack of involvement at the specific stage of the stress response that was analysed or due to the presence of back-up mechanisms that might mask their roles under the specific conditions used. In other words, such components might still play important roles under slightly different osmotic stress conditions.

One way to uncover such proteins would be to extend the 2-D gel analyses to a strain in which a regulatory system, known to be involved in the osmotic stress response, was disabled. The best-characterised system involved in osmo-adaptation is the EnvZ/OmpR two-component signal transduction system, which has been described in detail in Chapter One. Accordingly, it was decided to undertake a comparative 2-D gel analysis of the responses of SL1344 and an isogenic null *ompR*⁻ mutant, following salt-stress.

5.2 Comparison of Protein Expression Profiles of Wild Type and *ompR*⁻ Mutant *Salmonella* Under Non-Stress Conditions

To ensure that the 'base-line' protein profiles for the wild type and *ompR*⁻ mutant were not different under non-stressed conditions (mid-exponential phase, grown aerobically at 37°C), four paired batches of each strain were grown and whole cell lysates were prepared. The lysates were then separated by 2-D PAGE with a pH 4-7 first dimension and the resulting images analysed using Phoretix software. As indicated in Figure 5.1, there were no significant differences detected in the protein expression profiles of the two strains grown under these conditions.

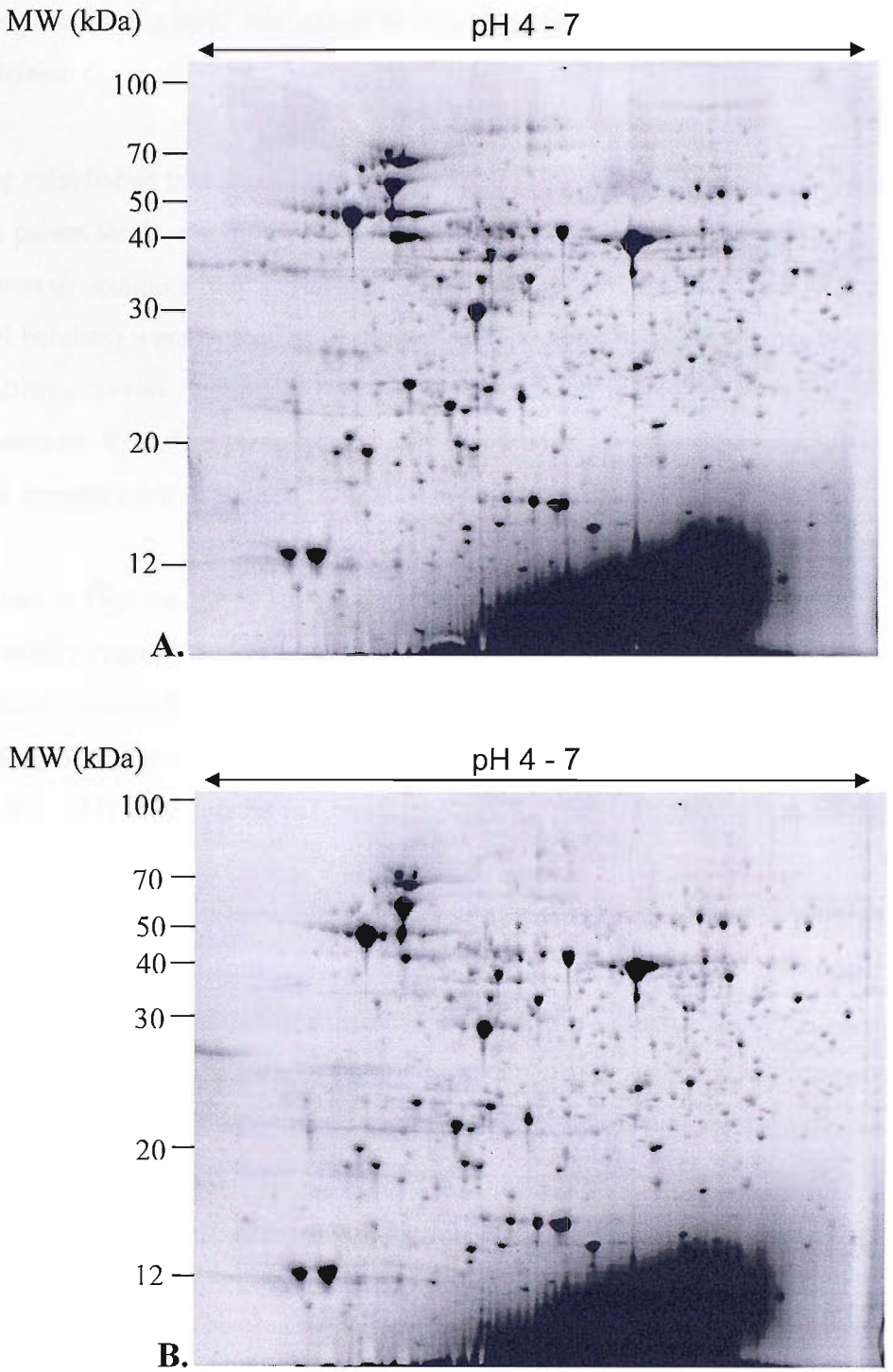


Figure 5.1 A representative set of 2-D gels (Coomassie brilliant blue stained) used to compare the protein profiles of whole cell lysate prepared from wild type **(A)** and *ompR*$^{-}$ mutant **(B)** cells of *S.* Typhimurium grown to mid-exponential phase under non-stressed conditions. Lysates were separated on the first dimension with a pI range of 4-7, second dimension gels are 12-14% SDS PAGE. 4 mg of protein was loaded on each gel.

5.3 Detection and Quantification of Proteins That Are Differentially Expressed in the Isogenic Null *ompR*⁻ Mutant of *S.* Typhimurium Under Hyper-Osmotic Conditions

Having established that there were no significant differences between the *ompR*⁻ mutant and its parent strain when they are grown under standard laboratory conditions, the next stage was to examine their behaviour following exposure to salt-stress. Accordingly, cells (4 batches) were treated as described previously to induce hyper-osmotic stress and the resulting lysates fractionated by 2-D gel electrophoresis using a pH range of 4-7. Replicates (n=4) were then subjected to image analysis as described in Chapter 2. Thus, 16 gels images were examined in total.

As shown in Figures 5.2, 5.3.1-5.3.5 and 5.4, several spots were found to be differentially regulated in the *ompR*⁻ mutant strain (13 up-regulated and 8 down-regulated). However, fewer spots were found to be differentially regulated than the overall number found in the original experiments studying the responses of the parent strain (SL1344) to salt stress (21 compared to 24, refer to Figures 4.1 and 5.2).

Interestingly, where the same proteins were differentially expressed in both the mutant and parent strain, the magnitudes of the changes were significantly higher in the former (Figure 5.5). The differentially expressed proteins were identified by mass spectrometry or, where they obviously mapped to previously identified spots, by comparison with previous results. In this way, it was found that disruption of *ompR*⁻ led to increased changes in the following proteins: DnaK, 30S ribosomal protein S1, MreB, alkyl hydroperoxide reductase c22 protein, YgaU, glyceraldehyde-3-phosphate dehydrogenase, heat shock protein A, and pyruvate kinase, relative to wild type cells.

It is also interesting to note that the proteins that were detectable on gels prepared from salt-treated cultures, but not on gels from control cultures, were the same for both strains (spots 21-24, Figures 4.1 and 5.2). The most highly up- and down-regulated proteins in the *ompR*⁻ mutant were 'unknown protein from 2-D gel' (spot 6) and pyruvate kinase (spot 17) respectively.

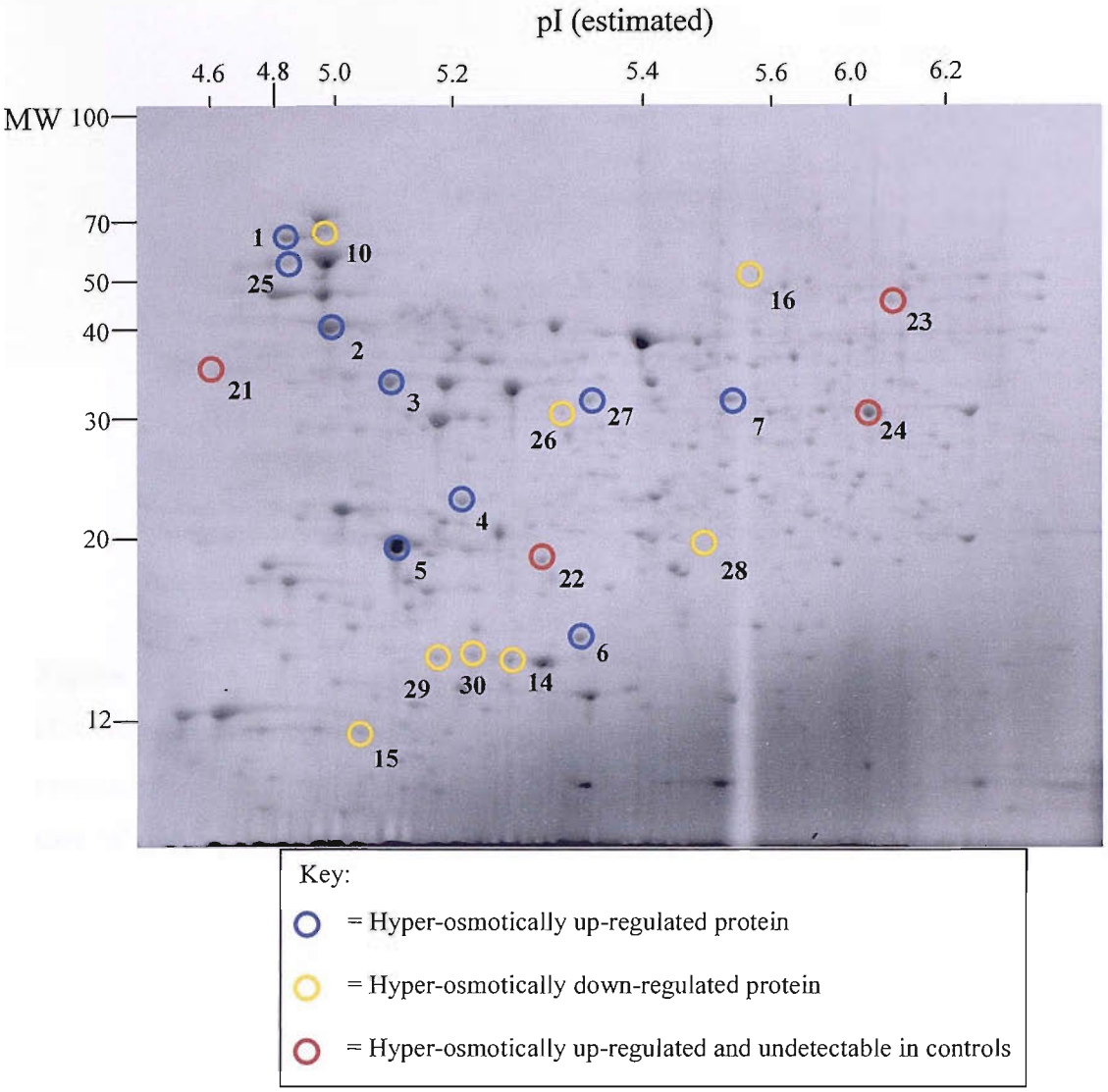
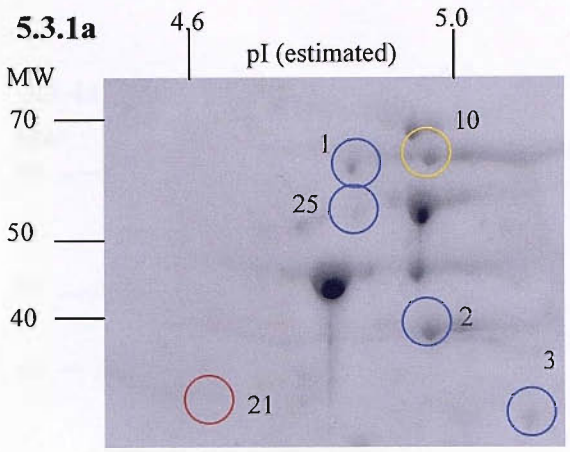


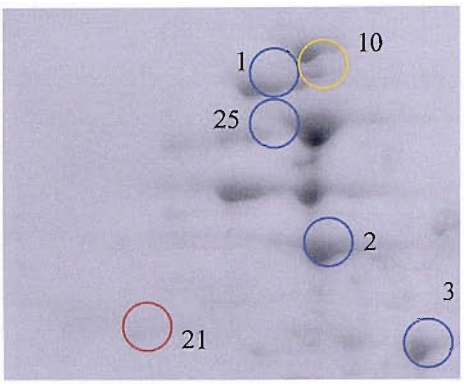
Figure 5.2 A representative 2-D gel (Coomassie brilliant blue stained) illustrating the effects of hyper-osmotic stress on protein expression in an *ompR*⁻ knockout strain of *S.* Typhimurium SL1344. The pI and MW (kDa) scales are on the top and left hand sides of the gel image respectively. Proteins that were up- or down- regulated, when cells are hyper-osmotically stressed, are ringed in blue and yellow respectively. Proteins that were up-regulated under the same conditions, but were undetectable in the cells grown in control conditions are ringed in red.

Figure 5.3.1a-5.3.5b (next pages) Enlarged sections of representative 2-D gels (Coomassie brilliant blue stained) illustrating the effects of hyper-osmotic stress on protein expression in an *ompR*⁻ knockout strain of *S.* Typhimurium SL1344. In each case 'a' corresponds to whole cell lysates from unstressed cultures and 'b' corresponds to whole cell lysates from osmotically stressed cells. Protein spot numbers relate to those on Figures 5.2 & 5.4 and Table 5.1.

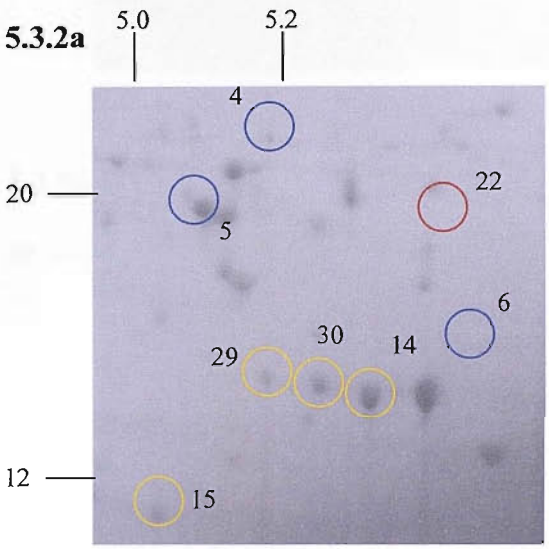
5.3.1a



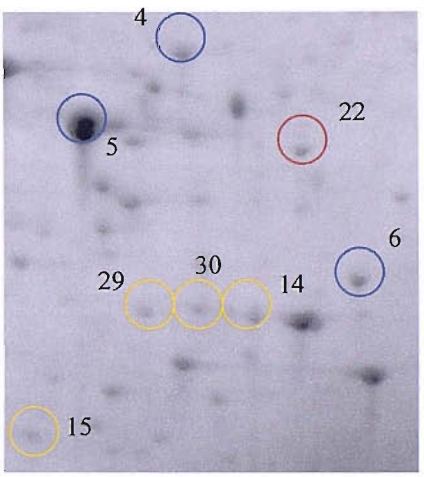
5.3.1b



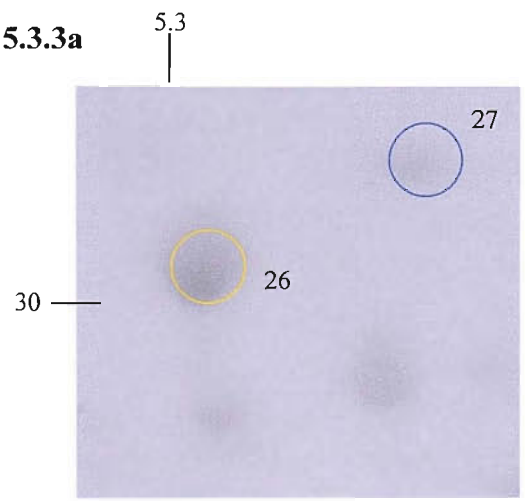
5.3.2a



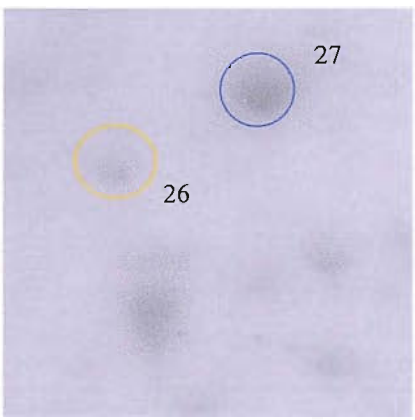
5.3.2b



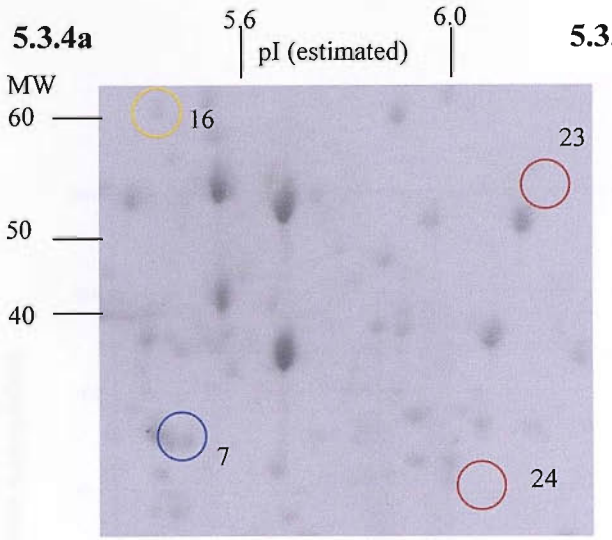
5.3.3a



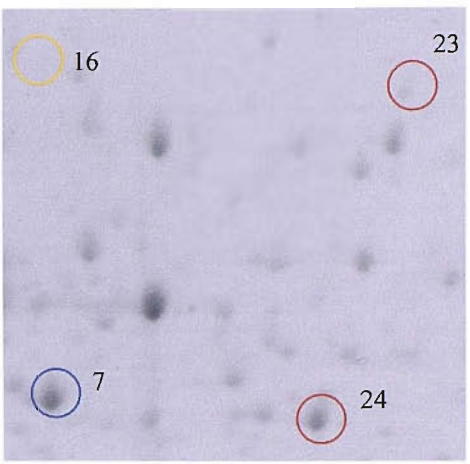
5.3.3b



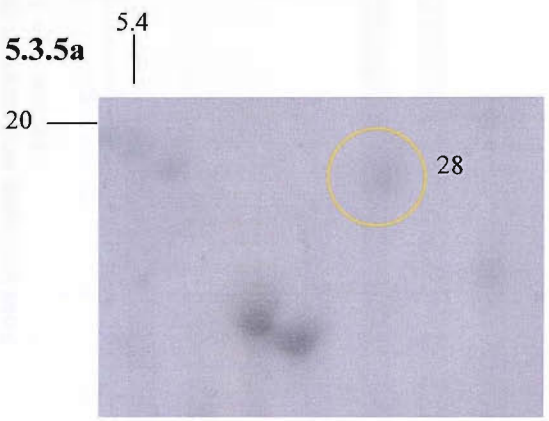
5.3.4a



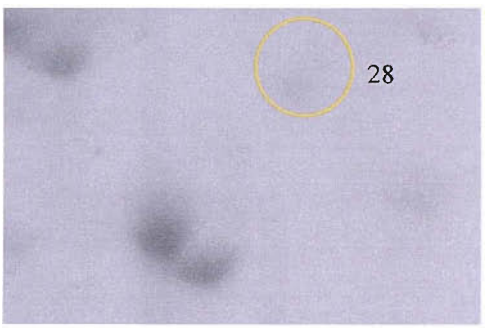
5.3.4b



5.3.5a



5.3.5b



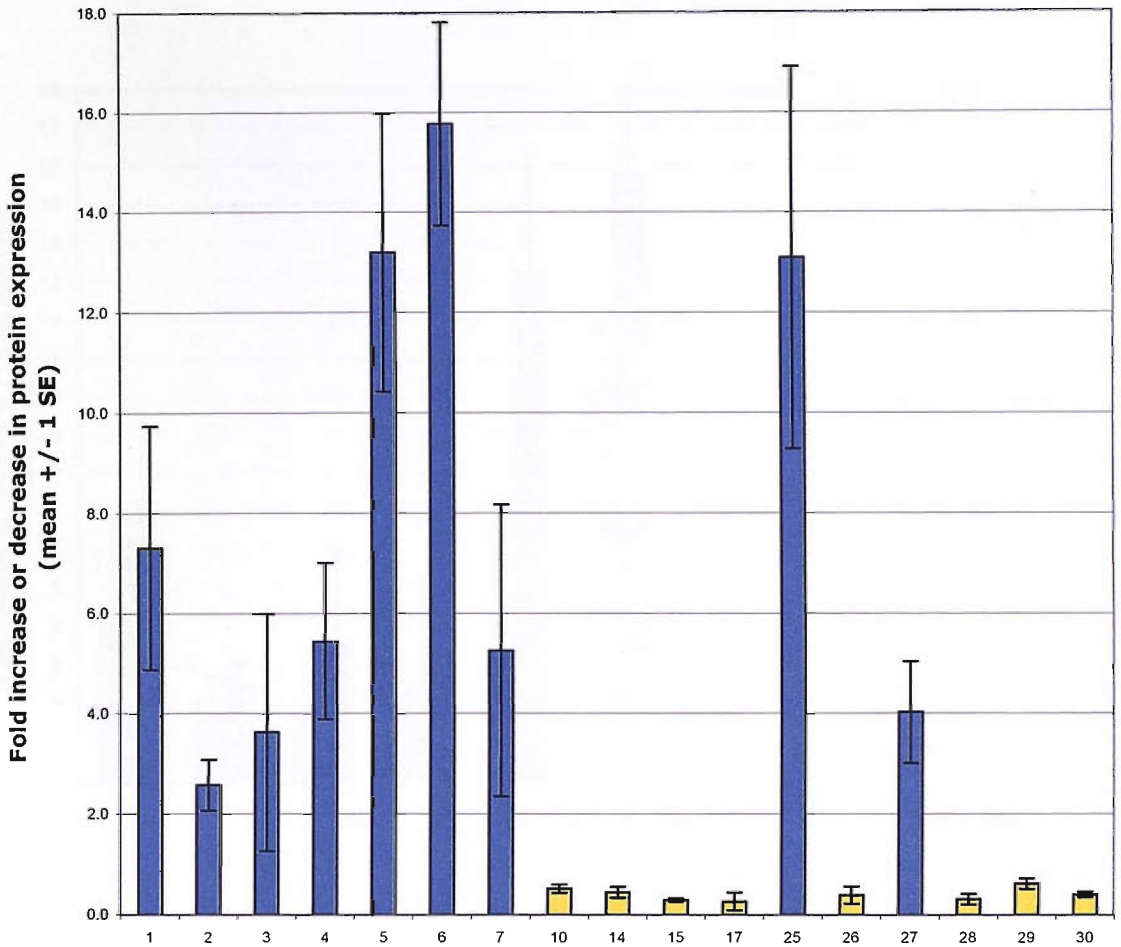


Figure 5.4 Quantification of changes in protein expression induced by salt-shock in an *ompR*⁻ knock out strain of *S.* Typhimurium SL1344 (blue bars up-regulated, yellow down-regulated). Proteins highlighted in red in Figure 5.2 cannot be illustrated, as these were not expressed in the control gels. The numbers along the x axis correspond to the protein numbers on Figure 5.2 and in Table 5.1.

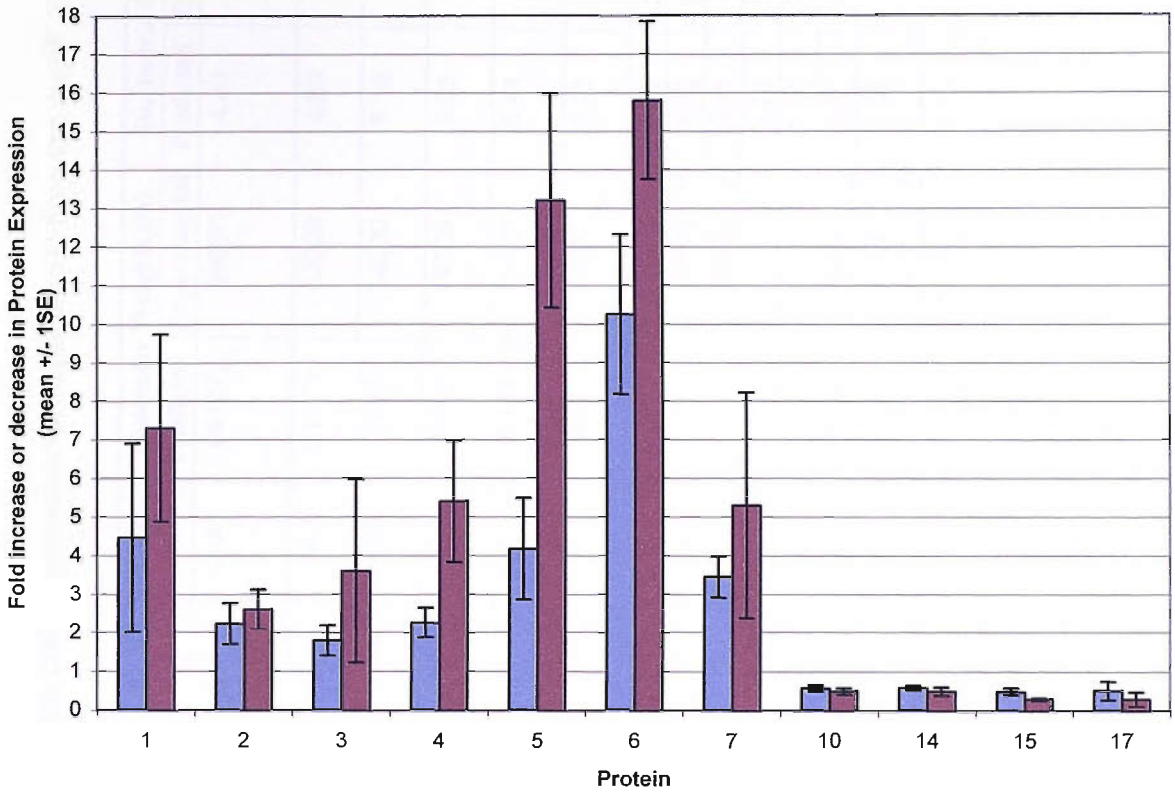


Figure 5.5 Comparison of proteins induced with salt-shock at 0.5 M with altered expression in both the wild type strain (blue bars) and the *ompR*⁻ knockout strain of *S.* Typhimurium SL1344 (red bars). The numbers along the x axis correspond to the protein numbers on Figures 4.1 & 5.2 and in Tables 4.1 & 5.1.

Table 5.1 (continued overleaf) Identified proteins with altered expression under hyper-osmotic stress in wild type and *ompR*⁻ *S.* Typhimurium SL1344.

Protein No[a]	Protein Name	% Peptide Coverage[b]	Score[c]	Main Method of Identification	STM[d]	Gene name	Molecular Weight (Da)		Isoelectric Point (pI)[e]		Accession Number
							Predicted	Experimental	Predicted	Experimental	
1	Chaperone Hsp70 in DNA biosynthesis/cell division (DnaK)	27	178	MALDI	0012	*dnaK*	69127	69000	4.83	4.8	Q56073
2	30s ribosomal protein S1 (cleavage fragment)	26	162	MALDI	0981	*rpsA*	61173	42000	4.89	5	Q8XGK9
3	Rod shaped determining protein	53	54.5	MALDI	3374	*mreB*	36952	34000	5.19	5.1	P13519
5	Alkyl hydroperoxide reductase c22 protein	42	79	MALDI	0608	*ahpC*	20616	19000	5.03	5.1	P19479
6	Unknown protein from 2-D gel (Putative LysM domain)	70	118	MALDI	2795	*ygaU*	16121	15000	5.44	5.3	Q8ZML9
7	Glyceraldehyde-3-phosphate dehydrogenase	96	132	MALDI	1290	*gapA*	35455	32000	6.32	5.6	P24165
10	30s ribosomal protein S1	32	242	MALDI	0981	*rpsA*	61173	70000	4.89	5	Q7CQT9
11	Flagellin	33	120	MALDI	1959	*fliC*	51481	47000	4.79	4.8	P06179
12	D-galactose binding protein	65	29	MALDI	2190	*mglB*	35814	30000	5.81	5.2	P23905
14	Heat shock protein A	50	69	MALDI	3809	*ibpA*	15750	14000	5.23	5.2	Q7CPF1
17	Pyruvate kinase (Kpy1)	80	177	MALDI	1378	*pykF*	50661	60000	5.66	5.6	P77983
20	Phosphofructokinase	-	-	MALDI	4062	*pfkA*	34915	33000	5.57	5.5	P65692
22	NADH dehydrogenase I chain E	50	70	MALDI	2325	*nuoE*	18602	18000	5.29	5.3	P33903
23	Oligopeptide transport ATP-binding protein OppD	40	50.4	MALDI	1743	*oppD*	36846	50000	5.84	6.1	P04285

Table 5.1 (continued from previous page).

25	GroEL protein	12	64	MALDI	4330	*groEL*	57155	55000	4.85	4.8	P48217
26	Acetyl-coenzyme A carboxylase carboxyl transferase subunit alpha	36	129	MALDI	0232	*accA*	35212	33000	5.38	5.4	P40674
30	Fels-2 prophage: similar to gpQ in phage 186	32	58	MALDI	2718	*STM2718*	17737	14000	5.26	5.2	Q8ZMT4

(Table 5.1 is Table 4.1 with additional protein identifications that were unique to the *ompR*$^-$ *S.* Typhimurium SL1344 i.e. proteins 25, 26 & 30). Proteins differentially regulated in both strains are highlighted in blue, those unique to the wild type *S.* Typhimurium are highlighted in red and those unique to *ompR*$^-$ mutant are in normal black typeface.

Footnote:

[a] Protein number refer to the spots highlighted in Figures 4.1 and 5.4

[b] % Peptide coverage indicates what % of the full-length sequence is accounted for by the tryptic peptides that were assigned to the protein.

[c] Mowse score from Mascot search results for proteins 1,2,5,10,11,14,22,25,26 & 30. Score from ProteinProbe search results for all other proteins.

[d] STM indicates the ordered locus name. A name used to represent an open reading frame in a completely sequenced genome, generally based on a prefix representing the organisms, e.g. *Salmonella* Typhimurium, and a number representing the sequential ordering of the genes on the chromosome.

[e] The pI values calculated using the amino acid sequence data and the pI/Mw tool on the ExPASy website (http://au.expasy.org/cgi-bin/pi_tool?).

5.4 Detection and Quantification of Proteins That Are Differentially Expressed in Wild Type and *ompR*⁻ *S.* Typhimurium Under Hyper-Osmotic Conditions, Using the Protein Expression System™

During the course of this study, there have been significant advances in proteomics technology, which have overcome a number of bottlenecks and limitations of more traditional approaches. Towards the end of the study, an opportunity arose to analyse the response of *Salmonella* cells to osmotic stress using a cutting edge proteomics approach developed by Waters Corporation known as the Protein Expression System™. It was hoped that a head-to-head comparison of the results obtained using this system and by 2-D gel electrophoresis would serve to confirm the identities of the proteins involved in the response. It was also possible that the new approach might uncover further components.

The Protein Expression System™ is a gel-free proteomics approach that enables the quantification of changes in protein expression alongside enhanced protein identification within a single LC/MS run. It is capable of quantitatively profiling protein and peptide components from complex samples without the need for isotope labelling. This is achieved by using a combination of exact mass measurement with parallel collision induced dissociation of tryptic peptide precursor ions to yield a Global Expression Dataset (GED) for each complex peptide mixture. Multiple GEDs can then be normalized and compared, for example, with a control.

In a conventional MS/MS strategy precursor peptides are selected for MS/MS analysis in series. This can be time-consuming and results in the instrument having a limited duty cycle. Therefore, co-eluting low abundance precursors are often missed from analysis. In contrast, the Protein Expression System™ enables precursor and fragment ions to be analysed simultaneously. Thus, more data is captured per unit time, resulting in increased proteome coverage.

As illustrated in Figure 5.6, the Protein Expression System™ LC/MS system conditions in the collision cell (which normally has an inert gas such as argon) rapidly alternate between low and high energy modes. When in the low energy mode, the mass spectrometer collects data on the masses (strictly speaking, the *m/z* values) of intact peptide ions. In contrast, in the high energy mode, the instrument collects data on

fragment ions derived from each parent peptide ion. Ultimately, this results in a time-resolved Global Expression Dataset containing:

- Conventional, low energy mass spectra *and*
- Mass spectra acquired at a high collision energy (MS^E)

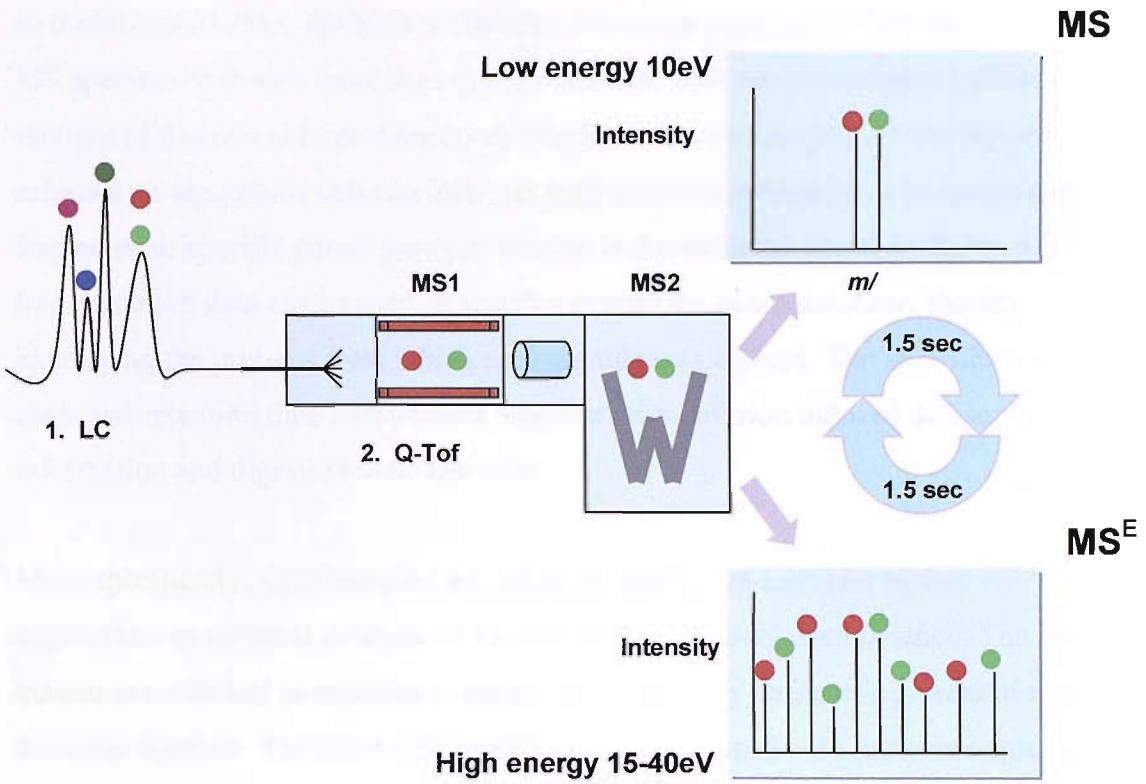


Figure 5.6 A schematic showing the principals of the Protein Expression System™ . As peptides elute from the LC column (1. LC) they are analysed by an electrospray Q-Tof mass spectrometer (2. Q-Tof) that cycles between low and high collision energies (MS and MS^E respectively). This approach has the advantage over traditional MS-MS in that co-eluting peptides can be simultaneously analysed. In this figure two co-eluting peptides are represented by a green and a red dot over the same LC peak. These peptides are then analysed simultaneously in the Q-Tof . In the low energy mode (MS), the mass spectrometer collects data on the masses of intact peptide ions, whilst in the high energy mode (MS^E), the instrument collects data on fragment ions derived from each parent peptide ion.

In traditional LC/MS, the high probability that many peptides will co-elute can lead to MS spectra containing more than one ion and MS^E spectra containing a complex mixture of fragments from these co-eluting ions. The Protein Expression System^{TM} uses informatics algorithms that can interpret such complex spectra so as to assign sets of fragments to specific parent peptides present in the collision chamber. Subsequently, the fragmentation data can be used to search a protein sequence database, thereby identifying the proteins from which each peptide was derived. The algorithms use exact mass and retention time information, together with collision induced dissociation (CID) information and digestion cleavage rules.

More specifically, time-resolved sets of exact masses are searched against an appropriate exact mass database of known proteins, pre-digested *in silico*. The detected masses are matched to peptides to assign the most likely sequences present in a given retention window. The identified peptides are then mapped onto proteins sequences in the database and eventually when multiple peptides are mapped onto a known protein, the proteins' identities can be assigned with high confidence.

In relative protein expression analyses, multiple normalised GEDs are compared and contrasted. Exact Mass Retention Time (ERMT) signatures enable all detected peptides to be uniquely identified and then ERMT signatures within any two GEDs can be matched for comparison. Each protein in a GED will be represented by multiple peptides, so relative expression can be calculated by comparing multiple EMRT signatures (Silva et al., 2005).

The overall workflow is summarised in Figure 5.7.

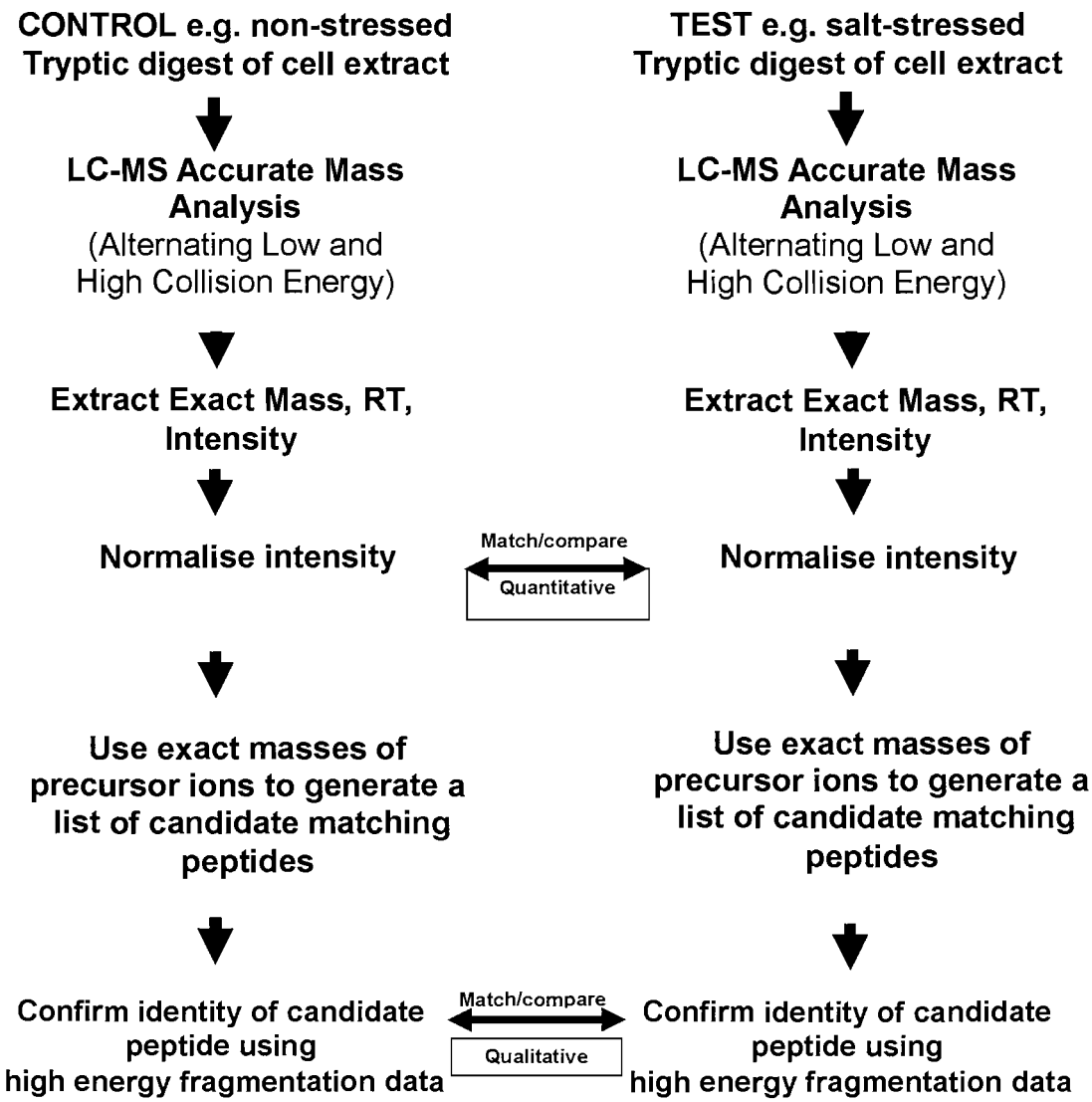


Figure 5.7 A summary flow diagram of the steps involved in analysing the relative expression of proteins in two samples, using the Protein Expression System™. Note that test and control samples are run sequentially, as are replicates. This is possible because the LC system employed is extremely accurate, producing very reproducible run profiles.

To compare the protein expression profiles of salt-stressed and non-stressed, whole cell lysates from wild type and *ompR*⁻ *S.* Typhimurium, were prepared as described in section 2.9. The subsequent processing (also described in section 2.9) and analyses using the Protein Expression System™ were carried out by Drs Mark Ritchie and Therese McKenna at Waters. The following summary of results are discussed here with their permission.

- Thirty-nine proteins were found to be up-regulated under salt-stress in the *ompR*⁻ mutant and thirty-one proteins were down-regulated in the same strain. Similar numbers of proteins were detected as differentially expressed when wild type cells were used for the experiment. Overviews of the major up-regulated and down-regulated proteins are shown on Figures 5.8 and 5.9 respectively and a summary table of all the proteins identified as differentially expressed under salt stress, whether identified by 2D gel analyses combined with mass spectrometry or the Protein Expression System™ can be found in Appendix A.

- YgaU was only found in salt treated samples for both strains. No YgaU peptides were detected in control samples for either strains, but 6-8 YgaU peptides (i.e. derived from 6-8 EMRTS) were detected in the salt treated for both strains.

- As was noted for the changes detected by 2-D gel analyses (Figures 4.1 & 5.4), the magnitude of the down-regulation of protein expression was much reduced compared to the magnitude of up-regulation.

- Again, as reported by the 2-D gel analyses, an exaggerated osmotic stress response was observed for *ompR*⁻ *S.* Typhimurium compared to wild type *S.* Typhimurium.

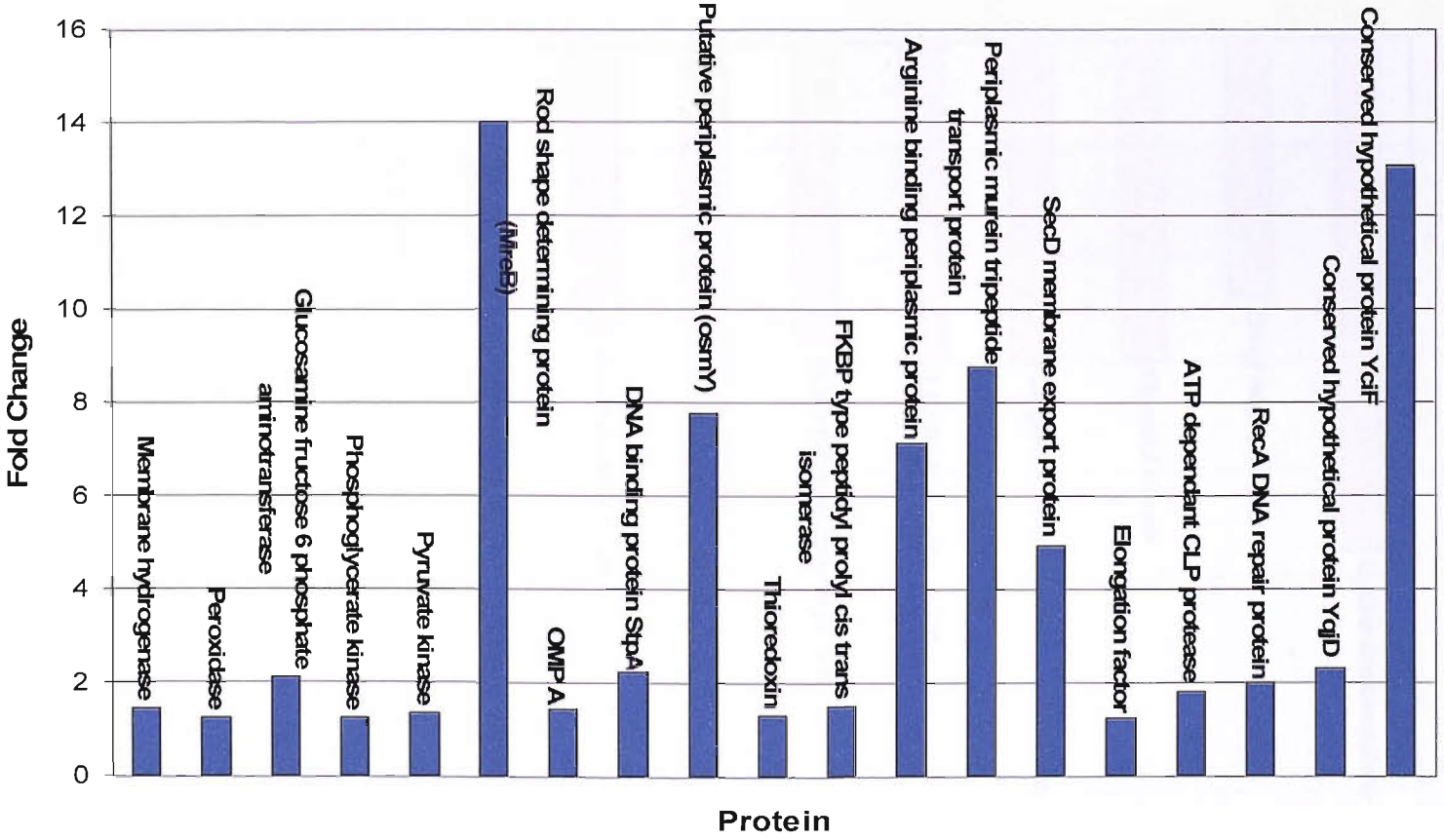


Figure 5.8 An overview of proteins detected as up-regulated under salt-shock in an *ompR*⁻ knock-out strain of *S.* Typhimurium SL1344 by the Protein Expression System™

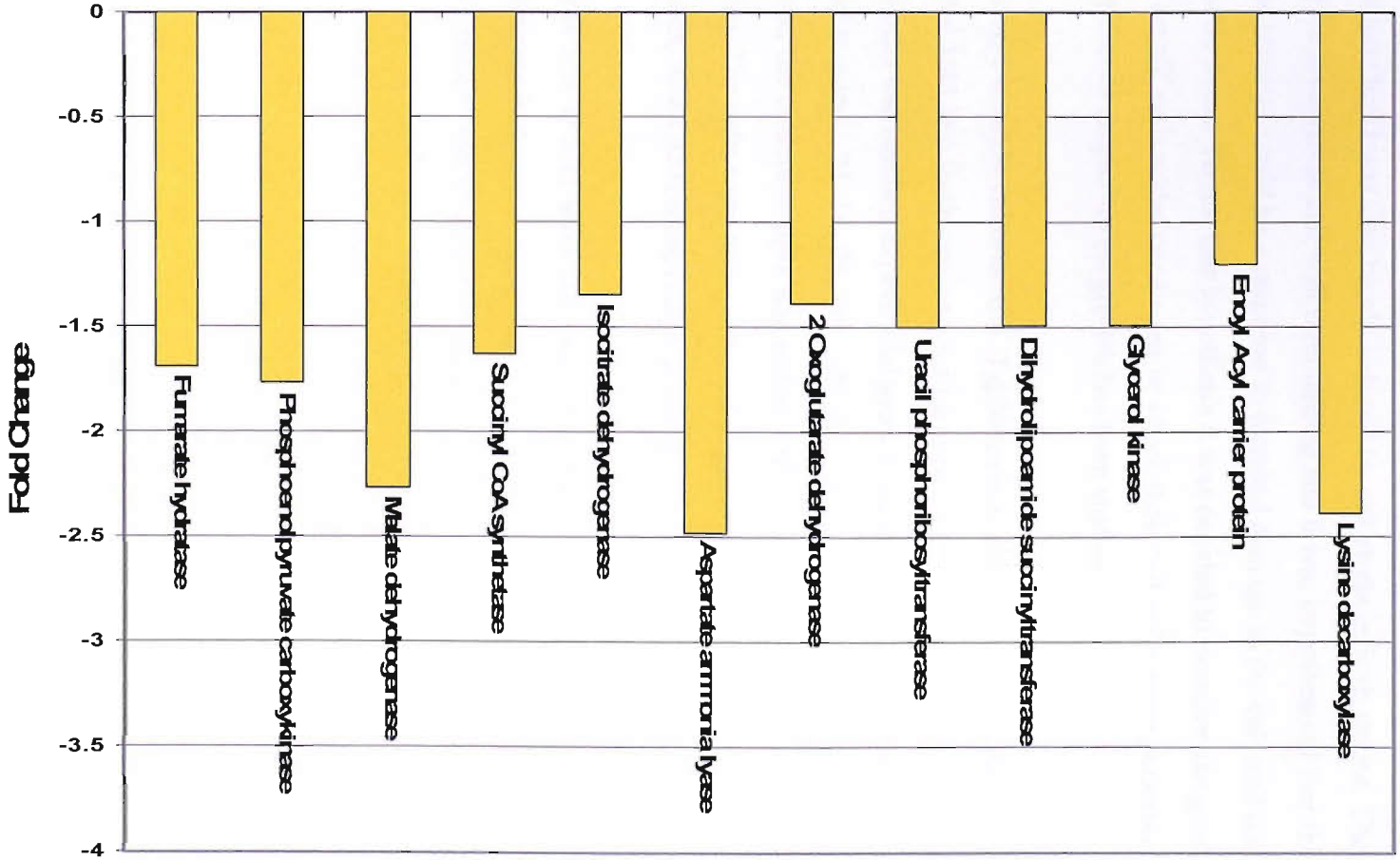


Figure 5.9 An overview of proteins detected as down-regulated under salt-shock in an *ompR*⁻ knock-out strain of *S.* Typhimurium SL1344 by the Protein Expression System™

5.5 Comparison of Wild Type and *ompR*⁻ *S.* Typhimurium Growth Responses to Cell Wall Damage.

As described, YgaU was the most strongly induced protein under salt stress in both the wild type and *ompR*⁻ mutant *S.* Typhimurium (~10 fold and ~16 fold respectively). In addition OppD was also highly induced by salt stress in both strains. These proteins may be involved in cell wall remodelling and it was hypothesised that the induction of these proteins could be a response to physical damage to the cell wall under hyper-osmotic stress. To test this hypothesis it was decided to examine the growth of wild type and *ompR*⁻ cells under conditions in which their cell walls were perturbed. Accordingly, the effect of ampicillin on growth has been studied.

In brief, overnight cultures of *S.* Typhimurium SL1344 and its *ompR*⁻ derivative were diluted 1:40 into fresh pre-warmed LB media and grown with vigorous aeration at 37°C until cells had reached exponential growth (A_{600} = 0.3). At this point, ampicillin was added to give 0, 25, 50, 75, 100, 125 and 150 μg/ml concentrations (for the 0μg/ml sample, the control sample was spiked with a corresponding volume of pre-warmed LB media). The cells were then permitted to grow aerobically at 37°C for a further 150 minutes, with OD readings taken at regular intervals.

Figures 5.10 & 5.11 show the resulting growth curves of SL1344 and SL1344 *ompR*⁻ cells respectively under the different ampicillin concentrations. It can be seen that the *ompR*⁻ mutant was markedly more susceptible to the effects of ampicillin, with a reduction in cell density indicative of lysis at lower concentrations and at a slightly earlier time relative to the wild type cells.

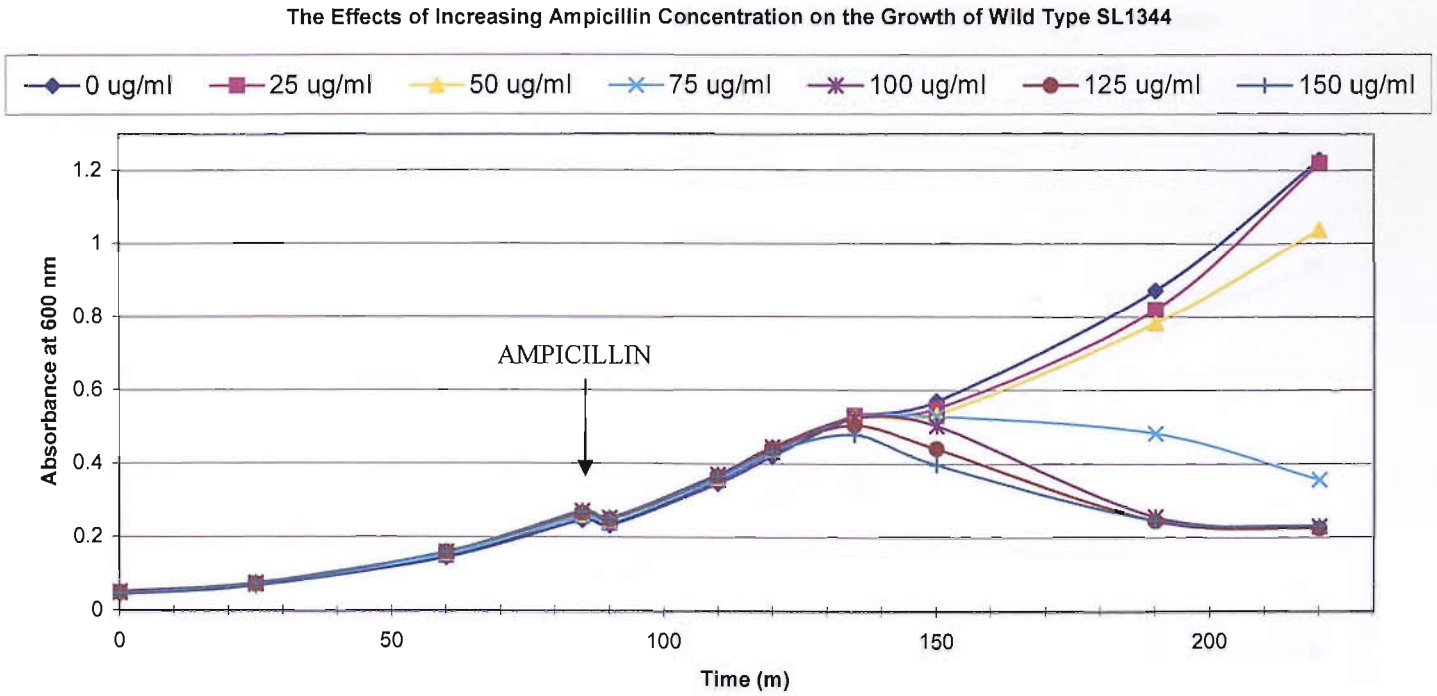


Figure 5.10 The growth responses of wild type *S.* Typhimurium, to differing ampicillin concentrations.

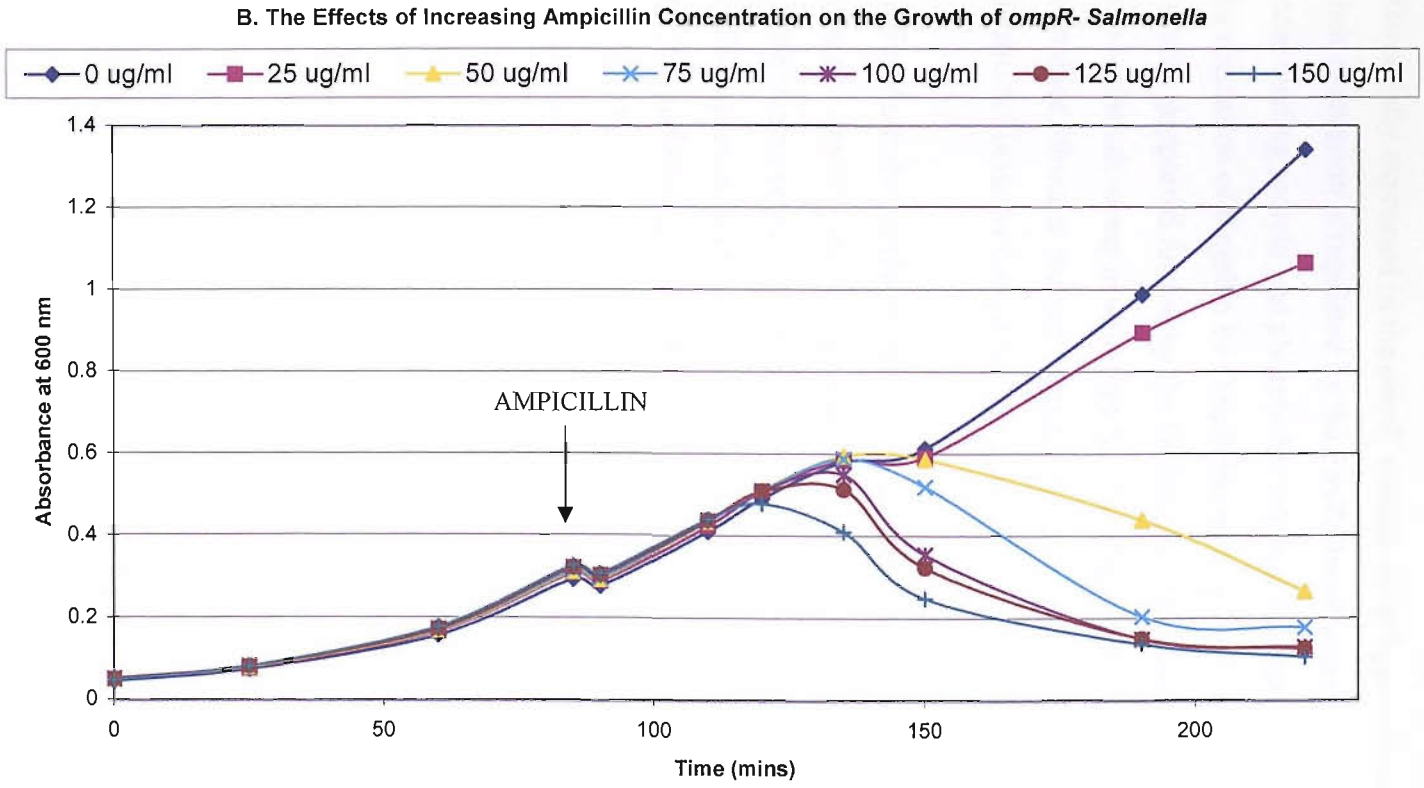


Figure 5.11 The growth responses of *ompR*⁻ *S.* Typhimurium, to differing ampicillin concentrations.

5.6 Discussion

This chapter has described the identification, by 2-D gel analyses, of a number of proteins that are differentially expressed when *ompR*⁻ mutant cells of SL1344 are exposed to osmotic up-shift.

Some of the osmo-regulated proteins identified in wild type SL1344 were not found to be differentially expressed in the *ompR*⁻ mutant under hyper-osmotic stress, suggesting that their expression is regulated by the EnvZ/OmpR system. These include flagellin, D-galactose binding protein and phosphofructokinase (for further details refer to Chapter 4). The regulation of flagellin by OmpR has already been described (Shin and Park, 1995) and is supported further by the finding here that there is differential expression of flagellin under salt-stress in wild type SL1344 but not the *ompR*⁻ mutant. As far as can be ascertained, direct or indirect regulation of D-galactose binding protein and phosphofructokinase by OmpR has not been reported. It therefore merits further study.

A significant number of the proteins were differently regulated in the same way in both wild type and *ompR*⁻ strains. The induced proteins included the chaperone DnaK, the shape determining protein MreB, glyceraldehyde-3-phosphate dehydrogenase and YgaU. The repressed proteins included pyruvate kinase and heat shock protein A (HspA). It is therefore suggested that such proteins must be controlled not by EnvZ/OmpR, but by alternative osmo-sensing mechanisms. In the case of DnaK it has been shown by other workers that increased osmolarity activates σ^{32}-dependent *dnaK* promoters, with no dependence upon EnvZ/OmpR (Bianchi and Baneyx, 1999). This also appears to be the case for YgaU which has recently shown to be part of the RpoS regulon (Ibanez-Ruiz et al., 2000).

As shown in Figure 5.5, when the degree of increase or decrease in the abundance of proteins differentially expressed in both strain was compared between wild type and *ompR*⁻ SL1344 the differential expression in the latter was more extreme. This finding suggests that the presence of a functional EnvZ/OmpR system damps down the level of expression of these proteins following exposure to osmotic stress. While this could be interpreted as OmpR directly or indirectly repressing expression of these proteins, it could also be that the *ompR*⁻ cells are more stressed than wild type following hyper-osmotic shock. Hence, other osmo-responsive proteins exhibit an exaggerated response.

This latter theory is further supported by the fact, that unique to the *ompR*⁻ mutant, there was a strong induction of the GroEL chaperone system (in addition to a protein encoded by the Fels-2 prophage and 3 unidentified proteins). This could be taken as an indication that the mutant has to up-regulate additional components to cope with hyper-osmotic stress in the absence of the EnvZ/OmpR system.

An alternative proteomics approach using the Protein Expression System™ produced data that strongly supported that of the 2-D analyses and provided further information regarding the systems affected by osmotic up shift.

As was noted for 2-D gel analyses, the Waters approach also detected an exaggerated osmotic shock response in the *ompR*⁻ strain of *S.* Typhimurium. Thus, the suggestion that the *ompR*⁻ strain is more 'stressed' upon osmotic up shift that wild type *S.* Typhimurium is further supported.

Among the down-regulated proteins under salt-stress in the *ompR*⁻ mutant, as detected using the Protein Expression System™, were: lysine decarboxylase, aspartate ammonia lyase, malate dehydrogenase, phosphoenolpyruvate carboxykinase and succinyl CoA synthetase. Although the magnitude of protein down-regulation was far smaller that that of up-regulation under salt stress (whether the 2-D gel *or* the Waters approach was used), the grouping of down-regulated proteins is suggestive of a reduction in metabolic enzymes, particularly those involved in the aerobic TCA cycle. This could reflect energy conservation under conditions of stress or be the result of reduced energy requirements. However, an attractive alternative explanation is that osmotic stress increases the metabolic demands on the cell as it struggles to contain the influx of sodium and chloride ions and its consequences. This increased metabolic demand in turn results in increased levels of reactive oxygen species, which are generated within the cell because of normal aerobic processes associated with the TCA cycle and respiratory chain. Therefore, to limit such free radical production, the cell down-regulates the TCA cycle enzymes (and possibly other components involved in aerobic metabolism).

There is now good evidence that when such cells are growing aerobically, the levels of oxidants that are generated is sufficient to threaten the fitness of the cells, e.g. via damage to DNA (Park et al., 2005). Thus it is plausible that *Salmonella* cells would try

to limit the production of oxidants under conditions of increased metabolic demands (e.g. due to stress) by switching to a partial anaerobic metabolism. In support of this proposal, it is interesting to note that another component that is significantly up-regulated under these condtions is AhpC, which is involved in peroxide scavenging.

Using the Protein Expression System^{TM}, the most highly up regulated proteins under salt stress in the *ompR*$^{-}$ cells were MreB, the conserved hypothetical protein YciF, periplasmic murein tripeptide transport protein MppA, OsmY and arginine binding periplasmic protein. Additionally, YgaU was also found to be uniquely expressed under salt stress in both wild type and *ompR*$^{-}$ *S.* Typhimurium.

The function of YciF is currently unknown but it has been reported to be up-regulated by exposure of *S.* Typhimurium to bile. YciF is encoded by the yciGFE-katN operon, which is known to be regulated by Rpos and also encodes YciE, a known acid shock protein. Taken together these facts would suggest that YciF could be a stress protein (Prouty et al., 2004). The arginine binding periplasmic protein ArtJ is known to be increased upon exposure of *E. coli* to human urine and is required for *E. coli* to be urovirulent (Russo et al., 1999). As this protein has been up-regulated under osmotic stress it could be of further interest for study as a virulence factor in *S.* Typhimurium.

MreB (discussed in detail in Chapter 6), MppA, OsmY and YgaU all appear to be involved in cell wall remodelling or maintenance of cell shape. As previously discussed, OsmY is a periplasmic *E. coli* protein that is expressed in response to stress conditions, in particular, osmotic shock and may prevent shrinkage of the cytoplasmic compartment by contacting the phospholipid interfaces surrounding the periplasmic space, as it contains two BON domains (Yeats and Bateman, 2003). OsmY was not detected as differentially expressed using 2-D gel analyses. However YgaU was readily detected and it is interesting to note that this hypothetical protein also contains a BON domain, along with a LysM motif known to be present in enzymes that degrade cell walls. Another protein, detected by the Waters approach was MppA, which is known to be involved in recycling of cell wall peptides in *E. coli* . The process of cell wall recycling also involves products of the *oppBCDF* locus and it is therefore noteworthy that the 2-D gel analyses revealed OppD to be up-regulated under salt stress in both *Salmonella* strains. It appears that periplasmic MppA binds the murein tripeptide, which is then

transported into the cytoplasm via membrane-bound and cytoplasmic OppBCDF (Park et al., 1998).

Thus, by two different proteomic approaches, several of the proteins exhibiting the greatest changes under hyper-osmotic stress are implicated in cell shape determination. Since the peptidoglycan is integral to cell shape, the effect of a cell wall disruptor was studied in wild type and *ompR*$^-$ cells. The effects of ampicillin on the growth of the wild type and *ompR*$^-$ mutant suggests that the *ompR*$^-$ mutant is more susceptible to the effects of ampicillin. While further studies are required to explore this further, it is known that ampicillin is taken up through OmpF (Nestorovich et al., 2002). Thus the differences in susceptibility of the two strains may reflect differences in the level of OmpF in wild type and *ompR*$^-$ cells. OmpF is the most abundant of the two outer membrane porins under conditions favourable for normal exponential growth. However, OmpC is constitutively expressed in *ompR*$^-$ cells (Figure 3.10) and hence such cells should have less OmpF (as the two porins are inversely regulated). It therefore seems unlikely that differences in ampicillin penetration can account for the observed differences in sensitivity. Thus, the possibility that cell wall metabolism is perturbed in *ompR*$^-$ cells merits serious consideration.

CHAPTER SIX

FURTHER REGULATORY AND PHENOTYPIC STUDIES ON SELECTED COMPONENTS THAT ARE DIFFERENTIALLY EXPRESSED IN *S.* TYPHIMURIUM SL1344 CELLS UNDERGOING HYPER-OSMOTIC STRESS

CHAPTER 6. FURTHER REGULATORY AND PHENOTYPIC STUDIES ON SELECTED COMPONENTS THAT ARE DIFFERENTIALLY EXPRESSED IN *S.* TYPHIMURIUM SL1344 CELLS UNDERGOING HYPER-OSMOTIC STRESS

6.1 Introduction

The results described in Chapters 4 and 5 refer to several novel components implicated in *Salmonella* responses to osmotic up-shift. One of these is the product of the previously hypothetical gene *ygaU*, which was the most highly up-regulated component under salt-stress conditions, as judged by analyses involving 2-D gel and the Protein Expression System™. In view of the novel character of the *ygaU* gene product, it is clearly of interest to further define its role in osmotic adaptation and also how it is regulated. Some clues about the latter process are already available due to the studies in the previous chapter that established that it is up-regulated still further in an *ompR*⁻ mutant. However, this phenomenon may be simply a reflection of the exaggerated osmotic stress responses of this strain. Moreover, we do not yet know if differential regulation of YgaU occurs at the transcriptional or post-transcriptional level. Clearly, its functional role is another key question that remains open at this time.

Another key component that surfaced in the proteomic studies was MreB. Again, it is of interest to determine its mode of regulation and its role in responses to hyper-osmotic stress. Additionally, recent work with the Gram-positive bacterium *Bacillus subtilis* suggests that it is involved in shape determination. It is therefore also of interest to find out if MreB in *Salmonella* has a similar role. This chapter describes further studies on such key components with the general aim of defining their mode of regulation and function.

6.2 Transcriptional Level Analysis of Three Proteins With Altered Expression Under Hyper-Osmotic Stress

Three proteins: rod shaped determining protein 'MreB', unidentified protein from 2-D gel 'YgaU', and D-galactose binding protein 'MglB', identified by the proteomic work described in Chapters 4 and 5, with altered expression under hyper-osmotic stress were investigated at the transcriptional level. Accordingly, cultures of wild type and *ompR*⁻ mutant cells were grown to mid-exponential phase under non-stress and hyper-osmotic stress conditions. Total cell RNA was extracted from paired samples (stressed and non-stressed) using the Trizol method and reverse transcription-polymerase chain reaction (RT-PCR) of RNA preparations was carried out, as described in Chapter 2, to quantify expression of mRNA of interest. mRNA levels of each protein were analysed in parallel with that of a house-keeping gene, glyceraldehyde-3-phosphate dehydrogenase (*gapA*). Glyceraldehyde-3-phosphate dehydrogenase protein expression was detected as up-regulated under osmotic stress in wild type and *ompR*⁻ S. Typhimurium. Despite this, the gene was still used as the house-keeping gene, as this concern was carefully considered, experiments were run that did not show *gapA* mRNA changing under osmotic stress *and* this was confirmed by co-workers and other experts in the field (Dr Jay Hinton, Institute for Food Research, *per comm.*). Furthermore this gene has successfully been used as a house-keeping gene in published work investigating gene expression under osmotic stress in *E. coli* (Michan et al., 1999). RT-PCR products were run out on agarose gels, stained with ethidium bromide and subjected to densitometric analysis (again as described in Chapter 2). Peak height was chosen as the parameter for band quantification. Adjusting for loading differences using the peak height results for the house-keeping mRNA bands, peak heights for the mRNA encoding proteins of interest were compared between treatments. A representative gel, showing the results of analysis for *ygaU* mRNA expression is shown in Figure 6.1 and numerical data is given in Table 6.1. It can be seen that there was a 4 to 5 fold increase in the levels of *ygaU* transcripts when either strain was salt-stressed. However, there was considerable variation between the replicates.

Using the same procedure, expression of *mglB* mRNA changed inconsistently between the two replicates for both strains (Table 6.2) and *mreB* mRNA expression was reduced under salt-treatment for both strains in both replicates but only minimally (Table 6.3). Again, however, only two replicates were analysed for each protein of interest and therefore statistical analyses could not be carried out. In addition, only raw peak height data was analysed, without background subtraction being optimised. Therefore, definitive conclusions could not be drawn from these results. Rather than a run a third replicate using this approach, the work was repeated using real-time, quantitative PCR (qPCR), as described in Chapter 2.

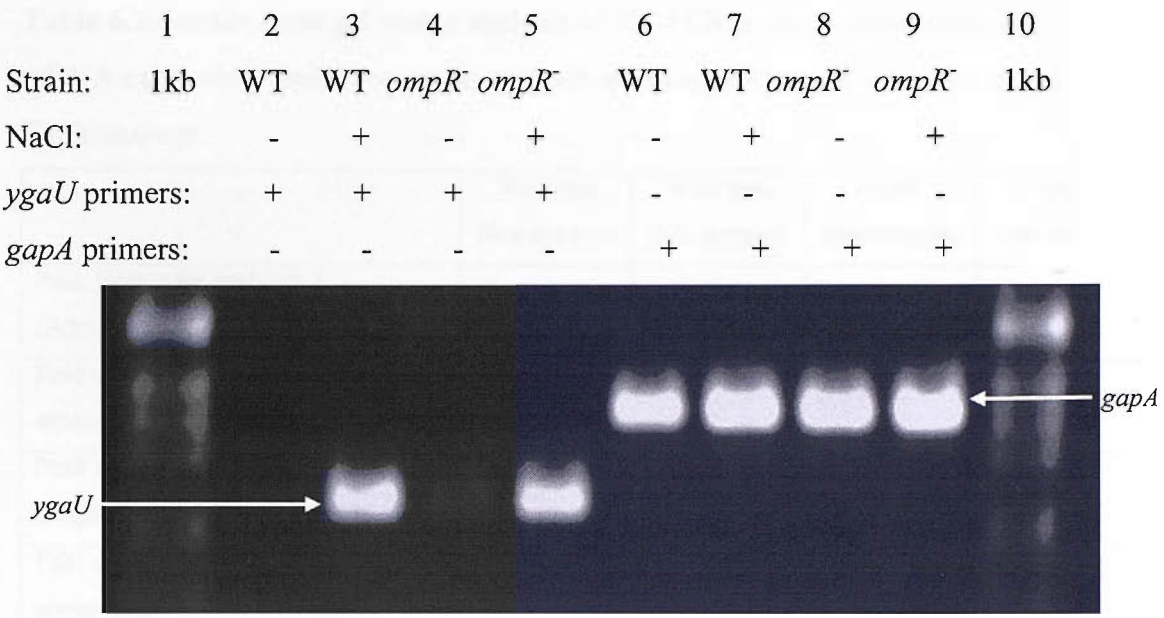


Figure 6.1 2.5% agarose gel separating RT-PCR product from RNA extracted from wild type *S.* Typhimurium cultured under non-stress (Lane 2 = *ygaU* product, and Lane 6 = housekeeping RT-PCR product) and salt-stress (Lanes 3 and 7) conditions, and *ompR*⁻ mutant *S.* Typhimurium cultured under non-stress (Lane 4 and 8) and salt-stress (Lane 5 and 9) conditions. Lanes 1 and 10 = 1 kb DNA ladder (Gibco BRL).

Table 6.1 Results from gel image analyses[a] of RT-PCR products comparing *ygaU* mRNA expression under non-stress and salt-stress conditions in wild type and *ompR*⁻ *S.* Typhimurium.

	Wild type Non-stressed	Wild type Salt-stressed	*ompR*⁻ Non-stressed	*ompR*⁻ Salt-stressed
Peak Height for replicate 1 (Adjusted for loading differences[b])	51.26	106.26	26.50	156.07
Fold change in expression under salt-stress (Rep 1)	2.07		5.89	
Peak Height for replicate 2 (Adjusted for loading differences)	21.94	137.05	24.26	97.66
Fold change in expression under salt-stress (Rep 2)	6.25		4.03	
Mean Fold change in expression under salt-stress	**4.16**		**4.96**	

[a] Gel images were analysed using the QuantityOne software package (BioRad) as described in Chapter 2.

[b] Loading differences were adjusted by normalising against levels of *gapA* mRNA

Table 6.2 Results from gel image analysis of RT-PCR products comparing *mglB* mRNA expression under non-stress and salt-stress conditions in wild type and *ompR*⁻ *S.* Typhimurium.

	Wild type Non-stressed	Wild type Salt-stressed	*ompR*⁻ Non-stressed	*ompR*⁻ Salt-stressed
Peak Height for replicate 1 (Adjusted for loading differences)	20.41	12.84	56.07	13.12
Fold change in expression under salt-stress (Rep 1)	0.63		0.23	
Peak Height for replicate 2 (Adjusted for loading differences)	23.94	24.02	43.27	45.74
Fold change in expression under salt-stress (Rep 2)	0		0	
Mean Fold change in expression under salt-stress	**0.32**		**0.12**	

Table 6.3 Results from gel image analysis of RT-PCR products comparing *mreB* mRNA expression under non-stress and salt-stress conditions in wild type and *ompR*⁻ *S.* Typhimurium.

	Wild type Non-stressed	Wild type Salt-stressed	*ompR*⁻ Non-stressed	*ompR*⁻ Salt-stressed
Peak Height for replicate 1 (Adjusted for loading differences)	45.84	33.72	85.28	76.49
Fold change in expression under salt-stress (Rep 1)	0.74		0.90	
Peak Height for replicate 2 (Adjusted for loading differences)	71.98	71.05	134.58	84.36
Fold change in expression under salt-stress (Rep 2)	0.99		0.63	
Mean Fold change in expression under salt-stress	**0.87**		**0.77**	

qPCR operates on the principle that the amount of fluorescence from a compound such as SYBR green is proportional to the amount of double stranded DNA present in a sample. Thus, as PCR of a reverse-transcribed template progresses, the fluorescence signal will increase and can be measured (qPCR using SYBR green is illustrated in Figure 6.2).

Relative quantification used the delta CT method (i.e. cycle number at which fluorescence exceeds the background level by 10-fold, which is dependent on the starting concentration of the target template) for analysis of the qPCR data. This was chosen as the method can be used for relative quantification, sample-to-sample, *and* permits comparisons between different assays within the same sample. This is the only method therefore that can be used to measure the ratios of different genes.

The delta CT method uses the difference in CT value obtained between a normalising gene (in this case *gapA*) and the gene of interest (*mreB, ygaU* or *mglB*) to calculate relative quantification. The normalising gene averages out sample to sample variations such that the changes in CT values between them reflects changes in expression level of genes of interest. This difference (delta CT) can then be converted into a fold change by assuming that each CT value increase between the target and normalising gene equals a relative halving of the target gene. Conversely a decrease in the delta CT by 1 unit equals a doubling of target gene. Relative quantification (between samples (stressed and unstressed) for each gene of interest) was achieved by separately normalising data from each target gene to the *gapA* control and then designating the unstressed sample as the delta delta CT reference. This analysis gives relative quantification of each target gene between the salt stressed and unstressed samples, with the unstressed sample being given the value 1 in each case.

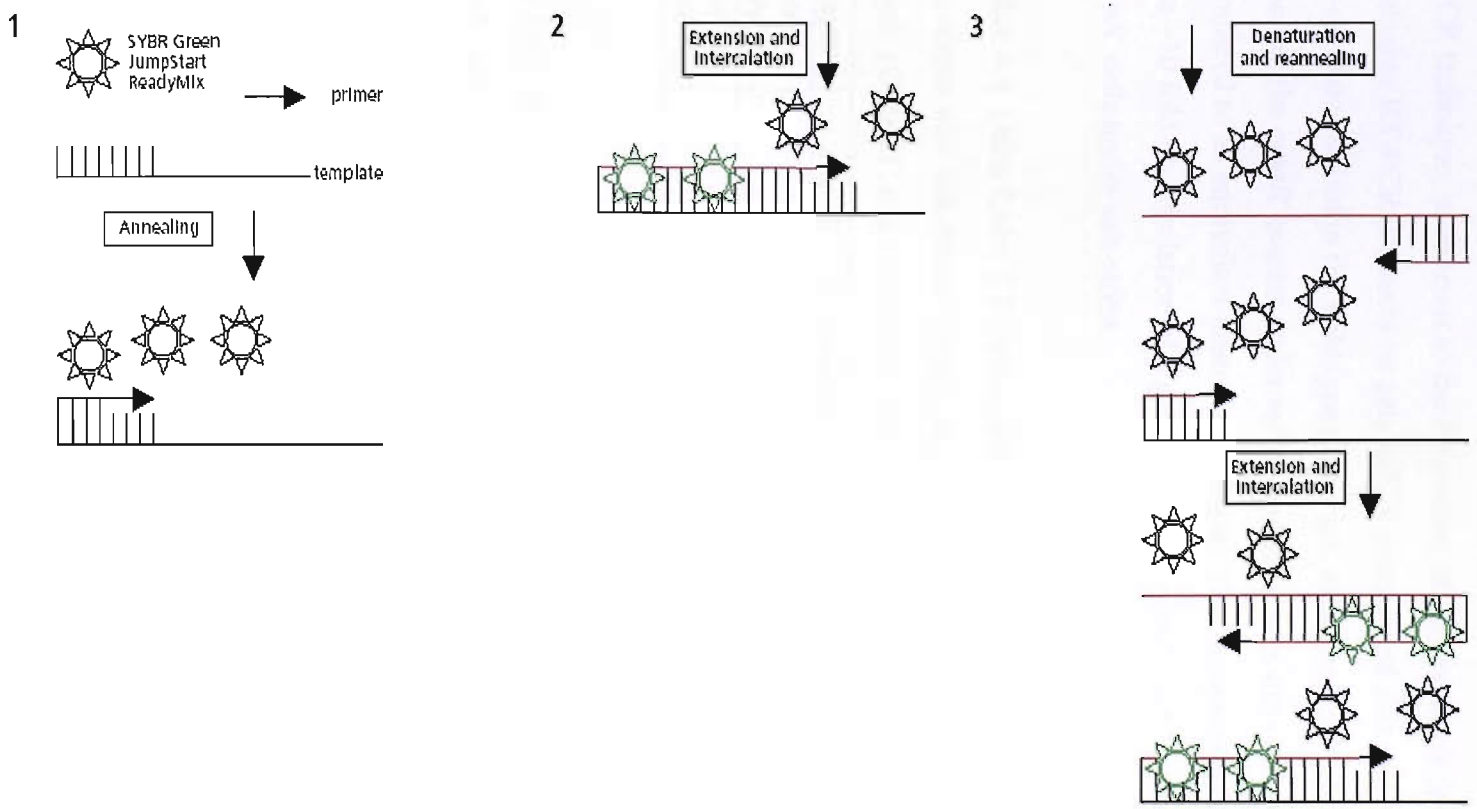


Figure 6.2 The process of qPCR using SYBR green (Adapted from the Sigma-Aldrich Website). 1. PCR reaction including SYBR green, Taq, dNTPs, buffer, primers and template. 2. As the reaction progresses, double stranded DNA is generated, the SYBR green intercalates into the products and fluoresces. 3. When enough products have accumulated, fluorescence levels rise and when a threshold level is exceeded the number of cycles (CT number) is recorded and used to quantitate the amount of starting template.

For *ygaU*, the results of the qPCR (Table 6.4) showed that *ygaU* mRNA transcripts were consistently and strongly up-regulated in both strains under salt-stress, though more so in the wild type *Salmonella* than in the *ompR*⁻ mutant. The latter finding differs from that described for RT-PCR and is likely to reflect the more accurate nature of the qPCR technique, in contrast to the limitations of densitometry (peak saturation) when analysing RT-PCR products on gels. qPCR determined that *ygaU* mRNA expression is up-regulated 9 fold in the wild type under salt stress compared to unstressed cells, whereas the *ompR*⁻ mutant under same conditions was only up-regulated 6.5 fold compared to an equivalent unstressed sample. This compares with protein level changes of a ~10 fold up-regulation of YgaU in wild type cells and ~16 fold up-regulation in *ompR*⁻ cells under salt-stress.

Table 6.4 Delta Delta CT values and fold changes in *ygaU* mRNA expression under non-stress and salt-stress conditions in wild type (WTC and WTS respectively) and *ompR*⁻ (OMPRC and OMPRS respectively) *S.* Typhimurium.

ygaU	ΔΔCT	Mean Change in Expression
WTS	-3.18	9.0
WTC	0	1
OMPRS	-2.71	6.5
OMPRC	0	1

For *mreB* the results of the qPCR (Table 6.5) were the same as for the RT-PCR i.e. *mreB* mRNA expression was reduced under salt treatment for both strains in both replicates but only minimally. qPCR determined that *mreB* mRNA expression was expressed at 0.9 fold in the wild type under salt-stress, compared to the unstressed cells and expression in the *ompR*⁻ mutant under the same conditions was expressed at 0.8 fold of the equivalent unstressed sample. This compares with protein level changes of a ~2 fold up-regulation of MreB in wild type cells and ~4 fold up-regulation in *ompR*⁻ cells under salt-stress.

Table 6.5 Delta Delta CT values and fold changes in *mreB* mRNA expression under non-stress and salt-stress conditions in wild type and *ompR*⁻ *S.* Typhimurium.

mreB	ΔΔCT	Mean Change in Expression
WTS	0.10	0.9
WTC	0	1
OMPRS	0.39	0.8
OMPRC	0	1

For *mglB* the results of the qPCR (Tables 6.6) showed that *mglB* mRNA expression was consistently and strongly down-regulated in the wild type *S.* Typhimurium under salt-stress. However in the *ompR*⁻ strain, expression appeared to be slightly increased. QPCR determined that *mglB* mRNA expression was down-regulated in the wild type *S.* Typhimurium under salt-stress with 0.2 fold expression compared to unstressed cells, whereas the *ompR*⁻ mutant under same conditions was up-regulated at 1.3 fold compared to an equivalent unstressed sample. This compares with protein level changes of a ~0.5 fold down-regulation of MglB in wild type cells and no change in MglB expression in *ompR*⁻ cells under salt-stress.

Table 6.6 Delta Delta CT values and fold changes in *mglB* mRNA expression under non-stress and salt-stress conditions in wild type and *ompR*⁻ *S.* Typhimurium.

mglB	ΔΔCT	Mean Change in Expression
WTS	2.70	0.2
WTC	0	1
OMPRS	-0.34	1.3
OMPRC	0	1

The raw data from the qPCR analyses are detailed in Appendix B.

6.3 Attempts to Make a 'Knock-out' Mutant of *ygaU*

To complement these studies it was decided to attempt to produce null mutants in components of interest using allelic replacement. However, certain difficulties were encountered that may have more general significance for the construction of further mutants in SL1344. The one-step inactivation method of Datsenko and Wanner (Datsenko and Wanner, 2000) was attempted (as described in Chapter 2 sections 2.13.6-2.13.8) to try and produce a mutant of *S.* Typhimurium in which chromosomal *ygaU* was inactivated.

In brief, the system developed by Datsenko and Wanner involves the transformation via electroporation, of cells containing a low-copy number plasmid inducibly expressing the red recombinase genes of bacteriophage lambda, with a selection determinant e.g. kanamycin resistance, flanked by 42bp homologous to selected regions upstream and downstream of the gene to be knocked out, brief incubation for recombination to occur, and selection plating (Figure 6.3).

Initially *S.* Typhimurium SL1344 (pKD46) i.e SL1344 transformed to express lambda red recombinase genes, was utilised as a host. Amplification of an FRT (FLP recognition target) flanked resistance gene was successfully carried out, both from a pKD3 and pKD4 plasmid. However, subsequent transformation with the digested PCR product yielded no colonies on the relevant selective media. Control transformations with plasmid DNA (pUC18) were successful, suggesting the cells were taking up DNA efficiently. Much time and effort was spent adjusting parameters and trying alternative strains such as LB5010 (pKD46) (a restriction negative version of SL1344), but the results remained negative.

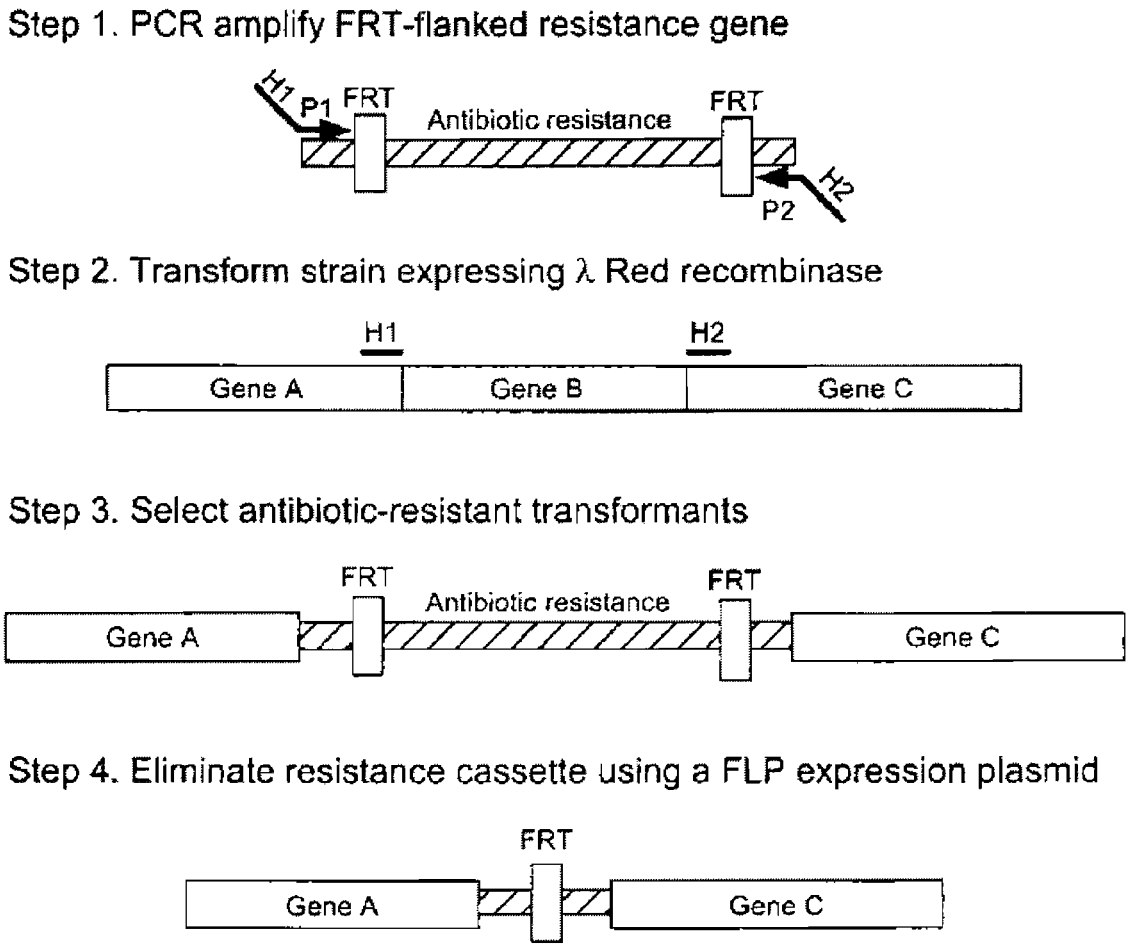


Figure 6.3 A summary diagram of the one-step inactivation method of Datsenko and Wanner (Datsenko and Wanner, 2000)

6.4 Cloning of the *ygaU* Gene Codon into pAJG38

Again to facilitate further studies, a clone of *S.* Typhimurium SL1344 that expressed *ygaU* under the control of an arabinose inducible promoter was engineered.

As described in Chapter 2, the *ygaU* gene was successfully amplified by PCR using SL1344 chromosomal DNA as a template then, via a digestion and ligation step, inserted into the plasmid pAJG38, thereby replacing a fragment carrying most of the *bipA* gene. In this way the *ygaU* gene was placed under control of the arabinose inducible promoter P_{BAD}. This cloning scheme is depicted in Figure 6.4. The resulting plasmid was designated pBC1 and was initially introduced in *E.coli* strain MACH-1. Sequencing of the plasmid DNA from the transformed *E.coli* MACH-1 (pBC1) confirmed that the plasmid contained the *ygaU* insert and gave 100% alignment with the predicted sequence. *S.* Typhimurium was then transformed using the plasmid preparation from the MACH-1 cells for subsequent expression studies.

Cultures of *E. coli* or *S.* Typhimurium cells bearing pBC1 were grown to various time points in 1% arabinose or 1% glucose in LB broth containing ampicillin, and protein extracts were prepared for analysis by SDS-PAGE to compare expression of YgaU.

Despite the successful cloning of *ygaU*, careful comparison of the protein bands from induced or non-induced samples in Coomassie stained gels failed to reveal a significantly denser band in the region of 16 kDa (the predicted molecular weight of YgaU is 16.121 kDa) that would have been consistent with the induction of *ygaU* expression (Figure 6.5).

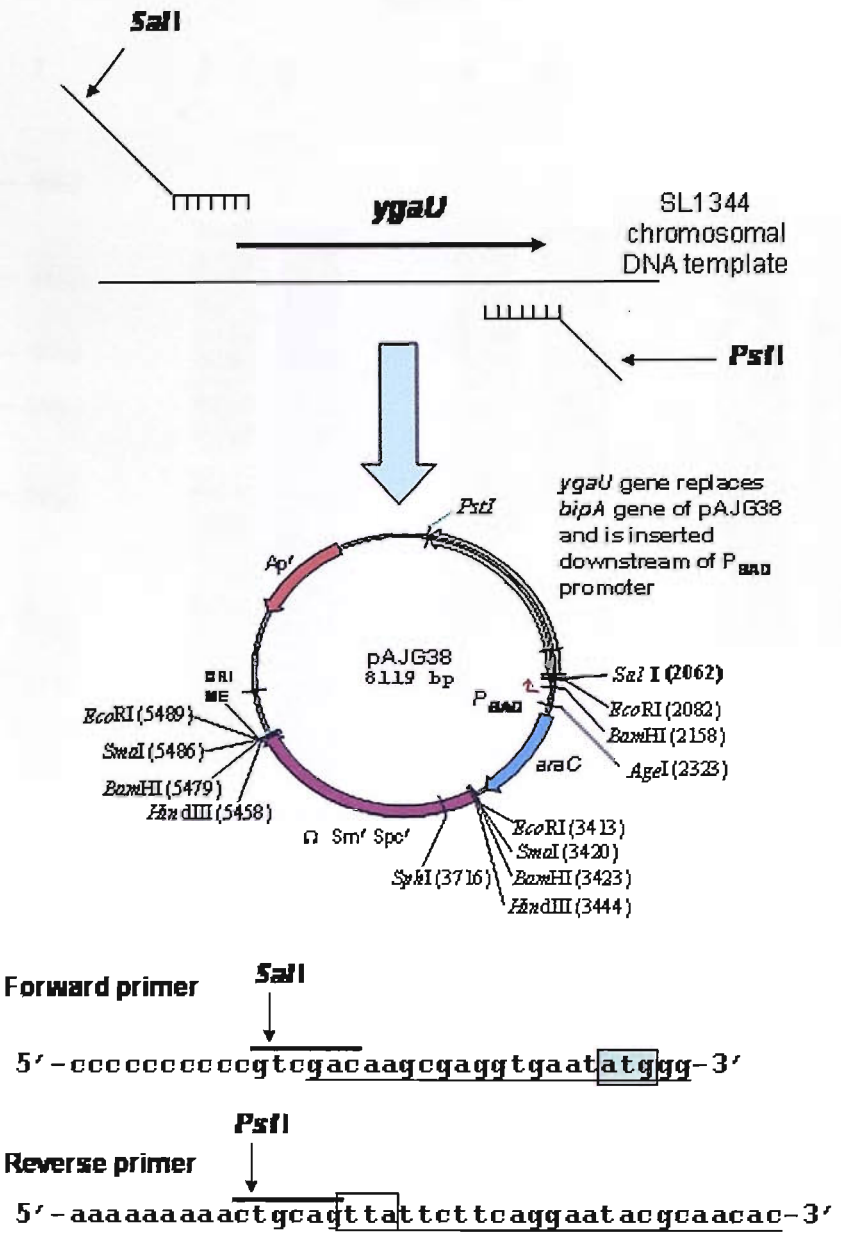


Figure 6.4 Cloning of the *ygaU* gene codon into pAJG38. Schematic showing trimming of the *ygaU* gene with *Sal* I and *Pst* I, prior to ligation into a similarly cut pAJG38 plasmid, replacing the *bipA* gene downstream of a P_{BAD} promoter.

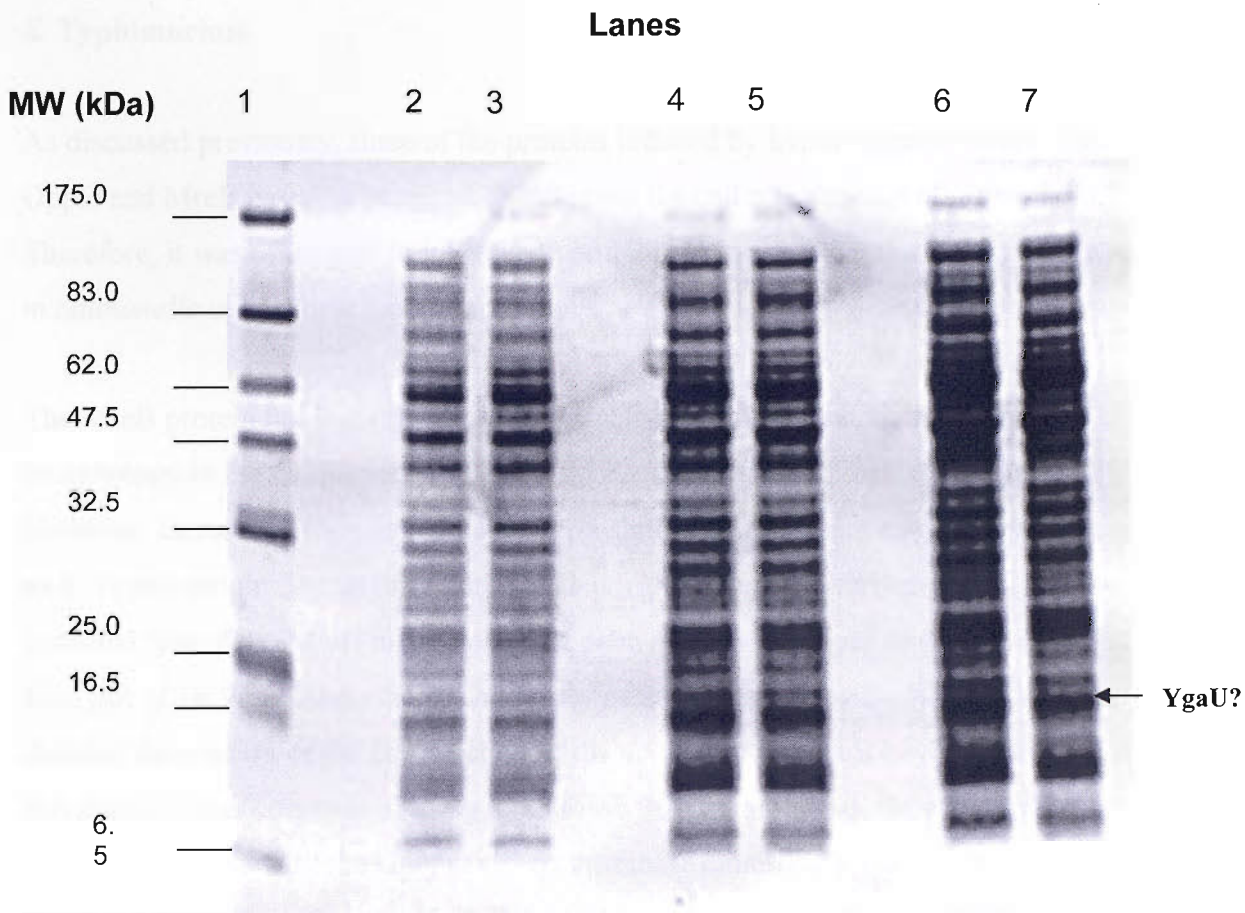


Figure 6.5 Coomassie brilliant blue stained, 12% NuPage Bis-Tris gels separating whole cell lysate from *S.* Typhimurium containing pBC1 cultured for 60 minutes with 1% glucose (Lane 2) or 1% arabinose (Lane 3), for 120 minutes with 1% glucose (Lane 4) or 1% arabinose (Lane 5), for 180 minutes with 1% glucose (Lane 6) or 1% arabinose (Lane 7).

6.5 Phenotypic Analyses of Wild Type, *ompR*⁻ Mutant and *mreB* Mutant *S.* Typhimurium

As discussed previously, three of the proteins induced by hyper-osmotic stress: YgaU, OppD and MreB have the potential to influence the cell morphology of *Salmonella*. Therefore, it was of interest to investigate possible shape changes that might take place in *Salmonella* under these conditions.

The MreB protein has been shown to play a role in the determination of cell morphology in the Gram-positive bacterium *Bacillus subtilis* (Jones et al., 2001). However, direct experimental evidence for a similar role in Gram-negative bacteria such as *S.* Typhimurium, is currently not available. Accordingly, to further investigate the potential role of the MreB in *Salmonella* a delta *mreB* null mutant of strain SL1344 (the kind gift of Dr Peter Alefounder, University of Southampton) was obtained. While a detailed description of the construction of the *mreB* null mutant is beyond the scope of this thesis, it was constructed using a modified version of the suicide vector pCVD442 that is compatible with the Gateway^{TM} recombinase cloning system (Invitrogen). Briefly, a 2.5kb fragment containing the *attP2*-Cm^{R}-*ccdB*-*attP1* region from the plasmid pDONR201 (Invitrogen) was inserted into the *Xba*I site of pCVD442 to create the vector pPA3065 (Grant et al., 2003). An *ΔmreB* gene with flanking *attB1* and *attB2* sites was then generated by PCR using appropriate primers. The fragment was inserted into pPA3065 using a Gateway BP reaction kit and introduced by transformation into *E. coli* S17-1(λ*pir*). The resulting plasmid was used for allelic exchange to produce Δ*mreB* derivative of SL1344 as previously described (Grant *et al.*, 2003). The genotype of the resulting strain was verified by PCR analysis of the relevant region in its chromosomal DNA (P. Alefounder, unpublished results).

The Δ*mreB* derivative of SL1344, designated PRA118, was then examined by phase contrast light microscopy to study its morphology. As shown in Figure 6.6, removal of MreB resulted in coccoid cells, strongly supporting the proposal that the protein plays an important role in determining cell shape.

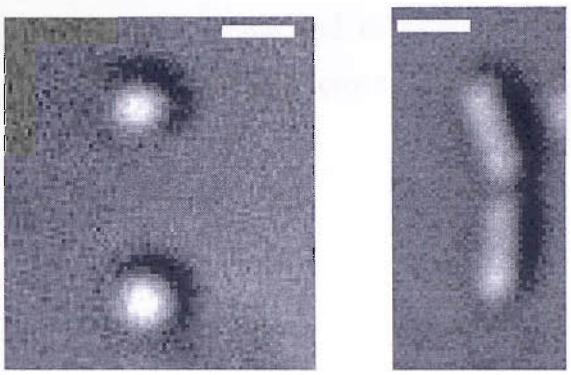


Figure 6.6 A representative picture of *S.* Typhimurium *mreB*$^{-}$ mutant cells (left-hand panel) and comparative picture of wild type *S.* Typhimurium (right-hand panel), both cultured in LB for 60 minutes and as viewed with a phase contrast microscope. Note the spherical form, rather than rod. Scale Bar represents 3μm.

Cells of wild type, *ompR*$^-$ mutant and *mreB*$^-$ mutant *S.* Typhimurium were cultured under non-stress and hyper-osmotic conditions, and analysed by phase contrast microscopy as described in Chapter 2. As it is known that there are morphological changes associated with different phases of bacterial growth curves, to elucidate the influence of hyper-osmotic stress, care was taken to ensure that all cultures were at the same point in their growth curves at the end of treatment i.e. mid-exponential. A representative image of the phase contrast microscope field of view of *ompR*$^-$ cells is shown in Figure 6.7.

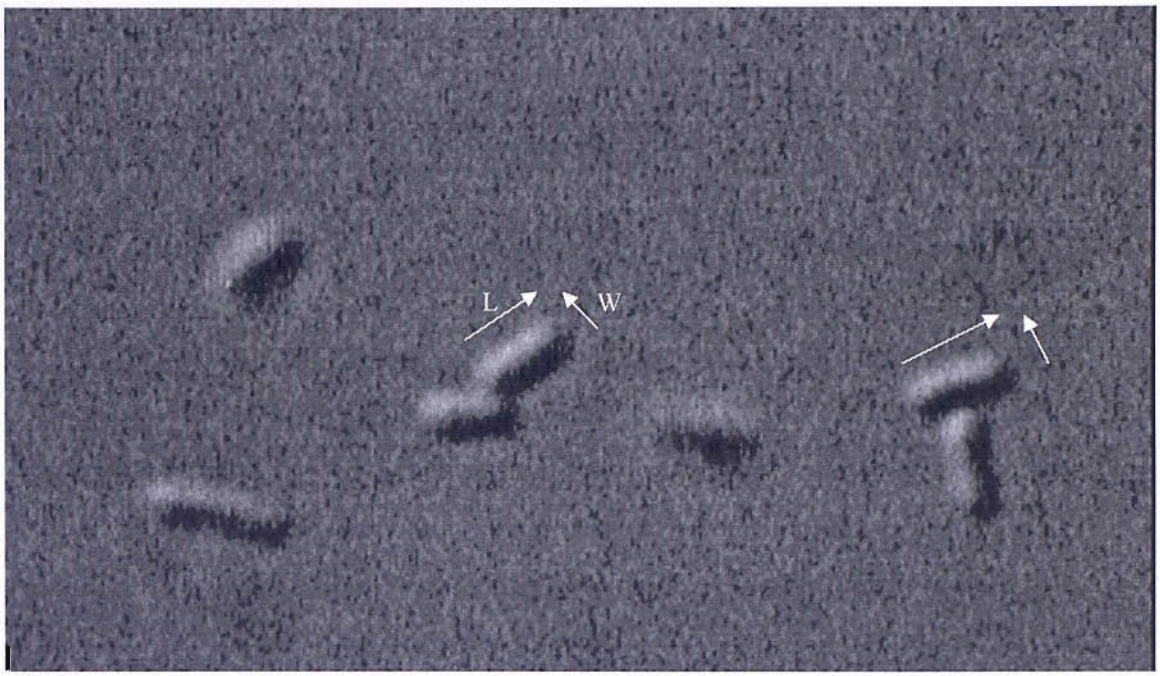


Figure 6.7 A representative picture of *S.* Typhimurium *ompR*$^{-}$ mutant cells (cultured in 0.5 M NaCl for 60 minutes) as viewed with a phase contrast microscope. The white arrows indicate the measurements taken from each cell. L=length and W=width. Magnification of whole cell x 5000

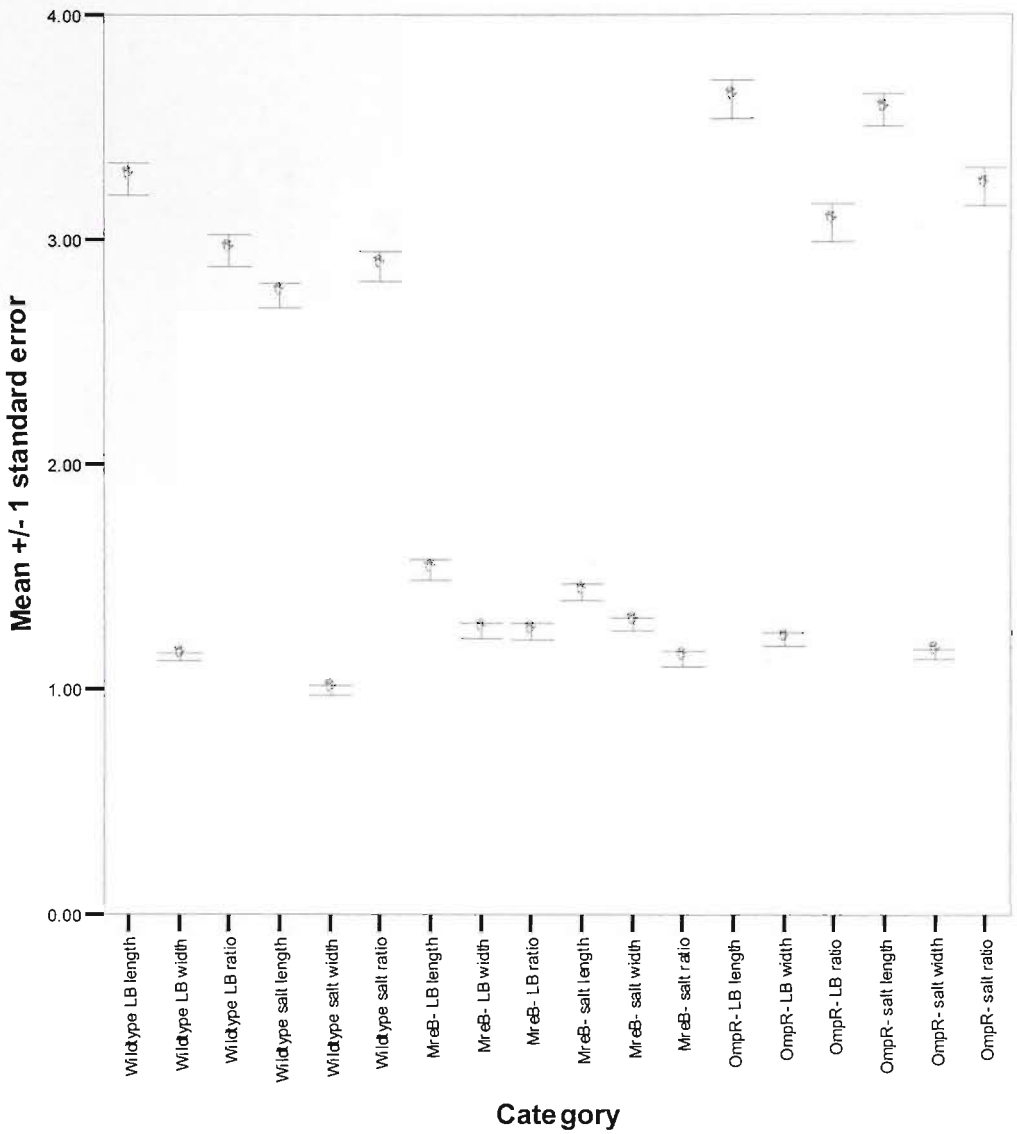
Length and width measurements of a minimum of 100 cells per treatment were recorded and entered, then ratios (length:width) were calculated and the results analysed using SPSS v11. For each strain, the experiment was repeated three times.

Graphs of the length, width and length:width ratio measurements (mean +/- standard error) for the wild type, *ompR*$^-$ mutant and *mreB*$^-$ mutant cells grown under control and hyper-osmotic stress conditions and sampled at 60 minutes, for the three experiments are shown in Figure 6.8 (A-C).

For each strain the lengths, widths and ratios at 60 minutes were compared for the control and salt-treated samples using a non-parametric Mann-Whitney U statistical test. This test was used as some of the data was not normally distributed and could not be transformed.

Wild type *S.* Typhimurium cells were found to be significantly reduced ($P<0.05$) in both their length and width dimensions following exposure to salt for 60 minutes in 2 of the 3 experiments. However, the third (Figure 6.8 C) revealed no significant change in morphology. The *ompR*$^-$ mutant was not significantly different between treatments for two of the three experiments. In the third experiment (Figure 6.8 C) the *ompR*$^-$ mutant in the salt treatment was found to be significantly longer and wider than that grown in just LB. Compared to the wild type and *ompR*$^-$ strains of *S.* Typhimurium the *mreB*$^-$ mutant was visually very different, showing coccoid cells (Figure 6.6). For the *mreB*$^-$ mutant two experiments revealed no significant difference between samples taken from controls cultures and those with NaCl added after 60 minutes. However, as with the other three strains one of the three experiments (Figure 6.8 B) gave a different result when the *mreB*$^-$ mutant cells under salt-stress were found to be larger than those incubated in just LB.

A


4.00
3.00
2.00
1.00
0.00
Mean +/- 1 standard error
Wildtype LB length
Wildtype LB width
Wildtype LB ratio
Wildtype salt length
Wildtype salt width
Wildtype salt ratio
MreB- LB length
MreB- LB width
MreB- LB ratio
MreB- salt length
MreB- salt width
MreB- salt ratio
OmpR- LB length
OmpR- LB width
OmpR- LB ratio
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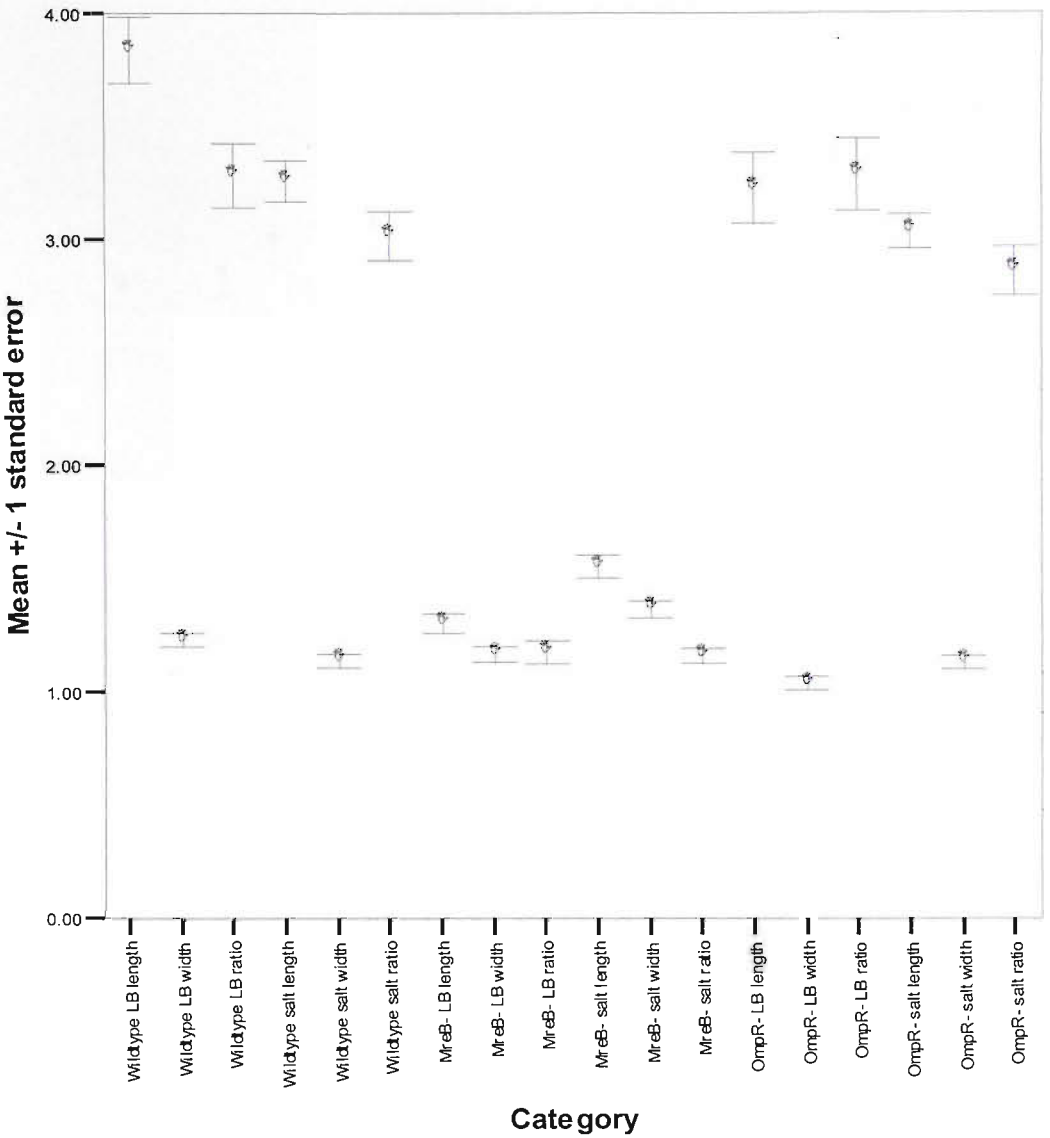
B


4.00
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Mean +/- 1 standard error
Wildtype LB length
Wildtype LB width
Wildtype LB ratio
Wildtype salt length
Wildtype salt width
Wildtype salt ratio
MreB- LB length
MreB- LB width
MreB- LB ratio
MreB- salt length
MreB- salt width
MreB- salt ratio
OmpR- LB length
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OmpR- salt width
OmpR- salt ratio
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C

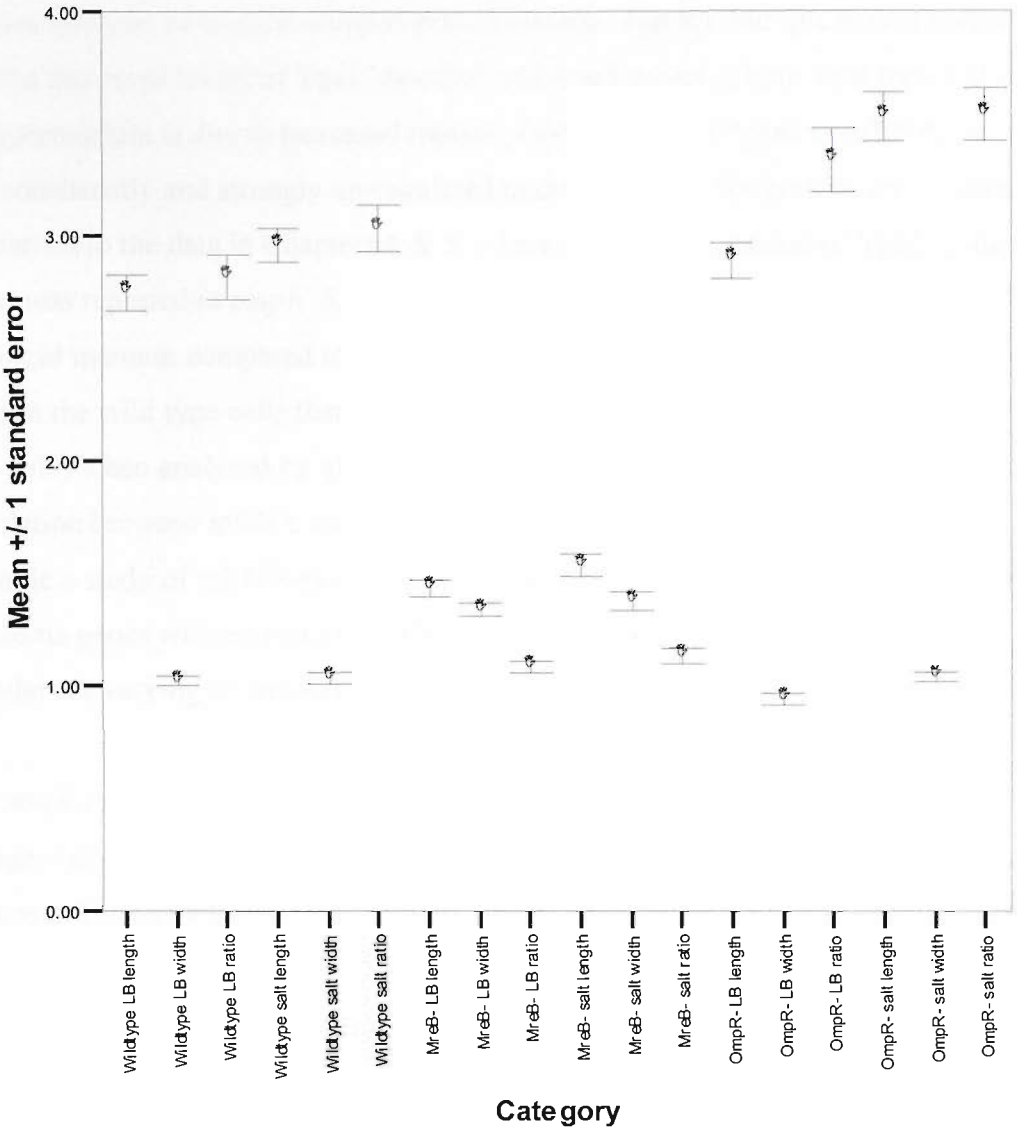

Figure 6.8 A-C The mean length, width and length:width ratio measurements (mean +/- standard error) for wild type, *mreB*⁻ and *ompR*⁻ strains of *S.* Typhimurium sampled after 60 minutes under control and hyper-osmotic stress conditions. Graphs A-C are the results for the 3 replicate experiments.

6.6 Discussion

Following the detection and identification of *Salmonella* proteins that were up- or down-regulated under conditions of salt-stress, this chapter has focussed on a more detailed analysis of specific components of interest. The RT and qPCR data suggests that the increased levels of YgaU detected under salt-stress in both wild type and *ompR*⁻ *S.* Typhimurium is due to increased transcription of the *ygaU* gene as mRNA expression was consistently and strongly up-regulated under salt stress for both strains. However, in contrast to the data in Chapters 4 & 5, where a higher expression of YgaU under salt stress was reported in *ompR*⁻ *S.* Typhimurium cultures compared to wild type cultures (~16 fold increase compared to ~10 fold), the *ygaU* mRNA expression was increased more in the wild type cells than *ompR*⁻ mutant under salt stress (~9 fold compared to ~6.5 fold) when analysed by qPCR. This is probably due to the well-documented lack of correlation between mRNA expression levels and protein expression levels. For example a study of mRNA-protein correlation in *Saccharomyces cerevisiae* reported that some genes with equivalent mRNA transcript levels translated into protein abundances varying by more than 50-fold (Haynes et al., 1998).

The *mreB* mRNA analyses both by RT- and qPCR detected no statistically significant changes (or even possible slight down-regulation) in mRNA expression under salt treatment for both strains. This does not concur with the clear up-regulation of MreB protein expression for both strains under salt treatment. While wild type cells had an increase in MreB expression under salt-stress that was far smaller than the changes reported for YgaU (2-4 fold changes for MreB compared to ~10-16 fold changes for YgaU), the *ompR*⁻ mutant showed a dramatic increase at the protein level that was not observed at the mRNA level. Therefore the up-regulated expression of MreB could be occurring post-transcriptionally.

Analyses of *mglB*, encoding the D-galactose binding protein were inconclusive. However, qPCR produced data supportive of the changes reported at the protein level. A down-regulation of D-galactose binding protein was detected in wild type *S.* Typhimurium under salt-stress, but no change was detected in the *ompR*⁻ strain. The qPCR data revealed a strong down-regulation in *mglB* mRNA expression in wild type *S.* Typhimurium, but only a minimal change (indeed, even a possible small up-regulation) in the *ompR*⁻ mutant. This suggests an influence of the *ompR* regulon on *mglB*

expression. Recently it has been observed that cultures of *E. coli* under high density or conditions of temperature up-shift leak many periplasmic proteins into the culture media. After a temperature up-shift it was observed that cell concentrations of many periplasmic proteins declined, with the periplasmic D-galactose binding protein MglB lost within the first hour (Rinas and Hoffmann, 2004). The observed loss of proteins of mainly periplasmic origin under stressful conditions could also have occurred during osmotic shock in this study and thus the reduced levels MglB detected by 2D SDS-PAGE under osmotic stress in the wild type cells could have been due to a combination of down-regulated mRNA transcription, leading to a fall in formation of MglB, and leakage of existing protein. It would be interesting to quantify levels of MglB over time in the media during osmotic stress to test this hypothesis.

The failure to detect colonies on selective media at the final stage of the attempt to produce a *ygaU* null mutant could have been for a number of technical reasons. However a concurrent transformation with a control plasmid was successful. Furthermore *ygaU* has recently been reported as an essential gene in the wild type *E. coli* MG1665 (Gerdes et al., 2003). Therefore it is highly possible that loss of *ygaU* may have proven lethal to *S.* Typhimurium SL1344. Further analyses are required to elucidate the issues relating to this work e.g. the construction of a conditional lethal mutant (Herring and Blattner, 2004) but due to time constraints this could not be pursued within this study.

E. coli and *S.* Typhimurium strains bearing *ygaU* under the control of the L-arabinose-inducible promoter P_{BAD} control were successfully made during this study. Unfortunately SDS-PAGE did not reveal a detectable increase in YgaU levels following induction of expression with L-arabinose. This does not rule out any change in YgaU expression in this system, as the SDS-PAGE experiment was not quantitative. However, further work, e.g. immunoblotting studies, is required to clarify whether the apparent lack of increased YgaU expression is due to the relative insensitivity of SDS-PAGE or issues such as a low copy number upon transformation. Again, due to time constraints further analyses of these constructs could not be undertaken within this study.

In view of the proteomic results, which uncovered components likely to be involved in determining cell morphology of *Salmonella*, it was of interest to study possible shape changes following osmotic up-shift. Unfortunately, it is difficult to draw any

conclusions from the phenotypic analyses due to the discrepancies observed with one of the three replicates. However, the observation that 2 of the 3 replicates of the wild type strain were significantly reduced in terms of length and width after 60 minutes in 0.5M NaCl, compared to no significant change for the *ompR*⁻ and *mreB*⁻ mutants, tentatively suggests that the loss of these proteins affects responses to shape change under hyper-osmotic stress. Ideally, another approach to analysing cell morphology needs to be investigated, as the method used had a lot of associated variability. Also, as the cells were approaching the limit of magnification despite the use of phase contrast microscopy, it was difficult to determine the plane in which the bacteria were lying, which may have decreased the accuracy of the measurements.

The spherical phenotype of the *mreB*⁻ mutant shows that loss of MreB had a clear effect on the basic morphology of *Salmonella*, suggesting it is integral to the maintenance of its 'normal' shape. A number of *E. coli* mutants are similarly spherical and most have changes to systems involved in cell wall synthesis e.g. peptidoglycan production (Spratt, 1975; Tamaki et al., 1980). This underscores the importance of the cell wall in maintaining cell shape. However, the induction of a spherical morphology in *mreB*⁻ *S.* Typhimurium strongly suggests a role for proteins beyond those involved in cell wall synthesis.

In recent years, it has become clear that bacteria synthesise homologues of both actin (e.g. MreB) and tubulin (FtsZ), the proteins that chiefly compose the eukaryotic cytoskeleton and that are required for cell division, maintenance of cell shape, and other functions. Information on actin homologues such as MreB, and how these proteins participate in cell shape formation, is now becoming clearer. While a role for MreB in cell shape determination was suggested almost two decades ago (Doi et al., 1988), it is only in the last four years that stronger evidence has emerged. In the Gram-positive bacterium *Bacillus subtilis*, *mreB* and a related gene, *mbl* (MreB like) have been shown to be required for cell morphogenesis, with each protein forming a filamentous helical structure close to the cell surface. Thus, it is currently proposed that MreB-like proteins have a cytoskeletal, actin-like role in bacterial cell morphogenesis (Jones et al., 2001) and this also appears to hold true for *S.* Typhimurium, as indicated here by the loss of rod shape when *mreB* is 'knocked-out'.

The phylogenetic distribution of MreB-like genes among the bacteria has revealed that bacteria with a non-spherical shape have one or more MreB-like genes (Jones et al., 2001). This adds further evidence to the theory that there is a MreB-based cytoskeleton in bacteria required to maintain cell shape.

Actin is a member of a larger superfamily of proteins, which includes Hsp70, cell division protein FtsA, and sugar kinases. MreB, among all proteins of the superfamily, has been shown to be most closely related to actin in overall size. It has also been shown that MreB purified from *Thermotoga maritima* can form polymers *in vitro* under similar conditions to eukaryotic actin (van den Ent et al., 2001) and a comparison of single protofilaments of actin and MreB has revealed that the subunits are in almost identical orientation (Figure 6.9). It appears that the MreB filaments formed in bacteria are dynamic structures and continuously re-modelled throughout the cell cycle and it has recently been suggested that components of the MreB cytoskeleton could serve as tracks for peptidoglycan-synthesising machinery, thus controlling cell wall formation and cell shape (Defeu Soufo and Graumann, 2004; Figge et al., 2004; Gitai et al., 2004; Gitai et al., 2005; Kruse et al., 2005; Daniel and Errington, 2003).

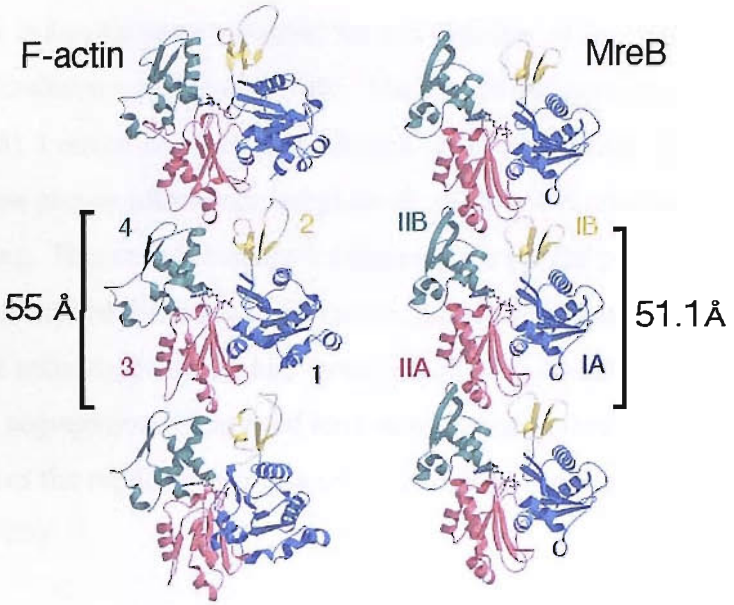

Figure 6.9 A comparison of single protofilaments of actin and MreB. (Adapted from Jan Löwe's website, http://www2.mrc-mb.cam.ac.uk/groups/JYL/frame_MreB.html)

The *mreB* gene is known to be essential for the viability of *Bacillus subtilis* but not *E. coli* (Abhayawardhane and Stewart, 1995; Varley and Stewart, 1992; Jones et al., 2001; Doi et al., 1988). Loss of *mreB* does produce a spherical mutant for both bacteria, however the loss proves ultimately lethal for *B. subtilis* with the cells swelling and eventually lysing. This could indicate a different role for the protein in these bacteria, or could simply reflect the difference in Gram-negative and positive cell envelope structure. More recently evidence has come to light that MreB is also involved in bacterial DNA segregation. Gitai *et al* have demonstrated that MreB binds specifically to and segregates the replication origin of the bacterial chromosome in *Caulobacter* (Gitai et al., 2005).

In terms of expression under stress, to date it has only been reported that *mreB* expression is up-regulated during recovery in heat injured *S. enteriditis* (Kobayashi et al., 2005) and in the heat stress response of *Bacillus cereus* (Periago et al., 2002).

In summary, the results presented in this chapter indicate that *ygaU* expression is transcriptionally up-regulated under salt-stress in both wild type and *ompR*$^-$ *S.* Typhimurium. In contrast, the increased MreB protein expression detected under salt stress for both strains appears to be due to post-transcriptional changes. MglB down-regulation in wild type *S.* Typhimurium under salt-stress appears to be due to a down regulation in mRNA transcription. However *mglB* mRNA was not affected in the same way in the *ompR*$^-$ mutant and therefore may be part of the *ompR* regulon. Loss of *mreB* led to clear changes in *Salmonella* morphology. In view of this and its up-regulation following salt-stress, it is suggested that the filaments formed by MreB are reinforced under these conditions, thereby helping to bolster the cell against a possible collapse in shape. It is further hypothesised that YgaU, which is likely to interact with peptidoglycan participates in re-modelling of the cell wall for a similar purpose.

CHAPTER SEVEN
CONCLUDING REMARKS AND FUTURE WORK

CHAPTER 7. CONCLUDING REMARKS AND FUTURE WORK

7.1 General Discussion

As unicellular organisms microbes encounter very wide fluctuations in the osmolarity of their environments and it is becoming clear that pathogenic species use these changes as cues for virulence gene expression. However, the components that mediate responses to shifts in osmolarity remain incompletely understood. This thesis describes the changes that occur in the *Salmonella* proteome under conditions of osmotic up-shift. It describes the detection and identification of proteins that have significantly altered levels of expression under such conditions and further shows which of these are likely to be directly or indirectly subject to regulation by the EnvZ/OmpR regulatory system. Because the criteria used for inclusion has been particularly rigorous, the numbers of proteins included in the final set is relatively small. Importantly, however, these proteins can be assigned with a very high level of confidence, which has not always been the case in other proteomic studies conducted elsewhere.

The two proteomic approaches used in this study (two-dimensional gels combined with peptide mass fingerprinting and analysis via the Protein Expression System™) enabled the identification of expression changes of a diverse range of proteins involved in the osmotic shock response in *S.* Typhimurium, including unexpected proteins that may not have been detected using more targeted, traditional approaches. However, it is recognised that the approaches suffer from a number of limitations, such as the failure to detect proteins that are poorly soluble, e.g. the hydrophobic membrane proteins and very small and very large proteins. The 2-D gel approach in particular is very time and labour intensive. During the life of this project, there have been huge advances in proteomics technology that have negated some of these issues, improving proteome coverage, simplifying the workflow and allowing a more rapid and complete dataset to be produced. These new techniques may eventually add to the data presented here. However, it is unlikely that many additional highly abundant proteins, that show major shifts in expression levels on salt-stress, will be added to the current data.

The functions of proteins determined, by 2-D gel analyses, to be differentially regulated under osmotic up-shift in wild type *S.* Typhimurium encompassed: the heat shock proteins (DnaK and HspA), at least one ribosomal protein, AhpC (which is known to

provide protection against oxidative stress), the major protein of the flagellar filament, flagellin, D-galactose binding protein, metabolic proteins (e.g. phosphofructokinase and glyceraldehyde-3-phosphate dehydrogenase), and finally, but perhaps most interesting of all, proteins with possible involvement in cell morphology (YgaU, MreB and OppD).

Use of an *ompR*⁻ mutant of *S.* Typhimurium revealed that, perhaps surprisingly, the majority of the differentially expressed proteins that were identified, by 2-D gel analyses, were not influenced by the EnvZ/OmpR two component system. Proteins that were identified as regulated by the EnvZ/OmpR two component system include: flagellin, D-galactose binding protein and phosphofructokinase. The EnvZ/OmpR regulon has previously been identified as an important regulon in bacterial adaptation to osmotic stress. However, the relatively limited involvement in the adaptation to the osmotic up-shift observed in this study underscores the importance of considering other regulatory systems. It should be noted that the *ompR*⁻ *S.* Typhimurium mutant still managed to adapt to the osmotic up-shift that was used. However, a clear difference between the wild type *S.* Typhimurium and *ompR*⁻ mutant was observed, with an exaggerated stress response occurring in the latter. This could reflect both a repression effect of the EnvZ/OmpR regulon and/or a higher degree of stress being experienced by the *ompR*⁻ mutant strain due to loss of the regulon.

These results were strongly supported by additional analyses using the LC/MS based Protein Expression System™, which also identified additional cell wall-associated proteins as up-regulated under osmotic stress (MppD and OsmY) and detected a subtle down-regulation of a number of components of the TCA cycle under the same conditions.

Further experiments, focussing on individual components, showed that changes in the expression of YgaU and MglB reflect changes at the transcriptional level. In contrast, the changes in MreB appears to be controlled at a later stage, i.e. post-transcriptionally. The precise transcriptional regulators involved however, were not identified, although *rpoS* has been previously implicated in the regulation of YgaU (Ibanez-Ruiz et al., 2000). Similarly, it is not yet known how MreB is up-regulated post-transcriptionally. The possibility that the results could be explained by an increase in the depolymerised form of MreB, following salt-stress, which may have increased solubility and/or more sensitivity to trypsin, merits exploration. It is interesting to note that glyceraldehyde-3-

phosphate dehydrogenase binds strongly to actin in eukaryotic cells (Cowan-Jacob et al., 2003; Yuan et al., 1999; Lowe et al., 2003). Given the high degree of structure conservation between these eukaryotic proteins and their prokaryotic counterparts (Lowe et al., 2001; Yun et al. 2000) it is tempting to speculate that the *gapA* and *mreB* gene products of *Salmonella* also interact. As indicated in Chapter 6, although the expression of glyceraldehyde-3-phosphate dehydrogenase was up-regulated at the protein level under osmotic stress, the level of the cognate mRNA was unaltered. The added possibility that this enzyme is also 'released' from a complex with the MreB polymers during salt-stress should also be considered

In addition, phenotypic analysis revealed the importance of MreB in determining the cell shape of *S.* Typhimurium, with an *mreB*$^-$ mutant exhibiting a spherical (coccoidl) growth form, rather than the usual rod-shape.

Consideration of all this data suggests some interesting hypotheses about *Salmonella* adaptation. Perhaps the most attractive of these arises from the novel observation that the most highly up-regulated protein under salt-stress conditions (as detected by 2-D gel proteomic analysis) was YgaU, which is strongly predicted to contain both a LysM peptidoglycan binding domain and a BON domain (previously described as a membrane anchor domain). That YgaU is up-regulated under salt-stress in both strains studied, suggests that *Salmonella* cells may remodel their cell walls as an integral part of adaptation to osmotic stress. This hypothesis is further supported by the detection of OsmY, OppD and MppD as up-regulated under salt-stress. In particular, the latter two proteins are known to play a role in the recycling of cell wall components. Also, as discussed in Chapter 6, the actin homologue MreB is implicated in both construction of a 'bacterial cytoskeleton' and bacterial DNA segregation. It would now be interesting to take the findings of this study forward and further elucidate the roles of MreB and YgaU in *S.* Typhimurium. However, elucidation of the precise role of YgaU was hampered in this study by the inability to create a null mutant. Concurrent studies in *E. coli* now suggest that *ygaU* may be an essential gene in *Salmonella*. Similarly, the *Δmreb* strain of *Salmonella* used in this study proved to grow extremely slowly although in contrast to its orthologue in *B. subtilis* was not an essential gene in the conditions used.

Recently Slovak *et al,* have reported that MreB is also essential in *Rhodobacter sphaeoides* and appears to play a role in elongating cells, in septation and, putatively, in the cytoplasmic control of peptidoglycan synthetic complexes (Slovak et al., 2005). Given that YgaU contains a LysM domain thought to be a general peptidoglycan-binding module, it could be hypothesised that the functions of MreB and YgaU may be inter-related. It is suggested that future studies pursue this interesting avenue of investigation. Construction of conditional lethal mutant could help identify the role of YgaU. An *mreB*⁻ mutant exists and could now be utilised for functional studies, along with the successfully created YgaU clone. Use of antibodies raised against MreB and YgaU could be utilised to identify localisation of these proteins within *Salmonella* cells under normal conditions and conditions of osmotic stress (both up-shift and down-shift). As the role of YgaU is particularly poorly understood , the creation of an affinity tagged form of YgaU could be used to 'fish out' interacting proteins within the cell, which would assist in the functional assignment of this potentially important protein. The possible use of other cell wall disruptors such as lysozyme, EDTA and ampicillin upon MreB and YgaU expression and localisation could also be enlightening.

An overview of the major systems that appear to be differentially regulated by hyper-osmotic stress and how they may inter-relate is depicted in Figure 7.1 and explained as follows:

- A number of confirmed and putative stress proteins (1) were up-regulated by hyper-osmotic stress, suggesting that these conditions damage cellular macromolecules and subsequently induce repair processes. One of the proteins up-regulated was AhpC, an oxidative stress protein. AhpC induction reflects a general cross-protection, as it is induced by many stresses (C.D.O'Connor, unpublished observations). One possible explanation for its induction is that diverse stresses are likely to trigger an increase in the level of reactive oxygen species (ROS) in the cell. This is because a stress will stimulate metabolism as the cell struggles to cope, which in turn will increase the level of endogenous oxidants produced as a consequence of normal processes, e.g. aerobic respiration. One prediction of this hypothesis is that proteins extracted from cells subjected to diverse stresses will contain elevated levels of carbonyl groups (a key indicator of oxidative stress). This proposal is easily testable.

- If the levels of oxidants increase within salt-stressed cells, this may explain the subtle down-regulation of TCA cycle components (2), as the cell tries to switch from aerobic to anaerobic metabolism to damp down the number of reactive oxygen species (ROS) that are produced. This presents a dilemma as anaerobic metabolism is far less efficient at producing ATP and also limits the number of compounds that can be synthesized. Thus, one would expect a modest down regulation of the TCA enzymes rather than a total switch.

- Other metabolic changes noted were changes in the levels of components of the glycolytic pathway (3). Here, the picture is mixed as two of the identified components, phosphofructokinase and pyruvate kinase, exhibited a modest decrease under salt-stress conditions, while glyceraldehyde 3-phosphate dehydrogenase showed a marked increase. Interestingly, the observed increase in the last enzyme seems to be due to post-transcriptional regulation, as RT-PCR and qPCR experiments showed that its mRNA level remained unchanged. The changes in the levels of glycolytic components are likely to reflect changes in the flux of metabolites flowing through this pathway.

- Another process that is likely to be significantly influenced, when *Salmonella* is subjected to hyper-osmotic stress, is cell motility. In keeping with previous observations flagellin isoforms were strongly down-regulated under these conditions. It is also likely that chemotaxis is affected. However, only indirect evidence for an affect on this process – down-regulation of D-galactose binding protein – was produced in this study.

- The observed increase in the proteins MreB, MppD, OppD, OsmY, and YgaU strongly suggests that cell architecture remodelling takes place when *Salmonella* undergoes osmotic up-shift (6). The precise nature of the events involved remains to be determined. However, it is likely that both peptidoglycan and also the newly-discovered actin-like cytoskeleton are key targets. The potential importance of such changes is underscored by the fact that two of the proteins (MreB and YgaU) were the most strongly up-regulated components in salt-stressed *Salmonella* cells, as determined by two different proteomics approaches.

As also indicated in Figure 7.1, it is interesting to note that three components linked to a possible reduction in chemotaxis are all EnvZ/OmpR regulated (flagellin, MglB and PFK). However, the identities of the other regulators involved in the osmotic stress response, with the notable exception of RpoS, are far from clear. Looking beyond the prokaryotes, it is well known that *Saccharomyces cerevisiae* adapts to osmotic stress by a rapid and reversible dissassembly of its actin cytoskeleton (Chowdry et al 1992, Yuzyuk & Amberg 2003, Levin 2005). Intriguingly, this process is regulated by the high-osmolarity glycerol (HOG) response pathway, which is positioned immediately downstream of a two-component system. Two-component systems, though common in prokaryotes, have only been discovered in plants and fungi relatively recently. Kultz & Burg (1998) referred to the EnvZ/OmpR two-component system in *E. coli* as a system homologous to the HOG cascade. However the detection of changes in MreB (and other cell morphogenesis proteins) in the *ompR*$^-$ mutant of *S.* Typhimurium would suggest that if an actin remodelling process is occurring in bacteria in response to osmotic stress (that may have evolved into a more sophisticated system in eukaryotic cells such as yeast) then a regulatory system other than EnvZ/OmpR is involved. This merits further investigation.

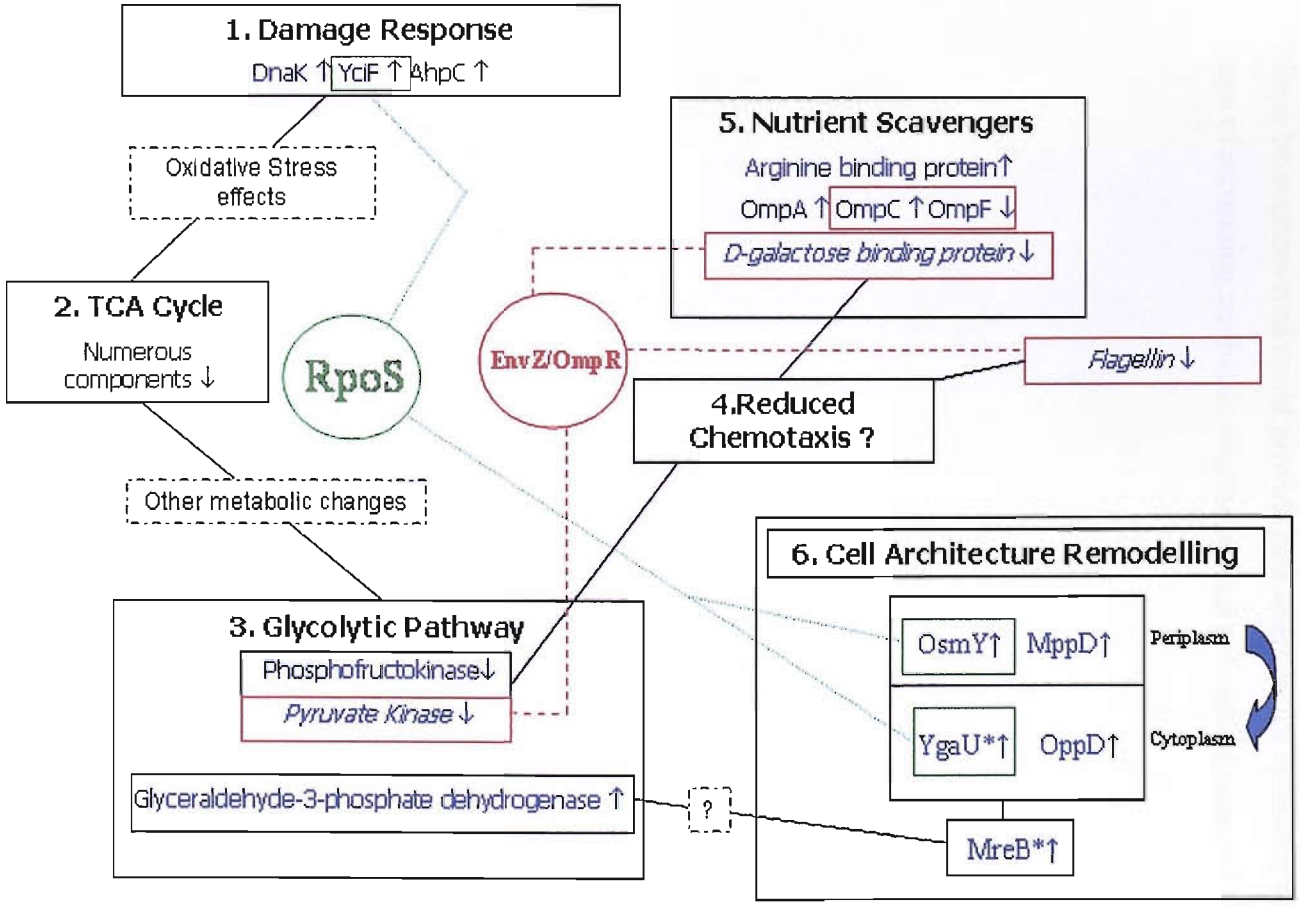

Figure 7.1 Schematic representation of key components and processes that are affected when *Salmonella* is exposed to hyper-osmotic stress. Known regulators of the stress response and their targets are indicated by red or green circles and boxes, respectively. Other components identified in this study or (in the case of OmpF (Alphen and Lugtenberg, 1977)) elsewhere are indicated by black boxes.* Most highly induced proteins. Further details are discussed in the text.

In summary, the findings of this study provide new perspectives into the mechanisms of osmo-adaptation of *S.* Typhimurium and, by extension, on similar mechanisms in related bacteria. A greater understanding of the roles and regulation of the proteins that have been uncovered could provide new avenues for antibiotic development and in an age of increasing antibiotic resistance such avenues of research are vital.

APPENDIX A

APPENDIX A.

The following table is a summary of all of the proteins identified as differentially expressed under salt-stress in *ompR*⁻ *S.* Typhimurium (with reference to chapter 5). This table covers the proteins identified by 2D gel analyses combined with mass spectrometry (2D PAGE/MALDI) and the Protein Expression System™ (PES).

Protein Name	Technology by which the protein was identified	Response under hyperosmotic Stress
Chaperone Hsp70 in DNA biosynthesis/cell division (DnaK)	2D PAGE/MALDI	Up-regulated
30s ribosomal protein S1 (cleavage fragment)	2D PAGE/MALDI	Up-regulated
Rod shaped determining protein	2D PAGE/MALDI & PES	Up-regulated
Alkyl hydroperoxide reductase c22 protein	2D PAGE/MALDI	Up-regulated
Unknown protein from 2-D gel (Putative LysM domain)	2D PAGE/MALDI & PES	Up-regulated
Glyceraldehyde-3-phosphate dehydrogenase	2D PAGE/MALDI	Up-regulated
NADH dehydrogenase I chain E	2D PAGE/MALDI	Up-regulated
Oligopeptide transport ATP-binding protein OppD	2D PAGE/MALDI	Up-regulated
GroEL protein	2D PAGE/MALDI & PES	Up-regulated
Membrane hydrogenase	PES	Up-Regulated
Peroxidase	PES	Up-Regulated
Glucosamine fructose 6 phosphate aminotransferase	PES	Up-Regulated
Phosphoglycerate kinase	PES	Up-Regulated
OMP A	PES	Up-Regulated
30S ribosomal proteins (mixed)	PES	Up-Regulated
50S ribosomal proteins (mixed)	PES	Up-Regulated
DNA binding protein StpA	PES	Up-Regulated
Putative periplasmic protein (osmY)	PES	Up-Regulated
Thioredoxin	PES	Up-Regulated
FKBP type peptidyl prolyl cis trans isomerase	PES	Up-Regulated
Arginine binding perisplamic protein	PES	Up-Regulated
Nitrate nitrite sensor NarX	PES	Up-Regulated
Periplasmic murein tripeptide transport protein	PES	Up-Regulated
SecD membrane export protein	PES	Up-Regulated
Elongation factor	PES	Up-Regulated
ATP dependent CLP protease	PES	Up-Regulated
RecA DNA repair protein	PES	Up-Regulated
Conserved hypothetical protein YqjD	PES	Up-Regulated
Conserved hypothetical protein YciF	PES	Up-Regulated
D erythrose 4 phosphatedehydrogenase	PES	Up-Regulated
Maltodextrin phosphorylase	PES	Up-Regulated
L Lactate dehydrogenase	PES	Up-Regulated
Phosphosugar binding protein	PES	Up-Regulated
GDP Mannose pyrophosphorylase	PES	Up-Regulated
Glucose 1 phosphate thymidytyltransferase	PES	Up-Regulated
SOS repair enzyme	PES	Up-Regulated

Penicillin binding protein 1C	PES	Up-Regulated
High affinity ribose transport protein	PES	Up-Regulated
Fimbrial usher protein	PES	Up-Regulated
Putative outer membrane usher protein	PES	Up-Regulated
Putative type 1 secretion protein	PES	Up-Regulated
Putative inner membrane protein	PES	Up-Regulated
RND family multidrug transport protein	PES	Up-Regulated
Putative flagellar biosynthesis type III secretory	PES	Up-Regulated
Hypothetical protein HCM2 0017c	PES	Up-Regulated
Orf proteins	PES	Up-Regulated
30s ribosomal protein S1	2D PAGE/MALDI	Down-regulated
Heat shock protein A	2D PAGE/MALDI	Down-regulated
Pyruvate kinase	2D PAGE/MALDI & PES	Down-regulated & Up-regulated respectively
Acetyl-coenzyme A carboxylase carboxyl transferase subunit alpha	2D PAGE/MALDI	Down-regulated
Fels-2 prophage: similar to gpQ in phage 186	2D PAGE/MALDI	Down-regulated
Fumurate hydratase	PES	Down-regulated
Phosphoenolpyruvate carboxylkinase	PES	Down-regulated
Malate dehydrogenase	PES	Down-regulated
Succinyl CoA synthetase	PES	Down-regulated
Isocitrate dehydrogenase	PES	Down-regulated
Aspartate ammonia lyase	PES	Down-regulated
2 Oxoglutarate dehydrogenase	PES	Down-regulated
Uracil phophoribosyltransferase	PES	Down-regulated
Dihydrolipoamide succinyltransferase	PES	Down-regulated
Glycerol kinase	PES	Down-regulated
Enoyl Acyl carrier protein	PES	Down-regulated
Lysine decarboylase	PES	Down-regulated
Lon protease	PES	Down-regulated
30S ribosome S3	PES	Down-regulated
50S ribosomal protein L24	PES	Down-regulated
Prolyl tRNA synthase	PES	Down-regulated
Inner membrane lipoprotein	PES	Down-regulated
Peptidoglycan associated lipoprotein	PES	Down-regulated
Tol protein	PES	Down-regulated
D galactose binding periplasmic protein	PES	Down-regulated
Glutamine binding periplasmic protein	PES	Down-regulated
Flagellin	PES	Down -regulated
Major outer membrane lipoprotein	PES	Down -regulated
Nucleoside diphosphate kinase	PES	Down -regulated
Histone proteins	PES	Down -regulated
YjgF translation inhibitor protein	PES	Down -regulated
Integral membrane protein	PES	Down -regulated
Membrane bound lytic murein tranglycosylase B	PES	Down -regulated
ParB	PES	Down -regulated
Putative cation transport ATPase	PES	Down -regulated

APPENDIX B

APPENDIX B.

The following 3 tables are the Raw CT, Mean CT and Delta CT values for the qPCR analyses comparing *ygaU*, *mreB* and *mglB* mRNA expression under non-stress and salt-stress conditions in wild type and *ompR*⁻ *S.* Typhimurium (see Chapter 6).

Table 1 Raw CT, Mean CT and Delta CT values for the qPCR analysis comparing *ygaU* mRNA expression under non-stress and salt-stress conditions in wild type and *ompR*⁻ *S.* Typhimurium.

	Wild Type Control			**Wild Type Salt Treated**		
	Raw CT	**Mean CT**	**ΔCT**	**Raw CT**	**Mean CT**	**ΔCT**
ygaU						
Rep1	25.82	25.45	6.06	22.76	22.57	2.88
	25.08			22.38		
Rep2	25.45	25.44		22.61	22.32	
	25.42			22.02		
Rep3	26.07	26.06		22.00	22.14	
	26.04			22.28		
gapA						
Rep1	19.81	19.71		19.62	19.64	
	19.61			19.65		
Rep2	19.64	19.66		19.42	19.41	
	19.67			19.39		
Rep3	19.42	19.39		19.14	19.34	
	19.35			19.54		
	***ompR*⁻ Control**			***ompR*⁻ Salt Treated**		
	Raw CT	**Mean CT**	**ΔCT**	**Raw CT**	**Mean CT**	**ΔCT**
ygaU						
Rep1	25.33	25.50	6.86	23.15	23.4	4.15
	25.66			23.65		
Rep2	26.33	26.43		23.31	23.32	
	26.52			23.32		
Rep3	25.04	25.22		23.99	23.92	
	25.4			23.84		
gapA						
Rep1	18.58	18.79		19.36	20.11	
	19			20.86		
Rep2	18.95	18.85		18.44	18.73	
	18.74			19.02		
Rep3	18.88	18.94		19.5	19.35	
	18.99			19.2		

Table 2 Raw CT, Mean CT and Delta CT values for the qPCR analysis comparing *mreB* mRNA expression under non-stress and salt-stress conditions in wild type and *ompR*⁻ *S.* Typhimurium.

	Wild Type Control			**Wild Type Salt Treated**		
	Raw CT	**Mean CT**	**ΔCT**	**Raw CT**	**Mean CT**	**ΔCT**
mreB						
Rep1	26.21	26.27	6.11	26.21	26.01	6.21
	26.32			25.81		
Rep2	26.52	26.68		26.67	27.06	
	26.83			27.45		
Rep3	27.51	27.08		26.93	26.49	
	26.64			26.04		
gapA						
Rep1	20.31	20.71		20.36	20.33	
	21.11			20.29		
Rep2	20.54	20.51		20.25	20.31	
	20.48			20.37		
Rep3	20.41	20.48		20.25	20.31	
	20.55			20.36		
	***ompR*⁻ Control**			***ompR*⁻ Salt Treated**		
	Raw CT	**Mean CT**	**ΔCT**	**Raw CT**	**Mean CT**	**ΔCT**
mreB						
Rep1	24.92	25.08	5.72	26.09	27.09	6.11
	25.24			28.08		
Rep2	25.16	25.61		25.69	25.53	
	26.06			25.36		
Rep3	25.12	25.25		26.51	26.75	
	25.37			26.98		
gapA						
Rep1	19.49	19.54		20.04	20.67	
	19.58			21.29		
Rep2	19.63	19.63		19.47	19.39	
	19.62			19.3		
Rep3	19.59	19.62		21.06	20.98	
	19.64			20.89		

Table 3 Raw CT, Mean CT and Delta CT values for the qPCR analysis comparing *mglB* mRNA expression under non-stress and salt-stress conditions in wild type and *ompR*⁻ *S.* Typhimurium.

	Wild Type Control			Wild Type Salt Treated		
	Raw CT	**Mean CT**	**ΔCT**	**Raw CT**	**Mean CT**	**ΔCT**
mglB						
Rep1	21.9	21.88	1.22	23.94	24.43	3.92
	21.86			24.92		
Rep2	21.91	21.77		24.01	24.07	
	21.62			24.13		
Rep3	21.73	21.71		24.26	24.19	
	21.68			24.12		
gapA						
Rep1	20.31	20.71		20.36	20.33	
	21.11			20.29		
Rep2	20.54	20.51		20.25	20.31	
	20.48			20.37		
Rep3	20.41	20.48		20.25	20.31	
	20.55			20.36		
	***ompR*⁻ Control**			***ompR*⁻ Salt Treated**		
	Raw CT	**Mean CT**	**ΔCT**	**Raw CT**	**Mean CT**	**ΔCT**
mglB						
Rep1	21.08	20.63	1.88	22.62	22.72	1.54
	20.17			22.82		
Rep2	22.23	22.02		21.34	21.05	
	21.81			20.76		
Rep3	22.12	21.84		20.68	21.33	
	21.55			21.98		
gapA						
Rep1	19.49	19.54		20.04	20.67	
	19.58			21.29		
Rep2	19.63	19.63		19.47	19.39	
	19.62			19.3		
Rep3	19.72	19.68		19.99	20.44	
	19.64			20.89		

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