UNIVERSITY OF SOUTHAMPTON

FACULTY OF MEDICINE, HEALTH & LIFE SCIENCES

School of Biological Sciences

Proteomic Analysis of the Oxidative Stress Response of *Salmonella enterica* serovar Typhimurium

by

Gillian Morag Howell Bsc (Hons)

Thesis for the degree of
Doctor of Philosophy

February 2004

UNIVERSITY OF SOUTHAMPTON

ABSTRACT

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PROTEOMIC ANALYSIS OF THE OXIDATIVE STRESS RESPONSE OF *SALMONELLA ENTERICA* SEROVAR TYPHIMURIUM

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Reactive oxygen species are of critical importance as a first line of host defence against bacteria. However, many pathogens have developed strategies to avoid, inactivate or tolerate such compounds, thereby allowing them to survive in the host and to cause disease. This thesis describes an analysis of the protein components involved in the oxidative stress responses of the facultatively intracellular pathogen *Salmonella enterica* serovar Typhimurium (strain SL1344), as well as a study of their control and integration with other key adaptive processes. Following dose response studies with adapted and non-adapted cells, benchmark lethal and sub-lethal dose values for H_2O_2 were derived for proteomic studies. Comparison of the protein expression profiles of cells of *S.* Typhimurium that has been exposed to H_2O_2 or left untreated showed that at least 15 proteins were differentially expressed. These included phase 1 and phase 2 flagellin, D-galactose binding protein and glycerophosphoryl diester phosphodiesterase. A putative peroxidase corresponding to the STM0402 gene, whose predicted sequence shares 42% identity with *ahpC* of *S.* Typhimurium, was strongly down regulated under the same conditions. The AhpC and STM0402 proteins of *Salmonella* are paralogues but show opposite responses to oxidative stress. Furthermore, STM0402 does not appear to be regulated by the OxyR or SoxRS global regulatory systems, in contrast to the AhpC protein.

The thesis also describes the construction and functional characterisation of a null mutant of *oxyS*, which codes for an RNA molecule that integrates responses to peroxide stress with other key adaptive processes. The putative *oxyS* gene product of *S.* Typhimurium is 110 nt in length and, like its *E. coli* counterpart, is predicted to contain three stem loops. In support of the structure of the OxyS RNA of *Salmonella*, base changes in a region predicted to be double stranded were accompanied by complementary base changes on the other strand. The *oxyS* mutant had an adaptive response to H_2O_2 that mirrored that of the wild-type but, surprisingly, it was much more resistant to H_2O_2. It is proposed that the observed phenotype is due to the derepression of translation of the mRNA transcript for the RpoS sigma factor, which controls the general stress response. The H_2O_2 stress regulon was also found to overlap with the PhoPQ and BipA regulons in *S.* Typhimurium. Evidence for the PhoPQ overlap is based on: (i) the co-regulation of common targets: (ii) cross-protection of a sub-lethal acid stress against an otherwise lethal H_2O_2 challenge and (iii) the hyper-resistance of a PhoP null mutant specifically to H_2O_2. Similarly, the putative global regulatory protein BipA was found to be crucial to the adaptive response to H_2O_2 in *Salmonella.* The absence of BipA resulted in a severely impaired adaptive response to this stress, although the response of non-adapted cells was normal. Collectively, the studies highlight the inter-relatedness of adaptive responses and define new regulators and target proteins involved in the oxidative stress response of this important pathogen.

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DECLARATION OF AUTHORSHIP

I, GILLIAN MORAG HOWELL declare that the thesis entitled

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and the work presented in it are my own. I confirm that:

- this work was done wholly or mainly while in candidature for a research degree at this University;
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- parts of this work have been published as:

Adams, P., Fowler, R., Howell, G., Kinsella, N., Skipp, P., Coote, P., & Connor, C. D. 1999, "Defining protease specificity with proteomics: a protease with a dibasic amino acid recognition motif is regulated by a two-component signal transduction system in *Salmonella*", *Electrophoresis*, vol. 20, no. 11, pp. 2241-2247.

Adams, P., Fowler, R., Kinsella, N., Howell, G., Farris, M., Coote, P., & Connor, C. D. 2001, "Proteomic detection of PhoPQ- and acid-mediated repression of *Salmonella* motility", *Proteomics*, vol. 1, no. 4, pp. 597-607.

Signed: .....

Date:.....

ACKNOWLEDGEMENTS

My most grateful thanks go to my supervisor Professor C. David O'Connor who has supported me tirelessly throughout my studies. His continued encouragement has been most invaluable and inspirational.

I would also like to thank past members of the DO'C group for their invaluable assistance and friendship, especially Dr Andrew Grant for his expert help.

Cross protection assays with *S.* Typhimurium SL1344 wild-type and PhoPQ mutant strains were carried out in collaboration with Dr Richard Fowler. Thank you Richard.

Thank you to Dr Gisela Storz for the OxyR antibody. I also wish to express my appreciation to Dr Hengge-Aronis for the antibody to RpoS.

Finally, my thanks go to my amazing husband Steve and my children Joe and Aimée for their unconditional support and encouragement throughout.

ABBREVIATIONS

AhpC	Alkyl hydroperoxide reductase small subunit
AhpF	Alkyl hydroperoxide reductase large subunit
Ap	Ampicillin
ASP	Acid shock proteins
ATP	Adenosine triphosphate
ATR	Acid tolerance response
BipA	BPI inducible protein A
bp	Base pair
BPI	Bactericidal/Permeability-Increasing
C. jejuni	*Campylobacter jejuni*
CAMP	Cationic antimicrobial peptide
cat	Chloramphenicol acetyltransferase
CFU	Colony forming units
CHP	Cumene hydroperoxide
Cm	Chloramphenicol
DNA	Deoxyribonucleic acid
E. coli	*Escherichia coli*
EF-Ts	Elongation factor Ts
EF-Tu	Elongation factor Tu
EPEC	Enteropathogenic *Escherichia coli*
FCCP	Carbonyl cyanide p-trifluoromethoxyphenylhydrazone
FSB	Final sample buffer
H. pylori	*Helicobacter pylori*
H_2O_2	Hydrogen peroxide
HCl	Hydrochloric acid
$HO^{\cdot}$	Hydroxyl radical
HOCl	Hypochlorous acid
HOONO	Peroxynitrile
kb	kilobase
kDa	kiloDalton
LB	Luria-Bertani
LPS	Lipopolysaccharide
MDR	Multidrug resistant
MOPS	Morpholinopropane sulphonate
mRNA	Messenger ribonucleic acid
MS	Mass Spectrometry
NADPH	Nicotinamide adenine dinucleotide phosphate (reduced)
NdhII	NADH dehydrogenase
$NO^{\cdot}$	Nitric oxide
NOS	Nitric oxide synthase
nt	Nucleotide
$O_2^{\cdot-}$	Superoxide radical
OD	Optical density
PAGE	Polyacrylamide Gel Electrophoresis
PBS	Phosphate Buffered Saline
PCR	Polymerase chain reaction
PMN	Polymorphonuclear cells
PQ	Paraquat

REP	Repetitive extragenic palindrome
RNA	Ribonucleic Acid
RNS	Reactive nitrogen species
ROS	Reactive Oxygen Species
RSNO	Nitrosothiols
S. Enteritidis	*Salmonella enterica* serovar Enteritidis
S. Typhi	*Salmonella enterica* serovar Typhi
S. Typhimurium	*Salmonella enterica* serovar Typhimurium
SCN^-	Thiocyanate ion
SCV	*Salmonella*-containing vacuole
SDS	Sodium Dodecyl Sulphate
SiRase	Sulphite reductase
SOD	Superoxide dismutase
SPI-2	*Salmonella* pathogenicity island 2
St	Streptomycin
Tc	Tetracycline
VFA	Volatile fatty acids

CHAPTER 1

INTRODUCTION

CHAPTER 1 Introduction

1.1 General Introduction

Oxygen began to accumulate in the atmosphere about 2 billion years ago. Most of the oxygen was probably produced by the first cyanobacteria as a by-product of photosynthesis (Koning, 1994), which uses light energy to split water molecules. Cyanobacteria were the first dominant organisms to use oxygenic photosynthesis (Koning, 1994). The released oxygen accumulated in the atmosphere and the iron in surface sediments was oxidised into red ferric oxide, marking the transition to an aerobic atmosphere (Koning, 1994).

Cyanobacteria produced an atmosphere of about 20% oxygen gas, which paved the way for the subsequent evolution of oxidative respiratory biochemistry (Koning, 1994). However, it created a serious problem insofar as oxygen related molecules collectively known as "reactive oxygen species" (ROS) are highly deleterious to many life processes if they are not controlled in some way. In short, the evolution of aerobic life had to go hand-in-hand with the evolution of mechanisms to avoid or to detoxify ROS.

The toxicity of ROS is highlighted by their exploitation in host defence systems to kill bacteria. For example, phagocytic cells such as macrophages pump millimolar amounts of superoxide into phagolysosomes containing an engulfed bacterium or virus. This is generally a highly effective means to inactivate such microbes.

By the same token, certain pathogenic microbes have not only evolved mechanisms to counter oxidative stress generated within them via normal aerobic metabolism, but have evolved additional strategies to cope with ROS produced by the host defence systems.

This thesis considers the oxidative stress response mechanisms of one such pathogen: *Salmonella enterica* serovar Typhimurium. Before reviewing what is known about the mechanisms by which *S.* Typhimurium copes with oxidative stress, it is appropriate to consider more general aspects of its biology as they relate to this study. These aspects are discussed in the following sections.

1.2 The health and economic consequences of *Salmonella* infections

Salmonellae are the etiological agents of a number of diseases, collectively known as salmonellosis. Human salmonellosis can de divided into four syndromes: typhoid fever, gastroenteritis, bacteraemia, and the asymptomatic carrier state (Nair *et al.*, 2002). Gastroenteritis is the most common manifestation of disease caused by *Salmonella*, and may be accompanied or followed by bacteraemia (Darwin and Miller, 1999).

Infections due to serovars of Sa*lmonella enterica* continue to be a major cause of mortality and morbidity worldwide. Recent years have seen a dramatic rise, both in terms of incidence and severity of cases of human salmonellosis. On a worldwide scale, there is an annual incidence of 1.3 billion cases of acute gastroenteritis or diarrhoea due to nontyphoidal salmonellosis, with 3 million deaths (Nair *et al.*, 2002). In many developing countries, typhoid fever, caused by *S.* Typhi, is still an important public health problem. The World Health Organisation has estimated that annually there are 16.6 million cases of typhoid fever, with nearly 600,000 deaths as quoted in Nair *et al.*, 2002.

Salmonellosis has a severe impact on farm economics as well as on public health. Serious problems in calves and adult cows such as septicaemia, diarrhoea and abortion are caused by various serovars of *Salmonella* (Veling *et al.*, 2001). The North-eastern United States, in particular, has experienced an increase of salmonellosis, which has spread throughout its veal and dairy beef-raising operations (McDonough *et al.*, 1999).

Salmonella serovars that primarily affect chickens have virtually been eradicated from intensively reared commercial flocks in Europe and North America. However, they still cause substantial economic losses of livestock in South America and Asia where intensification of the poultry industry is still in its infancy (Wigley *et al.*, 2001). In addition, other serovars of *Salmonella* with a broad host range continue to have a significant adverse economic impact on the egg industry, through decreased consumer confidence (Agron *et al.*, 2001).

The Food and Drink Federation of the United Kingdom calculated in its 1994 report that food borne illness, principally salmonellosis, costs the British economy approximately £1

billion annually. Similarly, a 1996 Economic Research Service report estimated that the annual total cost of food borne *Salmonella* infections in the United States in humans was between 0.6 to 3.5 billion dollars in medical expenses and productivity losses (Dargatz *et al.*, 1998; Buzby *et al.*, 1996).

Salmonella infections that afflict humans and animals continue to pose a serious medical and veterinary problem worldwide. Furthermore, salmonellosis has a severe impact on farm economics and places an unnecessary burden on the economy generally, as a result of productivity losses and medical expenses. The emergence of *Salmonella* strains that are resistant to antibiotics has hastened the need for a coordinated approach for disease monitoring and surveillance. Coupled with improved communication among animal industries, veterinary services, public health and government regulatory authorities, preventative and control measures for *Salmonella* infections can hopefully be successfully established.

1.3 Classification of *Salmonella*

Salmonella was first described by Eberth in 1880 and was cultured in 1884 by Gaffky (Burrows 1959; Darwin and Miller, 1999). *Salmonellae* are gram-negative, non-spore-forming rod-shaped bacteria belonging to the *Enterobacteriaceae* family. Most *Salmonellae* are motile, with the exception of the gallinarum-pullorum serotype (Hook, 1990). The *Salmonella* genus contains more than 2400 serovars, as differentiated by the Kauffmann-White scheme. The Kauffman-White scheme differentiates strains according to their reaction to sera. Classification is based on the lipopolysaccharide antigens of the cell wall (the O-antigens) and on the protein antigens of the flagella (the H-antigens), and strains identified in this way were given their own species designation e.g. *Salmonella typhimurium* (McWhorter-Murlin *et al.*, 1994; Darwin and Miller, 1999). However, it is now generally accepted that there is only a single species of *Salmonella*, *Salmonella enterica*, which is divided into six distinct subgroups (McWhorter-Murlin *et al.*, 1994; Darwin and Miller, 1999). Accordingly, the strain *Salmonella typhimurium* is officially designated *Salmonella enterica* serovar Typhimurium. However, throughout this study the nomenclature *S.* Typhimurium will be used.

1.4 Host range of *Salmonella*

Salmonella enterica serotypes differ widely in their host range. While some serotypes have a restricted host range, most are capable of causing disease in a broad-range of warm-blooded animals, and can also infect humans. The majority of the disease-causing *Salmonellae* belong to subgroup 1 (Hook, 1990; Tauxe and Pavia, 1998). Perhaps the most highly host-adapted serotype is *S.* Typhi, causing disease only in humans and higher primates (Rabsch *et al.*, 2002). Conversely, *S.* Typhimurium is considered to be the prototypical broad-host-range serotype, as it has been isolated from multiple species including humans, livestock, domestic fowl, rodents and birds (Rabsch *et al.*, 2002). *S.* Typhimurium causes gastrointestinal disease in humans, yet typically causes a lethal systemic infection in genetically susceptible mice (Morrow *et al.*, 1999).

With the advent of phage typing and other epidemiological typing methods, some *S.* Typhimurium variants have been found to have a very narrow host range. For example, *S.* Typhimurium variants that are associated with systemic disease in pigeons are considered to be highly host adapted (Rabsch *et al.*, 2002). Other highly host-adapted *S.* enterica serotypes include *S.* Dublin, which emerged for the first time in New York, Pennsylvania and Ohio in 1988. It is predominantly found in cattle and occasionally in swine, sheep, horses and zoological animals (McDonough *et al.*, 1999). *S.* Pullorum and *S.* Gallinarum are also restricted range serotypes, which cause pullorum disease, and fowl typhoid in chickens respectively (Kaiser *et al.*, 2000).

1.5 Disease state of *Salmonella*

1.5.1 Gastroenteritis

Gastroenteritis, or nontyphoidal salmonellosis is caused by *Salmonellae* other than *S.* Typhi. The two most common causes of this syndrome are *S.* Typhimurium and *S.* Enteritidis (Thong *et al.*, 1995). The incidence of non-typhoidal salmonellosis is increasing, and is the leading cause of food-borne disease in the United States, with an estimated 1.4 million cases annually (Kotetishvili *et al.*, 2002). The higher incidence of salmonellosis is thought to be due, in part, to an increase in the number of

immunocompromised or chronically ill people, changing agricultural and food distribution methods, and an increase in the proportion of the population older than 60 years (Darwin and Miller, 1999).

Although *S.* Typhimurium and *S.* Enteritidis cause gastrointestinal disease in a wide range of host species, they rarely cause systemic infections that are life-threatening (Clegg and Clegg, 1984). However, they can colonize the gut of poultry, thereby leading to horizontal transmission to other birds by the faecal-oral route. In turn, faeces may contaminate meat at the time of slaughter (Kaiser *et al.,* 2000). *S.* Enteritidis can also infect chicken ovaries before the eggshell is formed, as it may colonize the reproductive tract, thereby allowing transmission through intact eggs (Agron *et al.*, 2001; Kaiser *et al.*, 2000). Eggs can also be contaminated through cracks in the shell (Darwin and Miller, 1999). Internally infected eggs can achieve concentrations of 10^{11} *Salmonella* cells per yolk if left at room temperature, and as egg-laying hens are usually asymptomatic, control of transmission can be difficult (Darwin and Miller, 1999; Ziprin, 1994; St. Louis *et al.*, 1988). Rodents currently serve as an animal reservoir for *S.* Enteritidis. This serovar was once used as a rodenticide and this has contributed to its current prevalence (Agron *et al.*, 2001).

Incidence of nontyphoidal salmonellosis in humans is often associated with the consumption of raw, undercooked, or contaminated eggs or poultry meat (Kaiser *et al.*, 2000; Thong *et al.*, 1995). However human-to-human transmission and direct animal-to-human transmission can occur (Darwin and Miller, 1999; Prost and Riemann, 1967; Tauxe and Pavia, 1998; Wilson *et al*, 1982).

It is estimated that between 10^5 and 10^{10} bacteria are required to initiate infection (Darwin and Miller, 1999; Glaser and Newman, 1982). In mammals orally infected by *S.* Typhimurium, the bacterium must overcome the acid environment of the stomach and compete with the normal flora of the intestinal tract. Entry into intestinal epithelial cells is mediated by a type III secretion system encoded on *Salmonella* pathogenicity island I, (Darwin and Miller, 1999). As a result, pro-inflammatory cytokines are produced (Kaiser *et al.*, 2000). Polymorphonuclear cells (PMNs) infiltrate *Salmonella*-infected intestinal tissue and release prostaglandins, which in turn are thought to promote fluid secretion (Darwin and Miller, 1999).

The incubation period is typically 6 to 48 hours, and symptoms include headache, abdominal pain, vomiting, and diarrhoea, which can contain blood, lymphocytes and mucus (Darwin and Miller, 1999). Muscle aches, malaise and fever may also be experienced. Symptoms are normally alleviated within one week, but the disease tends to be more severe in children under 12 months of age and in adults over 60. Additionally, *Salmonella* may be shed in the faeces for up to twenty weeks (Darwin and Miller, 1999).

Salmonellosis caused by *S.* Typhimurium and *S.* Enteritidis infection is best treated by replacing the fluid and salts, which are lost in vomit and diarrhoea. Drugs to soothe the irritable and overactive bowel may also be used (Clegg and Clegg, 1984).

1.5.2 Typhoid fever

S. Typhi is the causative agent of typhoid fever. The disease remains an important public health problem in many developing countries (Thong *et al.*, 1996). Although the incidence of typhoid fever is decreasing, the disease is particularly pronounced in Indonesia, which has one of the highest incidences at over 1000 cases per 100,000 inhabitants (Thong *et al.*, 1995). The main source of *S.* Typhi infection is from swallowing infected water or food via the faecal/oral route. A relatively large number of organisms are usually required to initiate infection ($\sim 10^{6}$-10^{9}) in healthy individuals (Hook, 1990). Following ingestion, *S.* Typhi penetrates the epithelium of the small intestine and disseminates throughout the reticuloendothelial system (Leclerc *et al.*, 1998). An incubation period of 8 to 14 days, which is clinically asymptomatic, is followed by symptoms that include fever, malaise, headaches, abdominal pain and mental confusion (Clegg and Clegg, 1978). In severe cases, ulceration of the intestines may occur. Should the ulcers perforate, the contents of the small bowel can pour into the abdominal cavity, potentially leading to death (Clegg and Clegg, 1978).

The incidence of typhoid fever is decreased with the provision of clean water that has been chlorinated and filtered (Thong *et al.*, 1996). However, the use of raw river water is still practiced in many developing countries, and transmission by this pathway is the main source of infection (Thong *et al.*, 1996). Carriers of the disease must also be educated regarding matters of personal hygiene if the spread of typhoid fever is to be prevented.

Following an attack of typhoid fever, patients may excrete organisms in the faeces for several weeks. Additionally, chronic carriers may shed bacilli in their faeces for several months, or even years (Clegg and Clegg, 1978).

Before the use of antibiotics, about 15 to 20 per cent of cases of typhoid died, mainly due to perforation of the bowel wall. However, the mortality rate has fallen to below 5 per cent with the use of antibiotics such as ampicillin and chloramphenicol (Clegg and Clegg, 1978).

1.6 Multidrug resistant *Salmonella*

The emergence of bacteria possessing multiple resistance to antibiotics has become a worldwide health problem. As a result, the therapeutic advantage of antibiotics is now under threat. The extensive use, and misuse of antibiotics in human and veterinary medicine, and in livestock production for disease prevention or as growth-promoting feed additives, has lead to an alarming spread and increase in multiple antibiotic-resistant bacteria (Cruchaga *et al.*, 2001; Moellering, 1998).

The occurrence of multidrug resistant (MDR) *S.* Typhimurium DT104 (definitive type [DT] or phage type) has caused concern to public and animal health agencies (Dargatz *et al.*, 1998). MDR DT104 is resistant to at least five antimicrobics namely ampicillin, chloramphenicol, streptomycin, sulfonamides and tetracycline (R-type ACSSuT) (Hogue *et al.*, 1997). Some isolates of MDR DT104 in the UK have shown additional resistance to trimethoprim (R-type ACSSuTTm) and fluoroquinolones i.e. ciprofloxacin (ACSSUTCp) (Dargatz *et al.*, 1998;Threlfall *et al.*, 1997). This emerging food borne pathogen was first detected in the United Kingdom (UK) in 1984, and has since been detected in several other countries including the United States, Canada, Germany, France, Austria and Denmark (Hogue *et al.*, 1997). In this strain, antibiotic resistance genes, with the exception of trimethoprim, are chromosomally encoded, as opposed to being plasmid-mediated (Dargatz *et al.*, 1998; Threlfall *et al.*, 1996). Therefore, removal of the selective pressure of antibiotics is not expected to remove resistance, as it might with plasmid-mediated resistance (Hogue *et al.*, 1997).

The transmission of MDR DT104 to humans can occur directly by contact with infected farm animals (cattle and sheep) and indirectly by consumption of contaminated foods such as beef, poultry, and unpasteurised milk. Infection may also be caused by direct contact with sick pets such as cats and dogs (Dargatz *et al.*, 1998).

The number of reported human isolates of MDR DT104 (R-type ACSSuT) increased from 259 in 1990 to 4006 in 1996. In 1996 MDR DT104 (R-type ACSSuT) accounted for more than 10% of human *Salmonella* isolates in the UK, causing an estimated 30,000-300,000 human infections (Hogue *et al.*, 1997). In addition, the proportion of DT104 isolated that are also resistant to ciprofloxacin and trimethoprim is also increasing (Hogue *et al.*, 1997). DT104 is now the most prevalent *Salmonella* strain isolated from humans in England and Wales (Hogue *et al.*, 1997). Similarly, in the US, *Salmonella* MDR DT104 (R-type ACSSuT) increased from 9% in 1990 to 33% in 1996 (Hogue *et al.*, 1997). Clinical features of MDR DT104 infection in humans may be diarrhoea, fever, headache, nausea, bloody stools, and vomiting (Dargatz *et al.*, 1998; Hoseck *et al.*, 1997). Symptoms may be more severe than other *Salmonella* infections. In a UK study, 41% of patients were hospitalised and 3% died. The case-fatality rate for other nontyphoidal salmonellosis is approximately 0.1% (Hogue *et al.*, 1997).

Most of the MDR DT104 isolates from animals have been found in adult cattle, calves swine, sheep, and poultry. In the US it has been isolated from cattle, sheep, goats, swine, wild-birds, dogs, cats, mice and horses (Dargatz *et al.*, 1998). Direct animal-to-animal transmission of this pathogen is thought to occur via the faecal-oral route. Additionally, MDR DT104 may be indirectly transmitted by use of contaminated feed and water and grazing land contaminated by slurry or sewerage (Radostits *et al* 1994; Smith, 1990; Dargatz *et al.*, 1998). In cattle, clinical signs of the disease caused by *S.* Typhimurium DT104 include fever, depression and mental dullness, decreased milk production, anorexia, and diarrhoea progressing to dysentery. However, animals may be asymptomatic carriers of the disease and shed large numbers of bacilli for up to 18 months (Dargatz *et al.*, 1997).

Treatment of MDR DT104 infection is problematic, as it is resistant to a number of antimicrobial drugs, as discussed. Therefore, efforts to reduce, and control the incidence of infection in animals and humans are thought to be of extreme importance. The implementation of farm management practices that have already been shown to be

effective in reducing the risk of other *Salmonella* infections must be practiced by livestock producers, farmers and veterinarians (Wall *et al.*, 1994; Dargatz *et al.*, 1998). In addition, consumption of unpasteurised milk or products made with unpasteurised milk should be avoided (Dargatz *et al.*, 1998).

Multiple resistance to antibiotics has also become an important problem among *S.* Typhi strains (Thong *et al.*, 1996; Pang *et al.*, 1992), especially in developing countries where it is endemic (Hirose *et al.*, 2001). For approximately forty years, chloramphenicol has been used successfully, to treat typhoid fever, however, MDR *S.* Typhi, is resistant to ampicillin, chloramphenicol, and trimethoprim-sulfamethoxazole (Hirose *et al.*, 2001). Accordingly, fluoroquinolones have become the drug of choice for the treatment of typhoid fever caused by the MDR strain. However, some *S.* Typhi strains that are resistant to fluoroquinolones have been reported (Hirose *et al.*, 2001).

Antibiotic resistance in *S.* Typhi is generally plasmid-mediated (Shanahan *et al.*, 1998). Resistance to chloramphenicol, ampicillin, trimethoprim, sulphonamides and tetracycline is often encoded by large (~180 kb) conjugative plasmids that originate from Southeast Asia (Shahahan *et al.*, 1998).

Infection with *S.* Typhi generally requires effective antimicrobial chemotherapy if mortality is to be reduced (Shanahan *et al.*, 1998). Alarmingly, MDR *S.* Typhi strains have been reported in the Indian subcontinent, Latin America, Egypt, Nigeria, China, Korea, and the Philippines (Shanahan *et al.*, 1998).

1.7 Life-cycle of *Salmonella* in an animal host

Following evacuation from an animal host, *S.* Typhimurium usually enters a nutrient-rich aquatic environment. However, due to dilution, nutrient limitation may rapidly be experienced. If expelled from a mammalian or avian host, the organism will be subjected to a temperature downshift. Additional environmental conditions such as low osmolarity and fluctuating pH may possibly result in the organism entering a viable, but non-culturable state, in which it can survive for long periods of time (Foster and Spector, 1995).

Once ingested by the host via contaminated food or water, *Salmonella* is exposed to the acidic conditions of the stomach. In the intestinal tract, it must compete with resident flora for nutrients (Darwin and Miller, 1999), and survive conditions of increased osmolarity, low oxygen levels, weak acids and intestinal bile salts (Foster and Spector, 1995). Penetration of the intestinal epithelium by *Salmonella* is mediated by a type III secretion system (Darwin and Miller, 1999). The organism then enters the reticuloendothelial system, and escapes humoral defense mechanisms, by residing in *Salmonella*-containing vacuoles (SCVs) within host macrophages (Foster and Spector, 1995; Fang and Vazquez-Torres, 2002). Although macrophages provide protected sites for intracellular replication and a means of extraintestinal dissemination (Vazquez-Torres and Fang, 2001), *Salmonella* is likely to experience oxidative stress, low pH, limitations of certain nutrients and antimicrobial peptides within the macrophage (Foster and Spector, 1995). There is evidence that *Salmonella* may avoid exposure to the oxidative burst by re-routing the vesicles carrying the NADPH oxidase and this is discussed in Section 1.8.3.1.

1.8 *Salmonella* stress survival strategies

During its life cycle, a number of stresses are imposed on *Salmonella* (Figure 1.1). The ability of *Salmonella* to sense and quickly respond to unscheduled environmental extremes is crucial to its survival, as is its ability to overcome antimicrobial manoeuvres imposed by an infected animal host. The pathogen is able to survive such encounters due to the rapid initiation of various survival responses to stress. These are now considered in turn, with the obvious emphasis on what is known about responses to oxidative stress.

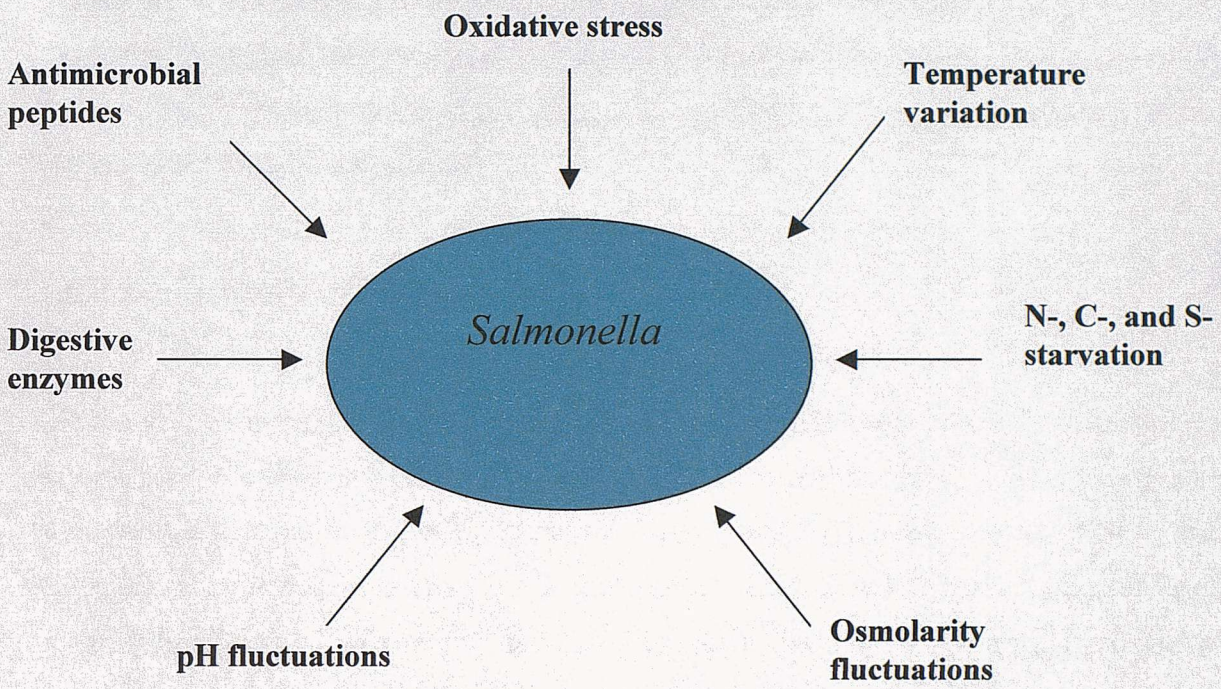

Figure 1.1. Stresses imposed on *Salmonella* during its life cycle. When expelled from a mammalian or avian host *Salmonella* experiences a temperature downshift and enters a nutrient-limited environment with low osmolarity and fluctuations in pH. Once ingested by a host it encounters the acidic environment of the stomach and digestive enzymes. Upon entering the intestine it is challenged by increased osmolarity and must compete with resident microorganisms for food. Within host macrophages *Salmonella* may also encounter the deleterious effects of antimicrobial peptides such as defensins. Furthermore, during its life cycle *Salmonella* experiences oxidative stress caused by exposure to reactive oxygen intermediates as a by-product of aerobic respiration, exposure to environmental agents and as a consequence of host invasion (Foster and Spector, 1995).

1.8.1 The acid tolerance response

Acidic pH is frequently encountered by *Salmonella.* While some bacterial species have evolved to the extent that their growth is optimal in extreme acid environments, *Salmonella* has developed survival systems that allow it to withstand and overcome exposure to low pH, even if growth is sub-optimal.

1.8.1.1 Sources of acid stress

During its lifecycle, *S.* Typhimurium, a neutralophile, encounters a broad range of acid stresses both in nature and during pathogenesis. Understandably, *Salmonella* prefers to live and grow at a pH near neutrality (Bearson *et al.*, 1998; Foster and Spector, 1995). The extreme low pH of the stomach is the first line of defence in the host armoury against *Salmonella* infection. Volatile fatty acids (VFA) such as butyrate, propionate, or acetate in the intestine and faeces present a further threat to the pathogen (Foster, 2000). Additionally, the acidic pH of the macrophage environment must also be endured during the infection process (Foster, 2000). *Salmonella* may also encounter VFAs in food products such as meat carcasses, salad dressing and mayonnaise, as acetate and propionate are used as preservatives due to their antibacterial properties (Kwon and Ricke, 1998).

1.8.1.2 Deleterious effects of acid stress

Acid stress is defined as the combined biological effects of H^+ ion and weak acid concentrations (Foster, 2000). The bacterial cell membrane has a low permeability to protons, however, in conditions of extreme low external pH (pH_o), H^+ will leak across the membrane. As a result, the internal pH (pH_i) is acidified, and biochemical reactions and macromolecular structures are, in turn, deleteriously affected (Foster, 2000). Even a mild acid stress (pH 5), can be potentially lethal to the cell when combined with 200 mM acetate. Acetate in its protonated form can permeate the bacterial cell membrane and dissociate, thereby acidifying the internal pH. Following dissociation, the organic acid is membrane-impermeable in its ionised form. It accumulates intracellularly, and causes further damage to the cell (Bearson *et al.*, 1998).

1.8.1.3 pH homeostasis

Enteric bacteria such as *S.* Typhimurium and *E. coli* have a remarkable ability to adapt to dramatic fluctuations in pH. Where a more moderate change in H^+ concentration above or below optimum growth pH i.e. 1 pH unit, occurs, the principal defense involves housekeeping pH homeostasis systems (Foster, 2000). A stable internal pH is maintained by pumps that generate pH gradients. At alkaline pH_o, sodium-proton antiporters move protons into the bacterial cell. Conversely, at acidic pH_i, potassium-proton antiporters extrude protons (Foster, 2000).

1.8.1.4 Mechanisms of acid tolerance in *Salmonella*

The acid tolerance response (ATR) of *S.* Typhimurium is an inducible acid survival system, in which exposure to a moderately acid environment produces a stress response that can protect the organism from more extreme acid conditions (Lee *et al.*, 1995). Two pH-dependent ATR systems exist in *S.* Typhimurium: A log-phase ATR and a stationary-phase ATR (Foster, 2000) (Figure 1.2).

1.8.1.4.1 The log-phase ATR

Induction of the log-phase ATR allows *S.* Typhimurium to withstand exposure to severe acid challenge (Hall and Foster, 1996). When exponential-phase cells are exposed to acid shock (pH 4.4) for 20 minutes or more, they can survive a subsequent, otherwise lethal acid shock (pH 3.3 to 3.0) 100- to 1,000-fold better than unadapted cells (Hall and Foster, 1996). Acid adaptation is thought to induce the differential expression of at least 50 polypeptides, termed acid shock proteins (ASPs), which are thought to contribute to subsequent acid survival (Adams *et al.*, 2001; Foster, 1999; Foster, 1995).

The log-phase ATR involves several regulatory genes, including the alternate sigma factor σ^s (encoded by *rpoS*), the iron regulator Fur and the two-component signal transduction system PhoPQ (Foster, 2000) (Figure 1.2). Each regulator is responsible for the induction

of a subset of ASPs. PhoP and σ^s are induced when exponential cells are acid shocked, but Fur is not known to be induced by acid (Foster, 2000).

S. Typhimurium *fur* mutants display an acid sensitive phenotype and are defective in the induction of several ASPs (Escolar *et al.*, 1999; Hall and Foster, 1996). The acid sensing and the iron-sensing mechanisms mediated by Fur are thought to be separate, as defined by mutations in the protein. Although a H90R Fur mutant maintains its ability to mediate acid induction of specific Fur-dependent ASPs, and hence acid tolerance, it cannot mediate iron regulation (Escolar *et al*, 1999; Hall and Foster, 1996). Conversely, a H144L Fur mutant displayed an acid-blind/iron-sensing phenotype (Foster, 2000).

The alternate sigma factor, σ^s plays an important role in the log-phase ATR, as it is required to survive exposure to volatile fatty acids and aids inorganic acid tolerance (Foster, 2000; Baik *et al.*, 1996). *Salmonella rpoS* mutants only transiently induce an ATR, therefore sustained induction of the ATR is said to be σ^s-dependent (Bearson *et al.*, 1998; Lee *et al.*, 1995). A number of genes that encode acid shock proteins in a σ^s-dependent manner have been identified, including *osmY*, which encodes a periplasmic protein of unknown function, and $sodC_{II}$, a periplasmic superoxide dismutase (Foster, 2000). Although levels of σ^s are barely detectable in exponential-phase cells, due to rapid proteolytic turnover by the ClpXP protease, σ^s levels rise following acid shock (Foster, 2000; Bearson *et al.*, 1996). The mouse virulence protein MviA is thought to signal decreased proteolytic turnover of σ^s, following acid shock, and thereby modulate σ^s levels via ClpXP (Bearson *et al.*, 1996).

The two-component PhoPQ system is the second σ^s-independent regulatory system that is implicated in the log-phase ATR, and is important for tolerance to inorganic acid stress (Bearson *et al.*, 1996). The PhoPQ system is directly activated by acid pH and in response to low external concentrations of Mg^{++} ions (Adams *et al.*, 2001). The response regulator protein, PhoP has itself been identified as an ASP, and is required for the expression of several other ASPs (Bearson *et al.*, 1996). Recently, two proteins that are down regulated during the log-phase ATR were identified as the major structural proteins of the flagellar filament, flagellin phase 1 and 2 (Adams *et al.*, 2001). In addition, acid repression of flagellin has been found to operate through the PhoPQ system (Adams *et al.*, 2001).

1.8.1.4.2 Stationary-phase acid tolerance response

The ATR in stationary phase cells involves σ^S-dependent and -independent systems (Foster, 2000) (Figure 1.2). *Salmonella rpoS* mutants exhibit a decrease in unadapted acid tolerance, during stationary phase, but are still able to adapt to normal levels in response to acid shock. Therefore, the σ^S-dependent system is considered to be pH-independent, and is not required for subsequent acid-induced tolerance of low pH. It appears to be part of a general stress response that provides a basal level of acid tolerance, which is induced by entry into stationary phase (Lee *et al.*, 1994).

The σ^S-independent stationary-phase ATR is induced by exposing stationary-phase cells to low pH (Bang *et al.*, 2000). Ten, unique stationary-phase ASPs have been identified (Lee *et al.*, 1994), one of which is OmpR (Bang *et al.*, 2000) (Figure 1.2). Acid shock has been found to lead to a significant increase in *ompR* message and OmpR protein (Bang *et al.*, 2000). OmpR is the response regulator of a two-component system that regulates the osmotically controlled genes *ompC* and *ompF* (Forst *et al.*, 1990). OmpR is considered to be active, only in its phosphorylated form. It is phosphorylated by the histidine kinase EnvZ (Aiba *et al.*, 1989). However, the cognate sensor kinase, EnvZ, and the porin genes, *ompC* and *ompF*, are not required for stationary-phase ATR (Bang *et al.*, 2000). In the absence of EnvZ, OmpR is phosphorylated by acetyl phosphate (Bang *et al.*, 2000). Evidence suggests that OmpR-P, formed from acetyl phosphate as the phosphodonor, is the form required to induce acid tolerance (Bang *et al.*, 2000).

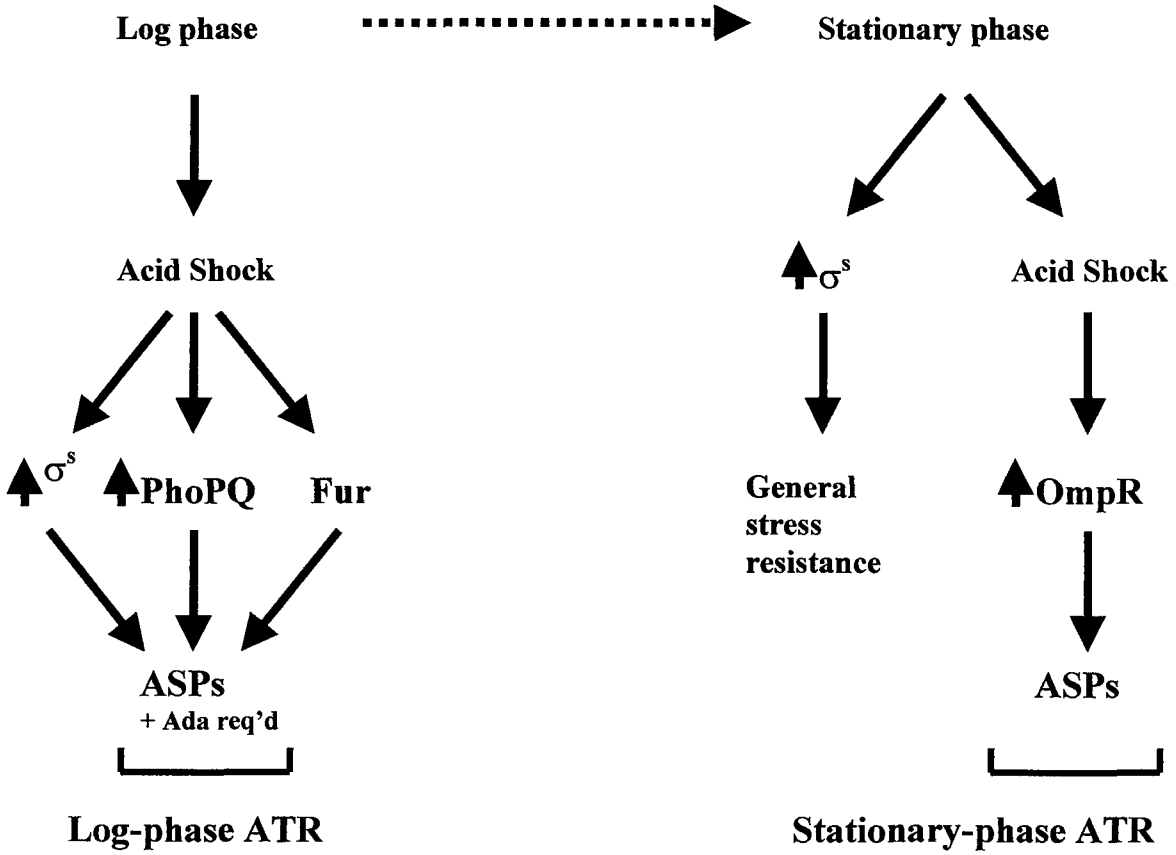

Figure 1.2. The log-phase and stationary-phase acid tolerance responses of *S.* Typhimurium. Log-phase cells treated with acid (pH 4.5) induces σ^s and PhoPQ. Fur is not known to be induced by acid. σ^s, PhoPQ and Fur each induce a subset of ASPs. The Ada protein is required for the log-phase ATR but does not appear to regulate the expression of other ASPs or to be induced by acid. A general stress resistance is induced on entry to stationary phase that is σ^s -dependent. Acid-shocked stationary phase cells also induce OmpR, which is required for the stationary-phase ATR. This diagram is adapted from Foster, (2000).

1.8.2 Antimicrobial peptides

As already discussed, facultative intracellular pathogens such as *S.* Typhimurium encounter a wide range of host defence mechanisms during their life cycles, which they must evade in order to cause disease. One such non-oxidative killing mechanism used by various animal species including mammals, insects and amphibians, is the production of cationic antimicrobial peptides (CAMPs) (Gunn and Miller, 1996). Antimicrobial, cationic peptides comprise between 12 and 50 amino acids, of which 50% are hydrophobic, with at least two excess positive charges (Hancock and Scott, 2000).

1.8.2.1 Mechanism of action of antimicrobial peptides

Over 500 such antimicrobial peptides have been discovered. They fit into at least 4 structural classes, namely, β-sheet, comprising 2 to 3 β-strands stabilised by disulphide bridges, amphipathic α-helices, extended structures, and loop structures (Hancock and Scott, 2000).

Examples of mammalian CAMPs include HNP-1 (α-defensin), HBD-2 (β-defensin), protegrin, bactenicin, LI-37, cecropin P1 and indolicidin (Hancock and Scott, 2000). Animal CAMPs include bactericidal permeability increasing protein (BPI), lactoferrin, transferrin, cathepsin G and cystatin (Hancock and Scott, 2000). CAMPs are found within neutrophil granules, macrophage phagosomes and secretions of mucosal epithelia (Gunn and Miller, 1996), as well as skin surfaces (Guina *et al.*, 2000).

Hancock and Scott (2000), have proposed that cationic peptides exert their deleterious effects on Gram-negative bacteria by initially interacting with and crossing both cell envelope membranes. Killing is thought to be by a multihit mechanism, that involves action on a number of anionic targets. They propose that peptides cross the outer bacterial cell membrane via self-promoted uptake (Hancock and Chapple, 1999; Sawyer *et al.*, 1988). The peptides are thought to interact with the polyanionic surface lipopolysaccharide (LPS), thereby causing disruption of the membrane, which, in turn, facilitates the passage of peptide molecules. Next, the peptides insert into the negatively charged phospholipid membrane. When they reach a critical concentration, they form informal transmembrane

channels. As a result, the cell may die due to severe permeability problems caused by the channels. Any peptides remaining in the inner monolayer following collapse of the channels may then dissociate and interact with cytoplasmic polyanions such as DNA (Zhang *et al.*, 1999; Hancock and Scott, 2000).

1.8.2.2 Resistance to antimicrobial peptides

The cationic antimicrobial peptide response is an important component of innate immunity. It allows animals to sense and control invading microbes (Guina *et al.*, 2000). Inducible peptide responses are less specific than other immune responses. Nor do they require clonal expansion (Hancock and Scott, 2000). As a result, CAMPs are able to effect a rapid antimicrobial response within minutes rather than days. Patients who have the condition, specific granule deficiency, almost completely lack defensins, and succumb to frequent, and often severe microbial infections (Groisman *et al.*, 1992; Ganz *et al.*, 1988).

Gram-negative bacteria are able to increase their resistance to CAMPs by altering the structure, immunogenic properties, and permeability of their surfaces (Guina *et al.*, 2000). The *S.* Typhimurium PhoPQ locus has been found to be involved in antimicrobial peptide resistance (Groisman *et al.*, 1992). *Salmonella* PhoP mutants are susceptible to a number of CAMPs, including the mammalian defensins, the frog-derived magainin 2, the insect-derived melittin and mastoparan, and polymixin B (Groisman, 2001). The PhoPQ two-component system is a major regulator of virulence in *Salmonella*. Indeed, PhoPQ mutants of *S.* Typhimurium are attenuated for virulence in mice (Adams *et al.*, 1999). The system mediates the adaptation to Mg^{2+}-limiting environments and regulates numerous cellular activities in *Salmonella* (Groisman, 2001). The inner membrane sensor, PhoQ senses environmental signals and transfers phosphate to the cytoplasmic regulator PhoP, which in turn activates genes termed *pag* (PhoP-activated gene) and represses genes termed *prg* (PhoP-repressed gene)(Gunn and Miller, 1996; Groisman, 2001). The PhoPQ system regulates the expression of over 40 proteins (Miller and Mekalanos, 1990; Gunn and Miller, 1996). Micromolar concentrations of Mg^{2+} activate transcription of *pag* in a PhoP- and PhoQ-dependent manner (Groisman, 2001). Additionally, Mg^{2+} can repress transcription of PhoP-activated genes. When wild-type *Salmonella* is grown in LB it is more than 1000-fold more resistant to the CAMP magainin 2, than when it is grown in LB

supplemented with 25 mM Mg^{2+} (Vescovi *et al.*, 1996). The system is also activated by acid pH (Adams *et al.*, 2001).

The primary defence mechanism against cationic peptides in Gram-negative bacteria is LPS (Groisman, 1994). Modifications to lipid A, the hydrophobic anchor for LPS in the OM has been found to be regulated by PhoPQ (Groisman, 2001). The PhoP-activated outer membrane protein PagP mediates the transfer of palmitate into the lipid A moiety of the LPS, thereby forming heptaacylated lipid A. *Salmonella pagP* mutants are hypersensitive to the synthetic α-helical peptide C18G, but display wild-type resistance to polymyxin and protegrin (Guo *et al.*, 1998; Groisman, 2001). The *pagP* gene is thought to encode an acyltransferase or a nonenzymatic inner membrane protein complex component that is essential for the transfer of palmitate to lipid A (Guo *et al.*, 1998). Although similarities exist between antimicrobial peptides, it appears that different PhoP-regulated determinants may mediate resistance to different CAMPs (Groisman, 2001).

The PhoPQ system of *S.* Typhimurium has been shown to regulate another two-component regulatory system known as PmrAB (Gunn and Miller, 1996). The *pmrAB* operon comprises three genes, the first of which is *pmrC* (previously identified as *pagB*) (Roland *et al.*, 1993; Groisman *et al.*, 1989; Miller *et al.*, 1998). The second and third genes in the operon are *pmrA* and *pmrB* respectively (Roland *et al.*, 1993). An increased resistance to the positively charged antibiotic polymyxin B has been displayed by a *S.* Typhimurium *pmrA* constitutive mutant (Vaara *et al.*, 1981). In addition, an increased resistance to the human neutrophil proteins CAP37 and CAP57 was also seen in the mutant (Vaara *et al.*, 1981). *S.* Typhimurium strains that constitutively express PmrA have been shown to have a high degree of substitution of the 4' phosphate of lipid A by 4-aminoarabinose (4AA) (Gunn *et al.*, 1998). This substitution reduces the net negative charge of the LPS and affords polymyxin B resistance (Groisman *et al.*, 1997; Gunn *et al.*, 1998;Groisman *et al*, 2001).

Another PhoPQ regulated gene, *pgtE,* which encodes an outer membrane protease, is also involved in resistance to CAMPs. (Guina *et al.*, 2000; Groisman, 2001; Adams *et al.*, 1999). PhoPQ is thought to regulate the expression of PgtE post-transcriptionally (Guina *et*

al., 2000). The membrane protease is thought to cleave the cationic peptide C18G, thereby conferring resistance (Guina *et al.*, 2000; Groisman, 2001).

Resistance to the cationic peptide protamine is mediated by a group of seven *sap* (sensitivity to antimicrobial peptides) proteins (Foster and Spector, 1995; Aspedon and Groisman, 2000). Antimicrobial peptides are thought to bind to SapA in the periplasm and be transported into the cytoplasm via the SapBCDF transporter, which belongs to the ATP-binding cassette (ABC) transporter family (Foster and Spector, 1995; Aspedon and Groisman, 2000). It is thought that cytoplasmic peptidases cleave protamine into inactive fragments (Foster and Spector, 1995; Aspedon and Groisman, 2000).

1.8.3 The Oxidative stress response

Oxidative stress may be defined as a disturbance in the prooxidant-antioxidant balance in favour of prooxidants (Sies, 1985; Storz and Zheng, 2000). Therefore, conditions that result in elevated levels of reactive oxygen species or the depletion of antioxidant molecules or enzymes can result in such an imbalance and can constitute an oxidative stress (Storz and Zheng, 2000).

1.8.3.1 Reactive oxygen species encountered and their sources

Reactive oxygen species encountered by *Salmonella* include hydrogen peroxide (H_2O_2), superoxide anion ($O_2^{\cdot-}$) and hydroxyl radical ($HO^{\cdot}$), which are derived from the sequential univalent reductions of molecular oxygen (Gonzalez-Fletcha and Demple, 1995). Molecular oxygen is a triplet species that can passively diffuse into cells (Imlay, 1999). Electron transfers to oxygen from singlet donors must occur univalently (Messner and Imlay, 1999) because a spin restriction kinetically impedes multivalent reduction (Imlay and Fridovich, 1991; Naqui *et al.*, 1986). The immediate product of univalent electron transfer to oxygen is $O_2^{\cdot-}$ (Messner and Imlay, 1999). The effects of these reactive oxygen intermediates have also been linked to the damage caused by hypochlorous acid (HOCl), and the reactive nitrogen intermediates nitric oxide ($NO^{\cdot}$), peroxynitrite (HOONO), and nitrosothiols (RSNO) (Storz and Imlay, 1999).

S. Typhimurium follows an aerobic lifestyle, which is not without jeopardy, because oxidative stress is an unavoidable by-product of respiration (Storz and Imlay, 1999). The intracellular steady state concentration of H_2O_2 is thought to be 0.15 μM in exponentially growing *E. coli* cells and can be assumed to be similar in *Salmonella* (Gonzalez-Fletcha and Demple, 1995). When *E. coli* cells are treated with carbonyl cyanide p-trifluoromethoxyphenylhydrazone (FCCP), which acts to uncouple electron transport, the H_2O_2 steady-state concentration, and the production rate of H_2O_2 decreases to approximately one-seventh the value of untreated cells (Gonzalez-Fletcha and Demple, 1995). Therefore, the respiratory chain is considered to account for approximately 87% of the H_2O_2 generation in intact bacteria (Gonzalez-Fletcha and Demple, 1995). The rate of H_2O_2 production in exponentially growing *E. coli* cells has been calculated to be ~4μM s^{-1}. This is thought to be in agreement with the theoretical 2:1 stoichiometry for superoxide: hydrogen peroxide, and therefore suggests that most of the H_2O_2 generation in *E. coli* is considered to arise as a by-product of $O_2^{\cdot-}$ generation by the reaction;

$2O_2^{\cdot-} + 2H^+ \longrightarrow H_2O_2 + O_2$

Gonzalez-Fletcha and Demple (1995) have shown that the inactivation of either NADH dehydrogenase 1 or NADH dehydrogenase 2 decreases the rate of hydrogen peroxide production by 25-30% (Gonzalez-Fletcha and Demple, 1995). In contrast, Imlay and Fridovich (1991) showed that inactivation of NADH dehydrogenase 2 decreased the production of $O_2^{\cdot-}$ in isolated membranes by 92% when compared to the parent strain. This deviation may be explained by the observation that reducing equivalents can still enter the respiratory pathway at the ubiquinone level via alternative dehydrogenases in intact bacteria lacking NADH dehydrogenases (Ingledew and Poole, 1984; Gonzalez-Fletcha and Demple, 1995).

Messner and Imlay (1999) have recently suggested that NADH dehydrogenase II is the primary site of electron transfer to oxygen in *E. coli*. Conversely, NADH dehydrogenase I, respiratory quinines, or cytochrome oxidases were found to form little or no H_2O_2 or $O_2^{\cdot-}$ (Messner and Imlay, 1999). This is based on the observation that null mutations in the gene encoding NADH dehydrogenase II averted autoxidation of vesicles prepared from *E. coli* cells, whereas, overproduction of NADH dehydrogenase II accelerated vesicle autoxidation (Messner and Imlay, 1999). Furthermore, it has been shown that when the reduced forms of the autoxidizing enzymes NADH dehydrogenase II, succinate

dehydrogenase, sulphite reductase, and fumarate reductase were exposed to molecular oxygen, each formed $O_2^{\cdot-}$ and H_2O_2 (Messner and Imlay, 2002). All of these enzymes contain flavins and either iron-sulfur clusters or quinones, and as a result are capable of univalent redox reactions (Messner and Imlay, 2001). However, the flavins have been found to be the predominant sources of $O_2^{\cdot-}$ and H_2O_2 (Messner and Imlay, 1999). Other moieties, such as the iron-sulphur clusters and the quinones may not react with oxygen because they are sequestered in environments that are hydrophobic or sterically inaccessible to oxygen (Messner and Imlay, 1999).

Fumarate reductase (Frd) acts as a terminal oxidase during anaerobic respiration (Imlay, 1995). The two enzymes NADH dehydrogenase II and Frd have been identified as significant sources of $O_2^{\cdot-}$ in respiring *E. coli* vesicles (Imlay, 1995). However when *E. coli* is grown in aerobic media, very little Frd is synthesised, and therefore is considered unable to contribute a significant source of $O_2^{\cdot-}$ (Imlay, 1995). It has been suggested, therefore, that Frd may confer particular oxidative stress when cells are transferred from anaerobic to aerobic environments, for example, following excretion from the anaerobic colon into oxygen-rich water surfaces (Imlay, 1995; Storz and Imlay, 1999).

Cytosolic redox enzymes are not thought to contribute significantly to the generation of superoxide anion, in part because they carry out the concerted transfer of a pair of electrons and are thought not to be prone to univalent autoxidation, unlike the components of the respiratory chain (Imlay and Fridovich, 1991). Reduced flavoenzymes such as the respiratory dehydrogenases have the tendency to generate superoxide anion due to the stability of the flavosemiquinone product (Massey *et al.*, 1969; Imlay and Fridovich, 1991). The majority of redox-active cytosolic enzymes are not flavoproteins thereby reducing the propensity of univalent autoxidation (Imlay and Fridovich, 1991).

Redox cycling drugs such as paraquat also generate intracellular $O_2^{\cdot-}$. Once intracellular, paraquat catalyses the transfer of electrons from redox enzymes such as sulphite reductase (SiRase) (SiRase comprises a second electron transport chain in *E. coli*) to oxygen (Figure 1.3) (Storz and Imlay, 1999; Messner and Imlay, 1999).

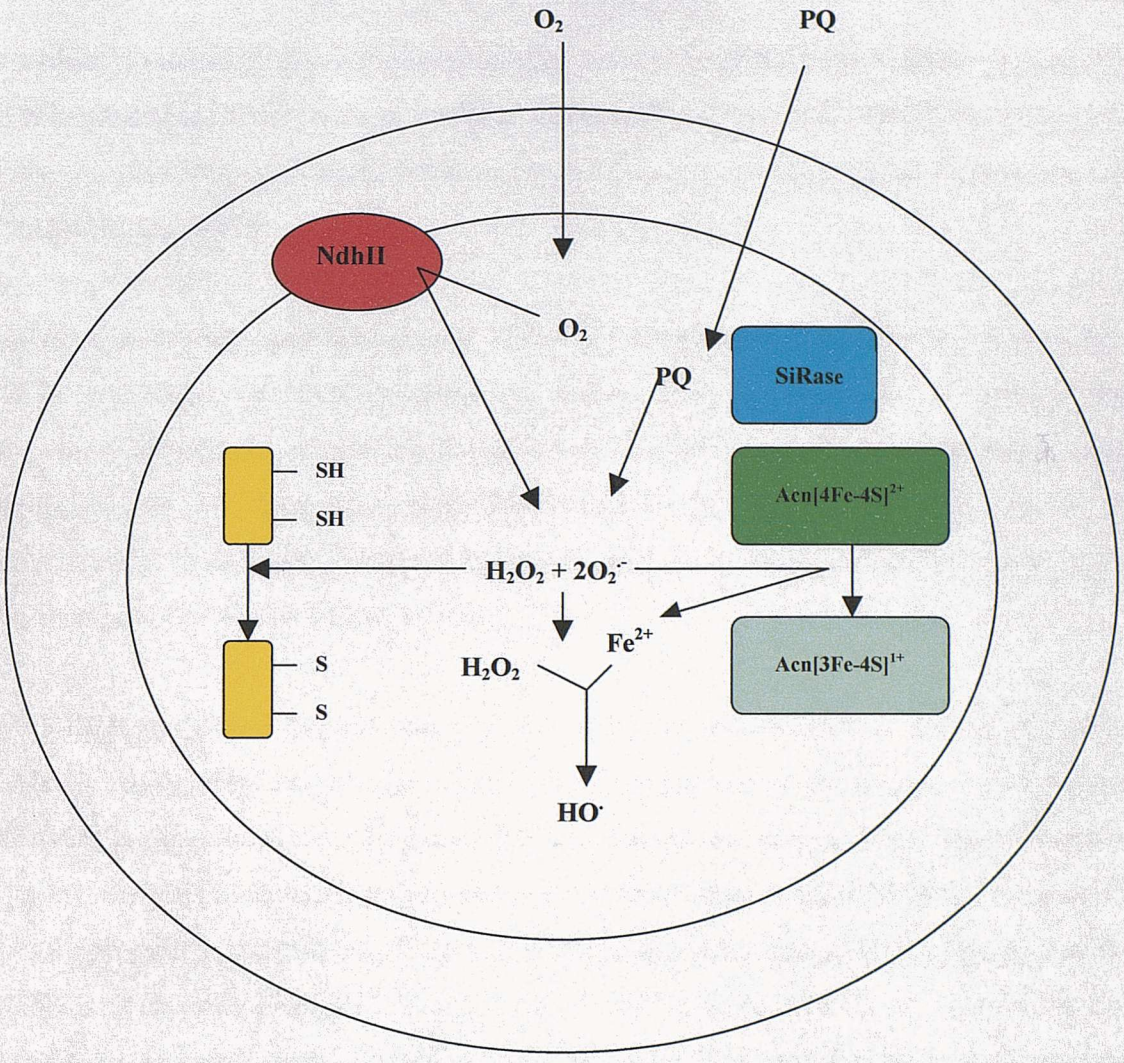

Figure 1.3. Mechanisms of oxidative cell damage by the generation of endogenous oxidants. Molecular oxygen enters the cell by passive diffusion and is converted to $O_2^{\cdot-}$ and H_2O_2 by the direct oxidation of flavoproteins, including NADH dehydrogenase (NdhII). Paraquat (PQ) accelerates the formation of reactive oxygen species (ROS) by catalysing the transfer of electrons from redox enzymes such as sulphite reductase (SiRase) to oxygen. Superoxide radical oxidatively destroys iron-sulphur clusters. The released iron can react with H_2O_2 to form hydroxyl radical, which directly damages DNA. H_2O_2 can also directly oxidise protein cysteinyl residues. This diagram is adapted from Storz and Imlay (1999).

The hydroxyl radical is only a transient species in most bacterial cells because the majority of it is produced upon exposure to ionising radiation (Madigan *et al*, 1997). However, $HO^{\cdot}$ can also be generated as a result of the reaction of H_2O_2 with iron, via the Fenton reaction (Storz and Imlay, 1999).

Phagocytes bombard engulfed bacteria with $O_2^{\cdot-}$, nitric oxide ($NO^{\cdot}$), hyperchlorous acid (HOCl), and their chemical by-products, H_2O_2, $HO^{\cdot}$, peroxynitrite (HOONO), and nitrosothiols (RSNO) by employing the action of NADPH phagocyte oxidase, nitric oxide synthase (NOS) and myeloperoxidase (Storz and Imlay, 1999). At neutral pH, $O_2^{\cdot-}$ is unable to cross membranes, however it may be able to in the acidic environment of the phagolysosome (Storz and Imlay, 1999).

The NADPH phagocyte oxidase catalyses the univalent reduction of molecular oxygen to $O_2^{\cdot-}$ which can be metabolised to a variety of toxic reactive oxygen species. As mentioned previously, patients with chronic granulomatous disease are deficient in NADPH oxidase and are susceptible to recurrent *Salmonella* infections (Mouy *et al.*, 1989; Mastroeni *et al.*, 2000). Furthermore, when the gp91*phox* gene that encodes an essential component of the NADPH oxidase was disrupted in mice, the oxidative burst activity of neutrophils and macrophages was eliminated, resulting in an increased susceptibility to *S.* Typhimurium (De Groote *et al.*, 1997).

NADPH oxidase is required for rapid, early killing of *Salmonella* by macrophages (Vazquez-Torres *et al.*, 2000). A sustained, subsequent bacteriostatic role is thought to follow, by the action of inducible nitric oxide synthase (iNOS) (Vazquez-Torres *et al.*, 2000). Nitric oxide ($NO^{\cdot}$) is generated by nitric oxide synthase (NOS) isoforms in a reaction that consumes NADPH, oxygen and L-arginine (MacMicking *et al.*, 1997; Vazquez-Torres *et al.*, 2000). $NO^{\cdot}$ is considered to have a weak antimicrobial effect on *Salmonella* (De Groote *et al.*, 1995). However, the autoxidation of $NO^{\cdot}$ produces reactive nitrogen species (RNS) that are cytotoxic to *Salmonella* (Fang, 1997; Vazquez-Torres, 2000). Peroxynitrite, which is formed by the reaction of $NO^{\cdot}$ with $O_2^{\cdot-}$ has been associated with the enhanced killing of *S.* Typhimurium and *E. coli* (Vazquez-Torres *et al.*, 2000).

As mentioned previously, recent evidence suggests that a type III secretory system encoded by *Salmonella* pathogenicity island 2 (SPI-2) enhances the survival of *Salmonella*

within macrophages (Vazquez-Torres and Fang, 2001). Following phagocytosis the NADPH phagocyte oxidase complex assembles in vesicles and produces toxic reactive oxygen species (Vazquez-Torres and Fang, 2001). However, SPI-2 appears to prevent the NADPH phagocyte oxidase-containing vesicles from trafficking towards SCVs (Figure 1.4). Instead, these vesicles are re-routed to the plasma membrane and to uninfected vacuoles, thereby reducing the oxidant stress encountered by *Salmonella* (Vazquez-Torres and Fang, 2001). In addition, *Salmonella* may also be protected from the antimicrobial actions of reactive nitrogen species. Recent evidence suggests that SPI-2 also interferes with the trafficking of vesicles containing inducible nitric oxide synthase (iNOS) to the SCV (Fang and Vazquez-Torres, 2002).

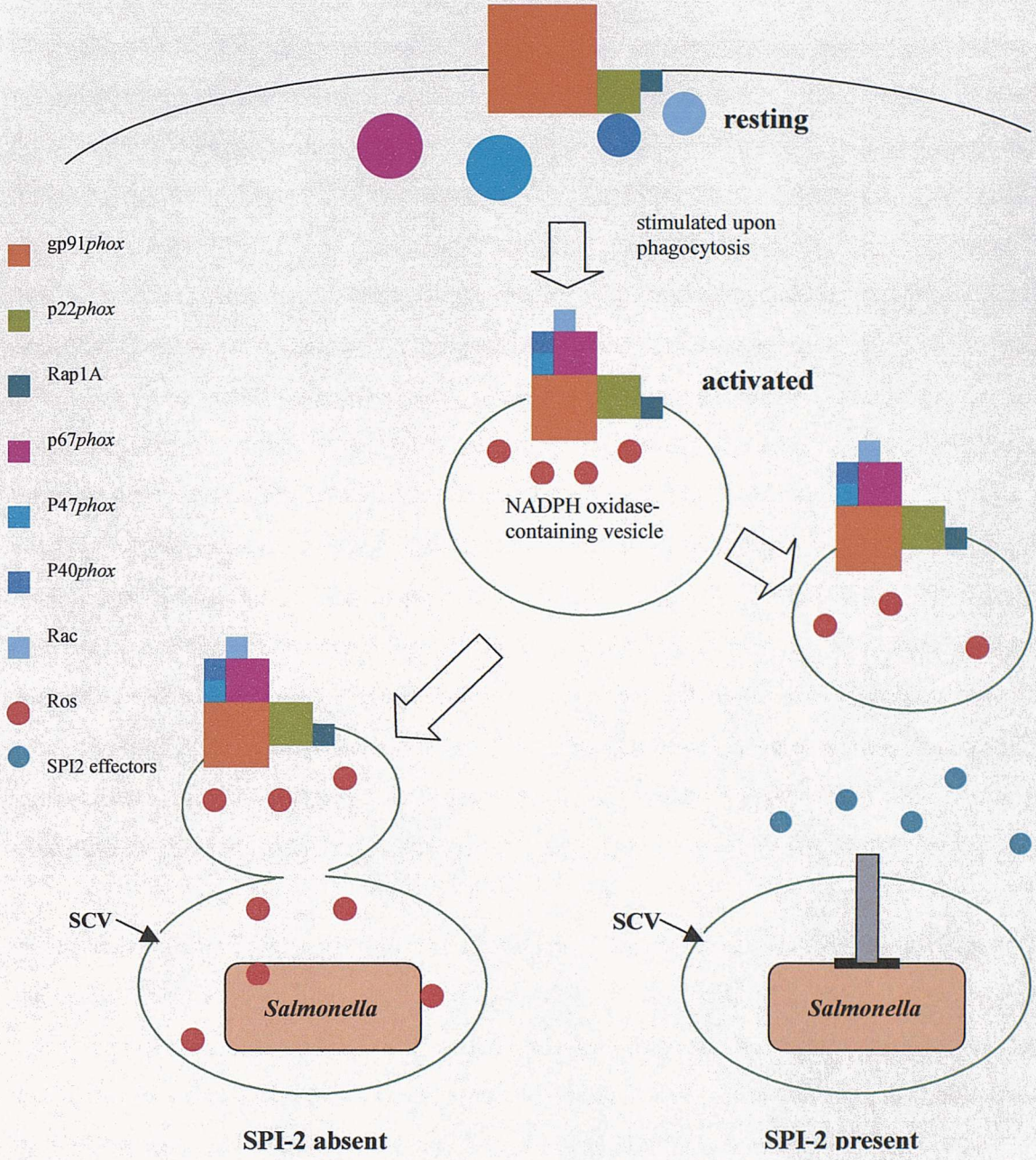

Figure 1.4. SPI-2-dependent evasion of NADPH phagocyte oxidase-containing vesicles. The components of the NADPH phagocyte oxidase are stimulated upon phagocytosis of *Salmonella* and the p67*phox*, p47*phox* and p40*phox* cytosolic components translocate via the cytoskeleton for association with the membrane-bound subunits gp91*phox*, p22*phox* and Rap1A together with cytosolic Rac proteins. Trafficking of NADPH oxidase-containing endosomes to the vicinity of the *Salmonella*–containing vacuole (SCV) is prevented by effector proteins secreted by the type III secretory system encoded by SPI-2. Adapted from Vazquez-Torres and Fang (2001).

1.8.3.2 Targets of oxidative stress

The cytosolic superoxide dismutases catalyse the conversion of superoxide radical to hydrogen peroxide and oxygen. Analysis of *E. coli* mutants lacking the cytosolic SODs has highlighted the targets of $O_2^{\cdot-}$ (Figure 1.3)(Gort and Imlay, 1998). When grown aerobically, SOD-deficient mutants are auxotrophic for branched-chain, aromatic, and sulphur-containing amino acids and can only catabolise fermentable carbon sources (Gort and Imlay, 1998; Carlioz and Touati, 1986; Imlay and Fridovich, 1992). In addition SOD-deficient mutants of *E. coli* exhibit a high rate of aerobic mutagenesis (Farr *et al.*, 1986), and when combined with attenuated DNA-repair systems, these mutants cannot grow aerobically at all (Touati *et al.*, 1995; Keyer *et al.*, 1995). The observed phenotypes arise because $O_2^{\cdot-}$ inactivates dihydroxyacid dehydratase (Gort and Imlay, 1998; Flint and Emptage, 1990; Kuo *et al*, 1987), aconitase (Gardner and Fridovich, 1991; Gort and Imlay, 1998), and fumarases A and B (Gort and Imlay, 1998; Flint *et al*, 1993; Liochev and Fridovich, 1993). These enzymes all have a common factor, in that they contain a distinctive [4Fe-4S] cluster, which helps to bind and dehydrate the substrate, due to its local positive charge (Gort and Imlay, 1998). In the absence of substrate, the cluster is exposed and can be oxidised by $O_2^{\cdot-}$. As a result, the cluster is destabilised causing the iron to dissociate, thereby resulting in the loss of enzyme activity (Gort and Imlay, 1998).

Superoxide anion is thought to be chemically incapable of directly damaging DNA molecules (Brawn and Fridovich, 1981; Lesko *et al.*, 1980; Keyer and Imlay, 1996). Previously it was believed that $O_2^{\cdot-}$ acted as a reductant for iron that was adventitiously bound to DNA (Beauchamp and Fridovich, 1970). The subsequent oxidation of the iron by H_2O_2 generates $HO^{\cdot}$, which can attack DNA (Keyer and Imlay, 1996).

$O_2^{\cdot-} + Fe^{3+} \longrightarrow O_2 + Fe^{2+}$

$H_2O_2 + Fe^{2+} \longrightarrow HO^{\cdot} + OH^{-} + Fe^{3+}$ (Fenton reaction)

Although this mechanism occurs *in vitro* (Brawn and Fridovich, 1981; Lesko *et al.*, 1980), the $O_2^{\cdot-}$ concentration required to rapidly reduce iron exceeds the estimated intracellular concentration by several orders of magnitude (Keyer *et al.*, 1995; Imlay and Fridovich, 1991; Keyer and Imlay, 1996). Furthermore, iron is thought to be reduced *in vivo* by more abundant intracellular reductants such as NADH and glutathione (Imlay and Linn, 1988; Keyer and Imlay, 1996). These conflicting observations have questioned the long-standing

hypothesis that $O_2^{\cdot-}$ promotes $HO^{\cdot}$ formation by the reduction of iron known as the Haber-Weiss scheme (Keyer and Imlay, 1996). Alternatively, Keyer and Imlay (1996) have suggested that the liberation of iron from enzymatic [4Fe-4S] by the oxidising action of $O_2^{\cdot-}$ elevates the internal pool of free iron (as already described). Cluster-repair processes continually replace lost iron atoms, thereby amplifying the impact of $O_2^{\cdot-}$ (Keyer and Imlay, 1996; Flint *et al.*, 1996). During exposure to elevated $O_2^{\cdot-}$ levels, cycles of oxidation and repair could potentially release many free iron atoms into the cytosol (Keyer and Imlay, 1996). The increase in free iron has been shown to increase the rates at which either exogenous or endogenous H_2O_2 damages DNA (Keyer and Imlay, 1996). Although $O_2^{\cdot-}$ can reductively leach iron from *E. coli* storage proteins such as bacterioferritin and ferritin, experiments using mutants lacking these two storage proteins confirmed that the bacterial ferritins are not the primary sources of free iron found in *E. coli* cells following $O_2^{\cdot-}$ stress (Keyer and Imlay, 1996). They have also proposed that the released iron might deposit along the phosphodiester backbone of nucleic acids, where it could catalyse the oxidation of DNA in conjunction with H_2O_2 by formation of hydroxyl radical. Therefore, the ability of superoxide to damage [4Fe-4S] clusters from a subset of dehydratases results in growth defects and DNA damage (Keyer and Imlay, 1996).

The role of H_2O_2 in contributing towards DNA damage has already been discussed. A wide variety of DNA lesions are formed by endogenous oxidants, and it is considered that cell death following exposure to H_2O_2 is mainly due to DNA damage (Imlay and Linn, 1988; Storz and Imlay, 1999). Damage to DNA bases can result in miscoding, and it has been suggested that lesions formed by endogenous oxidants could be a significant source of spontaneous mutagenesis in aerobically growing cells (Storz and Imlay, 1999). In agreement, Seaver and Imlay (2001) have reported that micromolar concentrations of H_2O_2 are mutagenic. Low levels of H_2O_2 (~30 μM) can inhibit the growth of *E. coli* (Seaver and Imlay, 2001). In addition, H_2O_2 can oxidise enzyme thiols, thereby possessing the capability to inactivate enzymes such as glyceraldehyde-3-phosphate dehydrogenase and other enzymes with active-site sulfhydryl residues (Storz and Imlay, 1999). Furthermore, Seaver and Imlay (2001) have recently suggested that that H_2O_2 can inactivate exposed [4Fe-4S] clusters of aconitase B and fumarase B.

The reactive nitrogen intermediates released by phagocytes, and the antimicrobial effects they exert, are thought to be integrally linked to reactive oxygen species, and therefore will be reviewed briefly. NO· has been shown to block bacterial respiration *in vitro* by inactivating cytochrome oxidase by binding the haem and/or copper sites (Storz and Imlay, 1996; Hori *et al.*, 1996; Butler *et al.*, 1997). Furthermore, Gardner *et al.* (1997) have demonstrated that NO· not only inactivates *E. coli* aconitase in a peroxynitrite-independent manner, but renders it poorly reactivatable. Because NO· has an affinity for haem and non-haem iron, it has been proposed that NO· binds to the iron atom of the aconitase [4Fe-4S] cluster (Gardner *et al.*, 1997). This conflicts with earlier work by Hausladen and Imlay (1994) which demonstrated that NO· does not inactivate aconitase at perceptible rates. However they propose, that inactivation of aconitase is mediated through peroxynitrite, which is the product of the reaction between $O_2^{\cdot-}$ and NO·.

Peroxynitrite, like NO·, can cross the cytoplasmic membrane of target cells (Keyer and Imlay, 1997). Unlike NO·, however, it can rapidly kill cells (Brunelli *et al.*, 1995; Keyer and Imlay, 1997). It is also capable of rapidly oxidising the [4Fe-4S] clusters of a subset of dehydrogenases *in vitro* (Hausladen and Fridovich, 1994; Castro *et al.*, 1994; Keyer and Imlay, 1997). Keyer and Imlay (1997) have demonstrated that peroxynitrite at 1% of its lethal dose almost fully inactivates the labile dehydrogenases in *E. coli*, and therefore suggest that the primary targets of this superoxide-derived RNS are these particular dehydrogenases. In addition, peroxynitrite oxidises protein thiols, albeit at a much slower rate (Keyer and Imlay, 1997). The free iron that is released from the destabilised [4Fe-4S] cluster could potentially lead to mutagenic or lethal DNA damage (Keyer and Imlay, 19970. However, it has been suggested that *E. coli* responds to this potential crisis by sequestering the free cytosolic iron very rapidly and reactivating the damaged enzymes (Keyer and Imlay, 1997).

Stevanin *et al.* (2002) have demonstrated that the flavohemoglobin protein (Hmp) of *S.* Typhimurium protects the organism against the cytotoxic effects of NO·. The *hmp* gene was first identified in *E. coli* (Vasudevan *et al.*, 1991), and is up-regulated by addition of NO· to *E. coli* cultures (Poole *et al.*, 1996; Vasudevan *et al.*, 2002). It encodes a monomeric protein whose C-terminal domain resembles ferrodoxin reductase (Stevanin *et al.*, 2002). *Salmonella* mutants defective in Hmp synthesis were shown to exhibit growth that was hypersensitive to nitrosating agents (Stevanin *et al.*, 2002). Moreover, NO·-

induced inhibition of respiration was increased in the mutant when compared to the wild-type (Stevanin *et al.*, 2002). It has been suggested by Stevanin *et al.* (2002) that Hmp protects *Salmonella* from killing within human macrophages, due to the NO·-detoxifying activity of Hmp.

1.8.3.3 Defences against oxidative stress

Endogenous levels of the reactive oxygen species H_2O_2 and $O_2^{\cdot-}$ are tolerated by organisms such as *Salmonella* and toxicity is avoided by the expression of low levels of antioxidant genes and repair activities. However, if a disturbance of the pro-oxidant-antioxidant balance arises in favour of prooxidants, then these defences are somewhat inadequate, and the organism differentially expresses specific proteins in order to cope with the oxidative stress. Different sets of proteins are induced/repressed depending on the reactive oxygen species present. The best studied responses are to H_2O_2 and $O_2^{\cdot-}$. Although the majority of studies have been carried out in *E. coli*, it is considered to be likely that *S.* Typhimurium possesses similar, if not identical systems (Foster and Spector, 1995). However, Pomposiello and Demple (2000) have recently suggested that although the *soxRS* genes of *E. coli* and *S.* Typhimurium are similar (nt sequences of soxRS genes show 97% identity), enzyme activity measurements suggest that the *soxRS* regulons from the two species are different. For example, incubation of *S.* Typhimurium cells with paraquat, induced expression of SodA and endonuclease IV, but not glucose-6-phosphate dehydrogenase or fumarase C, whereas all four proteins are regulated by SoxRS in *E. coli* (Table 1.1) (Martins *et al.*, unpublished data; Pomposiello and Demple, 2000).

Table 1.1 Examples of antioxidant activities in *E. coli* (Storz and Imlay, 1999).

Gene	Activity	Regulators
sodA	Manganese superoxide dismutase	SoxRS, ArcAB, FNR, Fur, IHF
fumC	Fumarase C	SoxRS, ArcAB, σ^s
acnA	Aconitase A	SoxRS, ArcAB, FNR, Fur, σ^s
zwf	Glucose-6-phosphate dehydrogenase	SoxRS
fur	Ferric uptake repressor	SoxRS, OxyR
micF	RNA regulator of ompF	SoxRS, OmpR
acrAB	Multidrug efflux pump	SoxRS
tolC	Outer membrane protein	SoxRS
fpr	Ferredoxin reductase	SoxRS
fldA	Flavodoxin	SoxRS
nfo	Endonuclease IV	SoxRS
sodB	Iron superoxide dismutase	σ^s, FNR
sodC	Copper-zinc superoxide dismutase	σ^s, FNR
katG	Hydroperoxidase I	OxyR, σ^s
ahpCF	Alkyl hydroperoxide reductase	OxyR
gorA	Glutathione reductase	OxyR, σ^s
grxA	Glutaredoxin 1	OxyR
dps	Non-specific DNA binding protein	OxyR, σ^s, IHF
oxyS	Regulatory RNA	OxyR
katE	Hydroperoxidase II	σ^s
xthA	Exonuclease III	σ^s

1.8.3.3.1 The superoxide stress response

Two-dimensional gel analysis of *E. coli* cells exposed to the superoxide-generating compounds plumbagin, paraquat, or menadione induces the synthesis of ~40 proteins, some of which are also induced following exposure to H_2O_2 (Greenberg and Demple, 1989; Storz and Zheng, 2000). In addition, when cells are treated with sub-lethal concentrations of plumbagin, they become resistant to subsequent, higher doses of this oxidant (Farr *et al.*, 1985; Storz and Zheng, 2000).

Greenberg *et al.* (1990) identified an oxidative stress regulon in *E. coli* that is controlled by a previously undescribed regulatory locus, *soxR.* The *soxR* locus is activated in response to intracellular $O_2^{\cdot-}$ and was defined by isolating mutant *E. coli* strains that were resistant to $O_2^{\cdot-}$generating compounds. The *soxR* locus was found to positively regulate nine superoxide-inducible proteins at the level of transcription (Greenberg *et al.*, 1990). DNA sequence analysis of the *soxR* locus revealed the presence of two genes involved in activating the *soxR* regulon, named *soxR* and *soxS,* which are arranged in opposite orientation, their 5' ends separated by 85 bp (Amabile-Cuevas and Demple, 1991). The SoxR protein (17.1 kDa) is significantly homologous to the MerR family of proteins, whereas SoxS (12.9 kDa) is homologous to the regulatory protein AraC (Amabile-Cuevas and Demple, 1991; Wu and Weiss, 1991). It was suggested by Amabile-Cuevas and Demple (1991) that SoxR and SoxS constitute a two-component regulatory system, in which both proteins act sequentially to activate members of the regulon genes in response to superoxide stress. Evidence for this was the observation that expression of the SoxS protein alone *in vivo* activated expression of at least three of the key regulon proteins, whereas expression of SoxR alone did not (Amabile-Cuevas and Demple, 1991).

Members of the *soxRS* regulon that have antioxidant roles include, the manganese superoxide dismutase (*sodA*) which destroys superoxide, endonuclease IV (*nfo*), which repairs DNA that has been damaged by free radicals, and glucose-6-phosphate dehydrogenase (*zwf*) which increases the reducing power of the cell (Greenberg *et al.*, 1990). The superoxide-resistant isozymes of fumarase (*fumC*) and aconitase (*acnA*), are also induced by SoxRS transcription factors in response to elevated levels of $O_2^{\cdot-}$ (Liochev and Lidovich, 1992; Gruer and Guest, 1994). Zheng *et al.* (1999) have shown that the

ferric uptake repressor, Fur is also regulated by SoxRS. The role of superoxide in accelerating the Fenton reaction, and its subsequent contribution to DNA damage induced by hydroxyl radical has already been discussed (Section1.8.3.2). Since the toxicity of superoxide is exacerbated by iron, Fur induction by SoxRS presumably protects against oxidative damage and mutagenesis (Zheng *et al.*, 1999). SoxRS induction of the MicF regulatory RNA, which represses the expression of the outer membrane porin OmpF, the *acrAB*-encoded drug efflux pump, and the *tolC*-encoded outer membrane protein is thought to exclude superoxide-generating compounds (Aono *et al.*, 1998; Chou *et al.*, 1993; Ma *et al.*, 1996). SoxRS regulates *fur* by activating the expression of a transcript encoding both flavodoxin and Fur (*fldA* is immediately upstream of *fur*) (Zheng *et al.*, 1999). Flavodoxin in conjunction with ferrodoxin/flavodoxin-$NADP^+$ reductase (encoded by *fpr*) acts to reduce metalloproteins (Zheng *et al.*, 1999). SoxRS also regulates the expression of the ferrodoxin/flavodoxin-$NADP^+$ reductase (Liochev *et al.*, 1994). Strains carrying *fpr* deletions have been shown to be hypersensitive to paraquat killing, while *fpr* over expressing strains are resistant (Bianchi *et al.*, 1995). Zheng *et al.* (1999) have speculated that the role of the flavodoxin reduction system might be to keep Fe-S clusters in their reduced state, thereby rendering them resistant to superoxide oxidation.

The cytosolic iron superoxide dismutase, SodB and the periplasmic copper-zinc superoxide dismutase, SodC also act to protect the cell against superoxide damage, but these enzymes are not regulated by SoxRS (Storz and Zheng, 1999).

Furthermore, in addition to its antioxidant role, the SoxRS regulon also provides resistance to organic solvents, many different antibiotics and drugs and reactive nitrogen species (Storz and Zheng, 2000).

Evidence of a direct role for the SoxR protein in the activation of *soxS* comes from band-shift assays and DNase I footprinting experiments by Nunoshiba *et al.* (1992), that demonstrate specific binding of SoxR to the *soxS* promoter in *E. coli* cell extracts. It has since been determined that regulation of the s*oxRS* regulon occurs by a two-stage process in which the SoxR protein is converted to an active form that enhances transcription of *soxS.* In turn, increased levels of SoxS activate the members of the regulon.

SoxR is a homodimer, and each monomer has a redox-active [2Fe-2S] cluster, which is necessary for the protein's function as a transcriptional activator (Hidalgo *et al.*, 1995). In agreement, the SoxR apoprotein has been shown to be transcriptionally inactive *in vitro* (Hidalgo and Demple, 1994). Iron-sulphur (FeS) proteins that are sensitive to oxidation by superoxide typically contain [4Fe-4S] centres, as previously discussed. Fe-SoxR must maintain its activity in the presence of increased levels of endogenous superoxide, and the [2Fe-2S] clusters of SoxR seem to be quite stable when in oxidised form (Hidalgo *et al.*, 1995). The oxidation of the reduced [2Fe-2S] $^{1+}$ form of SoxR to the oxidised [2Fe-2S]$^{2+}$ form appears to be the mechanism by which SoxR is activated (Storz and Imlay, 1999; Hidalgo *et al.*, 1995; Gaudu and Weiss, 1996; Ding *et al.*, 1996; Hidalgo *et al.*, 1997; Ding and Demple, 1998). Evidence for this mechanism comes from experiments that show the activity of the oxidised form of SoxR is lost when its [2Fe-2S] clusters are reduced by dithionite. Activity is rapidly restored by autoxidation (Gaudu and Weiss, 1996). In this instance, activity was defined by the ability of SoxR to activate transcription of its target gene, *soxS*. Furthermore, it has been demonstrated using electron paramagnetic resonance spectroscopy that over-produced wild-type SoxR protein is oxidised within 2 minutes following treatment with superoxide-generating compounds such as paraquat. Moreover, even in the absence of stress, constitutively active mutant SoxR proteins are predominantly in the oxidised form (Ding and Demple, 1997; Gaudu *et al.*, 1997; Hidalgo *et al.*, 1997).

Initially, superoxide was thought to be the signal detected by SoxR, because it could directly oxidise SoxR, and also because superoxide generators such as paraquat induce the *soxRS* regulon (Gaudu *et al.*, 2000; Hassan and Fridovich, 1977). However Liochev and Fridovich (1992) have proposed that superoxide does not induce the *soxRS* regulon directly. This is based on the effects of a *zwf* mutation that was expected to decrease the cellular production of NADPH and thus impair the production of $O_2^{\cdot-}$ by paraquat. However, the mutation enhanced the inducibility of the *soxRS* regulon (Gaudu and Weiss, 1996; Liochev and Fridovich, 1992). As a result, it was postulated that the inducing signal is a decreased NADPH/NADP$^+$ ratio caused by the consumption of NADPH during the production of $O_2^{\cdot-}$ (Gaudu and Weiss, 1996; Liochev and Fridovich, 1992). Other evidence that conflicts with the initial theory is that SoxR is capable of autoxidation *in vitro* (Hidalgo and Demple, 1994; Wu *et al.*, 1995; Gaudu *et al.*, 2000). Also, the *soxRS* regulon is induced by nitric oxide in the absence of oxygen *in vivo* (Nunoshiba *et al.*, 1993). These findings are consistent with the observation that SoxR is maintained *in vivo* in its reduced

inactive form by an unknown electron pathway (Gadua *et al.*, 2000; Gadua *et al.*, 1997; Hidalgo *et al.*, 1997).

In vivo electron paramagnetic resonance spectroscopy studies have shown that the oxidised, active form of SoxR is rapidly reduced once the oxidative stress is removed (Ding and Demple, 1997). The redox systems that might reduce/deactivate SoxR *in vivo* have not yet been elucidated (Gaudu and Weiss, 1996). The possibility that SoxS reduction is mediated directly by ferrodoxin, flavodoxin, or their reductase (Fpr) was explored by Gaudu and Weiss (1996). Although Fpr is part of the *soxRS* regulon, it did not catalyse the reduction of SoxR at a significant rate. Similarly, ferrodoxin and flavodoxin did not reduce SoxR effectively (Gaudu and Weiss, 1996).

Activity of SoxR is thought to be modulated by the assembly/disassembly of its [2Fe-2S] clusters (Storz and Zheng, 2000). Monothiols such as glutathione have recently been found to promote the disassembly of [2Fe-2S] clusters. Conversely, dithiols promote cluster assembly into apo-SoxR (Storz and Zheng, 2000; Ding and Demple, 1998).

1.8.3.3.2 The hydrogen peroxide stress response

The adaptive response to hydrogen peroxide was first recognised in *E. coli* (Demple and Halbrook, 1983) and was subsequently shown to exist in *S.* Typhimurium by Christman *et al.* (1985) (Section 3.1.1). Furthermore, two-dimensional gel analysis of *S.* Typhimurium cells that had been pre-treated with H_2O_2 revealed that at least 30 proteins are induced by H_2O_2 treatment (Section 3.1.1) (Christman *et al.*, 1985). Mutations in *S.* Typhimurium that not only led to the increased expression of a subset of H_2O_2–inducible proteins but also conferred increased resistance to H_2O_2 without pre-treatment, allowed the *oxyR* locus to be defined (Christman *et al.*, 1985). Additionally, in strains carrying an *oxyR* deletion, the nine proteins that are over expressed in *oxyR* constitutive mutants were shown to be uninducible by H_2O_2.

The majority of genes whose expression is regulated by OxyR following H_2O_2 treatment have antioxidant roles (Table 1.1). Examples include hydroperoxidase I (catalase, *katG*) and alkyl hydroperoxide reductase (*ahpCF*), which directly eliminate H_2O_2 and

alkylhydroperoxides respectively and therefore protect against toxicity (Christman *et al.*, 1985). The cellular thiol-disulphide balance is thought to be maintained by the induction of glutaredoxin 1 (*grxA*) and glutathione reductase (*gorA*), which are also OxyR-regulated (Christman *et al.*, 1985; Tao, 1997; Zheng *et al.*, 1998). Additionally, the induction of the Fur repressor (*fur*) is thought to prevent damage caused by the $HO^{\cdot}$ as previously discussed (Section 1.8.3.2) (Zheng *et al.*, 1998). OxyR also induces the expression of a small RNA molecule, denoted OxyS, which is described in detail later (Section 4.1). Briefly, OxyS acts as an antimutator and is thought to integrate the adaptive response to H_2O_2 (Altuvia *et al.*, 1994).

The σ^S-dependent gene *dps* encodes a non-specific DNA binding protein that is required for starvation-induced resistance to H_2O_2 and is highly abundant in *E. coli* stationary phase cells (Almiron *et al.*, 1992; Martinez and Kolter, 1997). In addition, *dps* is also expressed during exponential growth following treatment with sub-lethal amounts of H_2O_2 and is regulated by OxyR (Altuvia *et al.*, 1994; Lomovskaya *et al.*, 1994; Martinez and Kolter, 1997). It has been demonstrated by Martinez and Kolter (1997) that Dps binding to DNA can directly prevent oxidative damage, such as DNA strand breaks and certain types of base damage (Martinez and Kolter, 1997). DNA protection is thought to be due to the binding of Dps to the DNA because heat-inactivated Dps, which is unable to bind to DNA, offers no protection against oxidative damage (Martinez and Kolter, 1997). The crystal structure of Dps has revealed that it is a ferritin homologue, and therefore may protect against oxidative damage by sequestering iron (Storz and Imlay, 1999; Grant *et al.*, 1998). Interestingly, Valdivia and Falkow (1996) have shown that a *dps* homolog from *S.* Typhimurium is up regulated in macrophages, and therefore may be important for macrophage survival in this phagocytic cell.

Several OxyR-activated genes that code for H_2O_2-sensitive proteins have been identified in *E. coli*, such as *hemF* (encodes a coproporphyrinogen III oxidase), *rcsC* (encodes a regulator of capsular polysaccharide synthesis genes), and *f497* (an open reading frame that is similar to arylsulfatase-encoding genes) (Mukhopadhyay and Schellhorn, 1997). As yet the antioxidant roles of these proteins remain unclear. Possibly, up-regulation simply serves to compensate for H_2O_2-induced damage to the original pool of these proteins.

During exponential growth and following oxidative stress, OxyR acts as a repressor and negatively autoregulates its own expression (Christman *et al.*, 1989; Tao *et al.*, 1991). It also represses the gene for Antigen 43 protein, which localises to the outer membrane (Henderson and Owen, 1999). Phase variation of Ag43 in *E. coli* does not require a DNA sequence change (Henderson *et al.*, 1999; Low *et al.,* 2001; Wallecha *et al.*, 2002) but instead requires DNA modification by a deoxyadenosine methylase (Dam), which methylates the adenine residue of GATC sequences (Wallecha *et al.*, 2002). Phase variation of *agn43* also requires OxyR (Haagmans and van der Woude, 2000; Henderson and Owen, 1999). By binding to a region upstream of the coding sequence, OxyR represses *agn43* transcription and the Ag43 protein is not expressed (OFF). Dam-dependent methylation of the three GATC sequences in the regulatory region of agn43 abrogates OxyR binding and Ag43 is expressed (ON) (Henderson *et al.*, 1999;Wallecha *et al.*, 2002). Evidence for this model comes from experiments within an *oxyR* mutant and an isolate over-expressing Dam, where Ag43 expression is locked ON (Wallecha *et al.*, 2002). Moreover, in a *dam* mutant, expression of Ag43 is locked OFF (Haagmans and van der Woude, 2000; Henderson and Owen, 1999). Interestingly, OxyR also represses the Mu phage *mom* gene, which encodes a DNA modification function (Bolker and Kahmann, 1989; Hattman and Sun, 1997; Sun and Hattman, 1996). Binding of OxyR to *mom* is also Dam-dependent, but *mom* expression is not known to be phase variable (Wallecha *et al.*, 2002). The roles of OxyR repression of *mom* and *agn43* in the defence against oxidative stress remain unclear. However, it is interesting to note that OxyR in either reduced or oxidised form can bind to unmethylated *agn43* DNA (Wallecha *et al.*, 2002; Correnti *et al.,* 2002; Haagmans and van der Woude, 2000). Schembri and Klemm (2001) have recently suggested that that Ag43 expression is co-ordinately regulated with fimbrial expression. Furthermore they have proposed a model in which fimbrial synthesis may result in repression of Ag43 expression by causing a localised oxidative stress response (Wallecha *et al.*, 2002).

The *fhuF* gene of *E. coli* is also repressed by hydrogen peroxide in an OxyR-dependent manner. It has been suggested that RNA polymerase is prevented from binding to the *fhuF* promoter by OxyR, as it binds to a site that covers the +1, -10, and –35 sequences of the *fhuF* promoter. (Zheng *et al.*, 2001). Muller *et al.* (1988) have suggested that FhuF is a ferric iron reductase and is required for iron uptake. Repression of FhuF protein expression

by OxyR, as part of the oxidative stress response, may slow iron uptake and formation of $HO^{\cdot}$ via the Fenton reaction would be minimised (Zheng *et al.*, 2001).

The tetrameric OxyR regulatory protein is a member of the LysR family of bacterial regulators, and like other members of this family it possesses an amino-terminal helix-turn-helix motif that acts as a DNA binding domain (Christman *et al.*, 1989; Sambrook *et al.*, 1989; Kullik *et al.*, 1995). The OxyR protein exists in two forms, reduced and oxidised, and only the oxidised form of OxyR can activate transcription of genes belonging to the OxyR regulon (Storz *et al.*, 1990; Toledano *et al.*, 1994). In agreement, it has been demonstrated that direct oxidation of OxyR by H_2O_2 is the mechanism whereby cells sense oxidative stress. As a result, the OxyR regulon is duly induced (Storz and Imlay, 1999). Activation of OxyR is achieved by the direct oxidation of the C199 and C208 thiols to form an intramolecular disulphide bond (Figure 1.5) (Zheng *et al.*, 1998). Evidence for this comes from experiments in which mutation of either C199 or C208 residues abolished the ability of OxyR to sense H_2O_2 *in vivo* and *in vitro*. Moreover, mass spectrometric analysis, together with thiol-disulphide titrations of OxyR demonstrated that C199 and C208 are in a dithiol form in reduced OxyR. Conversely, the residues are in a disulphide bonded form in oxidised OxyR (Zheng *et al.*, 1998). Reduction of OxyR is thought to be effected by enzymatic reduction of the intramolecular disulphide bond (Figure 1.5)(Zheng *et al.*, 1998). Although glutaredoxin 1 and thioredoxin are able to reduce OxyR *in vitro*, glutaredoxin may be the reductant of choice by the cell (Zheng *et al.*, 1998). Evidence for this comes from experiments in which mutants lacking glutathione (GSH) or glutaredoxin 1 showed a prolonged OxyR response after H_2O_2 treatment, whereas mutants lacking thioredoxin or thioredoxin reductase did not (Zheng *et al.*, 1998). Furthermore, *gorA* and *grxA* are transcriptionally regulated by OxyR, therefore the response is autoregulated (Zheng *et al.*, 1998).

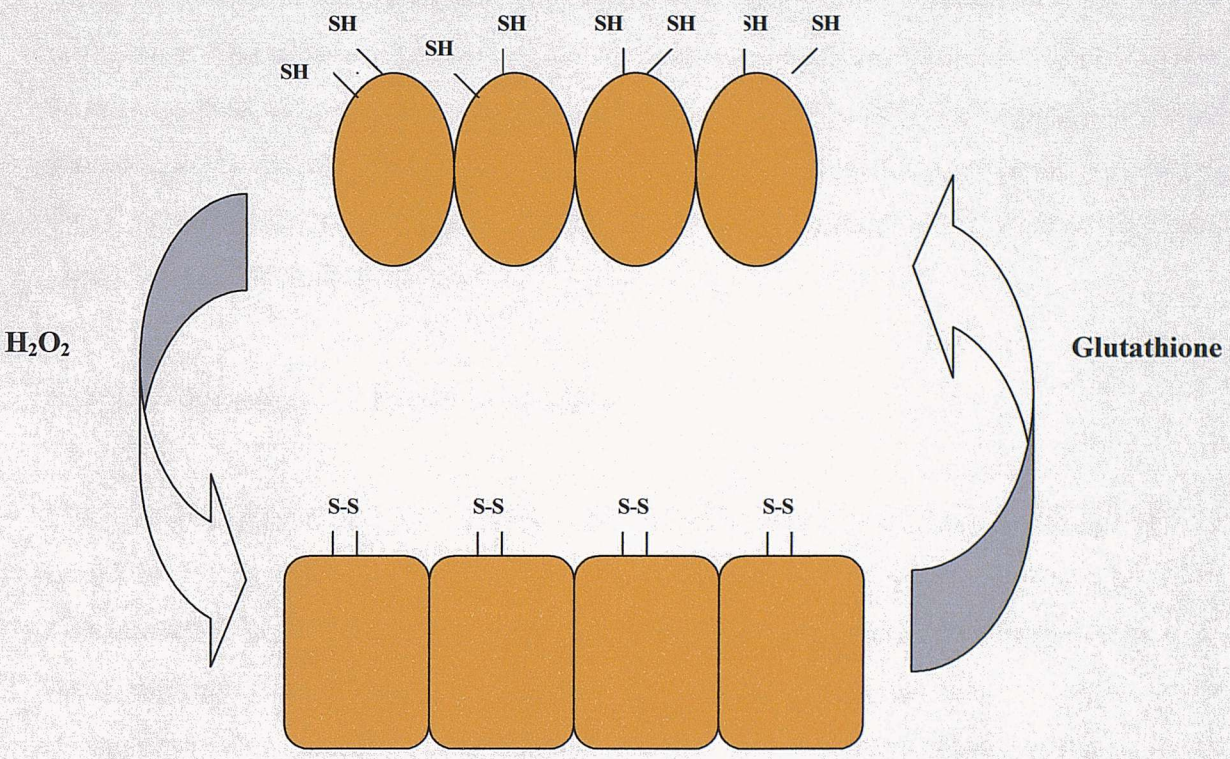

Figure 1.5. Mechanism of OxyR activation and reduction. H_2O_2 directly activates OxyR by oxidising cysteinyl residues to form an intramolecular disulphide bond. OxyR is deactivated upon reduction by glutaredoxin 1 with the consumption of glutathione. This diagram is adapted from Storz and Zheng, (2000).

A consensus motif for the binding of OxyR has been proposed (Toledano *et al.*, 1994). Although OxyR-DNA binding is based on a specific recognition code, the key interacting nucleotides are unusually dispersed (Toledano *et al.*, 1994). In addition Toledano *et al.* (1994) have shown that when in its oxidised state, OxyR binds to an ATAGnt motif on one face of the DNA helix at four adjacent major grooves. Conversely, reduced OxyR recognises ATAGnt motifs present in two pairs of adjacent major grooves separated by one helical turn. The different binding specificities are considered necessary to enable OxyR to function as both an activator and a repressor (Toledano *et al.*, 1994). For example, during normal growth OxyR can repress *oxyR* and *mom* promoters, but on exposure to H_2O_2, OxyR is activated and can induce the expression of antioxidant genes such as *katG* and *ahpCF* (Kullik *et al.*, 1995).

1.8.3.3.3 Overlap with other regulons

The *rpoS*-encoded σ^s subunit of RNA polymerase is present in exponential phase cells at very low levels. However, in response to various stress conditions such as starvation, osmotic stress, and acid stress, RpoS is up regulated and mediates the induction of over 50 stress response genes (Becker *et al.*, 1999). The cellular concentration of σ^s also increases during entry into stationary phase (Michan *et al.*, 1999). Stationary phase and starved cells are intrinsically resistant to a variety of different stresses including increased levels of H_2O_2 (Storz and Imlay, 1999). Members of the σ^s regulon include *katG* (Ivanova *et al.*, 1994), *gorA* (Becker-Hapak and Eisenstark, 1995), and *dps* (Altuvia *et al.*, 1992), which are also under the control of the OxyR regulatory protein. The expression of σ^s is tightly regulated at the transcriptional, translational and posttranslational levels (Lange and Hengge-Aronis, 1994). In addition, OxyS, which is activated by oxidised OxyR, has been shown to negatively regulate *rpoS* at a post-transcriptional level (Section 4.6.1).

Although the SoxRS regulon was previously found not to be induced by H_2O_2 (Nunoshiba *et al.*, 1992), Zheng *et al.* (2001) have demonstrated that members of the SoxRS regulon, including *sodA* and *fpr* (encodes ferrodoxin-flavodoxin reductase), and *soxS* were induced by 1mM H_2O_2. Furthermore using primer extension assays they found that a primary OxyR target, *oxyS*, was slightly induced by high concentrations of paraquat. These results add support to the proposed overlap between the superoxide and hydrogen peroxide stress

regulons (Zheng *et al.*, 2001) A further example of an overlap between these two regulons can be demonstrated by the iron-dependent repressor Fur. Following a hydrogen peroxide stress, OxyR induces the expression of *fur*. SoxRS also regulates *fur* by inducing the expression of a transcript that encodes both Fur and flavodoxin (Zheng *et al.*, 1999).

MarA and Rob are SoxS homologues, and they also regulate the expression of the majority of genes in the SoxRS regulon of *E. coli* (Miller and Sulavik, 1996; Storz and Imlay, 1999). The expression of *marA* is induced by certain antibiotics including tetracycline and phenolic compounds such as salicylate, and is part of an operon that confers multiple antibiotic resistance (Miller and Sulavik, 1996; Storz and Imlay, 1999). In addition, it is slightly induced by paraquat (Seoane and Levy, 1995; Pomposiello and Demple, 2000). Rob binds DNA in the region of the replication of origin of the *E. coli* chromosome (Skarstad *et al.*, 1993; Storz and Imlay, 1999). All three proteins share a DNA binding motif that is characteristic of the AraC family of transcriptional activators, and it has been proposed that each one can bind to and activate the same promoters. However, the reasons for the overlap between the *soxRS*, *marA* and *rob* regulons in *E. coli* are not fully understood (Storz and Imlay, 1999). It is not known whether such an overlap exists in *Salmonella*, but it is interesting to note that the *marA* gene in *S.* Typhimurium has been identified, and codes for a predicted polypeptide 86% identical to *E. coli* MarA (Sulavik *et al.*, 1997; Pomposiello and Demple, 2000).

1.9 Aims of the present work

With this background in mind, it is clear that a number of important questions remain about the oxidative stress response of bacteria such as *S.* Typhimurium. For example, it is not yet clear if other targets of the OxyR master regulon remain to be discovered or, indeed if other regulators of oxidative stress exist. Equally, it would be interesting to know if the OxyRS regulon overlaps with other key regulons in *Salmonella* and if co-regulation is reciprocal.

Finally, the role of particular oxidative stress regulators in the virulence of *Salmonella* remains to be further explored. These questions have been studied in the work described in this thesis.

CHAPTER 2

MATERIALS AND METHODS

CHAPTER 2 Materials and Methods

Materials

The following specialist materials were used:
Nitrocellulose transfer membrane (Schleicher & Schuell)
Fluorotrans transfer membrane (PALL)
Sterile disposable filters 0.22μm (Sartorius)
Phase Lock Gel™ III Light Extraction Tubes (5 Prime to 3 Prime)
X-ray film, blue sensitive (Genetic Research Instrumentation Ltd.)
IPG strips, pH 4-7 (Amersham-Pharmacia Biotech)
ExcelGel, 12 -14% SDS-acrylamide precast gels (Amersham-Pharmacia Biotech)

Reagents

The following specialist reagents were used:
DNA 1kb ladder size marker (Invitrogen Life Technologies)
Protein Broad Range Marker (New England Biolabs)
RNase Zap (Invitrogen Life Technologies)
SuperSignal Chemiluminescent Substrate (Pierce)
Qiaprep spin kits (Qiagen)
Radiochemicals (Amersham International plc)
Restriction endonucleases and DNA modifying enzymes (New England Biolabs; Promega)
IPG-Buffer pH 4-7L (Amersham-Pharmacia Biotech)
Unless otherwise stated, all other reagents were acquired from New England Biolabs, Promega or Sigma.

2.1 General Techniques

2.1.1 Bacterial strains, plasmids and media

S. Typhimurium and *E. coli* strains used in this study are listed in Table 2.1. Plasmids constructed and the cloning vectors used are listed in Table 2.2.

2.1.2 Bacterial growth media

Bacterial cultivation used the following media. Luria-Bertani (LB) medium consisted of 10g tryptone, 5g yeast extract, 5g NaCl per litre. By supplementing LB media with 1.5% agar, LB plates were produced. TB sucrose plates contained per litre, 10g tryptone, 5g yeast extract, 5% sucrose and 1.2% agar.

2.1.3 Antibiotics

When required, antibiotics were added at the indicated concentrations to the autoclaved medium, once it had cooled to below 50°C. Stock solutions were prepared with filter sterilised analytical grade water except where indicated. Stock solutions were stored at –20°C.

Ampicillin - 100 μg/ml

Chloramphenicol - 7 μg/ml (prepared in 100% ethanol)

Streptomycin - 50 μg/ml

Tetracycline - 100 μg/ml (prepared in 50% ethanol, 50% water)

Table 2.1. Bacterial strains used in this study

Strain	Genotype or characteristics	Source/Reference
SL1344	*his-* non LT2 isolate of *S.* Typhimurium	Wray and Sojka, 1978
SL1344 *ΔoxyS::cat*	SL1344 with *ΔoxyS::cat* inserted into chromosomally located *oxyS*, Cm^r	This study
SL1344 *phoP12*	*phoP12* allele transduced into SL1344 from LT2 strain RMA 1000	Adams *et al.*, 2001
SL1344 *pho-24*	*pho-24* allele transduced into SL1344 from LT2 strain RMA 1004	Adams *et al.*, 2001
SL1344 *bipA::cat*	*Δ bipA* derivative of SL1344	Adams *et al.*, 2001
SL1344 St^r	Streptomycin resistant derivative of SL1344	N. Kinsella
TA4100 (*oxyR1*)	OxyR constitutive mutant	G Storz
TA4129 (*oxyR1 zii614::*Tn*10*)	*oxyR1* linked to *zii614::*Tn*10* in LT2	G. Storz
SM10 λpir	Permissive strain for replication of plasmid pCVD442. *thi thr leu tonA lacY recA*::RP4-2-Tc::Mu Km *λpir*	M Donnenberg
E2348/69	Prototype O127:H6 EPEC strain	M. Donnenburg
AG1	E2348/69 with *ΔbipA::cat* inserted into chromosomally located *bipA*, Cm^r	A. Grant

Table 2.2. Plasmids used in this study

Plasmid	Genotype or characteristics	Source/Reference
PCVD442	π-dependent, *sacB*-containing positive selection suicide vector, Ap^r	Donnenberg and Kaper, 1991
pACYC184	Low-copy number cloning vector encoding Cm^r, Tc^r	New England Biolabs
pGMH	1.35 kb *XhoI-SacI ΔoxyS* fragment cloned into *SacI-SalI* digested suicide vector pCVD442, Ap^r	This study
pGMH1	*NotI* PCR fragment containing *cat* gene from pACYC184 cloned into *NotI* digested pGMH, Ap^r, Cm^r	This study

2.1.4 Maintenance of bacterial strains

All strains were stored at –70°C in 50% LB / 50% glycerol (v/v) and were revived by streaking a loopful of glycerol suspension prior to use on the appropriate solidified media and incubated overnight at 37°C. An overnight culture typically consisted of 10ml LB that had been inoculated with a single colony and incubated with shaking at 37°C.

2.2 DNA Techniques

2.2.1 Genomic DNA preparation

A 10 ml overnight culture was spun at 4000 rpm in a Beckman JA-20 rotor for 10 minutes to pellet the bacteria. The supernatant was removed and discarded. The pellet was resuspended in TE buffer pH 8.0 (10 mM Tris-HCl (pH 8.0), 1 mM EDTA, containing 1mg/ml proteinase K). The contents were shaken and then incubated for 30 minutes at 70°C. SDS (1% final concentration) was added and the contents were swirled gently, followed by a 60 minute incubation at 70°C. The solution was transferred to a phaselock tube (5 prime to 3 prime), into which was added 2 ml phenol (pH 7.5). The solution was gently agitated and spun at 4000 rpm in a Megafuge 1.0 Heraus 7570E. The supernatant was decanted into a new phaselock tube and the phenol extraction was repeated twice more. The supernatant was transferred into a Falcon tube into which was added 3 ml room temperature absolute ethanol. The tube was inverted until the precipitated DNA appeared fluffy. The DNA was removed using a sterile hook and was washed in –20°C, 70% ethanol. The residual liquid was removed by aspiration and the pellet was allowed to dry at room temperature for 30 minutes. The pellet was resuspended in TE buffer and stored at –20°C.

2.2.2 Plasmid DNA preparation

Bulk DNA preparations were obtained using the Qiagen Maxi kit following the manufacturer's instructions. Qiagen plasmid purification protocols were based upon a modified alkaline lysis procedure. DNA from a cleared lysate was bound to an anion-exchange resin under low-salt conditions and was eluted from the column in a high-salt

buffer then concentrated and desalted by isopropanol precipitation. Pelleted DNA was resuspended in sterile analytical grade water.

Small-scale plasmid DNA preparations were prepared from 10 ml overnight cultures using the Qiagen Mini prep spin kit, following the manufacturer's instructions. DNA was eluted with sterile analytical grade water.

2.2.3 Phenol, chloroform, ether extraction

Purification of DNA was carried out essentially as per Sambrook *et al.* (1989) and was achieved by the addition of equal volumes of phenol (pH 7.5) to the sample, which was vortexed thoroughly, followed by centrifugation in a micro-centrifuge to separate the layers. The aqueous layer was retained and the phenol extraction was repeated twice more. This was followed by three extractions in chloroform and finally three extractions in ether. For the ether stage the bottom layer was retained. Following ether extraction the sample was allowed to air dry for 5 minutes to remove any residual traces of ether. DNA was resuspended in TE or sterile analytical grade water.

2.2.4 Ethanol precipitation

This was carried out essentially as per Sambrook *et al.* (1989). DNA was precipitated by the addition of 0.1 volumes of 3 M NaAc (pH 5.6) and 2.5 volumes of –20°C absolute ethanol. The sample was incubated in a methanol dry-ice bath for 20 minutes. The DNA was pelleted by centrifugation at 15000 rpm for 10 minutes. The pellet was washed in 300µl of –20°C, 75% ethanol to remove salts before being left to air dry for 5 minutes. DNA was resuspended in sterile analytical grade water.

2.2.5 Estimation of DNA concentration and purity

DNA concentration was measured by taking absorbance readings at 260 nm against a suitable blank in quartz cuvettes using a UV spectrometer, which assumes that an A_{260} of 1.0 is equal to 50 µg DNA/ml (Miller, 1972). Protein contamination was measured at 280

nm. If the ratio of absorbance at 260 nm to 280 nm was below 1.75, DNA was further purified by sequential phenol, chloroform and ether extractions.

2.2.6 DNA modifications

Restriction endonuclease digestion of DNA, ligation reactions and DNA modifications were carried out as per Sambrook *et al.* (1989). Ligation reactions were performed in a Techne thermal cycler at 16°C for 16 hours followed by a one hour incubation at 4°C using T4 DNA Ligase (Promega).

2.2.7 Agarose gel electrophoresis

Electrophoresis of DNA was performed at room temperature on horizontal, 0.5-1.5% (w/v) agarose gels prepared as described by Sharp *et al.* (1973) and McDonnell *et al.* (1977). DNA samples were mixed with 0.2 volumes of gel loading buffer (30% v/v glycerol, 0.3% w/v bromophenol blue, made up in TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA)) and were electrophoresed at 100 V for 40 minutes in TAE buffer (0.04 M Tris-acetate, 0.001 M EDTA) containing 0.6μg/ml of ethidium bromide. The DNA molecular weight marker used was a 1 kb DNA ladder (Gibco BRL) (Table 2.3). DNA bands were visualised by transillumination with 300 nm UV light.

Table 2.3. Gibco BRL 1 kb DNA marker

DNA band	Size (kb)
1	12.21
2	11.19
3	10.18
4	9.16
5	8.14
6	7.12
7	6.01
8	5.09
9	4.07
10	3.05
11	2.03
12	1.63
13	1.01
14	0.51
15	0.50
16	0.39
17	0.34
18	0.29
19	0.22
20	0.20
21	0.15
22	0.13
23	0.04

2.2.8 Excision and purification of DNA from agarose

DNA fragments were excised from agarose gels using a clean scalpel blade. DNA was recovered from excised bands using the Qiagen Gel-Extraction kit following the manufacturer's instructions, and was eluted with sterile analytical grade water.

2.2.9 PCR amplification of DNA

Polymerase chain reactions (PCR) were performed essentially as described by Sambrook *et al.* (1989). Reactions were carried out in a Techne thermal cycler in 25µl reaction volumes using 0.5 ml eppendorf tubes. PCR buffer consisted of a final concentration of 1x Promega Buffer, 2 mM $MgCl_2$, 0.2 nM each dNTP, 20 pm of each primer and 2.5 U of *Taq* polymerase (Promega). The concentration of template DNA used was typically 200 ng. The PCR reaction mixture was denatured at 94^0C for 1 minute before the addition of the polymerase enzyme. Thermal cycler conditions typically consisted of: 94°C 1 minute, 55°C 1 minute, 72°C 2 minutes of which there were 30 cycles followed by a 10 minute hold step at 20°C. The oligonucleotides used in this study for PCR and sequencing were synthesised by Oswel and are listed in Table 2.4.

The crossover PCR method of Link *et al.* (1997) was used to create an *oxyS* deletion construct and is fully explained in Chapter 4.

Table 2.4. Oligonucleotides used in this study. Chromosomal coordinates of *oxyS* and flanking genes were taken from *S.* Typhimurium LT2 sequence (obtained from the Wellcome Trust Sanger Institute). Nucleotides that are complementary to chromosomal DNA or comprise a linker region are coloured red and blue respectively. Restriction sites are underlined.

Primer	Primer sequence (5'-3')
	4342264 4342284
GHoxyS-A	AAAAAAAACTCGAGAAGAAAAACCCGGATGCGC
	4343585 4343565
GHoxyS-D	GGGGGGGGGAGCTCTAGCCAGCATCATGGGCTCAT
	4342223 4342240
GHoxyS-A1	GCTTTCTGACCGCGTGAC
	4342913 4342893
GHoxyS-B2	TCGTTCTAGTAGGTCGCGGCCGCCTAAAGCCAACGTGAACTTTT
	4342950 4342967
GHoxyS-C2	GCGGCCGCGACCTACTAGAACGAGTGTCTTCAAGGGTTAAA
	4342816 4342796
GH-S2	TGGCGCTATGCTTATAAGGCT
AJG027	CCCCCCCCGCGGCCGCTGTGACGGAAGATCACTTCGCA
AJG028	TTTTTTTTGCGGCCGCCATAGCTGTTCCTCTAGTCAGTTACGCCC CGCCCTGCCACTC

2.2.10 DNA sequencing

When required, DNA sequencing was performed by Oswel using an ABI-100 sequencer.

2.2.11 Transformations

Electrocompetent cells and electroporation

Cells (500 ml) were grown to mid-exponential phase (an absorbance of 0.4-0.5 at 600 nm). Bacteria were pelleted at 6000 rpm at 4°C for 15 minutes in a Beckman JLA 10,500 rotor. The pellet was resuspended in a suitable volume of ice-cold sterile analytical grade water and cells were again spun as before. The pellet was resuspended in ice-cold sterile 10% glycerol, prior to a final spin at 6000 rpm for 15 minutes at 4°C in a Beckman JA-20 rotor. The supernatant was removed and the cells were aliquoted into 40 µl volumes, snap-frozen using liquid nitrogen and stored at –70°C.

Desalted DNA (typically between 10-100 µg) was added to the thawed 40 µl aliquot of electrocompetent cells. The sample was incubated on ice for 1 minute, prior to being transferred to an electroporation cuvette (1mm gap) and shocked with a voltage of 1.25 mV for 4.5-5 ms. Cells were resuscitated immediately by the addition of 1 ml of sterile SOC medium (10 mM NaCl, 2.5 mM KCl, 10 mM $MgSO_4$, 20 mM glucose, 20 g Bacto-tryptone and 5 g Bacto-yeast extract in 1 litre) and incubated at 37°C with light shaking for 1 hour, prior to being serially diluted and plated.

Calcium competent cells

Cells were grown to mid-exponential phase (an absorbance of 0.4-0.5 at 600 nm) and were pelleted at 6000 rpm for 15 minutes at 4°C in a Beckman JLA 10,500 rotor. The bacterial pellet was resuspended in 15 ml of ice-cold 0.1 M $CaCl_2$ and re-spun as before. Cells were resuspended in 1/25 volume of ice-cold 0.1 M $CaCl_2$ and were aliquoted into 100 µl volumes. DNA was added to the competent cells and the sample was incubated on ice for 45 minutes prior to a 60 second heat-shock at 42°C. The sample was again incubated on ice

for 60 seconds, after which 1 ml of LB was added. Bacteria were allowed to recover for 2 hours at 37°C with light shaking, prior to being serially diluted and plated.

2.2.12 Southern Blotting

Southern blotting was carried out essentially as per Sambrook *et al.* (1989)

Transfer of DNA

DNA to be probed was digested with the appropriate restriction enzymes prior to loading onto a 0.8% agarose gel, which was run at 120 V for 4 hours. The gel was washed in 0.1 M HCl for depurination with gentle rocking for 10 minutes. The gel was then immersed in alkali-denaturing solution (1.5 M NaCl, 0.5 M NaOH) for 30 minutes with gentle rocking. The gel was rinsed in dH_2O and then immersed in neutralisation solution (1 M Tris-HCl pH7.4, 1.5 M NaCl) for 30 minutes. The DNA was transferred onto nitrocellulose membrane by capillary action. The membrane was enveloped in Whatman 3 MM paper prior to incubation in an 80°C oven for 2 hours in order to fix the nucleic acids.

Preparation of probe

The PCR product to be used as the probe was denatured by heating to 95°C for 2 minutes, followed by transfer to ice. Radio labelling was carried out via random hexamers using the Prime-a-Gene Kit (Promega). The labelling reaction mixture contained a final concentration of 1x labelling buffer, 20 μM each of unlabelled dNTPs (dCTP, dGTP, dTTP), 500 ng/ml of DNA template, 400 μg/ml nuclease-free BSA, 1 mCi/ml [α ^{32}P] dATP (3000 Ci/mmol) (Amersham Corporation) and 100 μg/ml DNA polymerase Klenow fragment, added in the stated order. The reaction mixture volume was made up to 50 μl with nuclease free water, and was incubated at 37°C for 60 minutes. The reaction was stopped by heating to 95°C for 2 minutes followed by the addition of a final concentration of 20 mM EDTA and incubation on ice.

Hybridisation of probe

The membrane was washed in 2 x SSC (0.2 M NaCl, 0.03 M trisodium citrate (pH 7.0)) and transferred into a hybridisation bottle. The membrane was pre-hybridised in 10 ml of pre-warmed pre-hybridisation solution (5x SSC, 0.5% SDS, 5x Denhardt's solution (0.1% Ficoll, 0.1% Polyvinylpyrolidine, 0.1% Bovine Serum Albumin), 0.2% mg/ml Salmon Sperm DNA, made up in analytical grade sterile distilled water) for 1 hour at 65°C. The pre-hybridisation solution was replaced with hybridisation solution (as for pre-hybridisation solution, but with the addition of 50 µl labelled probe) and incubated at 65°C overnight. The membrane was washed for 1 minute in 50 ml of Wash Buffer 1 (2 x SSC and 0.5% SDS) pre-warmed to 65°C. The 1 minute wash was repeated and followed by two 15 minute washes in Wash Buffer 1, also at 65°C. The membrane was then washed twice in Wash Buffer 2 (1 x SSC and 0.1% SDS) for 15 minutes at 25°C, before being blotted dry and wrapped in cling film. It was then placed on a Phosphor screen for 1 hour and developed using a Molecular Dynamics Storm Imager in conjunction with Image Quant analysis.

2.2.13 Genetic Transduction

P22 phage propagation

A volume of 200 µl of P22 stock (~5 x 10^9 pfu/ml) that had previously been amplified on wild-type SL1344 strain, together with an equal volume of an overnight culture of donor cells (an SL1344 streptomycin resistant derivative) were added to 10 ml LB containing a final concentration of 10 mM $MgSO_4$. The solution was incubated at 37°C with light shaking for 16 hours, prior to being spun at 4000 rpm in a Megafuge 1.0 Heraus 7570E. The supernatant, containing the propagated phage was filter sterilised (0.2 µm filter) and 50 µl of chloroform was added prior to storage at 4°C.

Phage titration

The phage suspension was serially diluted with 10 mM $MgSO_4$ (10^0 to 10^{-10}) and 10µl of each dilution was pipetted onto LB plates containing bottom agar (1.5% agar, 10 mM

$MgSO_4$) and top agar (0.4% agar, 10 mM $MgSO_4$). Plates were incubated overnight at 37°C. Amplification of phage using the donor strain was repeated until the phage titre was ~5 x 10^9 pfu/ml.

Transduction with P22 bacteriophage

A 10 ml overnight culture of recipient SL1344 (Str^S) cells was spun at 4000 rpm in a Megafuge 1.0 Heraus 7570E for 10 minutes. The bacterial pellet was resuspended in 5 ml of 10 mM $MgSO_4$, 5 mM $CaCl_2$. Equal volumes (100µl) of recipient cells and phage lysate were mixed in a tube to which was added a final concentration of 20mM sodium citrate. The tube contents were spread onto LB agar plates containing the appropriate antibiotic and 20 mM sodium citrate. Plates were incubated overnight at 37°C. Negative phage and recipient controls consisted of tubes containing phage lysate and recipient cells respectively. In each case volumes were made up with LB. P22 transductants were streaked onto supplemented LB agar plates as above in order to remove any residual phage. This process was further repeated twice. Transductants were screened for streptomycin sensitivity and further analysed by PCR.

2.2.14 Bacterial conjugation

Bacterial conjugation experiments were performed essentially as described by Chikami *et al.* (1985). Briefly, 1.5 ml of an overnight culture of donor cells was spun in a micro-centrifuge for 1 minute. The bacterial pellet was resuspended in 1.5 ml of sterile PBS (8 mM Na_2HPO_4, 137 mM NaCl, 0.5 mM $MgCl_2$, 1.6 mM KH_2PO_4, 2.7 mM KCl). Cells were spun for 1 minute and resuspended in 600 µl sterile PBS. Recipient cells were prepared in the same way. 200 µl of the donor strain was mixed with 100 µl of the recipient strain. The mixture was pipetted onto a sterile 0.22 µm nitrocellulose filter (Millipore) on an LB plate without spreading. Plates were incubated for 4 hours at 37°C. Negative controls consisted of 300 µl of recipient cells only and 300 µl of donor cells only. After the incubation period, the filter was removed and bacteria were resuspended in 200 µl of sterile PBS. Transconjugants (recipients carrying the plasmid from the donor cell that has integrated into their chromosome due to a single cross-over event) were selected for on LB plates containing the appropriate antibiotic. Candidates having undergone successful transfer

were then screened for a second cross-over event by plating onto sucrose-TB medium containing the appropriate antibiotic. Plates were incubated at 37°C overnight.

2.3 Protein Techniques

2.3.1 Estimation of protein concentration

Protein concentrations were measured by UV spectroscopy taking absorbance readings in quartz cuvettes at 280 nm against a suitable blank. It was assumed that an absorbance reading of 1 was equivalent to a protein concentration of 1mg/ml.

2.3.2 Preparation of bacterial whole cell lysates

Unless otherwise stated, bacteria were harvested from 1 ml of a mid-exponential phase culture in a micro-centrifuge at 13000 rpm for 2 minutes. The bacterial pellet was resuspended in 200 ml 2 x Final Sample Buffer (FSB) (0.125 M Tris-HCl (pH 6.8), 4% SDS, 20% glycerol, 10% 2-Mercaptoethanol, 0.05% bromophenol blue). Samples were boiled for 5 minutes and then stored at –20°C.

2.3.3 SDS polyacrylamide gel electrophoresis of proteins

Unless otherwise stated, separation of protein samples by size was performed using 12% SDS gels as previously described by Laemmli, (1970) using the Bio-Rad mini-gel electrophoresis apparatus in accordance with the manufacturer's instructions. Gels were stained with Coomassie Blue (0.25% (w/v) Coomassie Brilliant Blue R-250, dissolved in 9% (v/v) glacial acetic acid and 45% (v/v) methanol) to enable visualisation of protein bands. Gels were destained in 5% (v/v) glacial acetic acid, 50% (v/v) methanol. Broad Range marker (New England Biolabs) was used for size approximation (Table 2.5).

Table 2.5. New England Biolabs Broad Range Molecular Weight Marker

Protein Broad Range Molecular Marker (NEB)	**Molecular Mass (Da) (to 3 sig.fig.)**
Myosin	212 000
MBP-β-galactosidase	158 000
β–galactosidase	116 000
Phosphorylase b	97 200
Serum Albumin	**66 400**
Glutamate dehydrogenase	55 600
Maltose-binding protein 2	42 700
Lactose dehydrogenase	36 500
Triose phosphate isomerase	**26 600**
Trypsin inhibitor	20 100
Lysozyme	14 300
Aprotinin	6 520
Insulin A, B chain	2 870

2.3.4 Two-dimensional gel electrophoresis

Preparation of whole cell lysates

For analysis of SL1344 and *ΔoxyS::cat* strains, overnight cultures were diluted 1:40 into fresh pre-warmed LB medium (pH 7.2) and grown to an A_{600}=0.5 at 37°C with light shaking. The culture (1L) was split and one half was treated with a non-lethal but inducing dose of 3 μM H_2O_2, whereas the remaining half was treated with an equal volume of sterile distilled water. Both samples were incubated for 60 minutes at 37°C. Cells were harvested at 37°C by centrifugation at 6000 rpm for 15 minutes in a Beckman J2-21 centrifuge. Bacterial pellets were resuspended in an equal volume of 0.9% (w/v) NaCl prior to a final spin at 6000 rpm for a further 15 minutes at 37°C. Cell pellets were resuspended in 4 ml of Lysis Buffer (2% (v/v) Triton X-100, 2% (v/v) β-mercaptoethanol, 2% (v/v) pH 4-7 IPG Buffer and 8 mM phenylmethylsulphonyl fluoride). Cells were sonicated using an MSE Soniprep 150 with 4 x 20 s cycles and 40 s cooling on ice. Urea was added to each sample to a final concentration of 9 M. Protein concentration was measured as previously described. Samples were then snap-frozen in liquid N_2 and stored at –70°C until use.

Immobiline DryStrip gel rehydration

Typically 4 mg of protein sample was mixed with an appropriate volume of rehydration solution (containing per 5ml: 8 M urea, 0.1 g CHAPS, 25 μl pH 4-7 IPG Buffer, 15 mg dithiothreitol, and a few grains of Orange G) to give a final sample volume of 400 μl. The sample was pipetted into a slot on the reswelling tray, taking care to remove any air bubbles. An Immobiline DryStrip gel (pH 4-7) (Amersham-Pharmacia) was placed gel side-down in the solution. The DryStrip was overlaid with 2ml of cover fluid (Amersham-Pharmacia) to minimise evaporation and crystallisation. The gel was allowed to hydrate in the covered reswelling tray overnight at room temperature.

First dimension run

The preparation of the Multiphor II Electrophoresis Unit (Amersham-Pharmacia) and application of the DryStrip were performed in accordance with the manufacturer's

instructions. The Thermostatic Circulator maintained the cooling plate at a temperature of 20°C.The equilibrated DryStrip was electrophoresed for a total of 75 kVh, which comprised of:

1 hour at 150 V	0.15 kVh
1 hour at 300 V	0.30 kVh
1 hour at 600 V	0.60 kVh
39 hours at 1896 V	73.95 kVh

Equilibration of Immobiline DryStrips

The DryStrip containing the focused proteins was transferred into a tube fashioned from a 10 ml pipette, to which was added 10 ml of Equilibration Solution 1 (containing per 10ml: 0.05 M Tris-HCl (pH 6.8), 6 M urea, 30% glycerol, 1% SDS, 25mg dithiothreitol). The tube containing the DryStrip was sealed with Parafilm and rocked at room temperature for 10 minutes, after which time the solution was poured off and replaced with Equilibration Solution 2 (as for solution 1 but the dithiothreitol was replaced with 0.45 g of iodoacetamide per 10ml and a few grains of Bromophenol Blue. After a further incubation period of 10 minutes at room temperature with gentle rocking, the DryStrip was removed from the glass tube and drained on its side on damp filter paper for between 3 and 20 minutes.

Second dimension run

The preparation of the Multiphor II Electrophoresis unit together with the application of the equilibrated DryStrip to a 12-14% gradient gel (Amersham-Pharmacia) was carried out in accordance with the manufacturer's instructions. The stages of electrophoresis were performed as follows:

Stage 1 1000 V, 20 mA, 40 W per gel - until the dye front had moved 4-6 mm from the DryStrip

Stage 2 1000 V, 40 mA, 40 W per gel – when the dye front had moved a further 2 mm, the cathode buffer strip was moved forward to the area of the removed DryStrip.

Stage 3 1000 V, 40 mA, 40 W per gel – until the dye front had reached the anodic buffer strip (approximately 2.5 hours).

The gel was removed from the electrophoresis unit and blotted to remove excess paraffin. Following electrophoresis, proteins were detected by staining with Coomassie when appropriate. When required, immobilised proteins were electroblotted onto polyvinylidene fluoride membrane for 45 minutes at a constant 500 mA, essentially as described by Matsudaira, (1987). Prior to electrotransfer, the plastic backing was removed with a film remover. The gel and the membrane were then equilibrated in 1 x CAPS Buffer (1 mM CAPS (pH 11.0). Transferred protein spots were detected by staining with Amido Black (0.1% (w/v) solution of Napthol Blue Black in 10% (v/v) methanol, 2% (v/v) acetic acid) and destained in 30% methanol prior to their excision for sequencing.

2.4 Immunological techniques

2.4.1 Carbonylation assays

Preparation of whole cell extracts

Overnight cultures (10 ml) were diluted 1:40 (final volume 40 ml) into fresh pre-warmed LB medium (pH 7.2) and grown until A_{600}=0.4. Cells were harvested at 4°C by centrifugation in a Beckman J2-21 centrifuge at 7000 rpm. Bacterial pellets were washed in 0.9% ice-cold NaCl and spun for a further 15 minutes at 7000 rpm at 4°C. Cells were resuspended in lysis solution (50 mM DTT, 100 mM Tris-HCl (pH 7.5)) and sonicated using an MSE Soniprep 150 for 15 s on, 40 s off for 3 cycles. Cells were spun down at 5000 rpm for 15 minutes at 4°C to remove cell debris. The supernatant was removed and the protein concentration was measured as previously described. Samples were snap-frozen and stored at –70°C until required.

Derivatization of proteins

Protein samples (20 μg) were denatured by the addition of a final concentration of 6% SDS. The carbonyl groups in the protein side chains of whole cell extracts were derivatized using the Oncor OxyBlot™ (Appligene) to 2,4 –dinitrophenylhydrazone (DNP-hydrazone) by reaction with 2,4-dinitrophenylhydrazine in accordance with the manufacturer's instructions. Derivatized proteins were spotted onto a nitrocellulose membrane and

detected with anti-DNP antibodies. Alternatively protein carbonylation was determined by a one-dimensional Western blot immunoassay. In both cases, the bound antibodies were detected using SuperSignal chemiluminescence substrate (Pierce).

2.4.2 Western blotting

Proteins were separated on a 12% SDS-polyacrylamide gel as previously described and then transferred onto nitrocellulose membrane using a Mini-Trans Blot Electrophoresis Transfer Cell (BioRad) in accordance with the manufacturer's instructions. Prior to transfer, the gel and the nitrocellulose membrane were equilibrated in ice-cold Transfer Buffer (25 mM Tris, 192mM glycine, 20% (v/v) methanol). Transfers were run at 100 V for approximately 3 hours, or until the current rose to 350 mA. Following transfer, the membrane was incubated in 150 ml of Blocking Buffer (10 mM Tris (pH 7.5), 1.975 mM Na_2EDTA (pH 8.0), 133 mM NaCl, 0.5% BSA (w/v), 0.5% Triton X-100 (v/v)) overnight at 4°C. After removal of the blocking buffer the membrane was incubated with the appropriate primary antibody for 2 hours at room temperature with gentle agitation. The membrane was washed three times in fresh blocking buffer prior to being incubated with the secondary horseradish peroxidase (HRP) labelled antibody diluted in blocking buffer for 2 hours at room temperature with gentle agitation. The membrane was washed three times in Tris-saline buffer (50 mM Tris-HCl (pH 7.5), 100 mM NaCl). Bound antibodies were detected with the SuperSignal chemiluminescence substrate (Pierce), the emitted light being detected with X-ray film. The membrane was then stained with amido black to reveal the efficiency of transfer.

2.4.3 Primary antibodies

Antibody	Dilution	Source
Anti OxyR polyclonal	1:10000	G. Storz
Anti σ^S polyclonal	1:5000	R. Hengge-Aronis
Anti-DNP	1:150	Appligene

2.4.4 Secondary antibodies

Antibody	Dilution	Source
Goat anti-rabbit HRP	1:3000	Autogene

2.5 Biological assays

2.5.1 Base-line studies

2.5.1.1 Assay to determine the effect of H_2O_2 on cell viability

For analysis of *Salmonella* and *E. coli* strains, 10 ml overnight cultures were diluted 1:40 (final volume 40 ml) into fresh pre-warmed LB (pH 7.2) and grown at 37°C with light shaking until A_{600} =0.5. 1.5ml of the mid exponential phase culture was pipetted sequentially into 5 universal tubes labelled A, B, C, D and E to which was then added a final concentration of freshly prepared 0, 0.15 μM, 0.3 μM, 3 μM and 30 μM H_2O_2 respectively. The cultures were incubated for a further 60 minutes at 37°C with light shaking. Cultures were serially diluted to 10^{-10} in ice-cold sterile PBS, prior to plating 100 μl aliquots onto LB agar. Plates were incubated overnight at 37°C and colonies were counted the following day.

2.5.1.2 Assay to test for protective induction of resistance to oxidative stress

For analysis of *Salmonella* and *E. coli* strains, 10 ml overnight cultures were diluted 1:40 (final volume 40 ml) into fresh pre-warmed LB (pH 7.2) and grown at 37°C with light shaking until A_{600} =0.4. 10 ml of the mid exponential phase culture was pipetted sequentially into 2 universal tubes labelled A and B to which was then added a final concentration of 0 and 0.3 μM H_2O_2 respectively (freshly prepared). Both cultures were incubated for a further 60 minutes at 37°C with light shaking after which time a 100μl aliquot was removed from each tube and serially diluted to 10^{-6} using ice-cold PBS to serve as a zero time point. A final concentration of 30 μM H_2O_2 was added to tubes A and B and cultures were incubated for 90 minutes at 37°C. During the incubation, 100μl aliquots were removed at 5, 15, 30, 60 and 90 minute time points and serially diluted as detailed above, prior to plating 100μl volumes of appropriate dilutions onto LB agar. Plates were incubated overnight at 37°C and colonies were counted the following day.

2.5.2 Cross-protection assays

2.5.2.1 Assay to test if an inducing acid stress of pH 5.0 can cross protect against an otherwise lethal hydrogen peroxide stress

Overnight cultures (10 ml) for analysis were diluted 1:40 (final volume 40 ml) into pre-warmed LB (pH 7.2) and incubated at 37°C with light shaking until A_{600}=0.4. 10 ml aliquots of the culture were decanted into universal tubes labelled A, B and C. A suitable volume of sterile distilled water was then added to tube A. A final concentration of 0.3 μM H_2O_2 and a suitable volume of HCl to lower the pH to 5.0 was then added to Tubes B and C respectively. All tubes were incubated for a further period of 60 minutes at 37°C with light shaking. An aliquot of 100 μl was removed from each tube, serially diluted in PBS to 10^{-6} and plated out onto LB agar to create zero time points for all three cultures. A final concentration of 30 μM H_2O_2 was added to tubes A, B and C which were incubated for 90 minutes, during which time 100μl aliquots were removed at 5, 15, 30, 60 and 90 minute intervals, serially diluted in PBS and plated onto LB agar. Plates were incubated at 37°C overnight. Colonies were counted the next morning and recorded as cfu/ml.

2.5.2.2 Assay to test if a sub-lethal hydrogen peroxide stress can cross protect against an otherwise lethal acid stress of pH 3.0

The assay detailed in Section 2.5.2.1 was repeated with the exception of the 30 μM H_2O_2 stress, which was replaced by an acid stress of pH 3.0. The pH of the cultures in tubes A to C was lowered by the addition of suitable volumes of HCl.

2.5.3 Assay to test if *Salmonella* viability is growth phase dependent following a hydrogen peroxide stress

Overnight cultures (10 ml) for analysis were diluted 1:40 (final volume 40 ml) into pre-warmed LB (pH 7.2) and incubated at 37°C with light shaking until A_{600}=0.4. A 100 μl

aliquot of the culture was removed, serially diluted in ice-cold sterile PBS to 10^{-6}. Appropriate dilutions were plated onto LB agar. 10ml aliquots of the culture were decanted into tubes A and B. Tube A was treated with a suitable volume of sterile distilled water whereas tube B was treated with a final concentration of 30 μM H_2O_2. Cultures were incubated for 60 minutes at 37°C with light shaking, during which time 100 μl aliquots were removed at 5, 15, 30 and 60 minute intervals, serially diluted and plated as above. LB agar plates were incubated at 37°C overnight, numbers of colonies being recorded the following morning as cfu/ml. The procedure was repeated, but modified by administration of the 30 μM H_2O_2 stress to cells at A_{600}=0.8 and 1.0.

2.5.4 Assay to determine the effect of paraquat on *Salmonella* Viability

For analysis of *Salmonella* strains, 10 ml overnight cultures were diluted 1:40 (final volume 40 ml) into fresh pre-warmed LB (pH 7.2) and incubated at 37°C with light shaking until A_{600}=0.4. 1.5 ml of the culture was dispensed sequentially into 2 universals labelled A and B. A final concentration of 0 and 100 mM paraquat was added to tubes A and B respectively. Cultures were incubated for 60 minutes at 37°C with light shaking, after which time a 100 μl aliquot was removed from each tube. Aliquots were serially diluted to 10^{-6} in sterile ice-cold PBS and 100 μl volumes of appropriate dilutions were plated onto LB agar. Plates were incubated overnight at 37°C. Colonies were counted the following morning and recorded as cfu/ml. The above assay was repeated using cultures grown to A_{600}=1.0 prior to the addition of paraquat.

CHAPTER 3

BASE LINE STUDIES AND THE DETECTION AND IDENTIFICATION OF PROTEINS THAT ARE DIFFERENTIALLY EXPRESSED ON TREATMENT OF *SALMONELLA* WITH HYDROGEN PEROXIDE

CHAPTER 3 Base-line studies and the detection and identification of proteins that are differentially expressed on treatment of *Salmonella* with H_2O_2

3.1 Introduction

3.1.1 Adaptation to hydrogen peroxide in *S. Typhimurium*

While bacteria display adaptive responses to many reactive oxygen species, the majority of such studies have used hydrogen peroxide because it is more stable than species such as superoxide anions and hydroxyl radicals (Christman *et al.*, 1985). The existence of a regulated adaptive response to H_2O_2 was first shown for *E. coli* (Demple and Halbrook, 1983). Christman *et al.* (1985) subsequently showed that exponentially growing cells of *S.* Typhimurium strain LT2 treated with 60 μM H_2O_2 for 60 minutes were resistant to an otherwise lethal dose of H_2O_2 (10 mM). Two-dimensional gel analysis of *S.* Typhimurium cells treated with H_2O_2 showed that this oxidant induces the synthesis of at least 30 proteins (Christman *et al.*, 1985). However, none were identified due to the absence of adequately developed protein MS technology at that time. Not withstanding such drawbacks, two major temporal classes of hydrogen peroxide-inducible proteins were shown to exist. Synthesis of the "early" H_2O_2-responsive proteins is maximal during the first ten minutes following treatment with H_2O_2. In contrast, synthesis of the "late" proteins is at a maximal rate starting ten to thirty minutes following H_2O_2 exposure (Christman *et al.*, 1985).

3.1.2 Proteomic analysis of *Salmonella*

The total complement of expressed proteins in *S.* Typhimurium, i.e., its proteome has not yet been fully characterised. *E. coli* is closely related to *S.* Typhimurium and therefore much of the knowledge that has been obtained about these two bacteria is interchangeable. However these two organisms differ, for example in their ability to utilise certain carbon sources and in their strategies to invade and survive in phagocytic cells (Qi *et al.*, 1996). Many of the genes are differentially expressed in response to particular conditions such as

exposure to reactive oxygen species. In order to identify proteins belonging to one or more of an increasing number of complex, overlapping regulons, a method to identify the products of open reading frames that are differentially expressed *in vivo* under particular conditions is essential.

The technique used in this study that potentially fulfils these requirements is two-dimensional gel electrophoresis. This method separates hundreds of polypeptides orthogonally by their isoelectric points and molecular masses (O'Farrell, 1975; Klose, J, 1975). Proteins that are differentially expressed in response to hydrogen peroxide stress can be detected by comparing the patterns of protein expression in cells grown in test and control conditions. Such proteins may subsequently be identified from a two-dimensional reference map if it exists. Alternatively, methods for identifying the proteins in gel spots such as mass spectroscopy (Wilm *et al.*, 1996) and N-terminal microsequencing can be used (Qi *et al.*, 1996).

3.2 Base-line studies

3.2.1 Determining a suitable lethal dose of hydrogen peroxide for unadapted *S.* Typhimurium SL1344 cells

As a pre-requisite to more detailed studies, *S.* Typhimurium SL1344 cells were treated with a range of hydrogen peroxide concentrations to assess its effects on *Salmonella* viability. Cells were exposed to the reactive oxygen species for 60 minutes as described in section 2.5.1.1. The graph at Figure 3.1 illustrates that a final concentration of 30 μM H_2O_2 was a lethal dose, as there was no survival after 60 minutes incubation, whereas a final concentration of 0.15 μM H_2O_2 appeared to have no deleterious effect on viability. Eighty per cent survival was achieved by doubling the H_2O_2 concentration to 0.3 μM, thus suggesting a benchmark for a quantifiable deleterious effect. A ten-fold increase in H_2O_2 concentration resulted in viability being reduced by approximately seventy five per cent when compared to unstressed cells.

Previous studies by Christman *et al.* (1985) indicated that a concentration of 60 μM H_2O_2 was used to pre-adapt *Salmonella* cells prior to a 10 mM H_2O_2 challenge. Although no reference was made to any deleterious effects on viability as a result of the pre-adapting concentration of H_2O_2, it never the less highlighted the apparent marked sensitivity to H_2O_2 displayed by the *Salmonella* strain SL1344 used in this study. To assess if SL1344 was in fact acutely sensitive to H_2O_2, *S.* Typhimurium KK1110 and *E. coli* MC4110 were also assayed for sensitivity to the reactive oxygen species. Exposure to a final concentration of 30 μM H_2O_2 for 60 minutes proved to be fatal for both strains tested. Therefore, it would appear that SL1344 is not unusually hypersensitive to hydrogen peroxide, and the particular assay conditions used in this study are responsible for any variation observed in survival when compared to other studies.

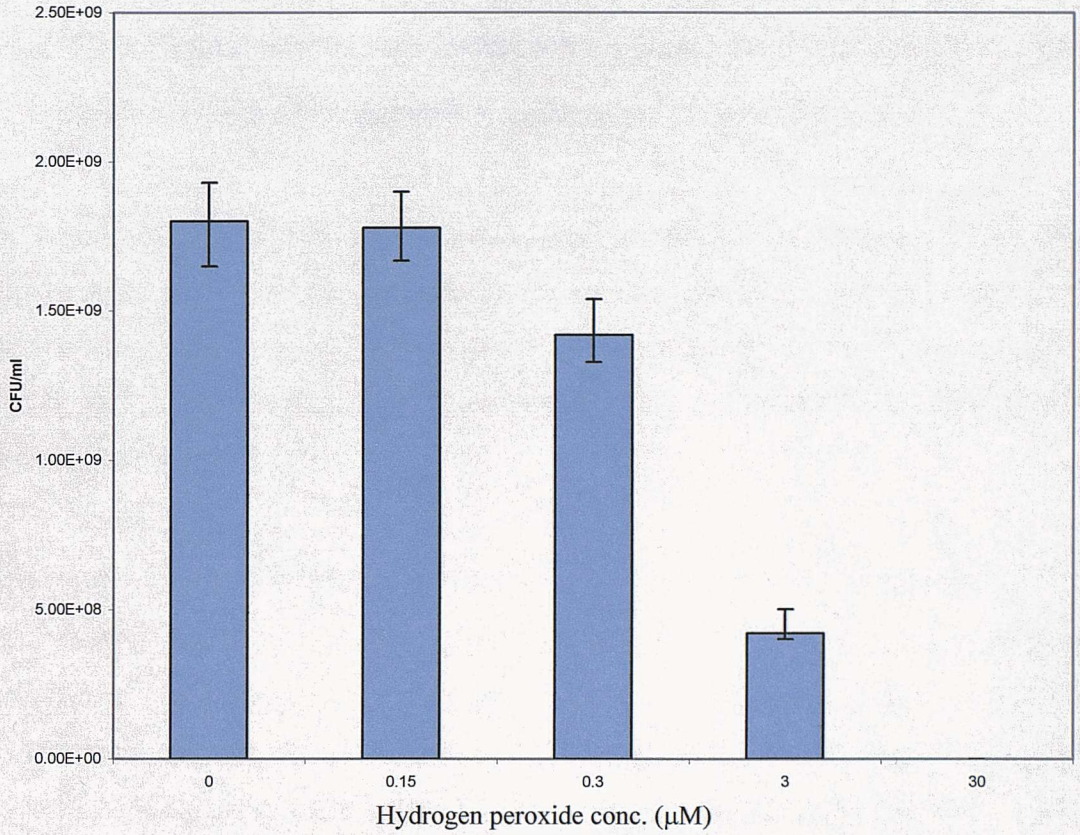

Figure 3.1. Effect of hydrogen peroxide on *Salmonella* viability. Mid-exponential phase (A_{600}=0.5) *Salmonella* cells were exposed to a range of hydrogen peroxide concentrations for 60 minutes. Cells were then diluted and plated onto LB agar. Plates were incubated overnight and colonies were then counted and recorded as cfu/ml. The data are the mean of three experiments carried out on separate occasions.

3.2.2 Prior exposure to sub-lethal concentrations of hydrogen peroxide offers protection against a subsequent lethal dose

To assess the regulation of defences against oxidative stress in *Salmonella* and subsequently to identify the components involved, it was considered important to analyse the adaptive response to hydrogen peroxide. Exponential phase cells, therefore, were pre-treated with a sub-lethal dose of hydrogen peroxide to initiate the adaptive response as detailed in section 2.5.1.2.

Salmonella cells that had been exposed to a pre-adaptive dose of 0.3 μM H_2O_2 for 60 minutes were resistant to a subsequent, otherwise lethal, stress of 30 μM H_2O_2 (Figure 3.2). In fact, pre-adapted cells remained viable at a 90 minute time-point after the 30 μM H_2O_2 stress had been administered, indicating that the response was long-lived. Conversely, unadapted cells were non-viable at 30 minutes under the same conditions. Although it has been shown that an inducing stress of 0.3 μM H_2O_2 deleteriously affected *Salmonella* viability by twenty per cent, this sub-lethal stress offered long-lived protection against a subsequent dose of 30 μM H_2O_2 and was therefore adopted for most subsequent studies.

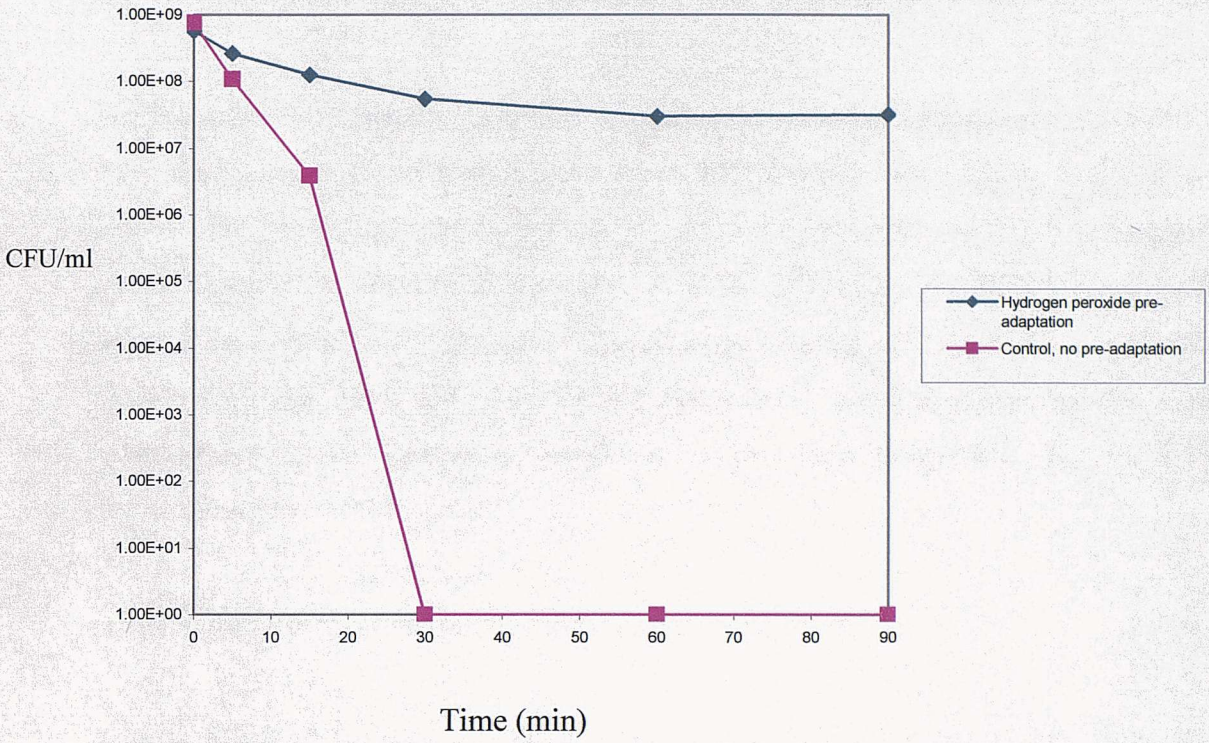

Figure 3.2. Analysis of the adaptive response to hydrogen peroxide in *Salmonella*. Mid-exponential phase (A_{600}=0.4) *Salmonella* cells that had been exposed to an inducing stress of 0.3 μM H_2O_2, or left unadapted, for 60 minutes were subsequently exposed to a 30μM H_2O_2 stress for 60 minutes. Cells were then diluted and plated onto LB agar. Plates were incubated overnight and colonies were then counted and recorded as cfu/ml. The data are the mean of three experiments carried out on separate occasions. Standard errors are all less than ten percent of the mean.

3.3 Two-dimensional gel analyses of wild-type SL1344 whole cell lysates

Having established suitable base-line conditions for inducing adaptation to H_2O_2 stress, the next stage was to detect proteins that were differentially expressed in response to such conditions. Accordingly, two-dimensional gel electrophoresis was used to compare the protein profile of unstressed wild-type SL1344 cells with that of hydrogen peroxide stressed cells (final concentration of 3 μM) in accordance with section 2.3.4. Preliminary studies indicated that the majority of *Salmonella* proteins detectable by 2-D gel electrophoresis were located in the pH 4-7 range. Accordingly, this was used for the studies described below.

3.3.1 Detection of differentially expressed proteins

Figures 3.3 and 3.4 illustrate the protein profiles obtained with whole cell lysates of SL1344 unstressed and H_2O_2 stressed cells respectively. For each growth condition at least four independent batches of cells were prepared and analysed. Numbered spots signify proteins that were differentially expressed consistently. It should be noted that quantitation of spots in 2-D gels is notoriously difficult without exact replicates as spot intensities may vary by as much as twenty per cent from batch to batch (Quadroni and James, 1999). In the absence of adequate image analysis software (at the time), it was considered prudent to wait until quantitation techniques were improved before attempting a more detailed analysis of the gel images. Accordingly, differentially expressed proteins were identified by eye by comparing the protein profiles obtained for unstressed cells with that of stressed cells. Only proteins whose intensities were consistently altered were recorded.

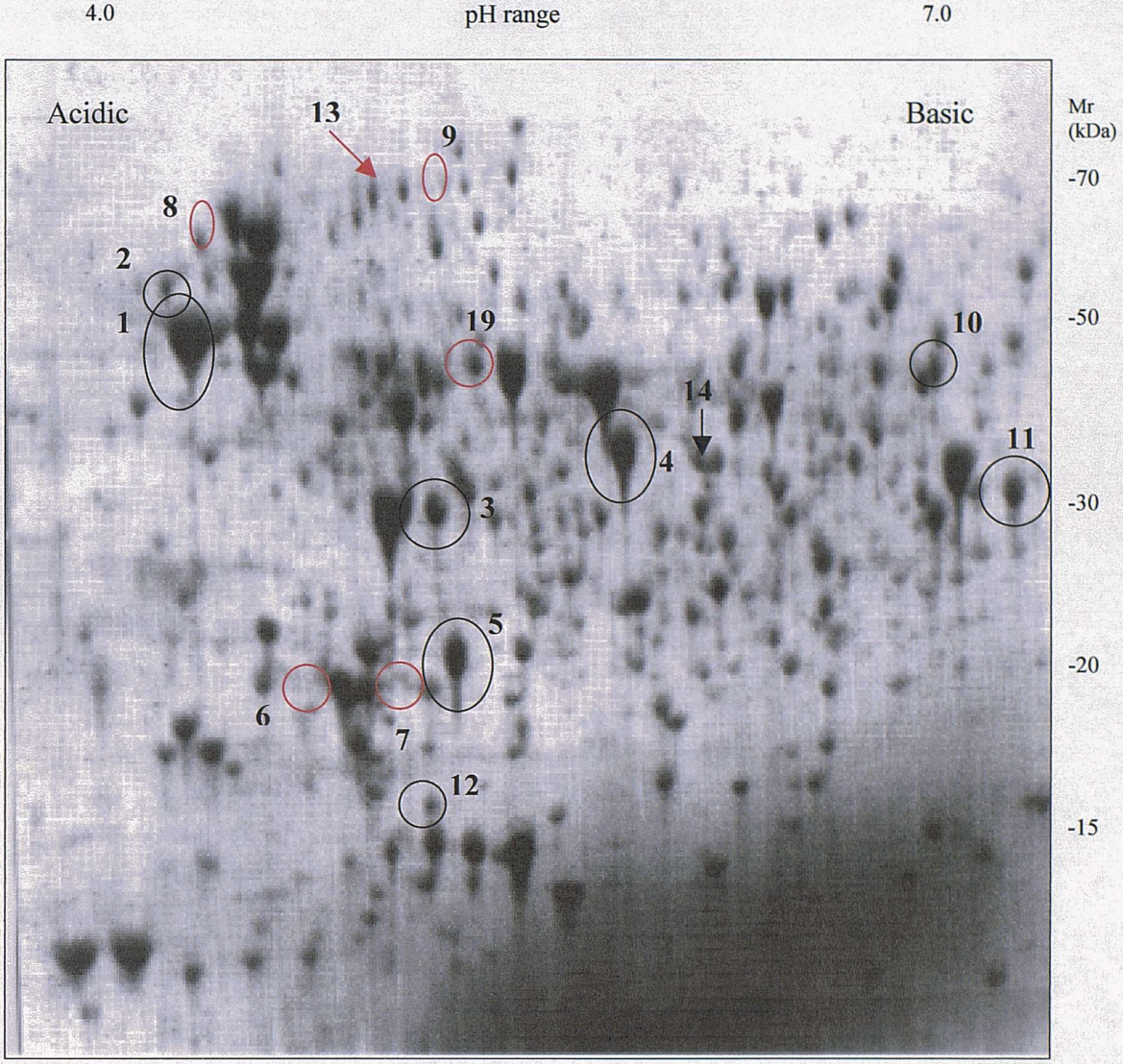

Figure 3.3. 2-D gel of unstressed *Salmonella* SL1344 whole cell extracts. Whole cell extracts were fractionated by 2-DE and stained as described in Section 2.3.4. This protein profile was compared with that of stressed cells (Figure 3.4). Open black circles and black arrows represent proteins that were down-regulated following H_2O_2 stress. Open red circles and red arrows represent proteins that were up-regulated following H_2O_2 stress.

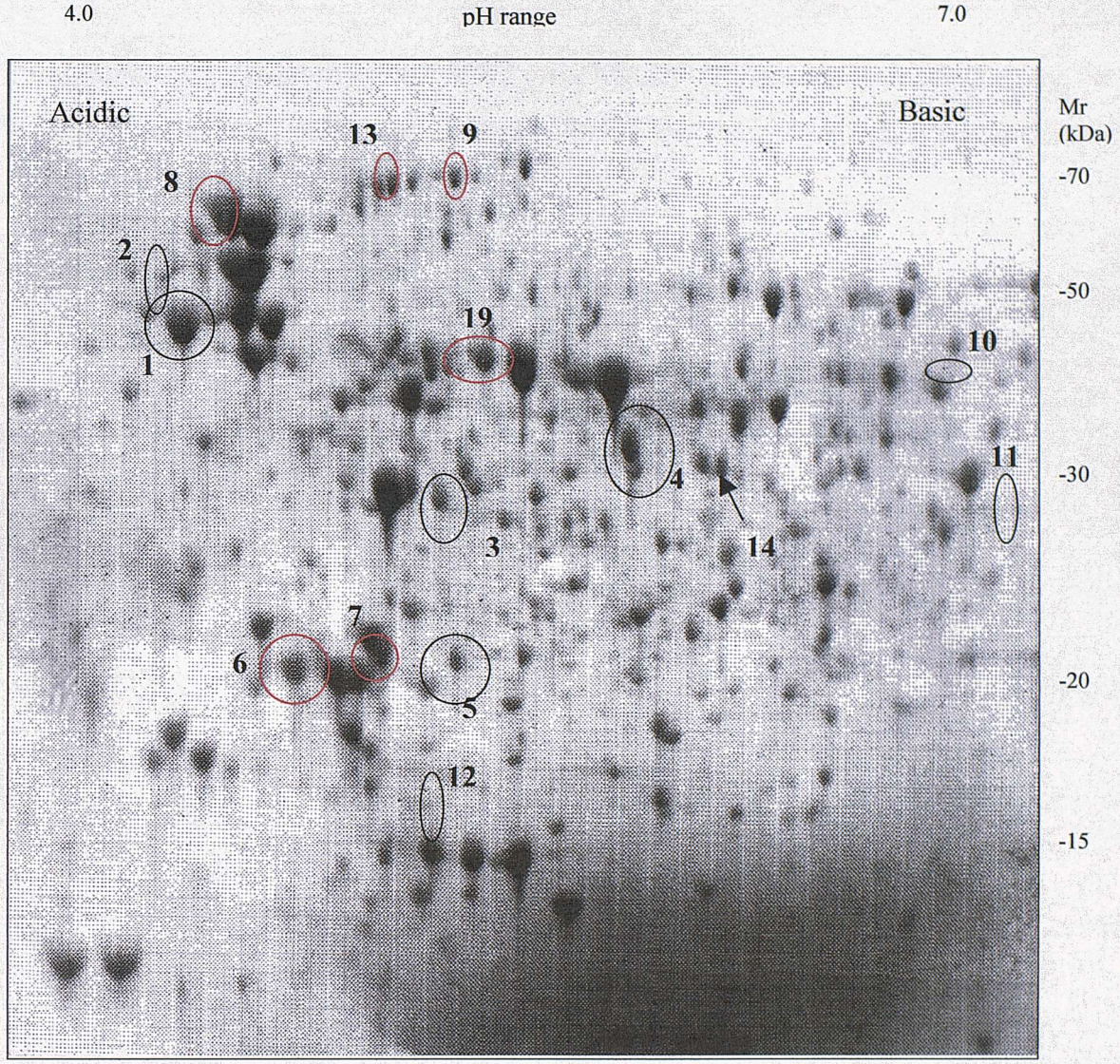

Figure 3.4. 2-D gel of H_2O_2 stressed *Salmonella* SL1344 whole cell extracts. Protein extracts from cells that had been exposed to a final concentration of 3 μM H_2O_2 for 60 minutes were fractionated by 2-DE and stained as described in Section 2.3.4 This protein profile was compared with that of unstressed cells (Figure 3.3). Open black circles and black arrows represent proteins that were down-regulated following H_2O_2 stress. Open red circles and red arrows represent proteins that were up-regulated following H_2O_2 stress.

Proteins that were down-regulated in the presence of hydrogen peroxide, their estimated molecular weights and isoelectric points are shown at Table 3.1. Up-regulated proteins are indicated at Table 3.2.

Table 3.1. Detection of proteins that were down-regulated in response to hydrogen peroxide. The protein profiles obtained for whole cell lysates of *Salmonella* SL1344 unstressed and H_2O_2 stressed cells were compared (Figures 3.3 and 3.4). Only proteins that were consistently down-regulated following H_2O_2 treatment were recorded.

Spot No.	pI[a]	M_r[a] (kDa)
1	4.7	50
2	4.7	52
3	5.2	30
4	5.5	35
5	5.2	21
10	6.2	41
11	6.4	30
12	5.2	16
14	5.6	35

Spot numbers relate to Figures 3.3 and 3.4.

[a] Values were estimated from the in-house two-dimensional reference map (compiled by Dr. P Adams and members of the DO'C group).

Table 3.2. Detection of proteins that were up-regulated in response to hydrogen peroxide. The protein profiles obtained for whole cell lysates of *Salmonella* SL1344 unstressed and H_2O_2 stressed cells were compared (Figures 3.3 and 3.4). Only proteins that were consistently up-regulated following H_2O_2 treatment were recorded.

Spot No.	pI[a]	M_r[a] (kDa)
6	4.9	19
7	5.0	21
8	4.8	70
9	5.2	80
13	5.0	80
19	5.3	45

Spot numbers relate to Figures 3.3 and 3.4.

[a] Values were estimated from the in-house two-dimensional reference map (compiled by Dr. P Adams and members of the DO'C group).

3.3.2 Identification of differentially expressed proteins

Two main approaches were used to identify the differentially expressed proteins. The first relied on the availability of a partial reference map for the SL1344 proteome derived from cells grown to mid-exponential phase in LB medium. This map was also used to estimate their isoelectric points and molecular weights. Proteins were also identified by searching the Swiss-Prot database with the N-terminal sequences derived by microsequencing (Qi *et al.*, 1996; Harrison *et al.*, 1992).

Spots 1 and 2 had previously been identified as Phase 1 and Phase 2 flagellin respectively (Qi *et al.*, 1996) and therefore were readily assignable from the in-house two-dimensional reference map. D-galactose binding protein and glycerophosphoryl diester phosphodiesterase had also previously been identified (P. Adams, unpublished data). The identified proteins are listed at Table 3.3.

To identify unassigned proteins, two-dimensional gels of unstressed and hydrogen peroxide stressed SL1344 whole cell lysates were electroblotted onto polyvinylidine fluoride membrane as described in Section 2.3.4. Following staining in amido black, spot number 5 was excised and N-terminally sequenced by Dr. A. Moir (Sheffield). The N-terminal sequence was used to search the Swiss-Prot database for protein matches.

Table 3.3. Identification of *Salmonella* proteins that were differentially expressed following exposure to hydrogen peroxide.

Spot	Protein identification Swiss-Prot accession number	M_r[a] (kDa)	pI[a]	M_r[b]	pI[b]
1	Flagellin phase 1 [a] (P06179)	50	4.7	51210	4.75
2	Flagellin phase 2 [a] (P52616)	52	4.7	52453.5	4.75
3	D-galactose binding protein [b] (P23905)	30	5.2	32997.3	5.07
4	Glycerophosphoryl diester phosphodiesterase [b] (P09394)	35	5.4	38200	5.22
14	Asparaginase 1 [b] (P18840)	35	5.6	37127	5.52

[a] Proteins were identified from the in-house two-dimensional reference map (compiled by Dr. P Adams and members of the DO'C group)

[b] Proteins were identified by searching the Swiss-Prot database with the N-terminal sequences derived by microsequencing (obtained from Dr. A. Moir).

[a] Values were estimated from the two-dimensional reference map as above

[b] Theoretical values were obtained from the Swiss-Prot data base.

3.3.3 Spot 5 shares homology with a 26-kDa antigenic protein from *Helicobacter pylori*

The N-terminal sequence obtained from Spot 5 was used to search appropriate sequence databases (Swiss-Prot, GSC Bacterial Blast) for possible homology to known proteins. The 25 amino acid residue stretch of N-terminal sequence shared 52 % identity with that of the 26-kDa AhpC protein from *Helicobacter pylori* (Figure 3.5 and Table 3.4). It was reassuring to learn that the homology between the *Salmonella* protein at Spot 5 and the 26-kDa *H. pylori* protein had previously been recognised by Qi *et al.*, 1996. Also, Spot 5 was shown to share 56% identity with the AhpC protein from *C. jejuni* (Figure 3.5 and Table 3.4). The *H. pylori* protein has been shown to be homologous to the alkyl hydroperoxide reductase small subunit AhpC protein found in *E. coli* (Storz *et al.*, 1989). In view of this finding, a further alignment of the N-terminal sequence of Spot 5 was performed with the AhpC proteins from *E. coli* and *S.* Typhimurium (Figure 3.6). This subsequently showed that the N-terminal sequence of Spot 5 shared 20% identity with these proteins (Table 3.4).

Figure 3.5. Alignment of N-terminal sequence from Spot 5 with AhpC proteins from *H. pylori* (Hp) and *C. jejuni* (Cj). Swiss-Prot/TrEMBL accession numbers are indicated. Asterisks denote identical residues, and dots indicate conservative substitutions. Sequences were aligned using Clustal Format for T-Coffee Version 1.41 (Notredame *et al.*, 2000).

Table 3.4. Percentage identities between the 25 amino acid N-terminal sequence from Spot 5 and AhpC proteins.

Alignment between Spot 5 and	**Identity (%)**
H. pylori AhpC (P21762)	52
C. jejuni AhpC (Q9ZI13)	56
S. Typhimurium AhpC (P19479)	20
E. coli AhpC (P26427)	20

Figure 3.6. Alignment of N-terminal sequence from Spot 5 with AhpC proteins from *S.* Typhimurium (SALTY) and *E. coli* (ECOLI). Swiss-Prot accession numbers are indicated. Asterisks denote identical residues, and dots indicate conservative substitutions. Sequences were aligned using Clustal Format for T-Coffee Version 1.41. (Notredame *et al.*, 2000).

3.4 Discussion

This chapter has described the determination of suitable conditions for induction of adaptation to H_2O_2 stress in *S.* Typhimurium SL1344 cells. It has also reported the identification of several proteins with altered levels under such conditions. The possible roles of these proteins are now considered on a case-by-case basis.

3.4.1 Spot 5 is a subunit of a peroxidase-like enzyme, related to AhpC, which is down-regulated in response to H_2O_2 stress.

The 25 amino acid residue stretch of N-terminal sequence that was obtained for Spot 5 strongly suggests that this protein is related to AhpC, a subunit of alkyl hydroperoxide reductase. The enzyme consists of two subunits, AhpC and AhpF. The AhpC subunit in its reduced form, reduces alkyl hydroperoxides to alcohols and in the process is oxidised. The AhpF subunit is thought to transfer electrons from NAD(P)H to the oxidised AhpC subunit thereby re-reducing it (Baillon *et al.*, 1999). AhpFC has been implicated in the detoxification of organic hydroperoxides (Tartaglia *et al.*, 1990). It may therefore have an anti-mutator role, although it does not significantly regulate cellular H_2O_2 levels *in vivo* in *E. coli* (Gonzalez-Flecha and Demple, 1997).

Subsequent to the experimental work described here, the genome sequences of two serovars of *Salmonella enterica* became available (Wellcome Trust Sanger Institute). Further sequence analysis using the BLAST similarity search algorithm showed that the gene for Spot 5 corresponds to STY0440 in *S.* Typhi and STM0402 in *S.* Typhimurium LT2, with a 100% sequence match between the N-terminal sequence for Spot 5 and for these predicted proteins. The full-length Spot 5/STM0402 amino acid sequence was aligned with AhpC sequences from other Gram-negative bacteria, namely *S.* Typhimurium, *E. coli*, *H. pylori* and *C. jejuni* (Figure 3.7). The alignment revealed two highly conserved cysteine residues toward the N and C termini, which in the Spot 5/STM0402 protein are found at residues 50 and 171. The sequences surrounding the conserved cysteine resides at positions 50 and 171 are DFTFVCP and VCPA respectively in all aligned proteins. The

biochemical alteration of the N-terminal cysteine residue has been shown to be important in the antioxidant activity of *S.* Typhimurium alkyl hydroperoxide reductase (Jacobson *et al.*, 1989). Overall, the Spot 5/STM0402 protein has 42% sequence identity with AhpC of *S.* Typhimurium (Figure 3.7 and Table 3.5). This suggests that the two proteins are paralogues, i.e. they are evolutionarily related through an ancestral gene duplication but do not now necessarily perform the same biochemical function. While it is therefore likely that Spot 5/STM0402 is a peroxidase, it cannot be assumed that it uses the same substrates as AhpC or that it has the same biological role.

The possible different roles of the Spot 5/STM0402 and AhpCF are underscored by the differences in their responses to oxidative stress. The AhpCF system has been shown to be induced by H_2O_2 and the cognate genes are positively regulated by the OxyR regulator (Christman *et al.*, 1985). In contrast, the results obtained here with Spot 5/STM0402 indicate that the protein is down-regulated following exposure of cells to H_2O_2. Despite these differences, it is still useful to consider how the AhpCF system is regulated in detail, as it is possible that the same or similar regulators may control the expression of Spot 5/STM0402, albeit in the opposite direction.

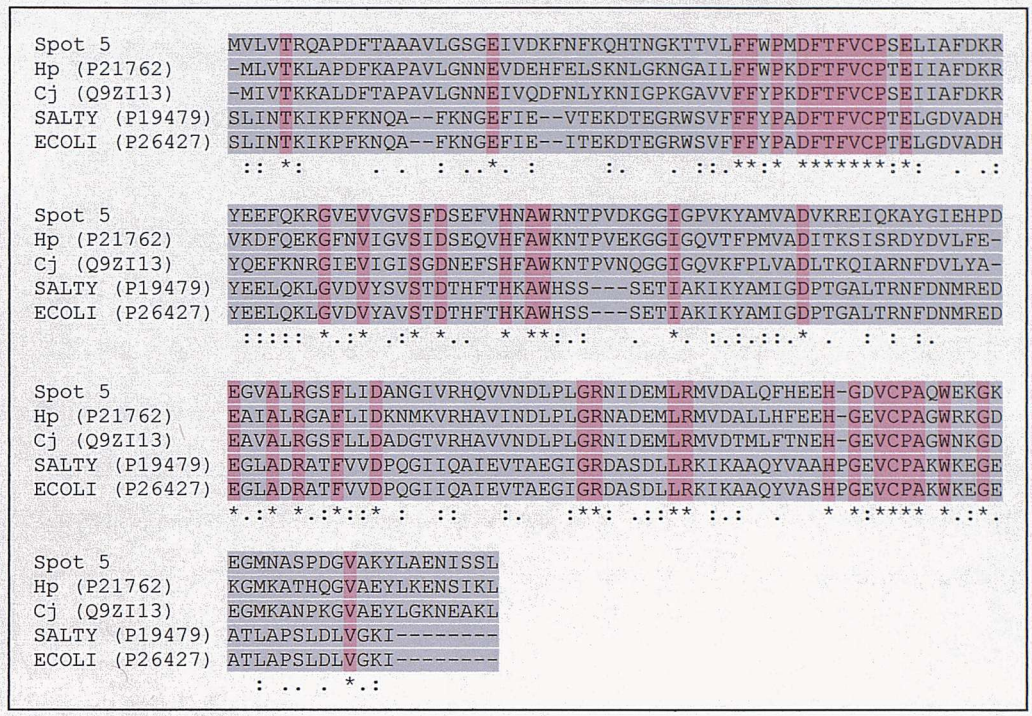

Figure 3.7. Alignment of full-length amino acid sequences of AhpC proteins from *H. pylori* (Hp), *C. jejuni* (Cj), *S.* Typhimurium (SALTY), *E. coli* (ECOLI) with Spot 5/STM0402. Swiss-Prot accession numbers are indicated. Asterisks denote identical residues, and dots indicate conservative substitutions. Sequences were aligned using Clustal Format for T-Coffee Version 1.41 (Notredame *et al.*, 2000).

Table 3.5. Percentage identities between the full-length amino acid sequence from Spot 5.

Alignment between Spot 5 and	Identity (%)
H. pylori AhpC (P21762)	57
C. jejuni AhpC (Q9ZI13)	61
S. Typhimurium AhpC (P19479)	42
E. coli AhpC (P26427)	42

3.4.1.1 Regulation of the AhpCF system and its possible implications for the control of Spot 5/STM0402

The *ahp* locus in *S.* Typhimurium contains two structural genes encoding both AhpC and AhpF proteins (Tartaglia *et al.*, 1989). The two genes are transcribed in the same direction with the *ahpC* gene located upstream of the *ahpF* gene. The synthesis of both proteins is positively regulated by OxyR, in response to hydrogen peroxide and an *oxyR*-regulated promoter has been identified just upstream of the *ahpC* gene (Tartaglia *et al.*, 1990; Christman *et al.*, 1985). It is considered likely that the *ahpC* and *ahpF* genes comprise, or are part of, an operon, the promoter upstream of the *ahpC* gene regulating the expression of both genes (Tartaglia *et al.*, 1990).

OxyR-DNA binding is based on a specific recognition code, however, the key interacting nucleotides are unusually dispersed (Toledano *et al.*, 1994) (Section 1.8.3.3.2 and Figure 3.8). The OxyR binding site in the regulatory region of *ahpC* in *S.* Typhimurium has also been mapped and similarly shows that interacting nucleotides have an unusual distribution (Toledano *et al.*, 1994) (Figures 3.8 and 3.9). Accordingly, the promoter region of the Spot 5/STM0402 gene was screened for a putative OxyR binding site. Although the unusually dispersed nature of the key nucleotides was taken into consideration, no putative OxyR binding site was identified.

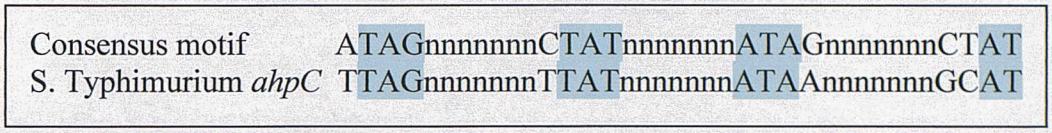

Figure 3.8. Comparison of the nucleotides that comprise the OxyR binding site upstream of the *S.*Typhimurium *ahpC* gene with the consensus sequence motif for the binding of oxidised OxyR (Toledano *et al.*, 1994). Shaded nucleotides match the consensus sequence. Eleven out of sixteen nucleotides match.

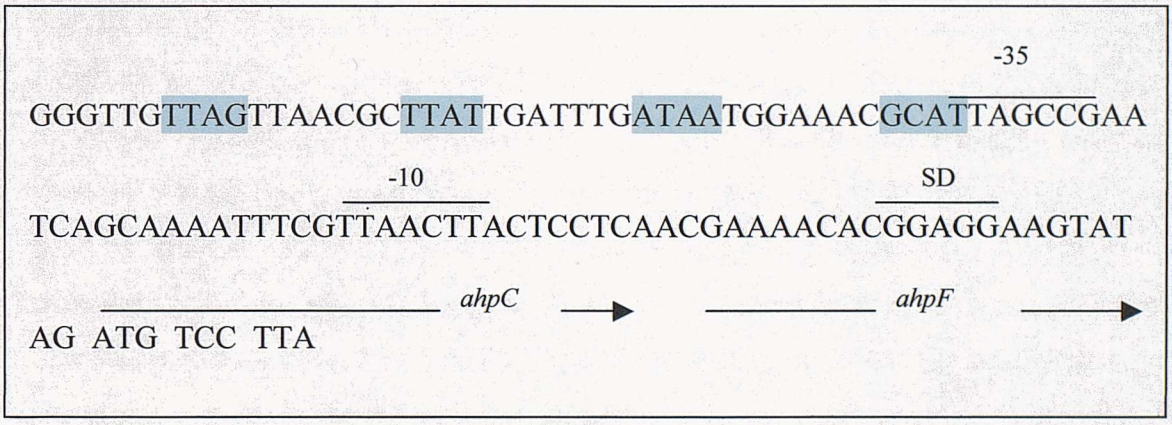

Figure 3.9. *S.* Typhimurium *ahpC* promoter region. OxyR binding motif is shown blue. Putative Shine-Dalgarno (SD) and sigma 70, –10 and –35 regions are marked by a line drawn above the relative nucleotides. Orientation of *ahpC* and *ahpF* genes are indicated by an arrow (Toledano *et al.*, 1994).

Baillon *et al.* (1999) have reported that the *C. jejuni* AhpC protein is expressed at a significantly higher level in cells grown under iron-restricted conditions. Conversely, expression has been shown to be iron repressible, a basal level of AhpC being expressed under iron replete conditions. They also reported that an *ahpC* null mutant was no more sensitive to hydrogen peroxide than its wild-type counterpart. However the mutant was found to be hypersensitive to cumene hydroperoxide (CHP) and displayed reduced aerotolerance. Whereas the expression of *ahpC* is usually mediated through the OxyR transcription factor in response to oxidative stress, no OxyR homologue has been identified in *C. jejuni* or *H. pylori* (Christman *et al.*, 1985; van Vliet *et al.*, 1999). Although Fur has been shown to mediate bacterial iron-responsive gene regulation in *E. coli* and *S.* Typhimurium, in *C. jejuni* iron regulation of AhpC and KatA is Fur independent, despite the presence of Fur box-like sequences in the *ahpC* promoter region (vanVliet *et al.*, 1999). Instead, iron regulation of AhpC is mediated by a Fur homologue, designated PerR and the product of *perR* negatively regulates the products of *ahpC* and *katA*. It can be regarded therefore, as a functional analogue for OxyR in *C. jejuni* (van Vliet *et al.*, 1999). Also, an *ahpF* homologue has not been identified in *C. jejuni*, thereby highliting the need for an alternative mechanism to recycle oxidised AhpC (Baillon *et al.*, 1999). Instead, the *ahpC* gene is flanked upstream by an *fdxA* (*fdxA* encodes a ferrodoxin) homologue and downstream by an *flhB* homologue (*flhB* encodes FlhB and is involved in both the biogenesis and regulation of biogenesis of flagella) (Figure 3.10). For comparison, genes flanking the Spot 5/STM0402 gene were identified (Figure 3.11).

As no Fur or OxyR homologue has been identified in *H. pylori*, regulation of *ahpC* expression in this organism remains unclear (van Vliet *et al.*, 1999).

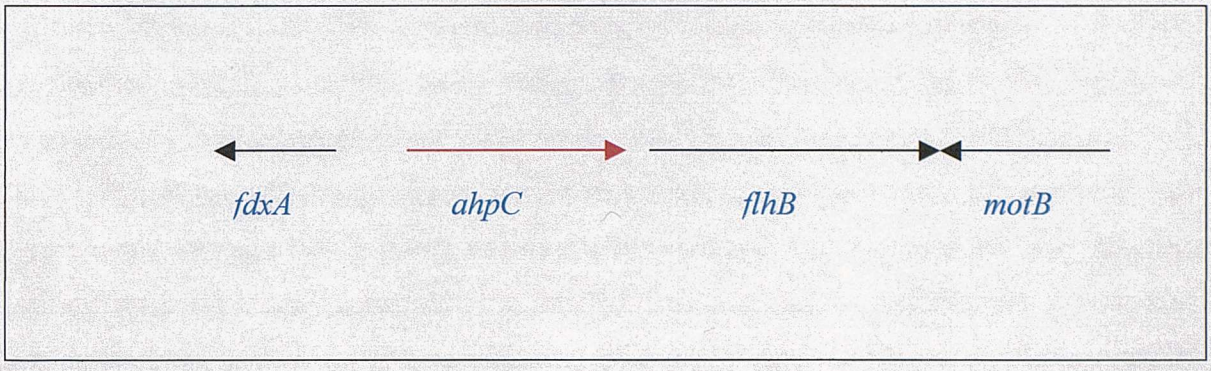

Figure 3.10. *C. jejuni* genomic region containing the *ahpC* gene. The *fdxA* gene encodes a ferrodoxin homologue that is divergently transcribed. The *flhB* homologue is independently transcribed. The FlhB protein is involved in both the biogenesis and regulation of biogenesis of flagella (Adapted from Baillon *et al.*, 1999).

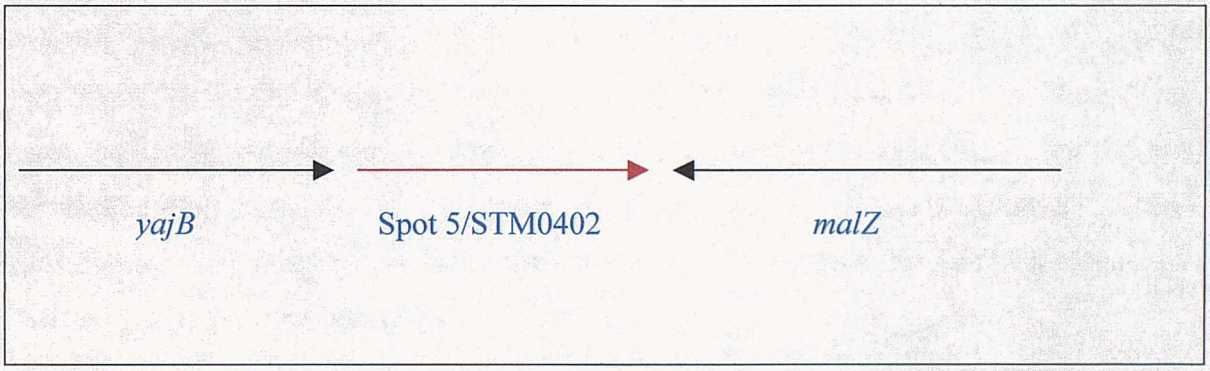

Figure 3.11. *S.* Typhimurium genomic region containing the Spot 5/STM0402 gene. The *yajB* gene encodes a putative cytoplasmic protein. The *malZ* gene encodes a maltodextrin glucosidase and is divergently transcribed.

In view of the intimate link between iron-regulated gene expression mediated by Fur and control of AhpCF, it would be of some interest to determine if Spot 5/STM0402 is controlled by Fur. In the presence of an Fe(II) cofactor, the Fur protein dimerises and binds to a 19 bp palindromic sequence in the promoter region of iron-regulated genes in *E. coli* (de Lorenzo *et al.*, 1987). More recent studies indicate that the minimal unit that Fur recognises is a hexameric sequence, 5' NAT(A/T)AT 3', the sequence is repeated at least three times, regardless of orientation, for efficient binding of Fur to promoter regions (reviewed by Escolar *et al.*, 1999). Interestingly, the regulatory region upstream of STM0402 (the gene encoding Spot 5) has three putative Fur sites (Figure 3.12). However, no genes with genuine Fur sites separated by such long sequences of DNA have yet been identified, and one of the putative sites in STM0402 is only in partial agreement with the Fur consensus. Clearly, further characterisation and analysis of the Spot 5/STM0402 gene and its regulatory region is required.

The possibility that Spot 5/STM0402 is controlled via the SoxRS system in addition to, or instead of some other regulator, must also be considered. Pomposiello *et al.* (2001) have recently shown that AhpC is indeed under such control. A SoxS-binding consensus of AN2GCAYN7CWA (where N is any base, Y is a pyrimidine, and W is A or T) has been used to identify several genes potentially regulated by the SoxRS system in *E. coli* (Li and Demple, 1996). Accordingly, the regulatory region upstream of the gene encoding Spot 5/STM0402 was analysed for the presence of such a sequence, but no potential SoxS-binding consensus was identified.

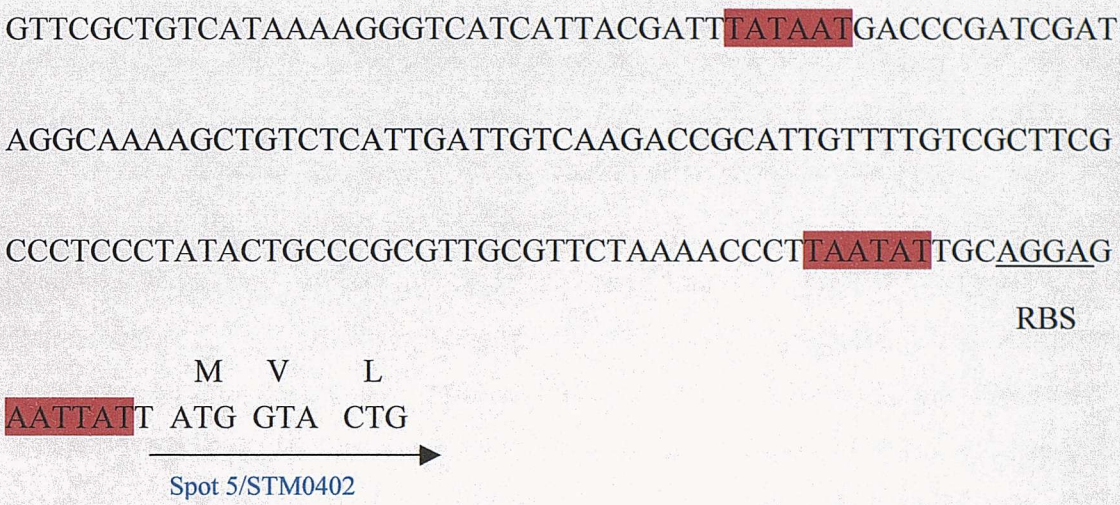

Figure 3.12. Analysis of the regulatory region upstream of the Spot 5/STM0402 gene. Putative Fur hexameric recognition sequences are in red.

3.4.2 Down-regulation of phase 1 and phase 2 flagellin – possible energy conservation in hydrogen peroxide challenged *Salmonella*

As reported in section 3.3.2, both forms of the major structural protein of the flagellar filament, were markedly down-regulated in cells exposed to hydrogen peroxide. Related results were obtained by Adams *et al.* (2001) who showed that phase 1 and phase 2 flagellin (and also motility) are negatively regulated by low pH via the PhoPQ system. Flagella can increase or decrease their rotational speed in relation to the strength of the proton motive force. Flagella rotation can move bacteria through liquid media at speeds up to 60 cell lengths per second (Madigan *et al.*, 1997). Therefore, flagella-mediated cell motility is a potentially important virulence associated process as it enables *Salmonella* to evade a potentially dangerous environment by use of negative chemotaxis. However, flagella require the expression of around 50 genes, and the synthesis of a flagellum consumes approximately 2 % of the cell's total energy (Madigan *et al.*, 1997). It is possible therefore, that phase 1 and phase 2 flagellin may be down-regulated following hydrogen peroxide stress in an attempt to save ATP so that it can otherwise be used in protective responses against reactive oxygen species. It should be noted, however, this putative energy-saving response is not observed generally. For example, osmotic stress only has a marginal affect on the levels of phase 1 and phase 2 flagellin (Adams *et al.*, 2001). It would be interesting to discover therefore, if treatment of *Salmonella* cells with substances that induce the superoxide stress response (such as paraquat) also result in the down regulation of the flagellin proteins. One clue comes from the work of Pomposiello *et al.* (2001), who recently conducted a DNA microarray analysis experiment to identify *E. coli* genes that are controlled via the SoxRS system. The flagellin gene, *fliC* was not found to be induced by paraquat. It seems likely, therefore, that the superoxide stress response does not explain the observed repression of flagellin synthesis in hydrogen peroxide-treated *Salmonella* cells. Instead, down regulation of phase 1 and phase 2 flagellin following hydrogen peroxide stress and acid stress suggests a possible overlap between the OxyR and PhoPQ regulons.

3.4.3 Down regulation of D-galactose binding protein – a possible conservation mechanism that does not jeopardise its chaperone-like characteristics

The binding protein-dependent galactose transport system of *S.* Typhimurium comprises four proteins. MglB is a periplasmic binding protein that interacts with and delivers its substrates, glucose and galactose to a membrane complex. The membrane complex comprises two integral inner membrane proteins, MglC and MglE, and an energy-transducing ATPase, MglA. Galactose-import requires energy, which is provided by the hydrolysis of ATP. Whilst nutrient uptake is undoubtedly important, *Salmonella* may favour energy conservation when exposed to an environmental stress.

Newly synthesised MglB is exported to the periplasm via the sec-dependent type II secretion system (Harayama *et al.*, 1983; Muller *et al.,* 1985; Bassford, 1990) and there is an accumulation of pre-MglB in *secB*, *secA* and *secY* mutants (Yaagoubii *et al.*, 1996). Moreover, a *dnaK* temperature sensitive mutant of *E. coli* also displayed a reduced transport activity and this correlated with a reduction in the quantity of MglB. Although these findings are of interest, a link between them and hydrogen peroxide stress appears tenuous at present.

MglB also acts as an initial receptor in the process of chemotaxis. In *E. coli* MglB interacts with an inner membrane chemotransducer Trg, that transmits the chemotactic signal to the flagellar apparatus via cytoplasmic components (Richarme and Caldas, 1997). However as Flagellin phase 1 and phase 2 expression is repressed following exposure to oxidative stress, the expenditure of energy to potentiate chemotaxis appears futile. Repression of MglB following exposure to hydrogen peroxide therefore may be an attempt to conserve energy. It is interesting to note that MglB levels are also repressed during the acid tolerance response (R.Fowler, personal communication) as are flagellin phase1 and phase 2 (Adams *et al.*, 2001).

Richarme and Caldas (1997) have shown that the MglB protein of *S.* Typhimurium and the proteins MalE (maltose-binding protein) and OppA (oligopeptide-binding protein) of *E. coli* display chaperone-like functions, and these occur at binding protein concentrations

that are ten to one hundred-fold lower than their high periplasmic concentrations. The *mglB* mRNA is produced by 3'-5' degradation of the polycistronic *mglBAC* mRNA. The *mglB* mRNA is more stable and abundant as it is protected against exonuclease attack by a Repetitive Extragenic Palindrome (REP) sequence (Yaagoubi *et al.*, 1996). As most of the known bacterial molecular chaperones are located in the cytoplasm i.e. DnaK and GroEL, MglB and other periplasmic binding proteins may be implicated in protein folding and protection from stress in the periplasm (Richarme and Caldas, 1997). Although MglB is less abundant following exposure to hydrogen peroxide stress, it may still be able to carry out its chaperone-like duties due to the low concentration required for this function.

3.4.4 Down-regulation of GlpQ suggests reduced transport of glycerophosphodiesters in *Salmonella* following hydrogen peroxide stress

When fats such as triglycerides and phospholipids are broken down during bacterial metabolism, the glycerol moiety is used in the form of glycerol-3-phosphate (G3P), glycerol or glycerophosphodiesters (Shang *et al.*, 1997). Glycerophosphodiesters in the periplasmic space are hydrolysed to G3P and alcohol by the periplasmic glycerophosphodiester phosphodiesterase (GlpQ), a component of the *glp* regulon, which encodes the major uptake system for G3P. G3P is then actively transported to the cytoplasm by the transmembrane inner membrane protein, G3P transporter protein encoded by the *glpT* gene. G3P can either be used in phospholipid synthesis or it can be converted into dihydroxyacetone phosphate by G3P dehydrogenase (GlpD) and used in the glycolytic pathway (Shang *et al.*, 1997).

Organisation of the genes encoding GlpT and GlpQ into a transcriptional unit provides for their co-regulation. The *glpTQ* operon is negatively regulated by the *glpR* repressor and induced by G3P (Larson *et al.*, 1983). The repressor's affinity for binding to the *glpTQ* operator is decreased in the presence of G3P, thereby resulting in derepression of the *glpTQ* operon. It follows that one explanation for the observed decrease in the abundance of GlpQ on *Salmonella* adaptation to H_2O_2 stress is that the metabolic rate may be lower under such circumstances, e.g. to conserve energy. This would result in a decrease of G3P synthesized and present in the periplasm. The decrease in G3P would in turn lead to

increased affinity of the GlpR repressor for its operator in front of *glpTQ* genes, thereby repressing the expression of GlpQ. Alternatively, GlpQ may be down-regulated by some other route and it may be this phenomenon that leads indirectly to a decrease in the level of G3P. Clearly, further studies are required to distinguish between these two possibilities.

3.5 Summary

While this survey of H_2O_2 stress-responsive proteins has been limited to the most dramatic and directly observable examples, the results obtained provide new insights on the processes that are affected during *Salmonella* adaptation to oxidative stress. Indeed, none of the components identified in this study had been previously implicated in adaptation to H_2O_2-induced stress. In summary, the results are in agreement with previous research confirming that *Salmonella* has the ability to sense and respond to unscheduled environmental changes (Foster and Spector, 1995). The ability to conserve energy by the regulation of stress survival genes in a co-ordinated manner is fundamental to the successful survival of any pathogenic microorganism.

CHAPTER 4

CONSTRUCTION AND ANALYSIS OF A *SALMONELLA ΔoxyS* MUTANT

4.1 Introduction

The importance of small RNA species as regulators of stress responses was highlighted by the discovery of OxyS, a 109 nt, untranslated RNA which is induced in response to hydrogen peroxide stress in *E. coli* (Altuvia *et al*, 1997). Christman *et al.* (1989) had previously suggested that mutations that constitutively activate OxyR, affect activity, rather than OxyR protein levels. Subsequently, whilst conducting experiments to compare the OxyR protein levels in wild-type and constitutive mutant strains, Altuvia *et al.* (1997) discovered that when Northern blots were probed with a fragment carrying the *oxyR* open reading frame and 200 bp of upstream sequence, the constitutive *oxyR* mutant expressed a distinct small RNA species, subsequently denoted *oxyS* that was not seen for the wild-type cells (Altuvia *et al.*, 1997). Other small RNA species have been connected to stress responses. In *E. coli,* transcription of the 93 nt *micF* RNA is controlled by many factors including the superoxide-response regulator SoxS (Chou *et al.*, 1993). The 85 nt *dsrA* RNA is required for the low temperature expression of RpoS during exponential growth in *E. coli* (Sledjeski *et al.*, 1996). RNA species may have been recruited as regulators of stress responses because they can be synthesised quickly with a relatively low input of energy, making them ideal regulators of rapid responses to changing environmental conditions.

Small RNA species appear to play critical roles in cellular metabolism, yet they are inherently difficult to identify by sequence analysis. For example, the region encoding *oxyS*, between the neighbouring *argH* and *oxyR* genes is only 266 nt (Altuvia *et al.*, 1997) and was thought to be devoid of genes (Gustafsson and Warne, 1992). Furthermore, small RNA species are poor targets for mutational screens and are equally difficult to detect with biochemical assays (Altuvia *et al.*, 1997).

OxyS RNA acts as a global regulator to activate or repress the expression of at least 40 genes in *E. coli*. Target genes include the *fhlA*-encoded transcriptional activator and the *rpoS*-encoded σ^S subunit of RNA polymerase (Altuvia *et al.*, 1998). OxyS also functions as an anti-mutator, thereby protecting cells from spontaneous and chemically induced mutagenesis (Altuvia *et al.*, 1989).

The *oxyS* gene of *E. coli* is located just upstream of, and is divergently transcribed from, the *oxyR* gene. During exponential growth, OxyR induces the expression of OxyS. Its secondary structure is predicted to contain at least three stem-loops and it is considered to be relatively stable (10-30 min half-life) and abundant (Altuvia *et al.*, 1997).

4.2 Construction of an SL1344 Δ*oxyS* mutant

In order to gain a greater insight into the role of *oxyS* in *Salmonella* with respect to the oxidative stress response, a Δ*oxyS* mutant was generated by allelic exchange of the wild-type *oxyS* gene on the chromosome with an inactivated *oxyS* gene.

4.2.1 Sequence analysis of the *oxyS* gene

Due to the unavailability of the *oxyS* gene sequence in *Salmonella* during the course of experimental studies, the *oxyS* sequence of *E. coli* K-12 was used to search *Salmonella* Washington University databases using the BLAST similarity search algorithm. The resultant, putative *oxyS* gene was found to be 110 nucleotides in length and flanked by the sequences encoding the proteins OxyR and arginino-succinate lysase (Figure 4.1). The *oxyS* gene from *S.* Typhimurium LT2 was shown to share 83% identity with its *E. coli* counterpart (Figure 4.2).

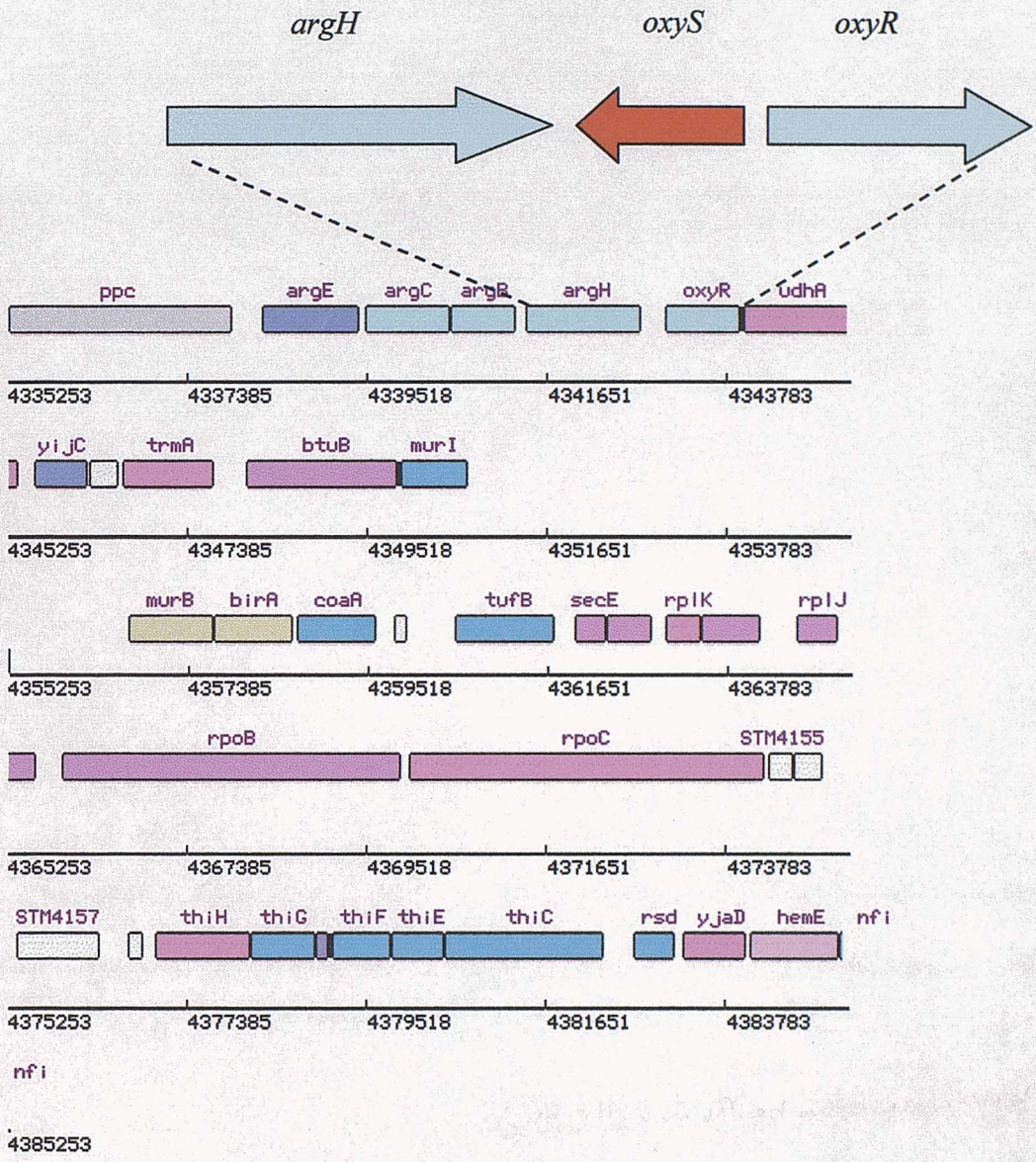

Figure 4.1. *S.* Typhimurium genomic region containing the putative *oxyS* gene. The *oxyR* gene encodes the hydrogen peroxide-inducible regulon activator, OxyR and is divergently transcribed. The *argH* gene encodes an arginino-succinate lyase. Graphics were obtained from the Wellcome Trust Sanger Institute.

A

B

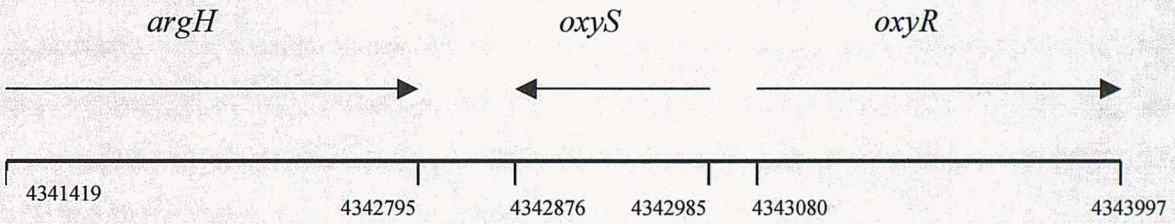

Figure 4.2. (A) Alignment of the *oxyS* gene from *S.* Typhimurium LT2 and *E. coli*. The putative *oxyS* nucleotide sequence has 83% identity with the sequence from *E. coli*. Shading denotes identical nucleotides. The predicted +1 nucleotide of *Salmonella oxyS* RNA transcript is indicated. Sequences were aligned using Clustal Format for T-Coffee Version 1.41. (Notredame *et al.*, 2000). (B) Chromosomal coordinates of *oxyS* and flanking genes taken from *S.* Typhimurium LT2 sequence (Obtained from the Wellcome Trust Sanger Institute).

4.2.2 Production of pCVD442Δ*oxyS*, a construct suitable for targeted disruption of the *oxyS* gene

Standard genetic techniques (Sambrook *et al.*, 1989), together with the modification of a crossover PCR method used to construct deletions (Link *et al.*, 1997), were used to construct the Δ*oxyS* mutant (see Chapter 2). Two asymmetric PCR reactions were used to generate fragments to the left and right of the sequence of *oxyS* to be deleted. A DNA sequence spanning part of the *argH* gene and the 3' region of *oxyS* was amplified from chromosomal DNA by PCR using the primers GHoxyS-A and GHoxyS-B2 (Table 2.4). Similarly, primers GHoxyS-C2 and GHoxyS-D were used to amplify the sequence that comprised the 5' region of the *oxyS* gene and part of *oxyR*. Primers GHoxyS-B2 and GHoxyS-C2 were complementary over a 15-nucleotide linker region, which contained an 8 base-pair recognition site for the restriction enzyme *NotI*. Therefore, when the two PCR products were mixed, the complementary regions annealed. The annealed structure was then amplified by PCR using the outer primers GHoxyS-A and GHoxyS-D, thereby generating a fragment in which the central region (36bp) of the *oxyS* gene had been deleted and replaced by a *NotI* restriction enzyme site (Figure 4.3). The resultant Δ*oxyS* fragment was cloned as an *XhoI-SacI* fragment into *SalI-SacI* digested suicide vector, pCVD442 (Donnenberg and Kaper, 1991) to create the plasmid pGMH.

To facilitate the selection of null mutants, the *cat* gene, together with its promoter region, was PCR amplified from the cloning vector pACYC184 using primers AJG027 and AJG028 (kindly supplied by A. Grant). The PCR product was cloned as a *NotI* fragment into *NotI* digested pGMH, thereby generating pGMH1.

The vector pCVD442 was used in this study as it requires the product of the *pir* gene for initiation of replication at *ori*R6K (Inzuka and Helsinki, 1978). Although pCVD442 does not specify the gene encoding this protein, a chromosomal copy is present in SM10 λ–*pir*, which was used as the donor strain. Therefore, pCVD422 plasmid derivatives such as pGMH1 were unable to replicate outside the donor strain, as the product of the *pir* gene must be supplied *in trans*. The donor strain chromosome also bears a conjugative plasmid, RP-4 that allows transfer of plasmid DNA from donor to recipient by a process known as

conjugative mobilisation. To aid selection of double cross-over events, pCVD442 additionally specifies *sacB*, which encodes a sucrase enzyme, levansucrase, that degrades sucrose to toxic metabolites, thereby resulting in cell death (Gay *et al.*, 1985). Thus, the *sacB* gene can be used as a negative selection to enrich for cells that have lost this gene.

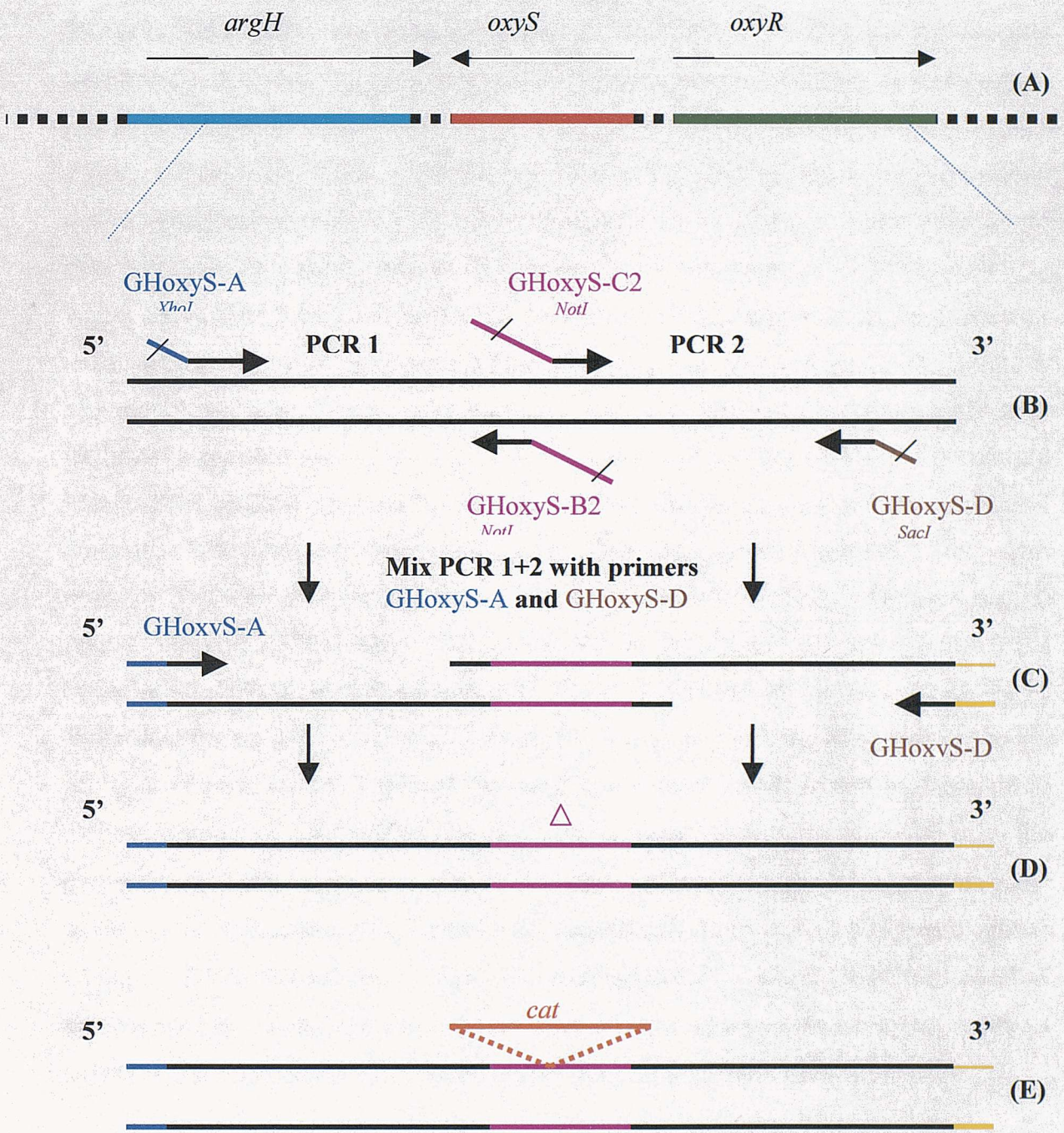

Figure 4.3. Targeted disruption of the putative *oxyS* gene of *Salmonella* strain SL1344, using a method adapted from Link *et al.* (1997).

(A) Region of chromosomal DNA containing the *oxyS* gene
(B) PCR reactions 1 and 2 produce Δ*oxyS* when fused
(C) PCR amplification of fused molecule with GHoxyS-A and GHoxyS-D
(D) Central region of *oxyS* is replaced by a *NotI* restriction enzyme site
(E) Insertion of *cat* cassette into engineered site

4.2.3 Replacing the wild-type *oxyS* gene on the *Salmonella* chromosome

The construct pGMH1 was transformed into *E. coli* SM10 λ-pir, and was subsequently transferred to a streptomycin-resistant (St^r) *S.* Typhimurium strain SL1344 by mobilisation. Recipient SL1344 St^r cells carrying the plasmid integrated into their chromosomes due to a single cross-over event were selected for on LB plates containing ampicillin, streptomycin and chloramphenicol. Selection for allelic exchange with the chromosomally located *oxyS* gene was performed using sucrose-TB medium. The resultant mutant SL1344 Δ*oxyS::cat* with a chromosomally located Δ*oxyS* was confirmed by PCR using primers complementary to chromosomal regions outside of the *oxyS* gene (GHoxyS-A1 and GHoxyS-D; Table 2.4; Figures 4.4 and 4.5). Finally, candidates were further verified by Southern hybridisation, using DNA digested with either *SalI, MluI* or *EcoRI* and probing with a 5' ^{32}P- labelled 610 bp PCR product, prepared by the PCR amplification of SL1344 chromosomal DNA upstream of the *oxyS* gene using primers GHoxyS-A1 and GH-S2 (Figure 4.6). Restriction enzymes *SalI* and *MluI* cut neither *cat* nor the chromosomal region complementary to primers GHoxyS-A1 and GHoxyS-D, comprising the *oxyS* gene. As expected, the probe bound to a *SalI/MluI* restriction fragment in SL1344 Δ*oxyS* that was approximately 850 bp larger than the corresponding band in the parent. Restriction enzyme *EcoRI* cut within the *cat* gene (Figure 4.6). As expected the probe bound to an *EcoRI* restriction fragment in SL1344 Δ*oxyS* that was smaller than the corresponding band in the parent. Finally, the chromosomal DNA containing the Δ*oxyS* gene was transferred into a streptomycin-sensitive (St^s) *Salmonella* SL1344 strain by genetic transduction using P22 bacteriophage (Section 2.2.14). The resultant mutant with a chromosomally located Δ*oxyS* was checked for streptomycin sensitivity and was confirmed by PCR using primers complementary to chromosomal regions outside of the *oxyS* gene (GHoxyS-A1 and GHoxyS-D; Table 2.4).

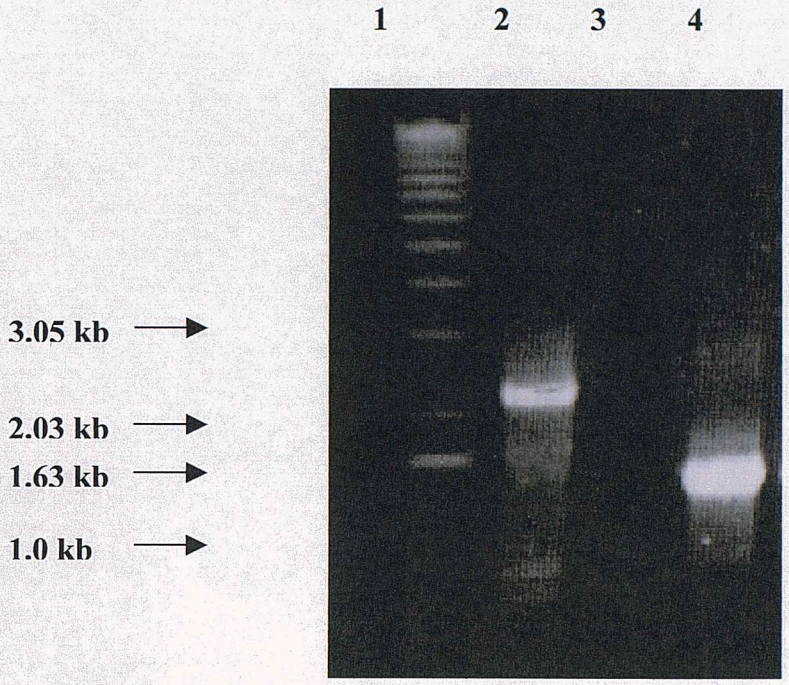

Figure 4.4. Agarose gel of PCR analysis of chromosomal DNA from *Salmonella* SL1344 wild-type and Δ*oxyS* strains using primers GHoxyS-A1 and GHoxyS-D. The amplified band was approximately 850 bp greater than that of the wild-type strain, as expected. Lane 1, 1 kb DNA ladder (BRL); lane 2, SL1344 Δ*oxyS*; lane 4, SL1344.

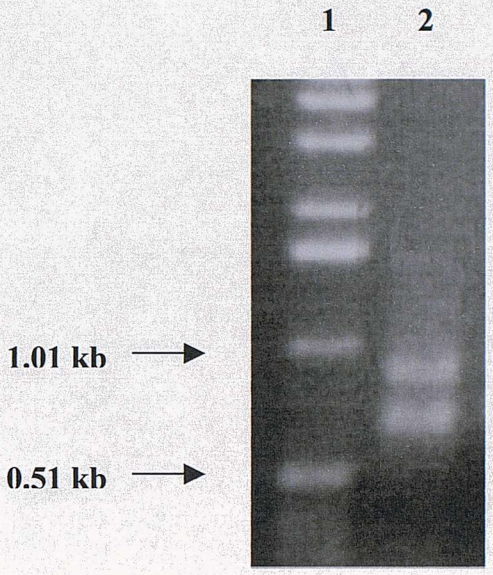

Figure 4.5. Agarose gel of PCR analysis of chromosomal DNA from *Salmonella* SL1344 Δ*oxyS* using primers GHoxyS-A1 and GHoxyS-D, cut with restriction enzyme *NotI*. Two bands were generated, an upper band of 855 bp i.e. the *cat* fragment, and a lower band of approximately 700 bp i.e. regions flanking the *cat* gene of 675 bp and 716 bp. Lane 1, 1 kb DNA marker (BRL); lane 2, SL1344 Δ*oxyS*.

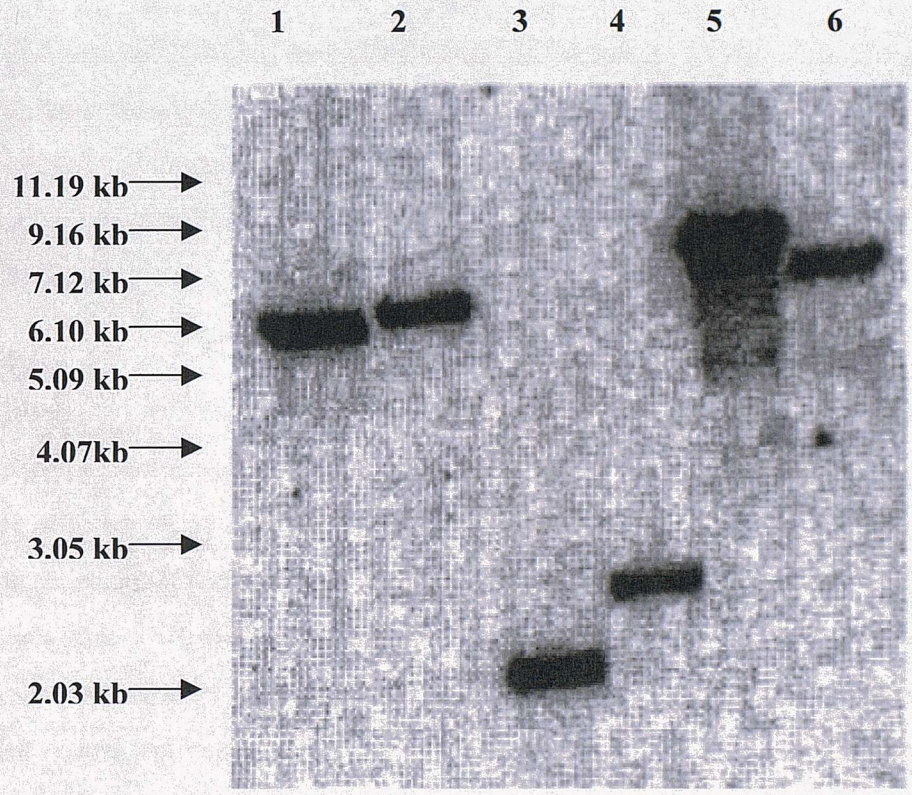

Figure 4.6. Southern hybridisation analysis using SL1344 wild-type and Δ*oxyS* strains. Chromosomal DNA was digested with *SalI, MluI* or *EcoRI* and probed with a 5' ^{32}P labelled PCR product. Lane 1, SL1344 *SalI* digested; lane 2, SL1344 Δ*oxyS SalI* digested; lane 3, SL1344 *MluI* digested; lane 4, SL1344 Δ*oxyS MluI* digested; lane 5, SL1344 *EcoRI* digested; lane 6, SL1344 Δ*oxyS EcoRI* digested.

4.3 Determining the effect of hydrogen peroxide on a *Salmonella ΔoxyS* mutant

Since the *oxyS* gene of *Salmonella* was only putative, it was considered important, not only to assess the effects of hydrogen peroxide on the mutant, but also to analyse any adaptive response to oxidative stress. A mid-exponential phase culture, therefore, was pre-treated with a sub-lethal dose of hydrogen peroxide to initiate the adaptive response as detailed in Section 2.5.1.2.

Salmonella oxyS mutant cells that had been exposed to a pre-adaptive dose of 0.3 μM H_2O_2 for 60 minutes were resistant to a subsequent, otherwise lethal stress of 30 μM H_2O_2. The adaptive response mirrors the adaptive response displayed by the wild-type, indicating that the response is not transient in either strain. Unadapted mutant cells, however, were totally non viable at 60 minutes following a 30 μM H_2O_2 stress, whereas the wild-type counterpart was non-viable at 30 minutes (Figure 4.7). Undoubtedly, this was a surprising result, as one would have expected the *oxyS* mutant cells to have an impaired response to hydrogen peroxide. However, the result was totally reproducible over the course of three separate experiments.

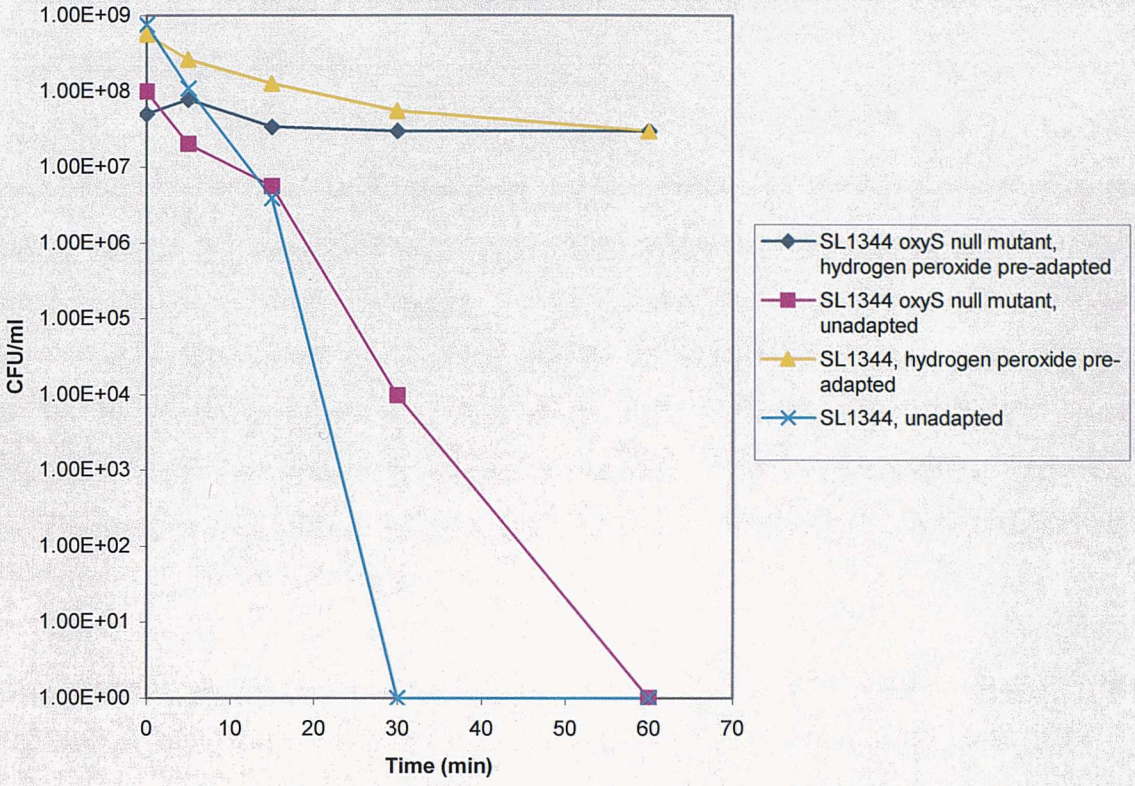

Figure 4.7. The effect of hydrogen peroxide on a *Salmonella ΔoxyS* mutant and analysis of the adaptive response to oxidative stress. Mid-exponential phase (A_{600}=0.4) *Salmonella* cells that had been exposed to an inducing stress of 0.3 μM H_2O_2, or left unadapted, for 60 minutes were subsequently exposed to a 30μM H_2O_2 stress for 60 minutes. Cells were then diluted and plated onto LB agar. Plates were incubated overnight and colonies were then counted and recorded as cfu/ml. Data for the wild-type cells was taken from Figure 3.2. The data are the mean of three experiments carried out on separate occasions. Standard errors are all less than ten percent of the mean.

4.4 Analysis of protein carbonylation levels in *Salmonella* wild-type and Δ*oxyS* mutant strains

Having established that the *oxyS* mutant was more resistant to hydrogen peroxide than its wild-type counterpart, the next stage was to compare protein carbonylation levels in both wild-type and mutant strains. The reason for this is that protein carbonyls are formed when reactive oxygen species such as hydroxyl radical and superoxide anion oxidise proteins. Thus, the extent of carbonylation is a direct measure of the degree of oxidative stress within cells of interest. The carbonyl groups can react with 2,4-dinitrophenylhydrazine, which is recognised by anti-2,4-dinitrophenol-antibodies. Accordingly, modified proteins can be detected by Western blotting.

Whole-cell extracts were prepared from mid-exponential phase cells. Protein samples were derivatized as described (Section 2.4.1). The carbonyl content of total proteins was determined by a one-dimensional Western blot immunoassay (Section 2.4.2). Protein carbonylation was found to be elevated in wild-type cells, when compared to the mutant (Figure 4.8).

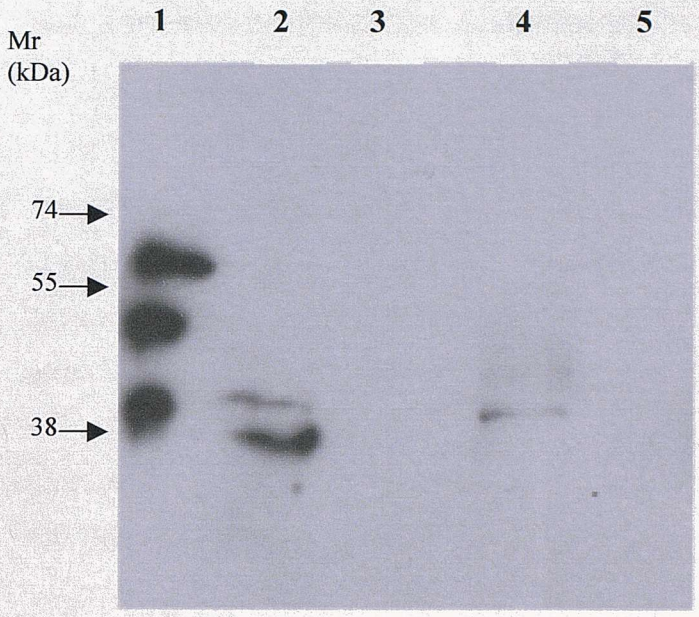

Figure 4.8. Protein carbonylation levels determined by one-dimensional Western blot immunoassay of mid-exponential phase unstressed *Salmonella* cells. Lane 1, pre-derivatized positive control; lane 2, SL1344; lane 3, SL1344 negative control (non-derivatized protein sample); lane 4, SL1344 Δ*oxyS*; lane 5, SL1344 Δ*oxyS* negative control (non-derivatized protein sample). Equal amounts of protein were loaded in lanes 2-5. Analysis was repeated three times to confirm reproducibility.

4.5 Two-dimensional gel analyses of SL1344 Δ*oxyS* mutant whole cell lysates

In view of the evidence of reduced oxidative stress in the *oxyS* mutant, it was considered important to compare the protein profile of unstressed Δ*oxyS* mutant cells with that of unstressed wild-type cells. Accordingly, two-dimensional gel electrophoresis was used to detect proteins that were differentially expressed (Section 2.3.4).

For each strain, three independent batches of cells were prepared and analysed. Differentially expressed proteins were detected as described in section 3.3.1. Numbered spots signify proteins that were differentially expressed consistently (Figures 4.9 and 4.10, Tables 4.1 and 4.2).

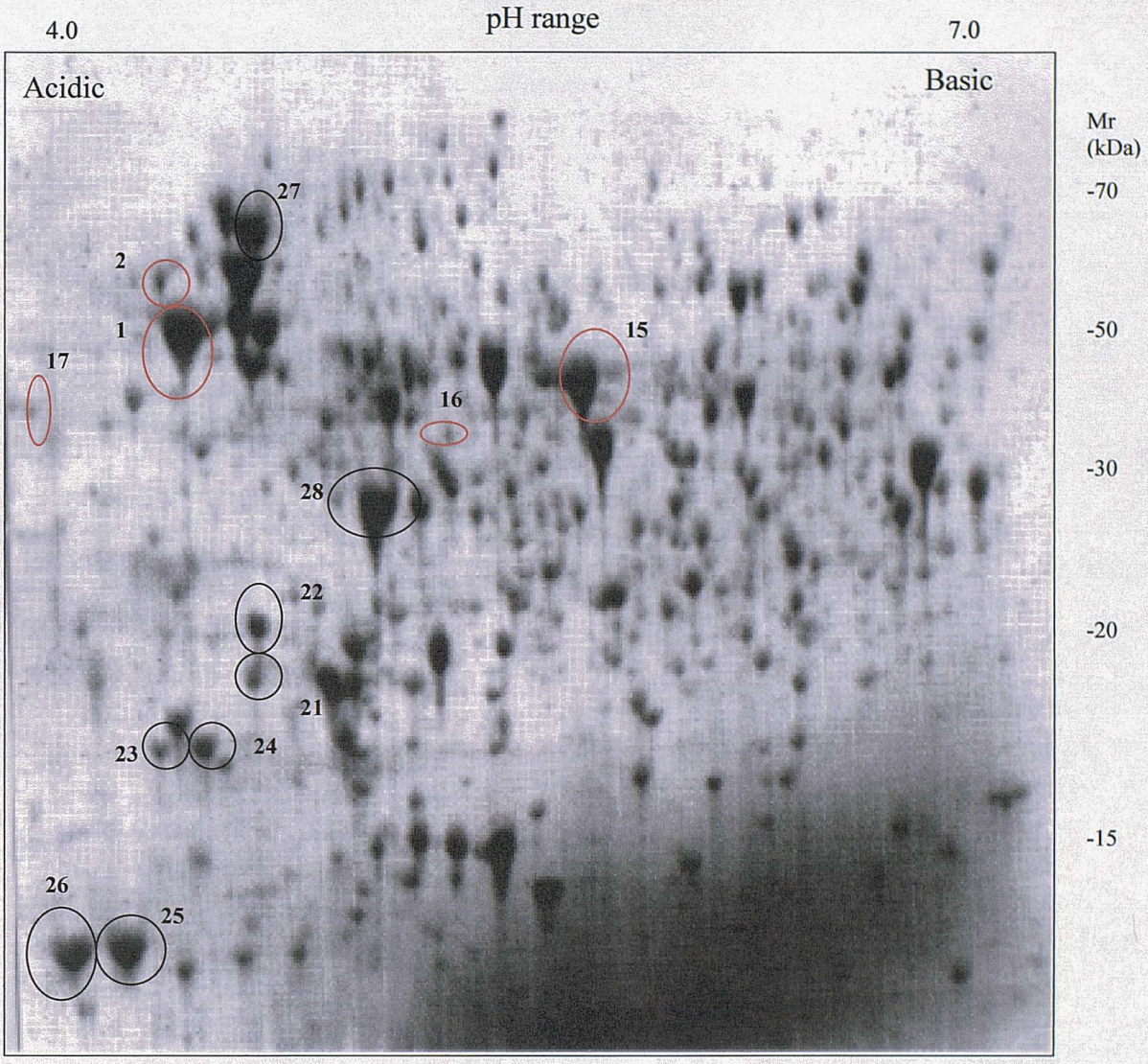

Figure 4.9. 2-D gel of unstressed SL1344 wild-type whole cell extracts. Whole cell extracts were fractionated by 2-DE and stained as described in Section 2.3.4. This protein profile was compared with that of unstressed Δ*oxyS* mutant cells (Figure 4.10). Open black circles represent proteins that were down regulated in the Δ*oxyS* mutant. Open red circles represent proteins that were up regulated in the Δ*oxyS* mutant. The 2-D gel for the wild-type cells was taken from Figure 3.3.

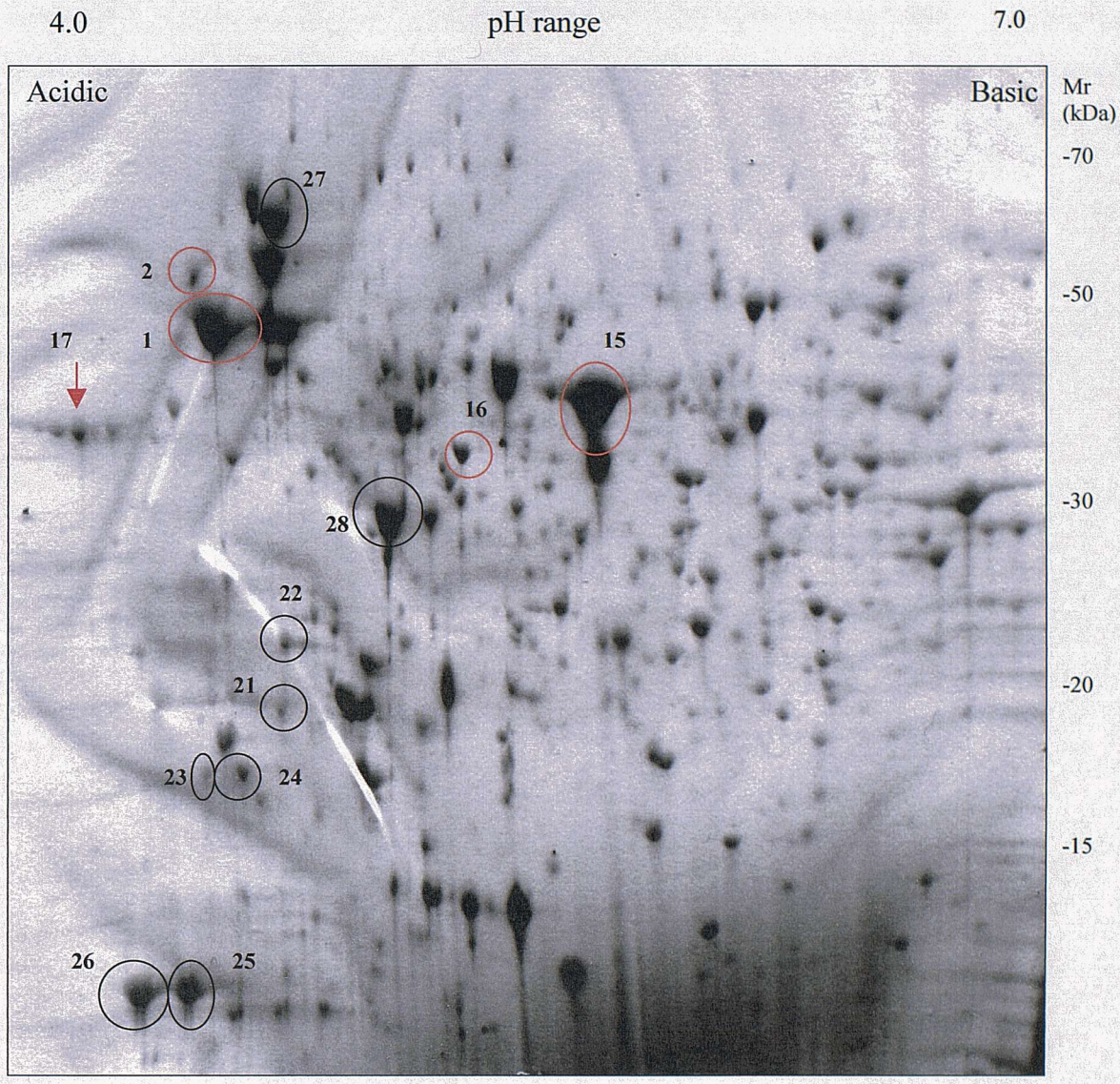

Figure 4.10. 2-D gel of unstressed SL1344 Δ*oxyS* whole cell extracts. Whole cell extracts were fractionated by 2-DE and stained as described in Section 2.3.4. This protein profile was compared with that of unstressed wild-type cells (Figure 4.9). Open black circles represent proteins that were down regulated in the Δ*oxyS* mutant. Open red circles and red arrow represent proteins that were up regulated in the Δ*oxyS* mutant.

Table 4.1. Proteins that were up-regulated in the SL1344 Δ*oxyS* mutant. The protein profile of unstressed wild-type cells (Figure 4.9) was compared with that of unstressed Δ*oxyS* mutant cells (Figure 4.10). Only proteins that were consistently up-regulated in the mutant were recorded.

Spot No.	**Protein identification Swiss-Prot accession no.**	**M_r [a] (kDa)**	**pI [a]**	**M_r [b] (Da)**	**pI [b]**
1	Flagellin phase 1 [a] (P52616)	50	4.7	51210	4.75
2	Flagellin phase 2 [a]	52	4.7	52453.5	4.75
15	Elongation factor Tu [a] (P21694)	42	5.5	43152.4	5.3
16	Unidentified	35	5.2	-	-
17	Unidentified	38	4.7	-	-

[a] Proteins were identified from the in-house two-dimensional reference map (compiled by Dr. P Adams and members of the DO'C group).

[a] Values were calculated from the two-dimensional reference map as above.

[b] Theoretical values were obtained from the Swiss-Prot data base.

Table 4.2. Proteins that were down-regulated in the SL1344 Δ*oxyS* mutant. The protein profile of unstressed wild-type cells (Figure 4.9) was compared with that of unstressed Δ*oxyS* mutant cells (Figure 4.10). Only proteins that were consistently down-regulated in the mutant were recorded.

Spot No.	**Protein identification Swiss-Prot accession no.**	**M_r [a] (kDa)**	**pI [a]**	**M_r [b] (Da)**	**pI [b]**
21	C-terminally truncated S1 protein [b] (P02349)	20	4.9	61158.1	4.89
22	C-terminally truncated S1 protein [b] (P02349)	28	4.9	61158.1	4.89
23	30S ribosomal subunit S2 protein [a] (P02351)	17.5	4.7	26612.5	6.69
24	30S ribosomal subunit S2 protein [a] (P02351)	17.5	4.7	26612.5	6.69
25	50S ribosomal protein L7 [a] (P18081)	12	4.5	12167.9	4.6
26	50S ribosomal protein L12 [a] (P18081)	12	4.6	12167.9	4.6
27	Protein S 1 [a] (P02349)	65	4.9	61158.1	4.89
28	Elongation factor Ts [a] (P02887)	29.5	5.1	30291.8	5.22

[a] Proteins were identified from the in-house two-dimensional reference map (compiled by Dr. P Adams and members of the DO'C group).

[b] Proteins were identified by searching databases with the N-terminal sequence derived from microsequencing (obtained by Dr. A. Moir).

[a] Values were calculated from the two-dimensional reference map as above.

[b] Theoretical values were obtained from the Swiss-Prot data base.

4.6 Two-dimensional gel analyses of hydrogen peroxide stressed SL1344 Δ*oxyS* whole cell lysates

The next stage was to compare the protein profiles of stressed *oxyS* mutant cells with that of unstressed mutant cells. Accordingly, two-dimensional gel electrophoresis was used to detect proteins that were differentially expressed (Section 2.3.4).

For each condition, three independent batches of cells were prepared and analysed. Numbered spots signify proteins that were differentially expressed consistently (Figures 4.11 and 4.12, Tables 4.3 and 4.4).

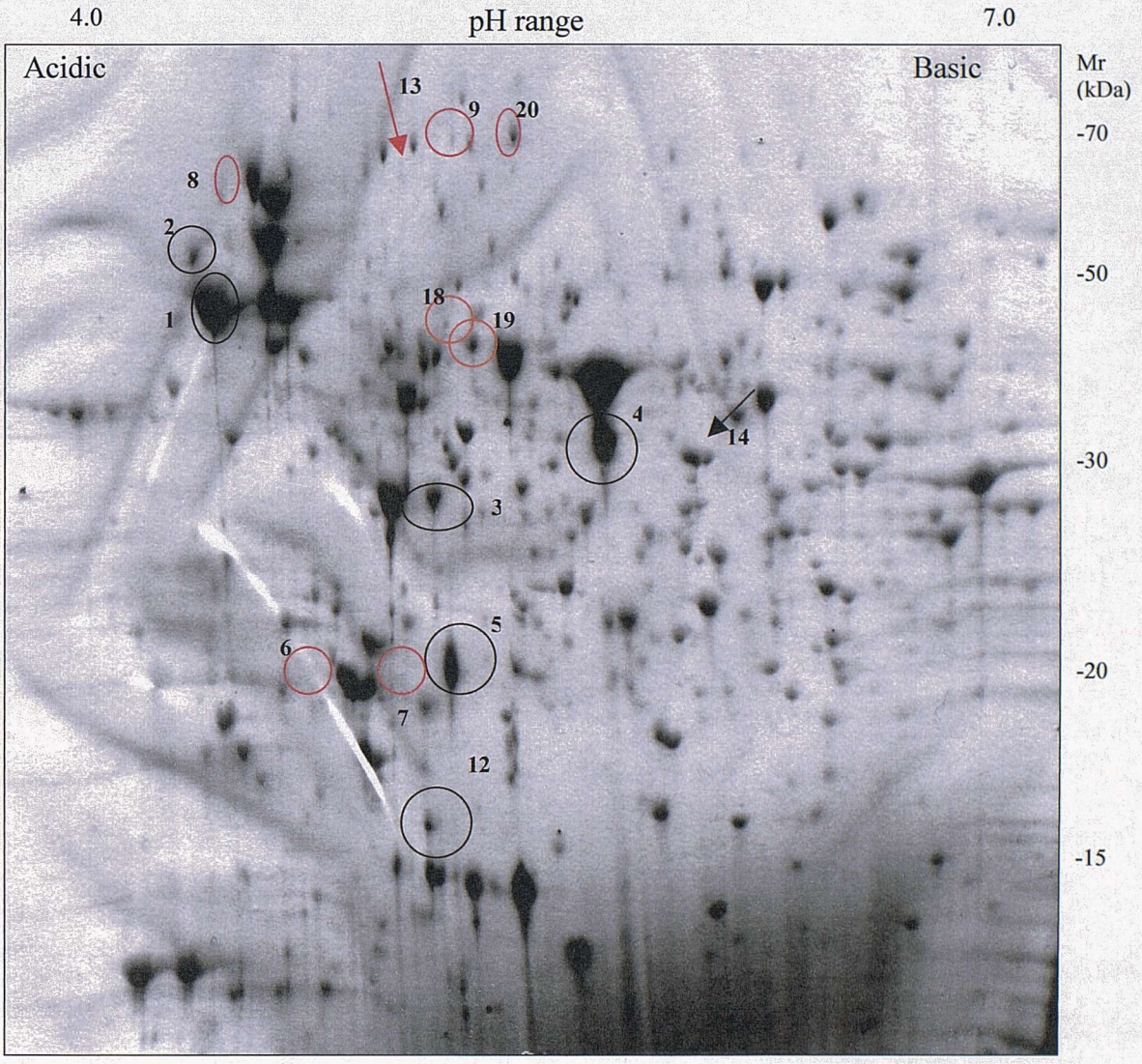

Figure 4.11. 2-D gel of unstressed *Salmonella* SL1344 Δ*oxyS* whole cell extracts. Whole cell extracts were fractionated by 2-DE and stained as described in Section 2.3.4. This protein profile was compared with that of stressed Δ*oxyS* mutant cells (Figure 4.12). Open black circles and black arrow represent proteins that were down regulated following H_2O_2 stress. Open red circles and red arrow represent proteins that were up regulated following H_2O_2 stress. The 2-D gel for the unstressed mutant cells was taken from Figure 4.10.

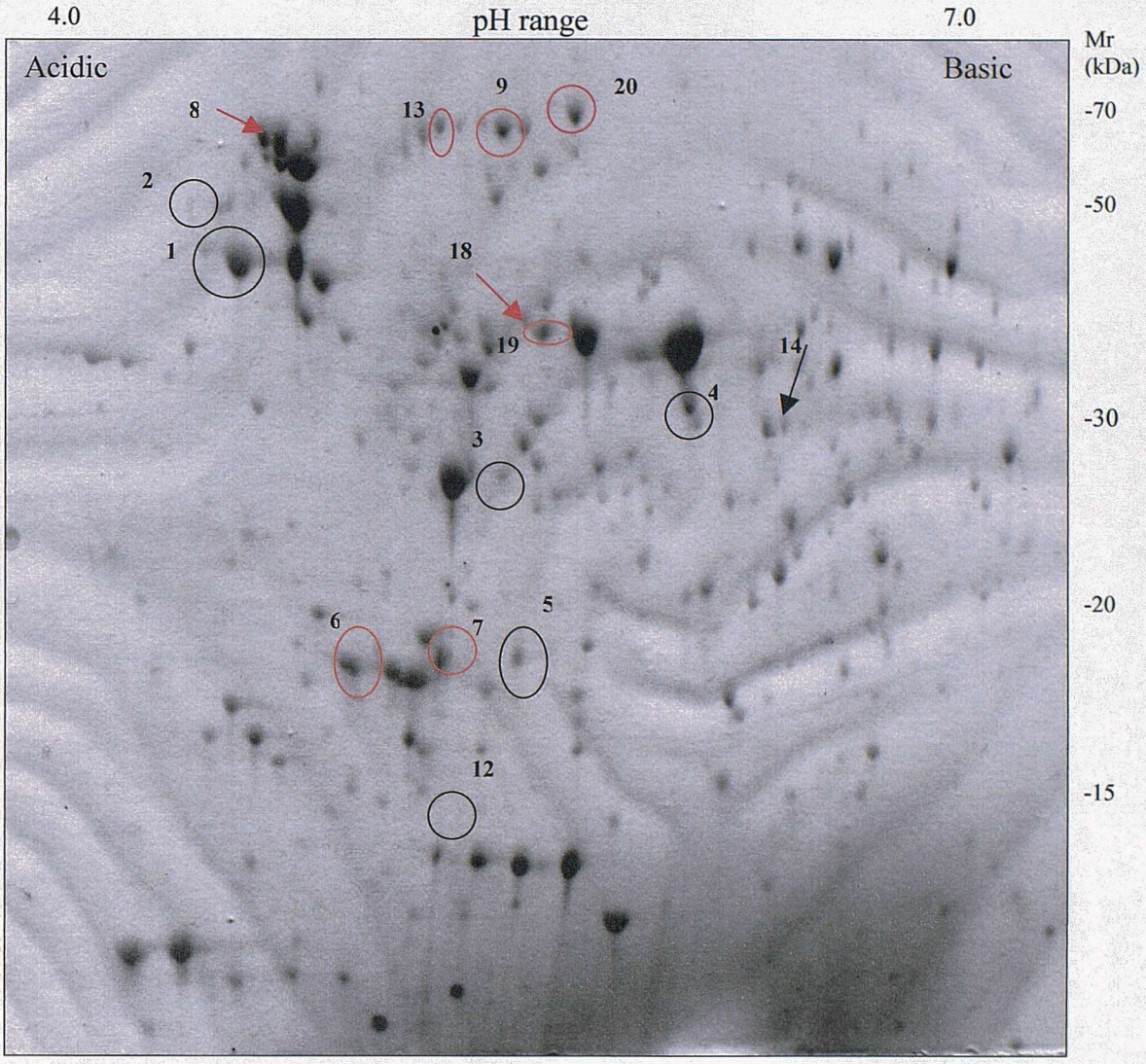

Figure 4.12. 2-D gel of H_2O_2 stressed *Salmonella* SL1344 Δ*oxyS* whole cell extracts. Protein extracts from cells that had been exposed to a final concentration of 3 μM H_2O_2 for 60 minutes were fractionated by 2-DE and stained as described in Section 2.3.4 This protein profile was compared with that of unstressed Δ*oxyS* mutant cells (Figure 4.11). Open black circles and black arrow represent proteins that were down regulated following H_2O_2 stress. Open red circles and red arrows represent proteins that were up regulated following H_2O_2 stress.

Table 4.3. Proteins that were down-regulated in the SL1344 Δ*oxyS* mutant when treated with hydrogen peroxide. The protein profile of unstressed mutant cells (Figure 4.11) was compared with that of stressed Δ*oxyS* mutant cells (Figure 4.12). Only proteins that were consistently down-regulated in the mutant were recorded.

Spot No.	Protein identification Swiss-Prot accession no.	M_r [a] (kDa)	pI [a]	M_r [b] (Da)	pI [b]
1	Flagellin phase 1 [a] (P52616)	50	4.7	51210	4.75
2	Flagellin phase 2 [a]	52	4.7	52453.5	4.75
3	D-galactose binding protein [b] (P23905)	30	5.2	32997.3	5.07
4	Glycerophosphoryl diester phosphodiesterase [b] (P09394)	35	5.4	38200	5.22
5	Putative peroxidase [b]	21	5.2	-	-
12	Unidentified	16	5.2	-	-
14	Asparaginase 1 [b] (P18840)	35	5.6	37127	5.52

[a] Proteins were identified from the in-house two-dimensional reference map (compiled by Dr. P Adams and members of the DO'C group).

[b] Proteins were identified by searching databases with the N-terminal sequence derived from microsequencing (obtained by Dr. A. Moir).

[a] Values were calculated from the two-dimensional reference map as above.

[b] Theoretical values were obtained from the Swiss-Prot data base.

Table 4.4. Proteins that were up-regulated in the SL1344 Δ*oxyS* mutant when treated with hydrogen peroxide. The protein profile of unstressed mutant cells (Figure 4.11) was compared with that of stressed Δ*oxyS* mutant cells (Figure 4.12). Only proteins that were consistently up-regulated in the mutant were recorded.

Spot No.	Protein identification Swiss-Prot accession no.	M_r [a] (kDa)	pI [a]
6	Unidentified	19	4.9
7	Unidentified	21	5.0
8	Unidentified	70	4.8
9	Unidentified	80	5.2
13	Unidentified	80	5.0
18	Unidentified	47	5.2
19	Unidentified	45	5.3
20	Unidentified	80	5.4

[a] Values were calculated from the two-dimensional reference map as before.

4.7 Discussion

This chapter has described the construction and characterisation of an *oxyS* deletion mutant in *Salmonella.* Deletion of the *oxyS* gene rendered the mutant more resistant to hydrogen peroxide than its wild-type counterpart. It has also reported the identification of differentially expressed proteins in the mutant, under normal conditions, and following hydrogen peroxide stress.

4.7.1 The *Salmonella* Δ*oxyS* mutant is less susceptible to damage caused by hydrogen peroxide

As reported in section 4.3, the *Salmonella* Δ*oxyS* mutant was more resistant to hydrogen peroxide than its wild-type counterpart. However, in *E. coli*, cells carrying a deletion of the *oxyS* gene were no more sensitive or resistant to hydrogen peroxide stress when compared to the parent (Altuvia *et al.*, 1997). Viability studies confirmed that the mutant had normal adaptive responses to hydrogen peroxide (Section 4.3). In addition, the results from the carbonylation assays, suggested that the mutant *Salmonella* cells had lower carbonyl levels compared to the wild-type (Section 4.4). Taken together, these results suggest that the Δ*oxyS* mutant is less susceptible to oxidative damage caused by exposure to hydrogen peroxide. A possible explanation for this profile will now be discussed.

The *rpoS*-encoded σ^s subunit of RNA polymerase is a central regulator of genes, induced by entry into stationary phase and other stresses (Zhang *et al.*, 1998). OxyS RNA represses *rpoS* at a post-transcriptional level. Repression is dependent on the *hfq*-encoded RNA-binding protein, which preferentially binds to sequences rich in adenines. The *E. coli* linker region between stem-loops b and c of OxyS contains five AA repeats (Figure 4.13 (A)) (Zhang *et al.*, 1998). Analysis of the *oxyS* gene of *Salmonella* confirmed that the same A-rich sequence was present in this species, suggesting that it too has the potential to bind Hfq (Figures 4.2 (A) and 4.13 (B)). OxyS represses translation of the *rpoS* mRNA by binding to the Hfq protein, which is essential for *rpoS* translation, thereby preventing Hfq from acting (Brown and Elliot, 1996: Zhang *et al.*, 1998). In strains carrying deletions of *oxyS*, *rpoS* expression is derepressed (Zhang *et al.*, 1998). Increased levels of σ^s, in turn

lead to the induction of many genes that play anti-oxidant roles. For example, *katE*, *xthA*, *sodA*, *katG*, *gorA* and *dps* are regulated by σ^S, and promote survival against oxidative stress (Storz and Imlay, 1999). Thus, a Δ*oxyS* mutant might be expected to have greater resistance to hydrogen peroxide. It should also be noted, however, that *katG*, *gorA* and *dps* are additionally regulated by OxyR, and therefore would already be induced by sub-lethal amounts of hydrogen peroxide under the conditions of the assay (Section 2.5.1.2) (Michan *et al.*, 1999). It has been proposed that OxyS RNA acts to prevent the redundant utilisation of oxidative stress genes (Altuvia *et al.*, 1997). In an *oxyS* deletion mutant, therefore, a duplication in the expression of antioxidant genes regulated by both OxyR and σ^S may not increase the mutant's resistance to hydrogen peroxide any further than is reported here.

4.7.2 Proposed OxyS RNA secondary structure

The secondary structure of OxyS RNA of *E. coli* is proposed to have three stem loops a, b and c (Zhang *et al.*, 1998). The secondary structure of the OxyS RNA of *S.* Typhimurium was predicted using the *mfold* program (Zuker *et al.*, 1999: Matthews *et al.*, 1999) and is shown at Figure 4.13. Like its *E. coli* counterpart, it is predicted to have three stem loop structures. Furthermore, and in support of the proposed structure, base changes in a region predicted to be double stranded were accompanied by complementary base changes on the other strand.

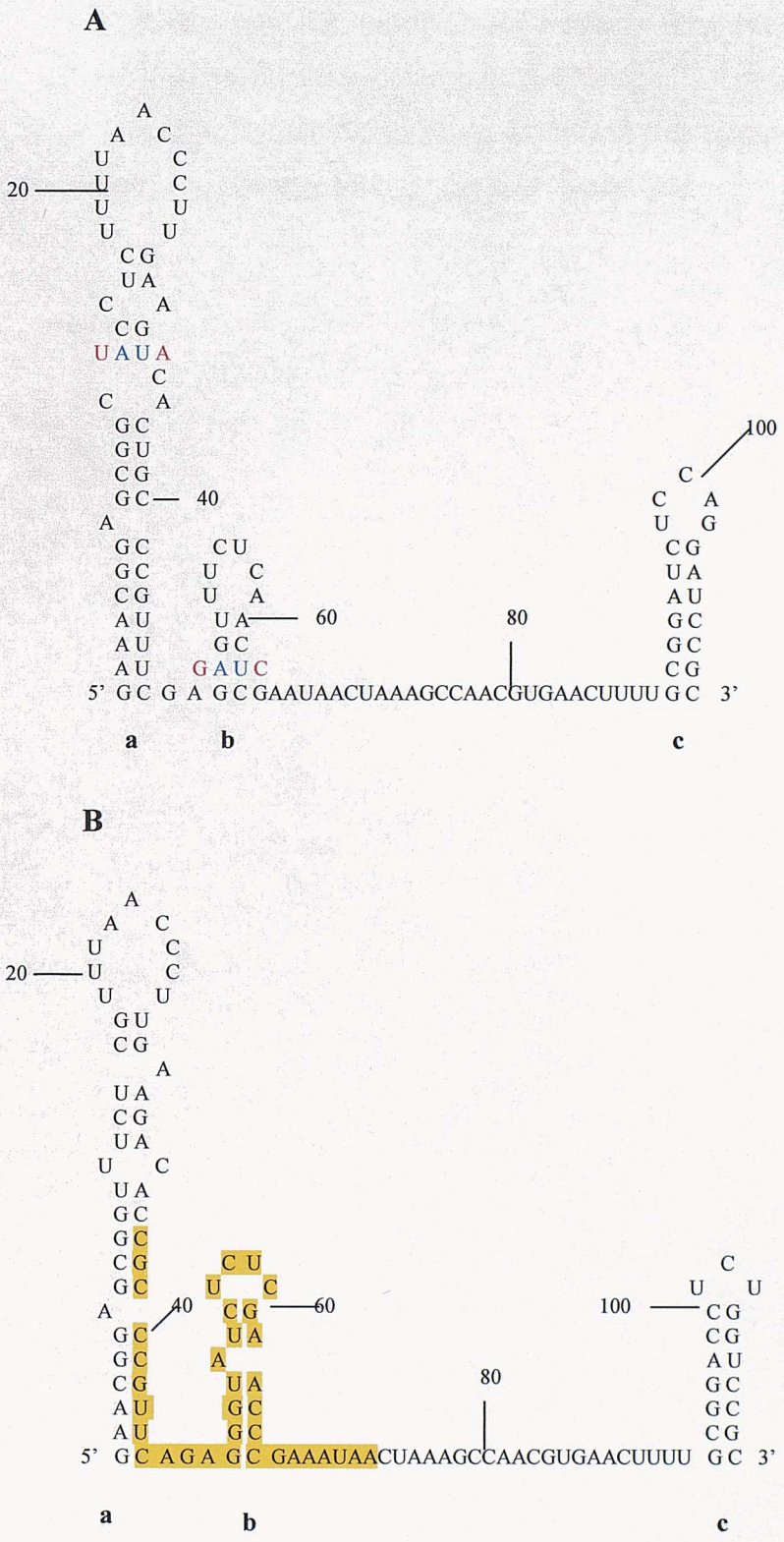

Figure 4.13. Proposed OxyS RNA secondary structure. (A) OxyS RNA of *E. coli* is proposed to have three stem loop structures, a, b and c. Red lettering indicates bases that occur in the OxyS RNA molecule of *S.* Typhimurium (*E. coli* residues are coloured blue),

in a region predicted to be double stranded. Furthermore, a complementary base change on the other strand also occurs in *Salmonella*. The proposed structure of the OxyS RNA of *E. coli* was obtained from Zhang *et al.* (1998). (B) Predicted secondary structure of the OxyS RNA of *S.* Typhimurium, based on the *mfold* program (Zuker *et al.*, 1999; Matthews *et al.*, 1999). Yellow lettering indicates bases that have been deleted in the *ΔoxyS* mutant.

4.7.3 Flagellin expression is up-regulated in the *Salmonella* Δ*oxyS* mutant under normal growth conditions

As reported in Section 4.5, phase 1 and phase 2 flagellin were up-regulated in the Δ*oxyS* mutant. The general stress sigma factor, RpoS, has been shown to stimulate the expression of the class 3 flagellar genes encoding phase 1 and phase 2 flagellin and possibly other flagellar genes too (Adams *et al.*, 2001). It is feasible, therefore, that elevated levels of these proteins in the Δ*oxyS* mutant may be due to the derepression of *rpoS* mRNA translation as already discussed (Section 4.7.1).

4.7.4 The elongation factor protein, EF-Tu, is up-regulated in the *Salmonella* Δ*oxyS* mutant-unstressed

Elongation factor Tu (EF-Tu) is coded for by two genes, *tufA* and *tufB*, located at 72 and 88 min of the *E. coli* chromosome respectively (Laursen *et al.*, 1982). The two gene products are identical, apart from residue 393 at the C- terminus, which can be either serine or glycine (Laursen *et al.*, 1981). EF-Tu promotes the binding of aminoacyl-transfer RNA to the A-site of ribosomes during protein synthesis. The EF-Tu, the aminoacyl-tRNA and GTP form a stable ternary complex that binds to the ribosome (Stark *et al.*, 1997).

The relative expression levels of the two proteins, EF-TuA and EF-TuB has been difficult to study as they co-migrate. However, the use of point mutations, which alter the isoelectric point of one of the proteins, has allowed expression levels to be examined (Neidhardt, 1996). Interestingly, exposure to the weak acid salt, sodium salicylate, which inhibits growth and induces the heat shock and *marA* regulons, produces a down-regulation of *tufA,* along with ribosomal proteins. Regulation of the *tufB* gene however, does not appear to be affected by sodium salicylate (Pomposiello *et al.*, 2001).

The synthesis of Ef-Tu is 7-10 times greater than that of ribosomal proteins and at growth rates of >1 per hour, the molar ratios of EF-Tu and EF-Tu/EF-Ts (elongation factor Ts, encoded by *tsf*) are virtually constant (Neidhardt, 1996). Under such conditions, *tufA*, *tufB*

and *tsf*, as well as the ribosomal genes are co-ordinately regulated, but at lower growth rates, coordination persists for *tufA* and *tufB* but expression of *tsf* and the ribosomal proteins drops much faster (Neidhardt, 1996). Accordingly, this scenario was observed for the Δ*oxyS* mutant. 2-D gel analysis indicated that expression of EF-Tu was up regulated in the mutant, whilst expression of the ribosomal proteins was down regulated (Figure 4.10 and Table 4.1). It may be possible that by knocking out the *oxyS* gene, the growth rate of the mutant is lowered in some way. This may be due to the loss of OxyS-dependent anti-mutagenic properties. OxyS RNA has been shown to protect against spontaneous and hydrogen peroxide-induced mutagenesis (Altuvia *et al.*, 1997), and loss of this protective mechanism is likely to prove deleterious to the cell.

4.7.5 Expression of Spot 5/STM0402 is not affected by OxyS

Expression of Spot 5/STM0402, the *Salmonella* putative peroxidase, did not appear to be affected by the *oxyS* deletion (Figures 4.11 and 4.12). This suggests that OxyS RNA does not play a direct role in the regulation of this protein.

4.7.6 Proteins that are differentially expressed in wild-type cells following H_2O_2 stress, are also differentially expressed in the stressed Δ*oxyS* mutant

2-D gel analysis of Δ*oxyS* mutant whole cell lysates, identified proteins that were differentially expressed following treatment with hydrogen peroxide (Figures 4.11 and 4.12). The proteins that were differentially expressed in the mutant following H_2O_2 stress, were also differentially expressed in the stressed wild-type, apart from Spot 20, which was moderately up-regulated in the mutant strain only. Possible explanations for differential expression of identified proteins have already been discussed (Sections 3.4.2, 3.4.3, 3.4.4 and 3.4.5). The moderate up-regulation of phase 1 and phase 2 flagellin observed in the unstressed Δ*oxyS* mutant was abolished following treatment with hydrogen peroxide (Section 4.5, Figures 4.11 and 4.12). When Δ*oxyS* mutant cells were treated with H_2O_2, both flagellin proteins were markedly down-regulated, thereby reverting to expression

levels observed for unstressed and stressed wild-type cells. It was suggested that both forms of flagellin were down-regulated following H_2O_2 stress in wild-type cells, in an attempt to save energy that might be required in the protective responses against reactive oxygen species (Section 3.4.2). The moderate up-regulation of flagellin proteins by RpoS, therefore, may be overridden by the need to save energy when in a stressful environment.

4.8 Summary

It has been proposed that the *oxyS* gene integrates the adaptive response to hydrogen peroxide with other cellular stress responses and protects against DNA damage in *E. coli* (Zhang *et al.*, 1998). However, the results of this chapter have shown, that despite deleting the *oxyS* gene, the *Salmonella* mutant not only remained able to counteract hydrogen peroxide treatment, but actually fared better than its wild-type counterpart, suggesting perhaps specific compensatory measures employed by this species. The mechanism by which the Δ*oxyS* mutant withstands oxidative stress, may involve the derepression of RpoS, as hypothesised here, but clearly, further studies are needed.

CHAPTER 5

EVIDENCE THAT THE HYDROGEN PEROXIDE STRESS REGULON OF *SALMONELLA* OVERLAPS WITH THE PhoPQ AND BipA REGULONS

Chapter 5 Evidence that the hydrogen peroxide stress regulon of *Salmonella* overlaps with the PhoPQ and BipA regulons

5.1 Introduction

The proteomic analyses described in section 3.3 indicated a possible overlap between the PhoPQ and hydrogen peroxide regulons. Proteins that were down-regulated following hydrogen peroxide stress, for example, flagellin phase 1 and phase 2, D-galactose binding protein, glycerophosphoryl diester phosphodiesterase and asparaginase, were also less abundant in acid treated cells. Protection against reciprocal challenge has already been demonstrated for the hydrogen peroxide stress response (Section 3.2.2). Similarly, reciprocal challenge has been observed in *Salmonella* for acid stress (Adams *et al*, 2001). In view of these results, cross-protection assays were performed, to further explore the putative relationship between these two important stress regulons in *Salmonella.*

Foster and Hall (1990) reported that the adaptive ATR system was not found to overlap with the hydrogen peroxide stress regulon in *Salmonella.* They attempted to trigger adaptation to 10 mM H_2O_2 by growth at pH 5.8. Furthermore, adaptation to 60 μM H_2O_2 was used to mimic the ATR. Adaptation was done at an OD_{600} of 0.2 followed by non-reciprocal challenge at an OD_{600} of 0.4, or at 60 minutes-post adaptation for H_2O_2. Conversely, Leyer and Johnson (1993) showed that acid-adapted *Salmonella* cells were more tolerant to the oxidative killing mechanism by the lactoperoxidase system. The antibacterial lactoperoxidase system generates a variety of short-lived toxic end products from the oxidation of the thiocyanate ion (SCN^-) by H_2O_2, and is thought to exert its action on the cytoplasmic membrane (Reiter and Harnluv, 1984). In view of this, it was proposed, that acid-adapted cells were more tolerant to the oxidative killing mechanism by the lactoperoxidase system due to changes in cell surface properties induced by treatment with acid (Leyer and Johnson, 1993).

This chapter first describes studies with *Salmonella* SL1344 cells to examine the non-reciprocal cross-protection phenomenon in more detail. It then describes studies on PhoPQ mutants of the strain before considering the possible involvement of BipA in the oxidative stress response.

5.2 Non-reciprocal challenge in *Salmonella*

5.2.1 A sub-lethal acid stress of pH 5.0 cross-protects against an otherwise lethal H_2O_2 stress

To assess the degree of overlap between the PhoPQ and hydrogen peroxide regulons, exponential phase SL1344 cells were pre-treated with an inducing acid stress of pH 5.0, prior to a subsequent dose of 30 μM H_2O_2, as described in section 2.5.2.1.

A sub-lethal acid stress of pH 5.0 offered short-lived cross-protection against a subsequent 30 μM H_2O_2 stress, whereas reciprocal challenge offered long-lived protection. Acid-adapted cells were non-viable at 60 minutes following a 30 μM H_2O_2 stress. Conversely, unadapted cells were non-viable at 30 minutes under the same conditions (Figure 5.1).

5.2.2 A sub-lethal hydrogen peroxide stress of 0.3 μM does not cross-protect against a lethal acid stress

To assess whether an inducing H_2O_2 stress offered protection against a subsequent acid stress, exponential phase *Salmonella* cells were pre-adapted with 0.3 μM H_2O_2 prior to a subsequent acid stress of pH 3.0 (section 2.5.2.2).

An inducing stress of 0.3 μM H_2O_2 offered no protection against a subsequent acid stress of pH 3.0 (Figure 5.2). Therefore, adaptation by growth in the presence of an inducing stress of hydrogen peroxide does not mimic the ATR under the standard conditions used. Reciprocal challenge, i.e. cells that had received an inducing stress of pH 5.0 for 60 minutes remained viable at 90 minutes under the same conditions.

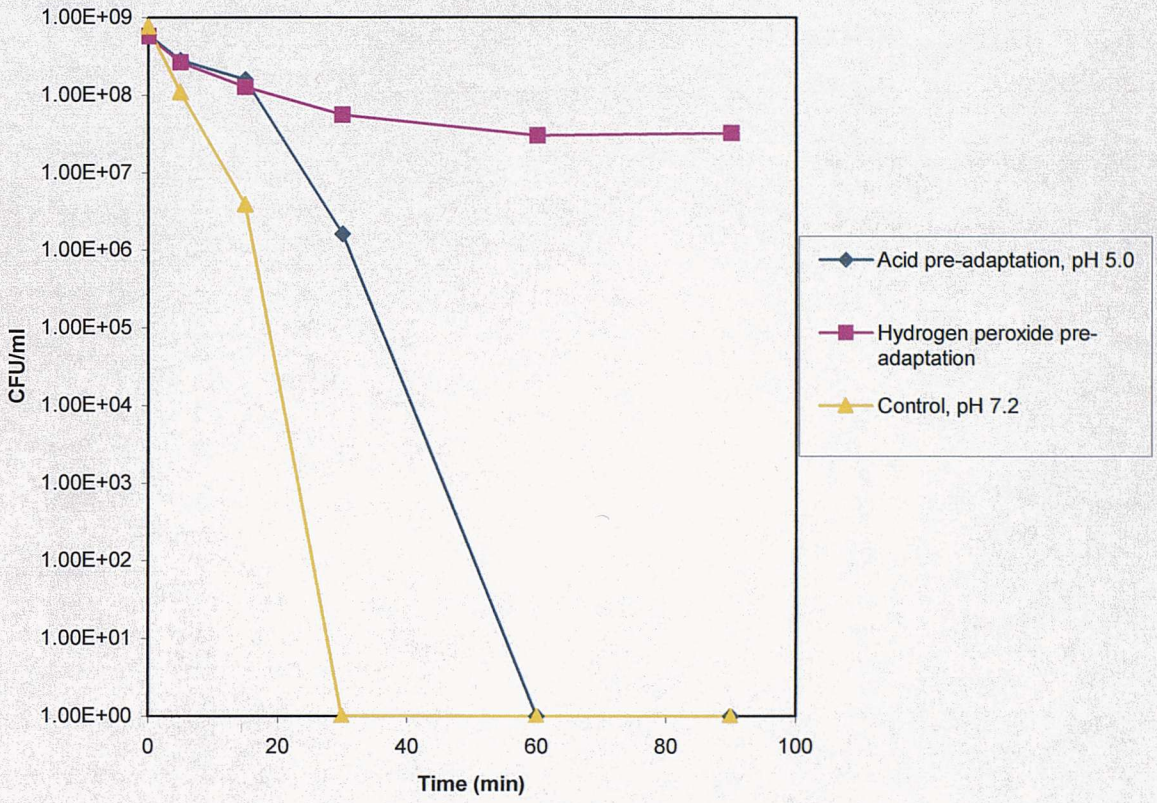

Figure 5.1. Acid pre-adaptation to hydrogen peroxide stress in *Salmonella.* Mid-exponential phase (A_{600}=0.4) *Salmonella* SL1344 cells that had been exposed to an inducing acid stress of pH 5.0, or left unadapted, for 60 minutes were subsequently exposed to 30 μM H_2O_2 in LB for 60 minutes. Cells were then diluted and plated onto LB agar. Plates were incubated overnight and colonies were then counted and recorded as cfu/ml. The data are the mean of three experiments carried out on separate occasions. Standard errors are all less than ten percent of the mean.

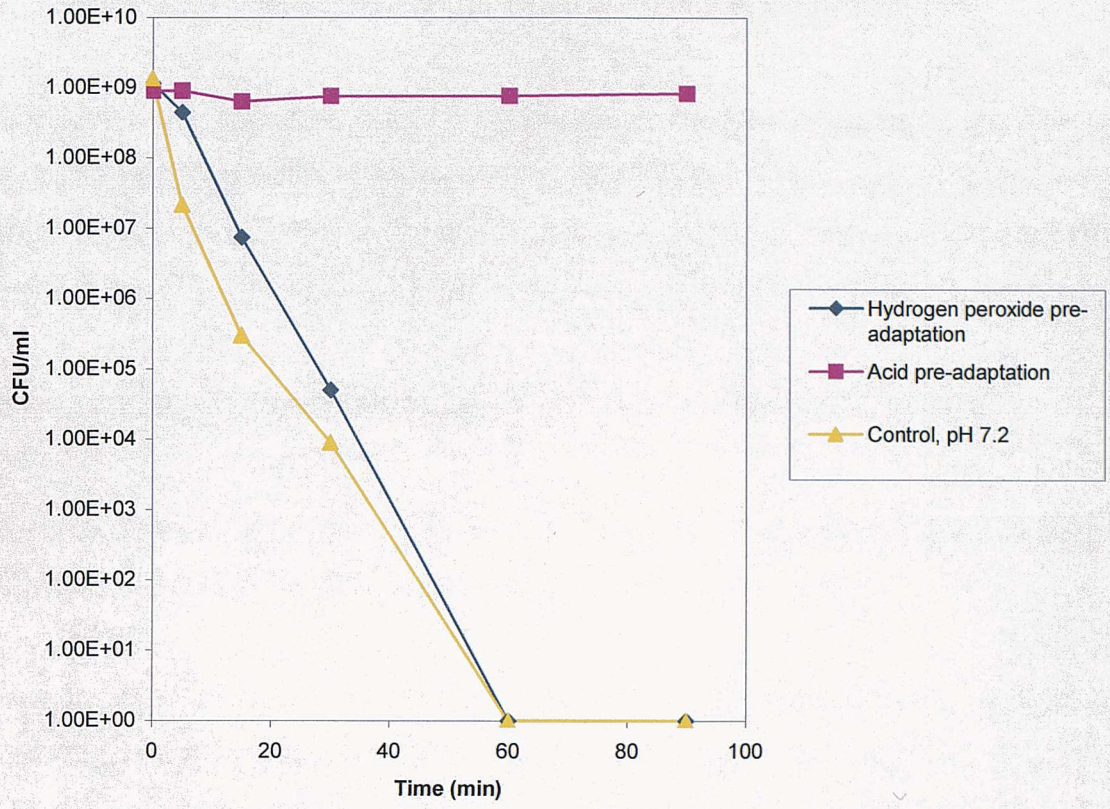

Figure 5.2. Hydrogen peroxide pre-adaptation to acid stress in *Salmonella*. Mid-exponential phase (A_{600}=0.4) *Salmonella* SL1344 cells that had been exposed to an inducing stress of 0.3 μM H_2O_2, or left unadapted, for 60 minutes were subsequently exposed to an acid stress of pH 3.0 for 60 minutes. Cells were then diluted and plated onto LB agar. Plates were incubated overnight and colonies were then counted and recorded as cfu/ml. The data are the mean of three experiments carried out on separate occasions. Standard errors are all less than ten percent of the mean.

5.3 Non-reciprocal challenge in PhoPQ mutants of *Salmonella*

The PhoPQ two-component system is an important regulator of virulence in *Salmonella* and is activated in response to low external concentrations of Mg^{++} ions and in response to low pH (Adams *et al.*, 2001). In view of this, the next stage was to test whether cross-protection could be achieved in a PhoP null mutant and a PhoPQ constitutive mutant.

5.3.1 Acid pre-adaptation to H_2O_2 stress in a PhoP null mutant

Mid-exponential phase *phoP12* cells were pre-treated with an inducing acid stress of pH 5.0, prior to a stress of 30 μM H_2O_2, as described in section 2.5.2.1.

Unadapted *phoP12* cells were more resistant to hydrogen peroxide than the wild-type parent (Figure 5.3). At 90 minutes following the 30 μM H_2O_2 stress, mutant cells were still viable, whereas wild-type cells were non-viable at 30 minutes under the same conditions (Figure 5.1). Mutant cells that had been pre-adapted with pH 5.0, were non-viable at 60 minutes following a 30 μM H_2O_2 stress, suggesting that acid pre-treatment was deleterious to the mutant's subsequent survival in 30μM H_2O_2. In short, the cross-protection that had been observed in the wild-type strain was not apparent in the mutant.

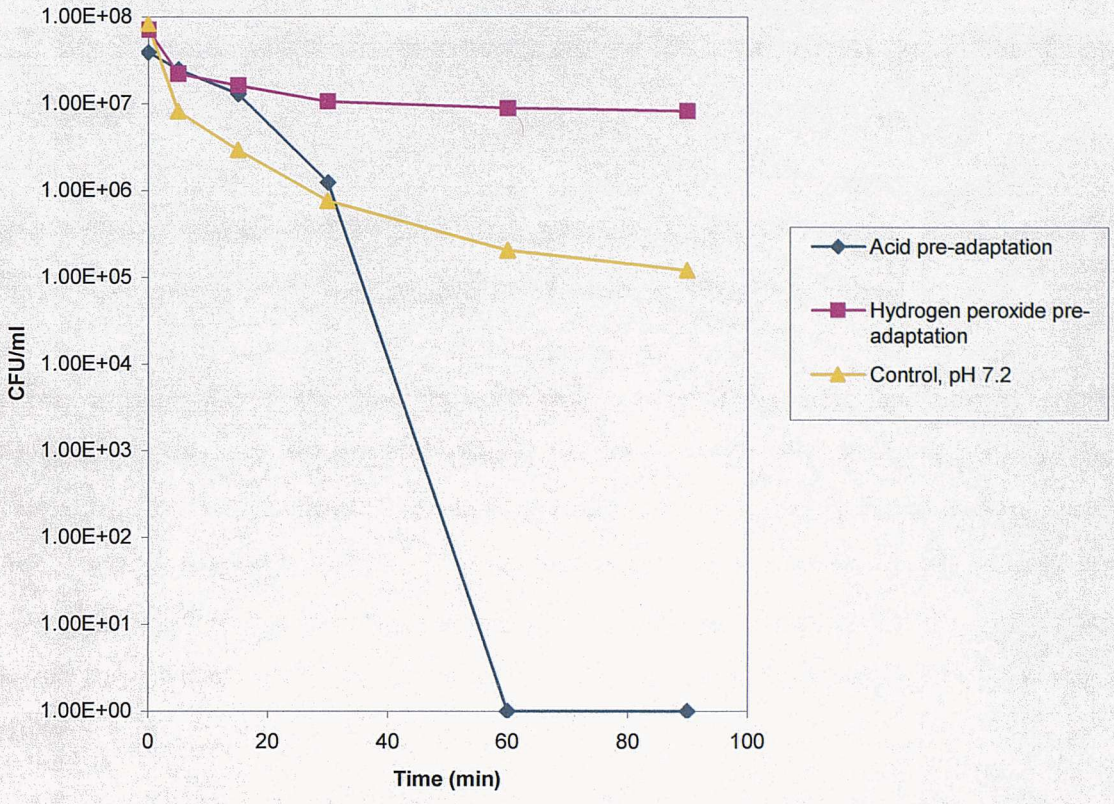

Figure 5.3. Acid pre-adaptation to hydrogen peroxide stress in a PhoP null mutant. Mid-exponential phase (A_{600}=0.4) *Salmonella phoP12* cells that had been exposed to an inducing acid stress of pH 5.0, or left unadapted, for 60 minutes were subsequently exposed to 30 μM H_2O_2 in LB for 60 minutes. Cells were then diluted and plated onto LB agar. Plates were incubated overnight and colonies were then counted and recorded as cfu/ml. The data are the mean of three experiments carried out on separate occasions. Standard errors are all less than ten percent of the mean.

5.3.2 Hydrogen peroxide pre-adaptation to acid stress in a PhoP null mutant

Mid-exponential phase *phoP12* cells were pre-treated with an inducing stress of 0.3 μM H_2O_2 prior to a subsequent acid stress of pH 3.0 as described in section 2.5.2.2.

Salmonella phoP12 cells that had been exposed to an inducing H_2O_2 stress for 60 minutes were not protected against a non-reciprocal acid stress of pH 3.0 and were non-viable at 30 minutes. In fact, the hydrogen peroxide pre-adaptation proved to be deleterious to the mutant's subsequent survival in acid, as unadapted cells remained viable up until a 60 minute time point under the same conditions (Figure 5.4). Reciprocal challenge, i.e. cells that had received an inducing stress of pH 5.0 for 60 minutes remained viable at 90 minutes.

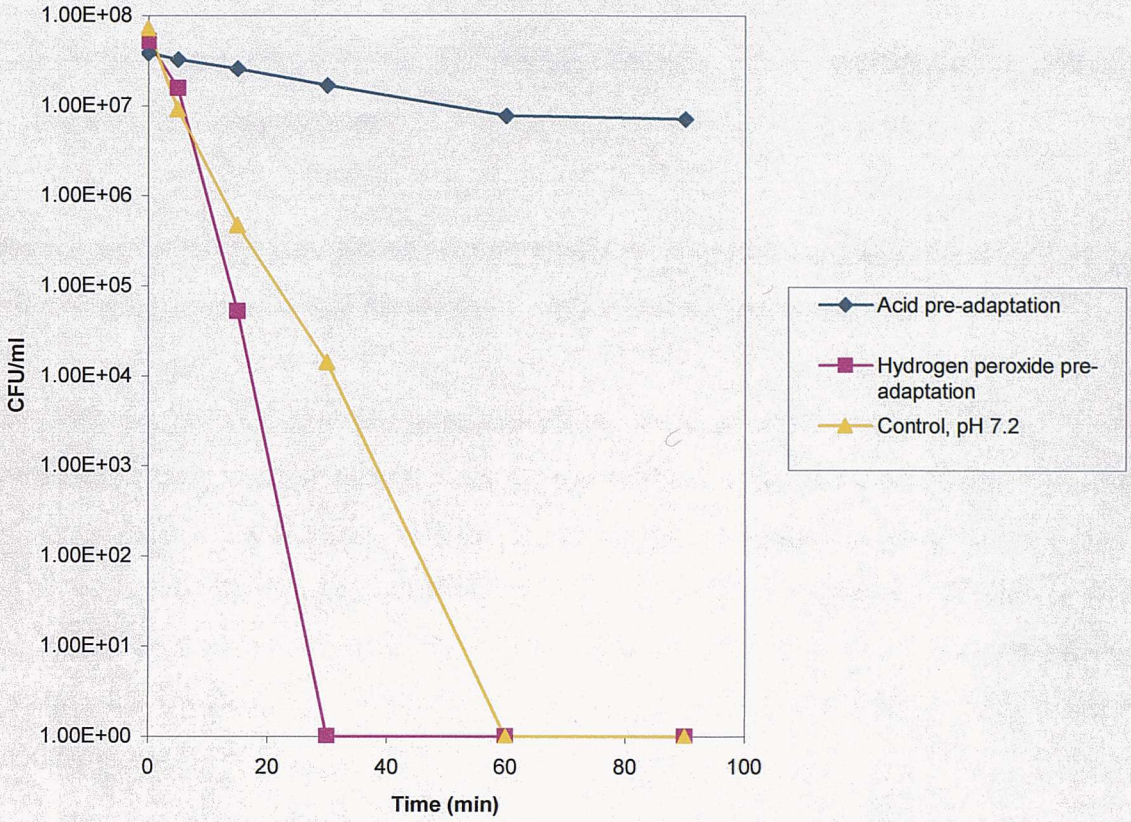

Figure 5.4. Hydrogen peroxide pre-adaptation to acid stress in a PhoP null mutant. Mid-exponential phase (A_{600}=0.4) *Salmonella phoP12* cells that had been exposed to an inducing stress of 0.3 μM H_2O_2, or left unadapted, for 60 minutes were subsequently exposed to an acid stress of pH 3.0 in LB for 60 minutes. Cells were then diluted and plated onto LB agar. Plates were incubated overnight and colonies were then counted and recorded as cfu/ml. The data are the mean of three experiments carried out on separate occasions. Standard errors are all less than ten percent of the mean.

5.3.3 Acid pre-adaptation to H_2O_2 stress in a *Salmonella pho-24* constitutive mutant

Mid-exponential phase *pho-24* cells were pre-treated with an inducing acid stress of pH 5.0 prior to a subsequent stress of 30 μM H_2O_2, as described in section 2.5.2.1.

Salmonella pho-24 cells that had been exposed to an inducing acid stress of pH 5.0 for 60 minutes were not protected against a subsequent non-reciprocal stress of 30 μM H_2O_2, and were non-viable at 30 minutes. Unlike the wild-type, acid pre-adaptation did not cross-protect against hydrogen peroxide stress in a *pho-24* constitutive mutant. Unadapted cells were non-viable at 30 minutes under the same conditions (Figure 5.5). Reciprocal challenge, i.e. cells that had received an inducing stress of 0.3 μM H_2O_2 for 60 minutes remained viable at 90 minutes.

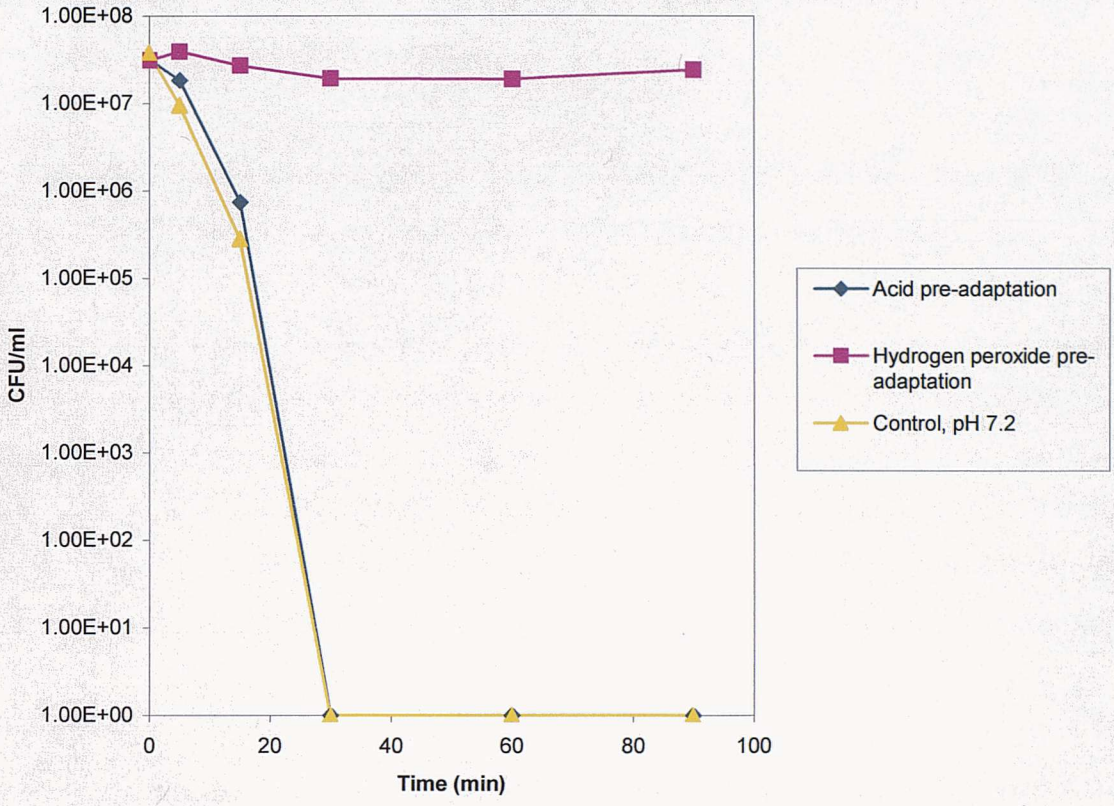

Figure 5.5. Acid pre-adaptation to hydrogen peroxide stress in a *pho-24* constitutive mutant. Mid-exponential phase (A_{600}=0.4) *Salmonella pho-24* cells that had been exposed to an inducing acid stress of pH 5.0, or left unadapted, for 60 minutes were subsequently exposed to 30 μM H_2O_2 in LB for 60 minutes. Cells were then diluted and plated onto LB agar. Plates were incubated overnight and colonies were then counted and recorded as cfu/ml. The data are the mean of three experiments carried out on separate occasions. Standard errors are all less than ten percent of the mean.

5.3.4 Hydrogen peroxide pre-adaptation to acid stress in a *Salmonella pho-24* constitutive mutant

Mid-exponential phase *pho-24* cells were pre-treated with an inducing stress of 0.3 μM H_2O_2 prior to a subsequent acid stress of pH 3.0 as described in section 2.5.2.2.

Salmonella pho-24 cells that had been exposed to an inducing H_2O_2 stress for 60 minutes were not protected against a non-reciprocal acid stress of pH 3.0 and were non-viable at 15 minutes. In fact, the hydrogen peroxide pre-adaptation proved to be deleterious to the mutant's subsequent survival in acid, as unadapted cells remained viable until a 90 minute time point under the same conditions (Figure 5.6). Reciprocal challenge, i.e. cells that had received an inducing stress of pH 5.0 for 60 minutes remained viable at a 90 minute time-point.

The results described in sections 5.2 and 5.3 are summarised in Tables 5.1 and 5.2. In brief:

1. Mild inorganic acid protects *Salmonella* from peroxide-induced oxidative stress.
2. Null and constitutive mutants of the PhoP-PhoQ system are not protected by mild acid. However, the null mutant survives better than wild-type cells when not pre-adapted.
3. Sub-lethal amounts of peroxide, capable of inducing a full oxidative stress response, do not protect wild-type *Salmonella* or its PhoP-PhoQ mutants from inorganic acid stress.

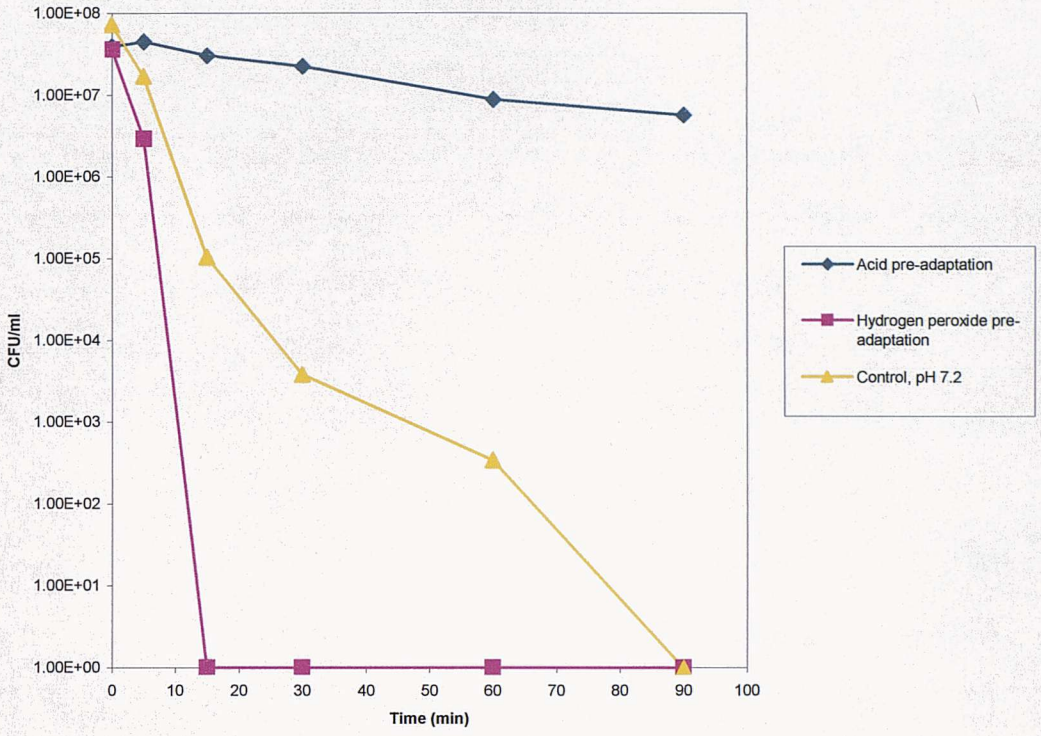

Figure 5.6. Hydrogen peroxide pre-adaptation to acid stress in a *pho-24* constitutive mutant. Mid-exponential phase (A_{600}=0.4) *Salmonella pho-24* cells that had been exposed to an inducing stress of 0.3 μM H_2O_2, or left unadapted, for 60 minutes were subsequently exposed to an acid stress of pH 3.0 in LB for 60 minutes. Cells were then diluted and plated onto LB agar. Plates were incubated overnight and colonies were then counted and recorded as cfu/ml. The data are the mean of three experiments carried out on separate occasions. Standard errors are all less than ten percent of the mean.

Table 5.1. Summary of viability studies and cross-protection data for hydrogen peroxide stressed cells.

Strain	**Pre-adaptation**	**Viability following exposure to H_2O_2 stress (30 μM) for 60 minutes**	**Pre-adaptation**	**Viability following exposure to H_2O_2 stress (30 μM) for 60 minutes**	**Cross-protection**
SL1344 wild-type	None	Non-viable at 30 minutes	pH 5.0	Non-viable at 60 minutes	Yes
SL1344 *phoP12*	None	Viable at 90 minutes	pH 5.0	Non-viable at 60 minutes	No
SL1344 *pho-24*	None	Non-viable at 30 minutes	pH 5.0	Non-viable at 30 minutes	No

Table 5.2. Summary of viability studies and cross-protection data for acid stressed cells.

Strain	**Pre-adaptation**	**Viability following exposure to Acid stress (pH 3.0) for 60 minutes**	**Pre-adaptation**	**Viability following exposure to Acid stress (pH 3.0) for 60 minutes**	**Cross-protection**
SL1344 wild-type	None	Non-viable at 60 minutes	0.3 μM H_2O_2	Non-viable at 60 minutes	No
SL1344 *phoP12*	None	Non-viable at 60 minutes	0.3 μM H_2O_2	Non-viable at 30 minutes	No
SL1344 *pho-24*	None	Non-viable at 90 minutes	0.3 μM H_2O_2	Non-viable at 15 minutes	No

5.4 Viability of *phoP12* cells following hydrogen peroxide treatment is growth phase dependent

Resistance to oxidative stress is increased as *Salmonella* cells enter stationary phase (Zhang *et al.*, 1998). The possibility that *phoP12* cells enter stationary phase early, thereby improving their survival following exposure to hydrogen peroxide was explored. Accordingly, *phoP12* cells were exposed to a hydrogen peroxide stress of 30 μM at OD_{600} readings of 0.4, 0.8 and 1.0 (Section 2.5.3). For comparison, the analysis was repeated with the wild-type parent.

The relative survival of the PhoP null mutant following hydrogen peroxide stress was found to be growth phase dependent (Figure 5.7). Viability was proportionally related to the OD_{600} value at which the H_2O_2 stress was administered. This phenomenon was observed in the wild-type for OD_{600} values of 0.4 and 0.8. However, at an OD_{600} value of 1.0, there was no increase in survival (Figure 5.8).

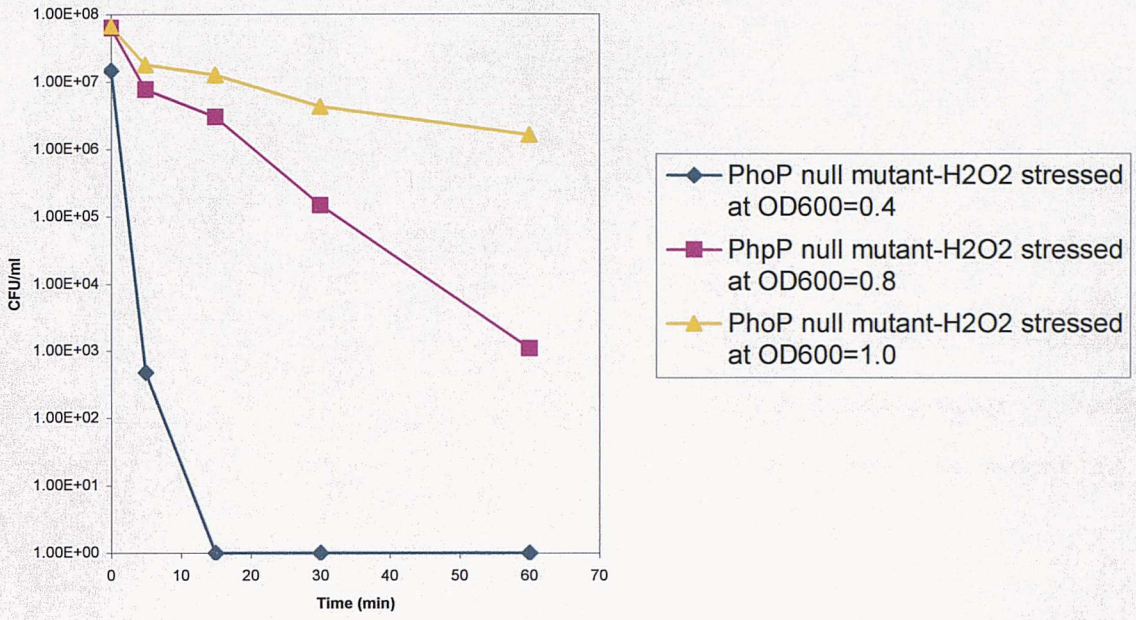

Figure 5.7. Viability of *PhoP12* cells following hydrogen peroxide stress. SL1344 *phoP12* cells were exposed to a hydrogen peroxide stress of 30 μM at OD_{600} values of 0.4, 0.8 and 1.0. Cultures were incubated for 60 minutes at 37°C. Cells were then diluted and plated onto LB agar. Plates were incubated overnight and colonies were then counted and recorded as cfu/ml. The data are the mean of three experiments carried out on separate occasions. Standard errors are all less than ten percent of the mean.

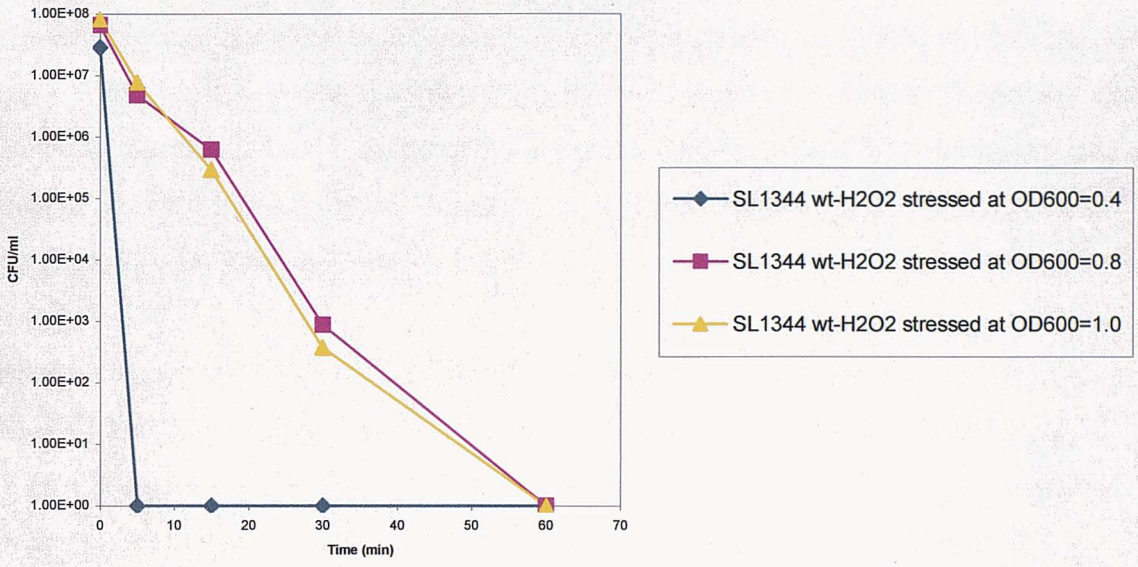

Figure 5.8. Viability of SL1344 cells following hydrogen peroxide stress. SL1344 wild-type cells were exposed to a hydrogen peroxide stress of 30 μM at OD_{600} values of 0.4, 0.8 and 1.0. Cultures were incubated for 60 minutes at 37°C. Cells were then diluted and plated onto LB agar. Plates were incubated overnight and colonies were then counted and recorded as cfu/ml. The data are the mean of three experiments carried out on separate occasions. Standard errors are all less than ten percent of the mean.

5.5 Derepression of the oxidative stress response in the PhoP null mutant does not appear to be due to a general stress response

The possibility that the PhoP null mutant's enhanced resistance to hydrogen peroxide was due to a general stress response, initiated by early transition into stationary phase, was also considered. RpoS levels are normally increased during transition into stationary phase (Zhang *et al.*, 1998). Accordingly, Western blot analysis was used to assess RpoS levels in wild-type and mutant strains (Section 2.4.2).

Western blot analyses suggested that RpoS levels in the PhoP null mutant were not elevated, when compared to the wild-type strain. This observation remained unaltered in hydrogen peroxide stressed and unstressed cells, irrespective of the OD_{600} value when cells were harvested or stressed, prior to sample preparation (Figures 5.9a, 5.9b and 5.10).

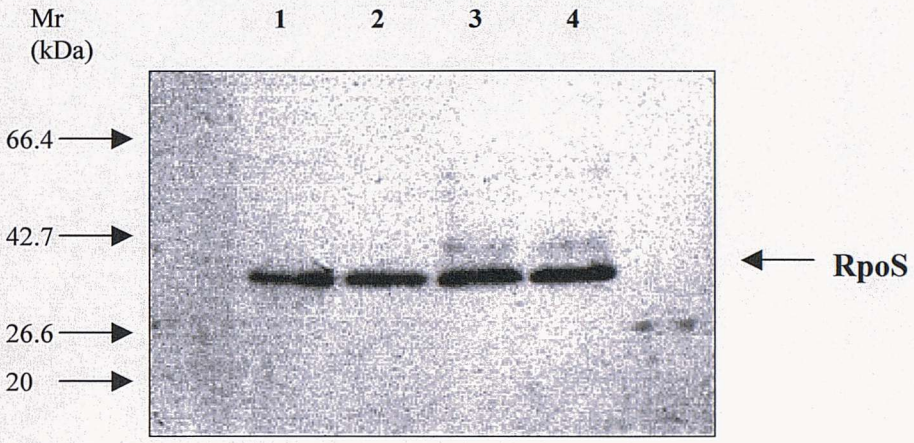

Figure 5.9a. RpoS-specific immunoblot of whole cell extracts from SL1344 wild-type and *phoP12* mutant strains. Unstressed cells were harvested at an OD_{600} of 0.4 and whole cell extracts were prepared as previously described. Equal amounts of protein samples were separated by polyacrylamide gel electrophoresis, transferred onto nitrocellulose and probed with antisera specific to RpoS. Lane 1, SL1344 (5 µl volume); lane 2, SL1344 *phoP12* (5 µl volume); lane 3, SL1344 (10 µl volume); lane 4, SL1344 *phoP12* (10 µl volume). Analysis was repeated three times to confirm reproducibility.

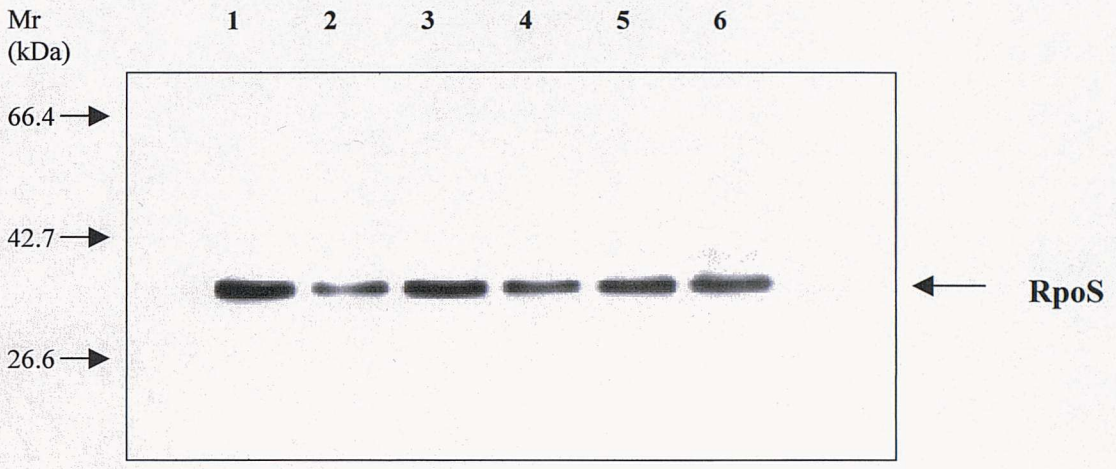

Figure 5.9b. RpoS-specific immunoblot of whole cell extracts from SL1344 wild-type and *phoP12* mutant strains. Unstressed cells were harvested at OD_{600} values of 0.6, 0.8 and 1.0. Whole cell extracts were prepared as previously described. Protein samples were separated by polyacrylamide gel electrophoresis, transferred onto nitrocellulose and probed with antisera specific to RpoS. Lane 1, SL1344 (OD_{600}=0.6); lane 2, SL1344 *phoP12* (OD_{600}=0.6); lane 3, SL1344 (OD_{600}=0.8); lane 4, SL1344 *phoP12* (OD_{600}=0.8); lane 5, SL1344 (OD_{600}=1.0); lane 6, SL1344 *phoP12* (OD_{600}=1.0). Analysis was repeated three times to confirm reproducibility.

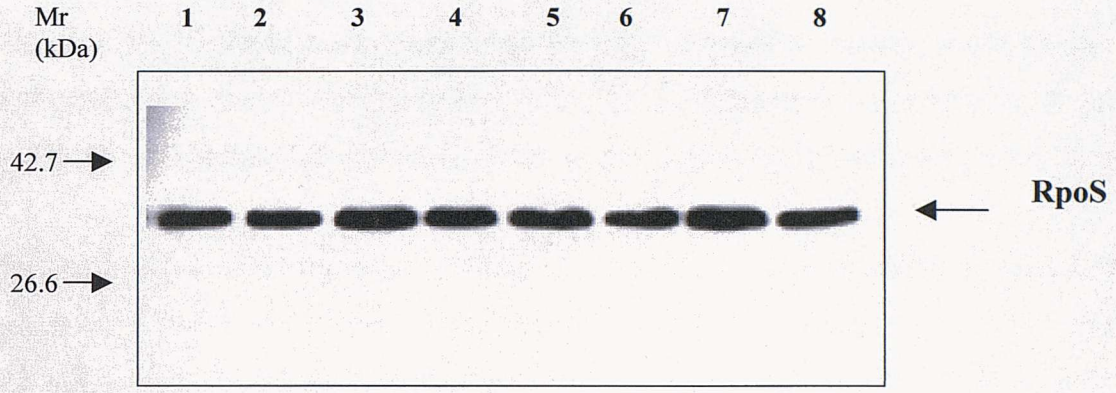

Figure 5.10. RpoS-specific immunoblot of whole cell extracts from SL1344 wild-type and *phoP12* mutant strains. Cells at OD_{600} values of 0.4, 0.6, 0.8 and 1.0 were stressed with 30 μM H_2O_2 for 60 minutes prior to harvesting. Whole cell extracts were prepared as previously described. Protein samples were separated by polyacrylamide gel electrophoresis, transferred onto nitrocellulose and probed with antisera specific to RpoS. Lane 1, SL1344 (OD_{600}=0.4); lane 2, SL1344 *phoP12* (OD_{600}=0.4); lane 3, SL1344 (OD_{600}=0.6); lane 4, SL1344 *phoP12* (OD_{600}=0.6); lane 5, SL1344 (OD_{600}=0.8); lane 6, SL1344 *phoP12* (OD_{600}=0.8); lane7, SL1344 (OD_{600}=1.0); lane 8, SL1344 *phoP12* (OD_{600}=1.0). Analysis was repeated three times to confirm reproducibility.

5.6 The oxidative stress response in the PhoP null mutant is specific to hydrogen peroxide

Although the PhoP null mutant was markedly more resistant to hydrogen peroxide than the wild-type parent, the specificity of the oxidative stress response was unknown. Therefore, sensitivity to paraquat was analysed in both mutant and wild-type strains (Section 2.5.4)

The PhoP null mutant was more sensitive to paraquat, when it was administered at OD_{600} values of 0.4 and 1.0, when compared to the wild-type (Figure 5.11). The increased resistance of the mutant to oxidative stress therefore appeared to be specific to hydrogen peroxide and the OxyR regulon. The SoxRS regulon did not appear to be involved.

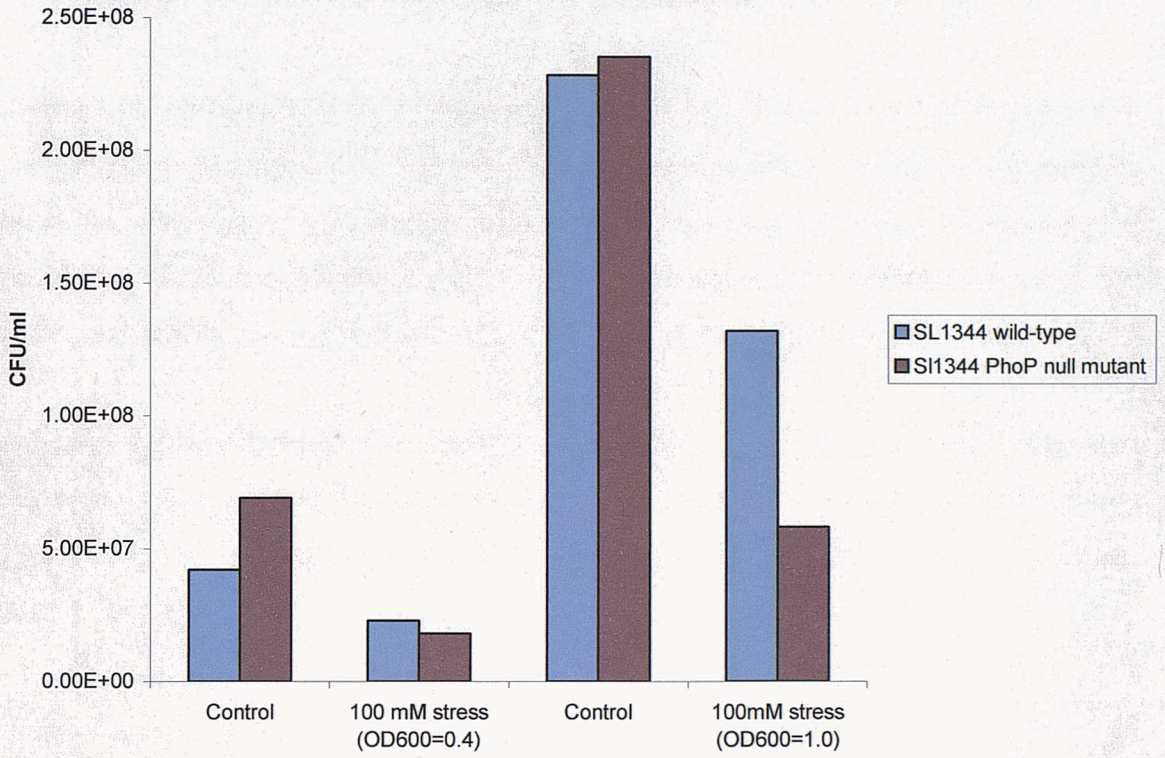

Figure 5.11. Sensitivity to paraquat. *Salmonella* cells at OD_{600} values of 0.4 and 1.0 were stressed with 100 mM paraquat in LB for 60 minutes prior to harvesting. Cells were then diluted and plated onto LB agar. Plates were incubated overnight and colonies were then counted and recorded as cfu/ml. The data are the mean of three experiments carried out on separate occasions. Standard errors are all less than ten percent of the mean.

5.7 Levels of OxyR are similar in the mutant and wild-type strains

The PhoP null mutant was shown to be more resistant to hydrogen peroxide than its wild-type counterpart (Section 5.3.1). Conversely, the mutant was less resistant to an oxidative stress in the form of paraquat than the wild-type, thereby suggesting that derepression of the oxidative stress response was specific to the OxyR regulon. Accordingly, Western blot analysis was used to assess OxyR levels in wild-type and mutant strains (Section 2.4.2).

The results obtained (figure 5.12) suggested that OxyR levels in the PhoP null mutant were not elevated, when compared to the wild-type strain, thereby suggesting that derepression of the oxidative stress response in the PhoP null mutant does not appear to be due to an up-regulation of OxyR.

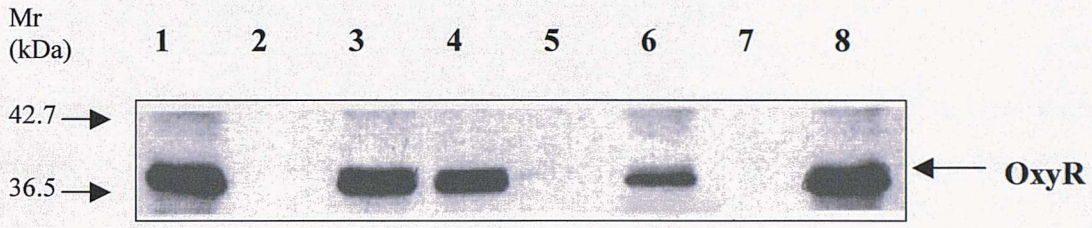

Fig 5.12. OxyR-specific immunoblot of whole cell extracts prepared as previously described from unstressed, mid-exponential phase (OD_{600}=0.4) *Salmonella* cells. Equal amounts of protein were separated by polyacrylamide gel electrophoresis, transferred onto nitrocellulose and probed with antisera specific to OxyR. Lane 1, TA4100 (*oxyR1*); lane 2, FSB; lane 3, SL1344; lane 4, SL1344 *phoP12*; lane 5, FSB; lane 6, TA4129 (*oxyR1zii614::*Tn*10*); lane 7, FSB; lane 8, TA4100 (*oxyR1*). Analysis was repeated three times to confirm reproducibility.

5.8 Analysis of protein carbonylation levels in *Salmonella* wild-type and *phoP12* mutant strains

The next stage was to compare protein carbonylation levels in both wild-type and mutant strains, in an attempt to measure oxidative damage, as previously described (Section 4.4).

Whole-cell extracts were prepared from mid-exponential phase cells. The proteins were then derivatized as described (Section 2.4.1), spotted onto a nitrocellulose membrane and detected with anti-DNP antibodies. Alternatively, the carbonyl content of total proteins was determined by Immuno-blotting after fractionation by SDS-PAGE (Section 2.4.2).

Protein carbonylation was found to be elevated in mutant cells, when compared to the wild-type. This suggested that the absence of a functional PhoP protein triggers increased oxidative damage (Figures 5.13 and 5.14).

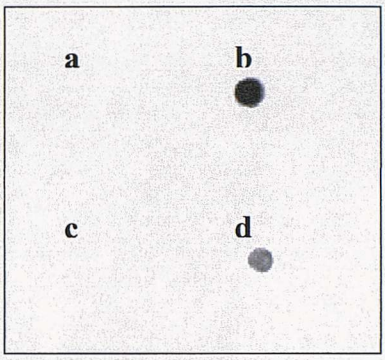

Figure 5.13. Protein carbonyl levels in mid-exponential phase unstressed *Salmonella* cells. Equal amounts of derivatized protein (20 μg) were spotted onto a nitrocellulose membrane and detected with anti-DNP antibodies. Bound antibodies were detected using SuperSignal chemiluminescence substrate (Pierce). Further details are given in Materials and Methods (Chapter 2, section 2.4.1). **a**, SL1344 *phoP12* negative control (non-derivatized protein sample); **b**, SL1344 *phoP12*; **c**, SL1344 negative control (non-derivatized protein sample); **d**, SL1344. Analysis was repeated three times to confirm reproducibility.

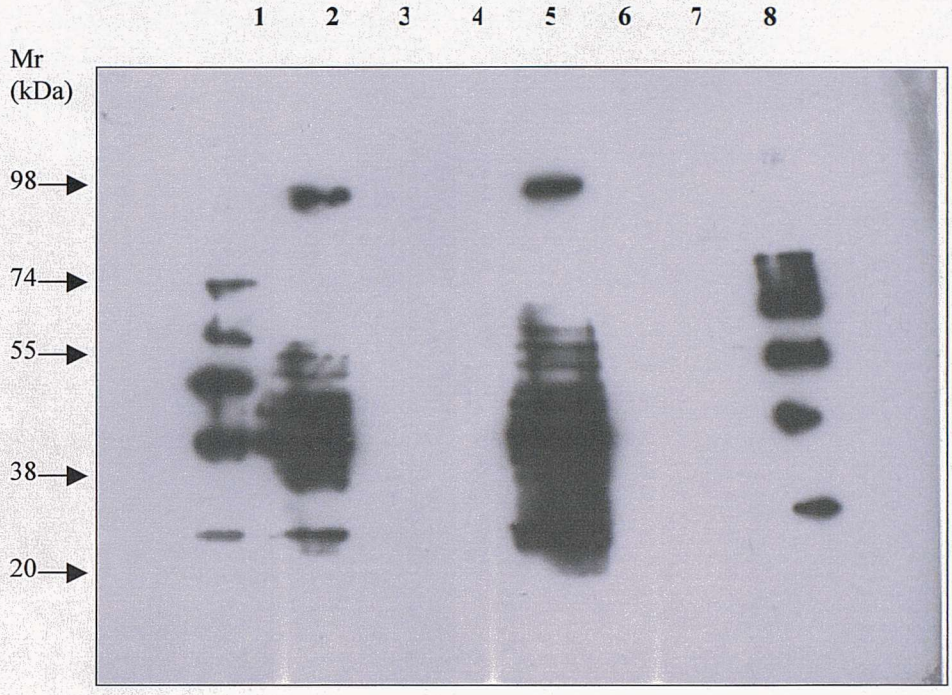

Figure 5.14. Protein carbonylation levels determined by one-dimensional immunoassay of mid-exponential phase unstressed *Salmonella* cells. Lane 1, pre-derivatized positive control; lane 2, SL1344; lane 3, SL1344 negative control (non-derivatized protein sample); lane 4, FSB; lane 5, SL1344 *phoP12*; lane 6, SL1344 *phoP12* negative control (non-derivatized protein sample); lane 7, FSB; lane 8, pre-derivatized positive control. Analysis was repeated three times to confirm reproducibility.

5.9 The involvement of BipA in the oxidative stress response

5.9.1 The adaptive response to H_2O_2 in a *Salmonella* BipA null mutant

In view of the global regulatory properties of the BipA GTPase, it was also of interest to assess the involvement, if any, of this protein in the hydrogen peroxide stress response in *Salmonella*. Accordingly, the susceptibility of mid-exponential phase wild-type and *bipA::cat* cells to hydrogen peroxide was compared under adapted and non-adapted conditions.

The unadapted, BipA null mutant was non-viable at 30 minutes following a hydrogen peroxide stress of 30 μM, which mirrored wild-type survival under the same conditions (Section 3.2.2). In contrast, the pre-adapted mutant was non-viable at 60 minutes following a 30 μM H_2O_2 stress, whereas the wild-type was viable for the duration of the experiment. These results suggest that the adaptive response to hydrogen peroxide is impaired in the BipA mutant (Figure 5.15).

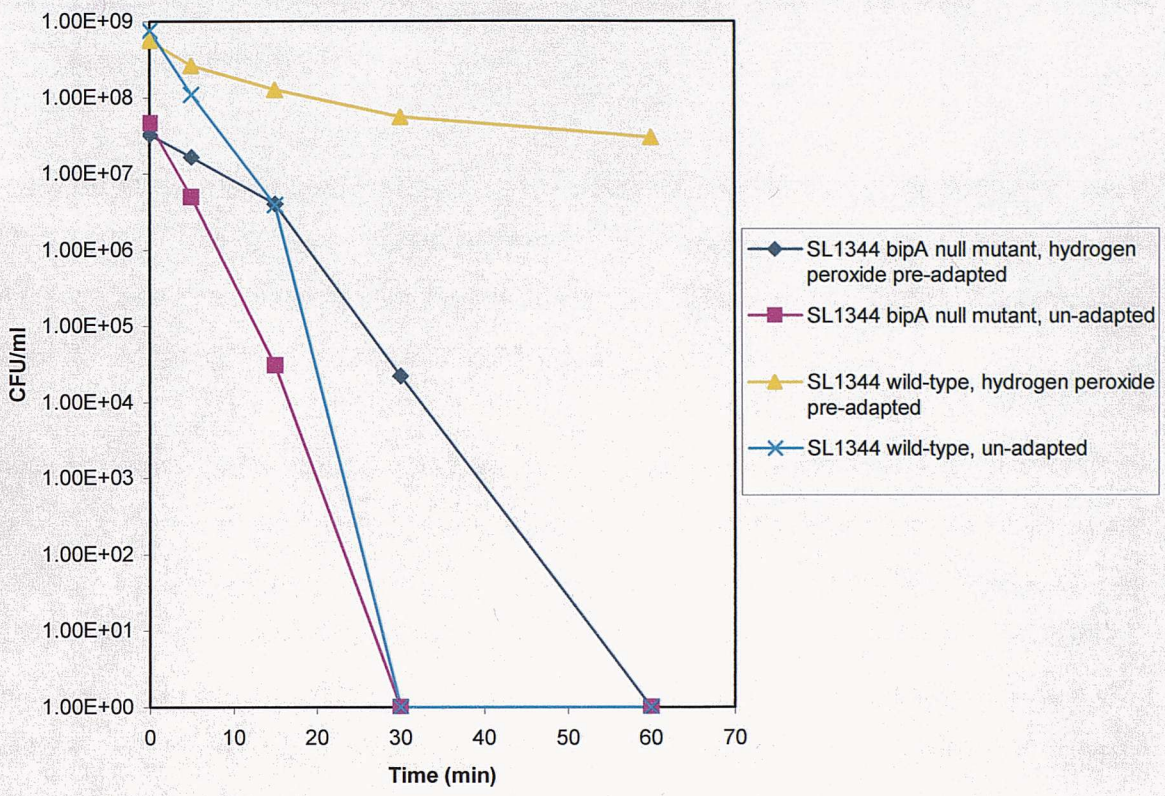

Figure 5.15. The effect of hydrogen peroxide on a *Salmonella bipA* null mutant under adapted and non-adapted conditions. Mid-exponential phase (A_{600}=0.4) *Salmonella* cells that had been exposed to an inducing stress of 0.3 μM H_2O_2, or left unadapted, for 60 minutes were subsequently exposed to a stress of 30 μM H_2O_2 in LB for 60 minutes. Cells were then diluted and plated onto LB agar. Plates were incubated overnight and colonies were then counted and recorded as cfu/ml. Data for the wild-type cells was taken from Figure 3.2. The data are the mean of three experiments carried out on separate occasions. Standard errors are all less than ten percent of the mean.

5.9.2 The adaptive response to H_2O_2 in EPEC wild-type and BipA null mutant strains

In view of the results obtained with the *bipA* mutant of *Salmonella,* it was decided to see if an *E. coli bipA* mutant had a similar phenotype. Mid-exponential phase E2348 wild-type and E2348 *bipA::cat* cells were pre-treated with a sub-lethal dose of hydrogen peroxide to initiate the adaptive response as described in Section 2.5.1.2.

Unadapted BipA mutant and wild-type cells were both non-viable at a 15 minute time point following a 30 μM H_2O_2 stress (Figure 5.16). The adaptive response to hydrogen peroxide was present in the wild-type strain, and long-lived protection was achieved. Furthermore, the level of viability remained essentially unchanged. However, in the BipA mutant, the adaptive response was impaired. Long-lived protection was achieved against a hydrogen peroxide stress, but viability was impaired, although cells appeared to make a partial recovery.

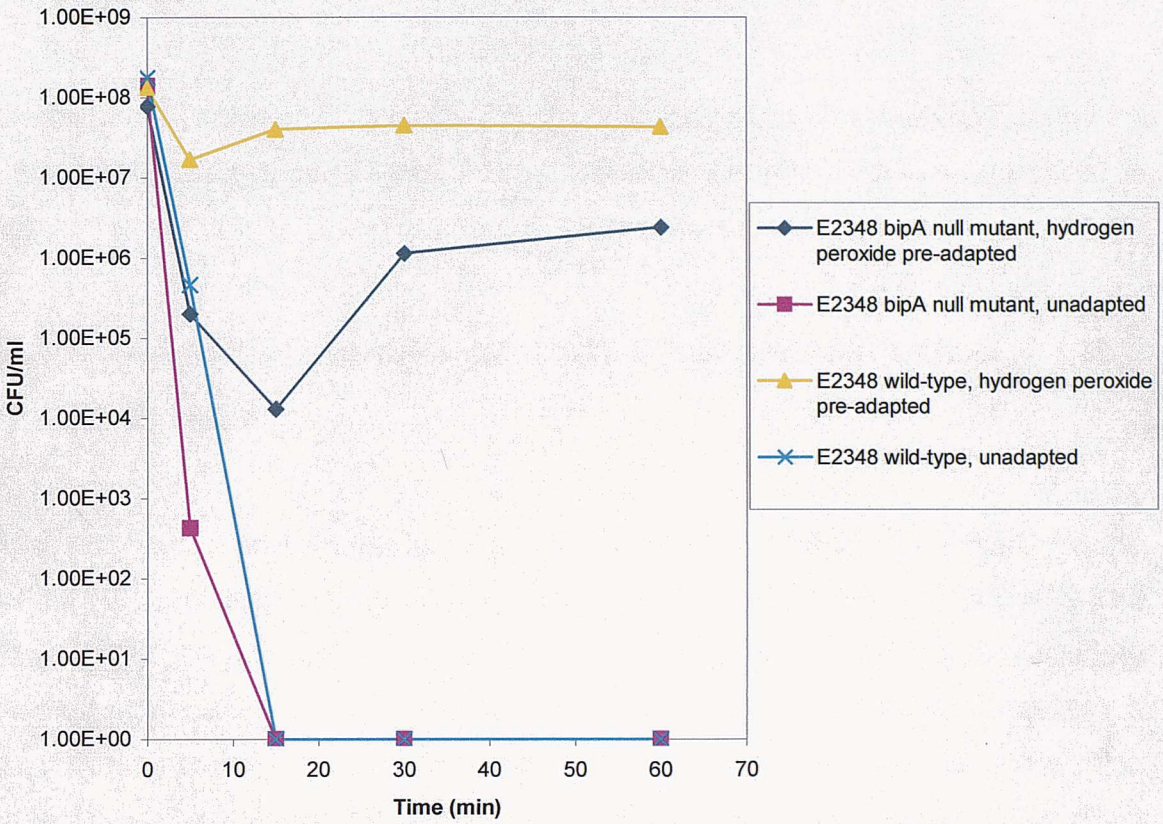

Figure 5.16. The effect of hydrogen peroxide on EPEC strain E2348 wild-type and *bipA* null mutant cells under adapted and unadapted conditions. Mid-exponential phase (A_{600}=0.4) *E. coli* cells that had been exposed to an inducing stress of 0.3 μM H_2O_2, or left unadapted, for 60 minutes were subsequently exposed to a stress of 30 μM H_2O_2 in LB for 60 minutes. Cells were then diluted and plated onto LB agar. Plates were incubated overnight and colonies were then counted and recorded as cfu/ml. The data are the mean of three experiments carried out on separate occasions. Standard errors are all less than ten percent of the mean.

5.10 Discussion

This chapter has reported an overlap with the hydrogen peroxide stress regulon and the PhoPQ and BipA regulons of *Salmonella*, explanations for which are now considered in detail.

5.10.1 A sub-lethal hydrogen peroxide stress protects against a lethal H_2O_2 stress

The hypothesis that the hydrogen peroxide stress regulon of *Salmonella* overlaps with the PhoPQ regulon, proposed as a result of the down-regulation of key proteins following acid and hydrogen peroxide stress, has been substantiated by the cross-protection analyses carried out as part of this study. Pre-adaptation of wild-type cells in mild acidic conditions offered cross-protection against a subsequent, otherwise lethal stress of H_2O_2. This suggests that a sub-lethal acid stress induces the hydrogen peroxide stress response, albeit transiently, as protection was short-lived when compared to reciprocally challenged cells. Conversely, hydrogen peroxide did not appear to induce the ATR as no cross-protection was observed when H_2O_2 adapted cells were treated with lethal amounts of HCl.

5.10.2 The PhoP null mutant is hyper resistant to hydrogen peroxide

Experiments with the null mutant of the PhoPQ system showed that it did not need to be pre-treated with sub-lethal amounts of hydrogen peroxide in order to survive a lethal dose. A simple interpretation of these results would be that activation of the PhoPQ system represses the expression of the OxyRS regulon. However, activation of PhoPQ, by incubation of wild-type *Salmonella* cells with sub-lethal amounts of HCl, did not block subsequent induction of the OxyRS system (Figure 5.1). Cells were cross-protected against a subsequent H_2O_2 stress and fared better than unadapted cells, as previously discussed.

If PhoPQ represses the expression of the OxyRS regulon, then one might also expect the PhoPQ constitutive mutant to be hypersensitive to hydrogen peroxide. However, unadapted, mid-exponential phase mutant cells were no more sensitive to 30 μM hydrogen peroxide than the wild-type under the same conditions.

A modified explanation would be that PhoPQ represses the OxyRS regulon in a growth-phase dependent manner, with little, or no repression when cells are in mid-exponential phase, but with an increased level of repression at higher OD_{600} values (Figure 5.17). This hypothesis is in agreement with the results showing that resistance of *phoP12* cells to H_2O_2 cells was proportionally related to the OD_{600} value at which the H_2O_2 stress was administered (Section 5.4). PhoP null mutant and wild-type cells exposed to H_2O_2 at an OD_{600} of 0.4 were both non-viable at 15 minutes. Repression of OxyRS regulated components by PhoPQ at this OD_{600} value in the wild-type would be weak, therefore derepression of OxyRS in the null mutant would have minimal impact on H_2O_2 survival. However, at higher OD_{600} values, derepression of the OxyRS regulon in the mutant would afford hyper resistance to H_2O_2, as was observed (Figure 5.7). OxyRS repression by PhoPQ would render the wild-type far more sensitive to H_2O_2 at higher OD_{600} readings than the null mutant (Figure 5.8).

The cross-protection achieved by pre-treating wild-type cells with sub-lethal amounts of HCl, presumably activated PhoPQ. However, activation of PhoPQ did not render the cell more sensitive to H_2O_2 as repression of OxyRS would have been weak during mid-

exponential phase if the hypothesis is correct (Figure 5.1). As predicted, cross-protection was abolished in the PhoPQ constitutive mutant, presumably due to enhanced repression of OxyRS (Figure 5.5).

In view of the results, PhoPQ may co-ordinate certain stress responses in *Salmonella*. In mid-exponential phase cells, OxyRS inhibits *rpoS* mRNA translation (Zhang *et al.*, 1998). Conversely, in cells approaching stationary phase, repression of the OxyRS system by PhoPQ may act to alleviate RpoS repression. By regulating the OxyRS system in a growth-phase dependent manner, the PhoPQ system possibly prevents the redundant utilisation of transcription factors.

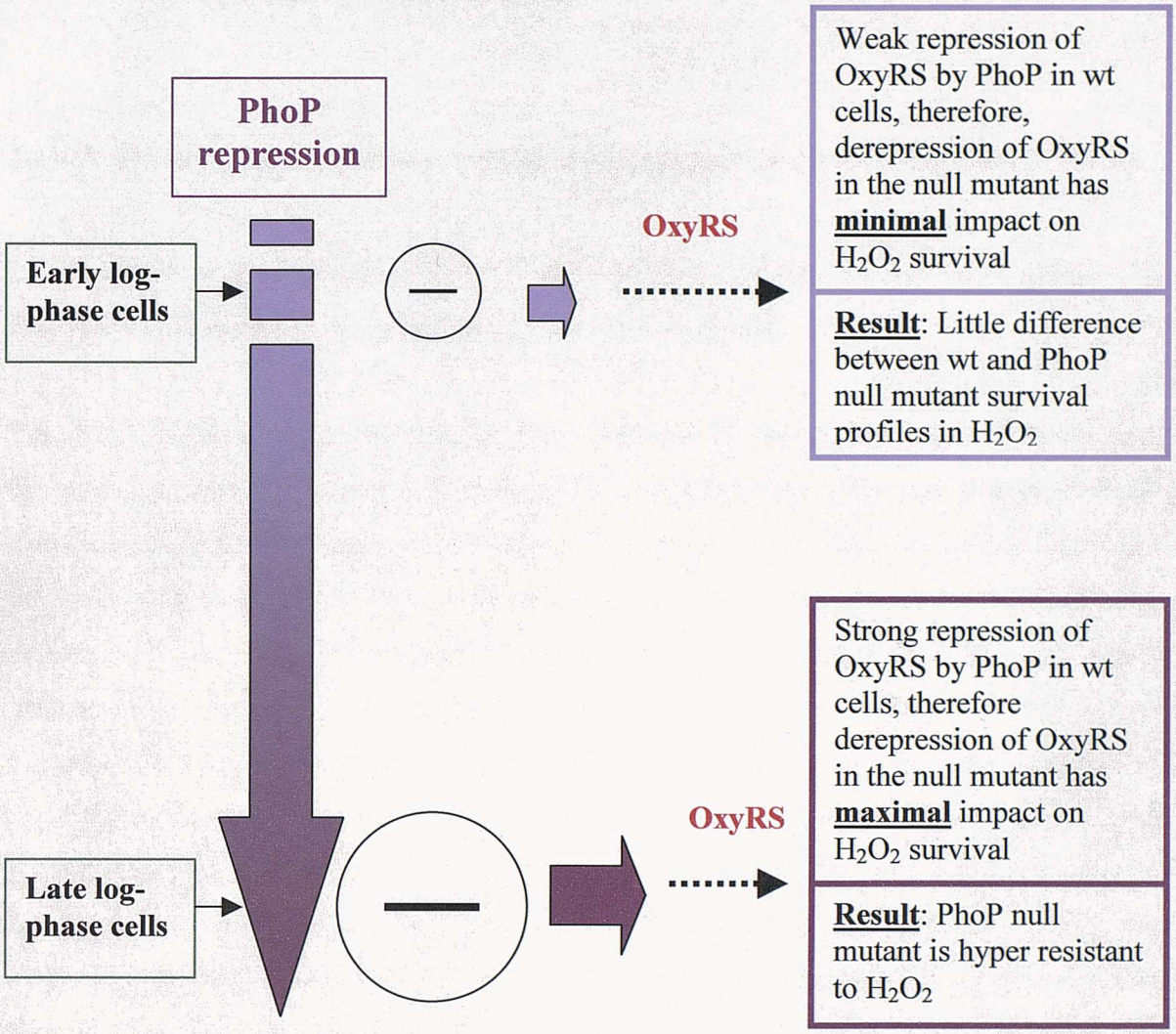

Figure 5.17. A possible model for the observed growth-phase-dependent increase in resistance to H_2O_2 in the PhoP null mutant. Assuming that there is little, or no repression of OxyRS by PhoP during early log-phase, wild-type and PhoP mutant cells will have similar levels of resistance to hydrogen peroxide. However, assuming that the OxyRS regulon is strongly repressed by PhoP during late log-phase, removal of PhoP has maximal impact on H_2O_2 survival, thereby rendering the PhoP mutant hyper resistant to this oxidant.

5.10.3 PhoP may positively regulate an oxidative stress response protein

An alternative explanation for the PhoP null mutant's resistance to hydrogen peroxide is that PhoP itself regulates an oxidative stress response protein.

"OxyBlot" analyses suggested that the PhoP null mutant had higher carbonyl levels than the wild-type, indicating increased levels of intracellular reactive oxygen species. If PhoP were to positively regulate a gene coding for a protein that protects against hydrogen peroxide under normal growth conditions, then oxidative stress levels in the PhoP null mutant would be elevated, as was observed (Figures 5.13 and 5.14). Catalase, which catalyses the dismutation of hydrogen peroxide to water and oxygen, would be an attractive candidate for co-regulation by PhoPQ because the oxidative stress response in the PhoP null mutant appeared to be specific to hydrogen peroxide (Section 5.6). An increased degree of oxidative damage may mimic pre-adaptation and prime the cell for subsequent oxidative stress. In turn, the resultant oxidising intracellular environment would activate OxyR, thereby affording the mutant greater resistance to hydrogen peroxide (Figure 5.18). It would be interesting to compare the redox status of OxyR in PhoP null mutant and wild-type cells. One would predict the mutant to have a higher proportion of OxyR in its oxidised form.

Nystrom (1999) has proposed that oxidative stress increases in senescent cells. Applying this to the hypothesis, loss of PhoP in early log phase cells (with only minor internal oxidative stress due to aerobic respiration), would lead to a minor increase in ROS generated and very little induction of OxyRS. However, in senescent cells with higher internal oxidative stress levels, removal of PhoP would have a greater impact. This would lead to a massive increase in ROS and hence a proportionally increased expression of the OxyRS regulon.

A further possibility is that PhoP is required for the synthesis of glutathione and/or glutaredoxin. Removal of the PhoP protein would shift the redox status of the cytosol making it more of an oxidising environment.

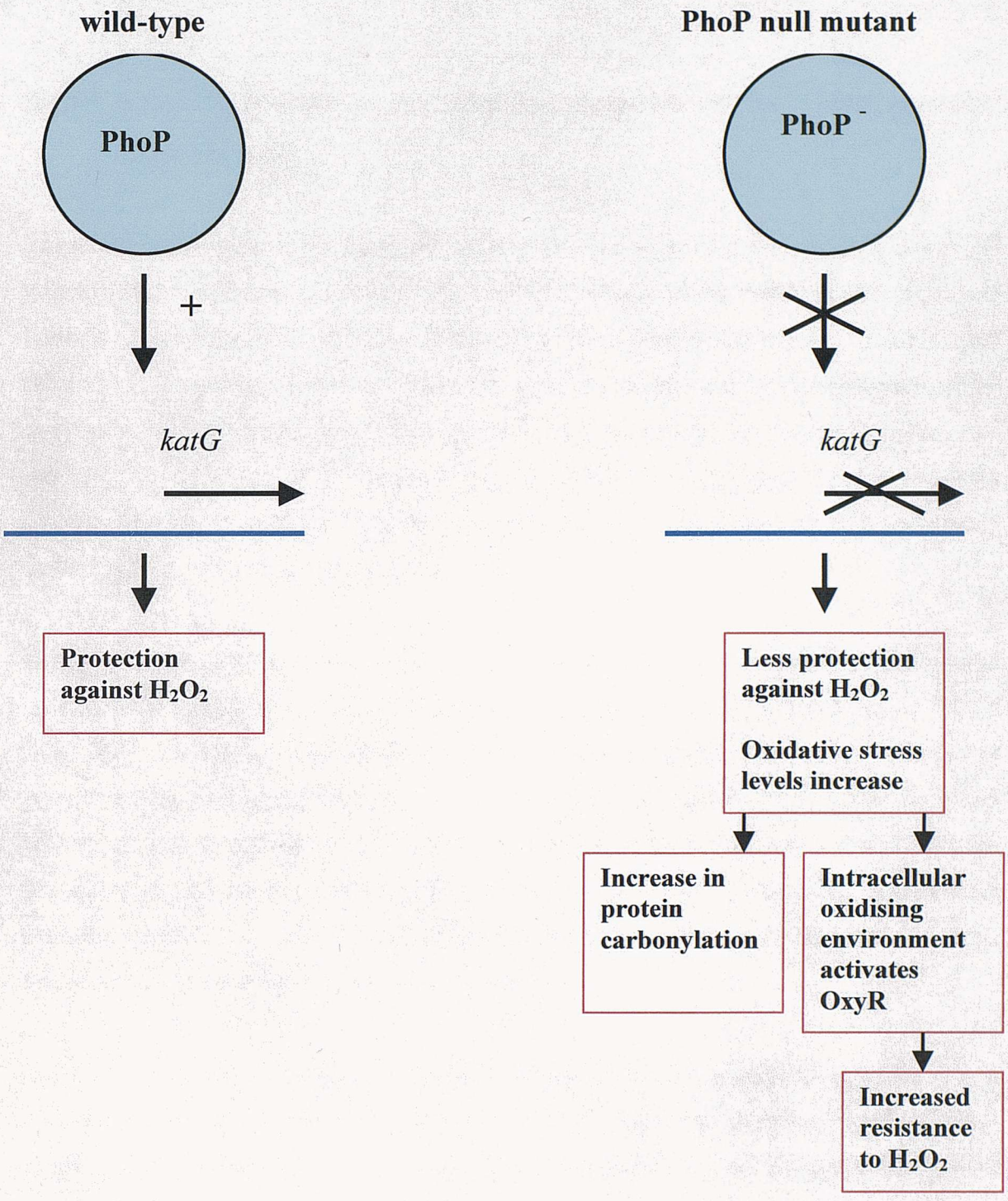

Figure 5.18. A possible model for the observed increase in oxidative stress responses in the PhoP null mutant. A disturbance in the prooxidant-antioxidant balance may result in an increased resistance to hydrogen peroxide in the PhoP null mutant. Assuming that PhoP activates the transcription of an antioxidant gene such as *katG*, then the null mutant would be exposed to higher levels of endogenous H_2O_2. The resultant oxidising intracellular environment would activate OxyR and thereby protect the cell from subsequent external hydrogen peroxide stress.

5.10.4 BipA is crucial to the adaptive response to hydrogen peroxide in *Salmonella*

The adaptive response to hydrogen peroxide was found to be severely impaired in a BipA null mutant of *Salmonella*. Whereas the unadapted BipA mutant behaved as wild-type in terms of its susceptibility to H_2O_2, pre-adapting with a sub-lethal amount of H_2O_2 only offered a short-lived protection against a subsequent 30 μM H_2O_2 challenge, when compared to the wild-type under the same conditions. In a similar null mutant in EPEC, the adaptive response was also impaired, but to a lesser extent; after 30 minutes cells recovered by two log orders. It is not yet clear if the initial drop in viability is attributable to a sub-population of hyper susceptible cells.

BipA, a GTPase, was first detected as a polypeptide that was strongly induced on exposure of *Salmonella* cells to bacterial/permeability-increasing protein (BPI) (Qi *et al.*, 1995). Since BPI is a cationic antimicrobial protein from neutrophils, which also use reactive oxygen species to kill microbes, it is possible that BipA coordinates *Salmonella* responses to both of these innate host defences. In EPEC, BipA has been implicated in the formation of actin-rich pedestal formation in host epithelial cells and resistance to host-defence peptides (Farris *et al.*, 1989). BipA also regulates cell motility in EPEC and *Salmonella* albeit with different effects (Adams *et al.*, 2001).

In view of its likely importance in EPEC infection, it has been proposed that BipA is a virulence regulator (Farris *et al.*, 1998). It is not surprising, therefore, that the BipA regulon overlaps with the hydrogen peroxide stress regulon. As yet, the method by which BipA aids the adaptive response to H_2O_2 remains unclear.

5.11 Summary

The PhoPQ and BipA regulons had not previously been linked to the hydrogen peroxide stress response in *Salmonella*. However, the results described here suggest that BipA is crucial to the adaptive response to H_2O_2. Similarly, it has been shown that the PhoPQ

system also overlaps with the hydrogen peroxide stress response and plays an important, but as yet, unclear role.

CHAPTER 6

GENERAL DISCUSSION

Chapter 6 General Discussion

1.11ntroduction

Oxidative stress is caused by exposure to reactive oxygen species. It is an unavoidable by-product of an aerobic life-style because $O_2^{\cdot-}$ and H_2O_2 are formed upon the auto-oxidation of components of the respiratory chain (Storz and Zheng, 2000). Although aerobic respiration is the main source of endogenous levels of $O_2^{\cdot-}$ and H_2O_2 in aerobically growing bacterial cells, exposure to ionising radiation, metals and redox-active drugs also causes increased levels of reactive oxygen species. Furthermore, in animals, reactive oxygen species are generated during phagocytosis to defend against bacterial invasion (Storz and Zheng, 2000).

Increased levels of $O_2^{\cdot-}$, H_2O_2 and $HO^{\cdot}$ are deleterious to cells as they can damage proteins, nucleic acids and cell membranes. It is not surprising therefore, that bacterial cells have developed adaptive responses to elevated levels of oxidative stress (Storz and Imlay, 1999).

1.2 Base-line studies and differential protein expression in response to H_2O_2

Initial base-line studies established the lethal dose of H_2O_2 (30 μM) for unadapted *S.* Typhimurium SL1344 cells. A benchmark of H_2O_2 (0.3 μM) was also suggested for a quantifiable deleterious effect. Following the elucidation of these parameters, the adaptive response to H_2O_2 was investigated. A sub-lethal stress of H_2O_2 offered a long-lived protection against a subsequent, otherwise lethal challenge, thus supporting previous work by Christman *et al.* (1985) who also demonstrated the existence of a regulated adaptive response to H_2O_2 for *S.* Typhimurium LT2.

Two-dimensional gel analysis of *S.* Typhimurium cells treated with H_2O_2, showed that at least fifteen proteins were differentially expressed following exposure to this oxidant. Examples of proteins that were down regulated under such conditions include flagellin

phase 1 and phase 2, D-galactose binding protein, glycerophosphoryl diester phosphodiesterase and asparaginase. Furthermore, a putative peroxidase, designated Spot 5/STM0402, whose predicted gene sequence was found to share 42% identity with *ahpC* of *S.* Typhimurium was also strongly down regulated in cells following exposure to H_2O_2. The AhpC sequences from *S.* Typhimurium, *E. coli*, *H. pylori* and *C. jejuni* all contain two highly conserved cysteine residues toward the N and C termini. The N-terminal cysteine residue is important in the antioxidant activity of AhpC in *S.* Typhimurium (Jacobson *et al.*, 1989). The conserved residues are also present in Spot 5/STM0402. Taken together, these findings support the proposal that Spot 5/STM0402 and the AhpC of *S.* Typhimurium are paralogues. However, their responses to oxidative stress are very different. Whereas the expression of *ahpC* is mediated by OxyR in response to hydrogen peroxide (Christman *et al.*, 1985), the promoter region of the Spot 5/STM0402 does not appear to contain a putative binding site for OxyR. Furthermore, no potential SoxS-binding consensus was identified, so it is unlikely that Spot 5/STM0402 is directly controlled via the OxyR or SoxRS systems. The regulatory region upstream of the Spot 5/STM0402 gene has three putative Fur binding sites, but the sites are separated by unusually long sequences of DNA. Analysis of the oxidative stress response to hydrogen peroxide in a *fur*$^-$ mutant of *S.* Typhimurium might help to determine if regulation of Spot 5/STM0402 is mediated by Fur. Additionally, it would be of interest to carry out genetic mapping experiments to locate the sequence elements responsible for the down regulation of the gene for Spot 5/STM0402, following oxidative stress.

1.3 Characterisation of an SL1344Δ*oxyS* mutant

A Δ*oxyS* mutant was generated by allelic exchange of the wild-type *oxyS* gene on the chromosome with an inactivated *oxyS* gene. Before the mutant could be constructed, the chromosomal region containing *oxyS* and its flanking genes had to be characterised in *Salmonella*. The resultant putative *oxyS* gene was found to be 110 nt in length and flanked by the genes encoding OxyR and arginino-succinate lyase. The putative secondary structure of the OxyS RNA molecule in *Salmonella* was determined, as based on the *mfold* program (Figure 4.9). This shows that some base changes that occur in *S.* Typhimurium, in a region predicted to be double stranded, are accompanied by a complementary base

change on the other strand. Such base changes preserve and support the proposed secondary structure of OxyS in *Salmonella.*

The adaptive response to H_2O_2 in the *oxyS* mutant was found to mirror the adaptive response displayed by the wild-type. However, deletion of the *oxyS* gene rendered the mutant more resistant to H_2O_2 than its wild-type counterpart. Furthermore, carbonylation assays suggested that the degree of oxidative stress was elevated in wild-type cells, when compared to the mutant. OxyS represses translation of *rpoS* mRNA by binding to the Hfq protein (Brown and Elliot, 1996; Zhang *et al.*, 1998). Therefore, the observed phenotype might be due to derepression of *rpoS* mRNA translation in the *oxyS* mutant. In support of this, 2-D gel analysis of *oxyS* mutant cells showed that phase 1 and phase 2 flagellin were up regulated. RpoS has recently been shown to induce the expression of the class 3 flagellar genes (Adams *et al.*, 2001), therefore up regulation of these genes in an *oxyS* mutant is in agreement with the hypothesis. Altuvia *et al.* (1997) have proposed that OxyS repression of RpoS prevents the redundant induction of oxidative stress genes, since RpoS and OxyR activate some of the same antioxidant genes. Removal of the *oxyS* gene might result in the induction of multiple antioxidant genes, thereby explaining the observed phenotype of the *oxyS* mutant. If derepression of *rpoS* mRNA translation is the mechanism by which the mutant is hyper-resistant to H_2O_2, one would expect the *oxyS* mutant to have elevated levels of RpoS, and this could easily be checked.

1.4 Overlap between the H_2O_2 stress regulon and the PhoPQ and BipA regulons

This thesis proposes that the hydrogen peroxide stress regulon overlaps with the PhoPQ regulon in *Salmonella.* Evidence in support of this idea includes the finding that a sub-lethal acid stress offered cross-protection against an otherwise lethal stress of H_2O_2. Furthermore, experiments with a PhoP null mutant showed that it was hyper-resistant to hydrogen peroxide in a growth phase dependent manner (Section 5.4). The PhoP null mutant's enhanced resistance to H_2O_2 was not due to a general stress response as RpoS levels were not elevated, when compared to the wild-type. Derepression of the oxidative stress response was specific for H_2O_2. Evidence for this came from experiments that showed that *PhoP12* cells were more sensitive to paraquat than the wild-type.

Carbonylation assays suggested that the absence of a functional PhoP protein triggers increased oxidative damage in *Salmonella* (Section 5.8). Taken together, these results suggest that PhoP may activate the transcription of one or more antioxidant genes. If the assumption is correct, removal of PhoP would result in increased levels of endogenous oxidative stress. Such an oxidising environment would activate OxyR, which in turn, would render the cell more resistant to H_2O_2. OxyR levels were not found to be elevated in the PhoP null mutant when compared to the wild-type, however, Storz *et al.* (1990) have suggested that increased expression of the *oxyR* regulon after treatment with H_2O_2 is not due to increased levels or synthesis of OxyR, but is the result of a modification of pre-existing OxyR.

The putative global regulatory protein BipA was found to be crucial to the adaptive response to H_2O_2 in *Salmonella* (Section 5.10.4). Removal of the BipA protein resulted in a severely impaired adaptive response to this oxidant. However, the mechanism by which BipA aids the adaptive response to H_2O_2 remains unclear. The previously unsuspected role of this protein in oxidative stress responses underscores how much there is yet to study in this area. It would be of some interest to dissect the regulatory mechanism in detail as the available evidence suggests that BipA is likely to operate via interactions with the ribosome. Such a mechanism is therefore likely to be novel but, in view of the ubiquity of BipA, widespread.

1.5 Final comments

The overall objective of this study was to gain a greater insight into the oxidative stress response of *S.* Typhimurium. Stress response systems in *Salmonella* have been shown to be complex and interconnected (Foster and Spector, 1995). *Salmonella* counteracts oxidative stress by expressing enzymes that detoxify reactive oxygen species and repair any damage that has been caused. In this way, toxicity from endogenous oxidants can be avoided. However, if intracellular levels of ROS are accelerated, these defences are inadequate (Storz and Imlay, 1999).

The production and characterisation of the *oxyS* deletion mutant has shown that *Salmonella* can still adapt to and survive oxidative stress, perhaps by using alternative compensatory

antioxidant mechanisms. Although the inducible system regulated by OxyR could act as a potential target for the inactivation of *Salmonella*, this pathogen's ability to induce compensatory defence strategies must surely be considered. The findings of this study strongly suggest the potential benefit of multiple strategies that could attack not only the OxyRS regulon, but also the PhoPQ system and BipA. Without furthering our knowledge of this diverse pathogen, *Salmonella* will continue to outwit its host and pose a threat to world health. The emergence of *Salmonella* strains possessing multiple resistance to antibiotics, which is a source of concern to both the public and animal health agencies, has reinforced the need for further research if we are to keep one step ahead of this pathogen.

CHAPTER 7
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