Stress proteins of *Salmonella enterica* serovar Typhimurium: control of expression and roles in infection and immunity

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DECLARATION

I declare that this thesis was composed by myself and the research presented is my own.

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June 1997.

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ABBREVIATIONS

Ab	antibody
Amp^R	ampicillin resistant
ADP	adenosine diphosphate
ATP	adenosine triphosphate
dATP	deoxyadenosine triphosphate
ddATP	dideoxyadenosine triphosphate
bp	basepairs
°C	degrees Celsius
c.f.u.	colony forming units
Ci	Curies
CMI	cell-mediated immunity
Cml^R	chloramphenicol resistant
CTAB	hexadecyltrimethyl ammonium bromide
dCTP	deoxycytidine triphosphate
ddCTP	dideoxycytidine triphosphate
Da	Daltons
DMSO	dimethylsulphoxide
DNA	deoxyribonucleic acid
DTH	delayed-type hypersensitivity
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetate
g	grams
g	standard acceleration of gravity
GM-CSF	granulocyte-macrophage colony-stimulating factor
dGTP	deoxyguanosine triphosphate
ddGTP	dideoxyguanosine triphosphate
dH_2O	distilled water
IFN	interferon
Ig	immunoglobulin
IL	interleukin
i.p.	intraperitoneally
IPTG	isopropyl-β-D-thiogalactoside
kb	kilobases
Kan^R	kanamycin resistant
l	litres
LB	Luria-Bertani medium
LD_{50}	50% lethal dose
LHFP	left hind footpad
LPS	lipopolysaccharide
LSLB	low salt Luria-Bertani medium
LSB	Laemmli (Loading) sample buffer
M	molar
mA	milliamps
mg	milligrams
ml	mililitres
mm	millimetres

MW	molecular weight
µl	microlitres
NAD	nicotinamide adenine dinucleotide
dNTP	deoxynucleotide triphosphate
OD	optical density
ONPG	o-nitrophenyl-β-D-galactoside
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PEG	polyethyleneglycol
PMN	polymorphonuclear leucocyte
PMSF	phenylmethylsulphonyl fluoride
RHFP	right hind footpad
RNA	ribonucleic acid
ROS	reactive oxygen species
SAP	shrimp alkaline phosphatase
s.c.	subcutaneously
SDS	sodium dodecyl sulphate
TEMED	N, N, N'-tetramethylethylenediamine
Tet^R	tetracycline resistant
TNF	tumour necrosis factor
dTTP	deoxythymidine triphosphate
ddTTP	dideoxythymidine triphosphate
Tris	tris(hydroxymethyl)aminomethane
UV	ultraviolet
V	volts
v/v	volume per volume
w/v	weight per volume
X-gal	5-bromo-4-chloro-indoyl-β-D-galactoside

ABSTRACT

OxyR-regulated genes are essential to the development of hydrogen peroxide resistance in *Salmonella enterica* serovar Typhimurium (*S. typhimurium*) and *Escherichia coli*. Classically, such regulation is dependent upon both OxyR and σ^{70}. Recently, in *E. coli*, a number of OxyR-dependent genes have been shown to be induced in the stationary phase of growth by the alternative sigma factor RpoS, independently of OxyR. In addition, RpoS has been shown to regulate a number of genes in *S. typhimurium* when within host cells. In a previous study of *S. typhimurium*, a hydrogen peroxide-inducible locus, *ahp*, encoding alkyl hydroperoxide reductase, was found to be induced during interaction with macrophages (K. P. Francis, PhD Thesis, 1993). In the present study, the role of growth phase and RpoS upon *ahp* was assessed in *S. typhimurium* using a Mu*dlux* reporter system and Western blotting. Although a basal level of protein was found to be present in the cells, Ahp was found not to be induced upon entry into the stationary phase. This makes *ahp* unique amongst those OxyR-regulated loci examined to date.

Preliminary experiments employing the Mu*dlux* reporter system indicated that the *ahp* locus was osmotically sensitive in *S. typhimurium*. Studies on other bacteria supported this view. The affect of the osmotic environment of the cell upon the expression of *ahp* was therefore addressed in greater depth. The subsequent use of immunoblotting techniques conclusively demonstrated that chromosomal expression of the *ahp* locus was not affected by the osmotic environment surrounding the cell. Instead, the Mu*dlux* element was found to alter the natural behaviour of the *ahp* promoter in such a way that it adopted an osmotically-regulated status and, this mode of regulation appeared to override regulation via the normal hydrogen peroxide-inducible mechanism.

S. enterica is an intracellular pathogen which is capable of surviving within macrophage cells. Macrophages are equipped with an arsenal of anti-microbial effector mechanisms, including a respiratory burst which generates reactive oxygen metabolites. Since *ahp* had previously been shown to respond to the respiratory burst

of macrophages, this study also assessed the role of oxidative stress resistance genes in the virulence of *S. typhimurium*. Strains of the mouse pathogen SL1344 were constructed in which the *ahp* and *oxyR* loci were disrupted and their virulence was assessed in LD_{50} studies. Disruption of the *ahp* or *oxyR* loci was found to have no affect upon the gross virulence of SL1344 for mice, suggesting that these loci were not essential for survival within the macrophage.

The ability to develop immunity against infection by *S. typhimurium* is thought to correlate with the development of immunity against bacterial antigens which are expressed *in vivo*. As a further part to this study, the immunological responses of mice to two *S. typhimurium*-derived polypeptides, AhpC and GroEL, following cloning and overexpression of these proteins, were examined. Mice previously infected with an attenuated strain of *S. typhimurium* were shown to elicit significant delayed-type hypersensitivity reactions following subcutaneous injection of these polypeptides 33 and 104 days post-infection. Moreover, AhpC- and GroEL-specific antibodies were detected during the course of infection of mice with *S. typhimurium*. These results demonstrated that immunity to *S. typhimurium* in the murine host was regulated by T_H1 cells. In addition, detection of both cell-mediated and humoral immune responses to AhpC and GroEL indicated that these polypeptides are targets for immunological recognition and may contribute to the protective immunity generated by mice following *S. typhimurium* infection.

CHAPTER 1

Introduction

1.1 GENERAL OVERVIEW

The species *Salmonella enterica* is composed of over 2, 000 different serovars. For the purpose of this thesis, serovars of *S. enterica* will be identified initially by their official designation but subsequently referred to by their commonly used serovar name (which will be displayed in adjacent brackets). However, the use of the species name, *S. enterica*, will be used when referring to or discussing general properties or features of the serovars that form the species.

The species *S. enterica* is an important agent of disease as many of its serovars are capable of infecting and causing disease in humans and animals. Some like *S. enterica* serovar Typhi (*S. typhi*), the causative agent of typhoid fever, only infects humans whereas *S. enterica* serovar Typhimurium (*S. typhimurium*) is capable of causing diseases in multiple hosts (Cohen and Tauxe, 1986; Finlay and Falkow, 1989a). The usual route of infection for *S. enterica* is by the ingestion of contaminated food and water, and the diseases associated with infection are collectively termed salmonellosis. Salmonellosis generally takes the form of diarrhoeal disease but this can vary from gastro-enteritis (food-poisoning), which is essentially a self-limiting infection, to a much more severe disease, such as typhoid fever, where the bacteria invade and replicate in the tissues of the body (Gulig, 1996).

Diarrhoeal diseases are some of the most important health problems faced by the world today (Pang *et al.*, 1995; Maurice, 1994). The contribution of salmonellosis to the incidence of diarrhoeal disease is highlighted by the World Health Organisation's (WHO) recent statistics which show that annually, there are approximately 16.6 million cases of typhoid fever with nearly 600, 000 deaths. Moreover, although gastro-enteritis is generally considered a non-fatal disease, approximately 3 million deaths result from the estimated 1.3 billion incidences of acute gastro-enteritis/diarrhoea (due to non-typhoidal salmonellosis) which occur *per annum* (Pang *et al.*, 1995; Ivanoff *et al.*, 1995). The incidence of *S. enterica*-related disease is particularly prevalent in developing countries where the problems of

infection are exacerbated by the poor sanitation and health care that exists. However, diarrhoeal diseases are also a problem for industrialised countries, but here the increase in incidence of disease has been associated with the demand for large scale food production and greatly increased dependence on convenience and fast foods (Maurice, 1994).

Infection by *S. enterica* is also a concern for animals including chickens, calves, swine and sheep (Coynault *et al.*, 1996), where diarrhoeal and systemic disease have economic consequences in farming and animal husbandry. For example, in the USA it has been estimated that approximately 57% of all cattle and 70% of all chickens are carriers of *S. enterica* (Jay, 1992). Importantly, such infected animals are considered to be a major factor in the spread of *S. enterica* through the food chain to humans (Maurice, 1994). In the Western world, the potential problems of *S. enterica* infection have been significantly reduced by the use of antibiotics. For example, antibiotics such as chloramphenicol have been used in farming practice to keep animals pathogen-free such that they show an optimal increase in mass (Cohen and Tauxe, 1986). Moreover, antibiotics have proven useful in treating infections in humans, caused by non-typhoidal *S. enterica* serovars such as *S. typhimurium,* or in rare instances by *S. typhi* (Pang *et al.*, 1995). However, multidrug resistance is emerging in *S. enterica* serovars and is spreading globally. For example, it has been estimated that around 50-70% of *S. typhi* strains in the Indian sub-continent are now resistant to chloramphenicol. Thus, the use of antibiotics in preventing serious disease in both humans and animals is becoming less effective (Pang *et al.*, 1995).

Many of the problems caused by *S. enterica* infection could be alleviated in both the developed and developing world by the use of vaccines (Ivanoff *et al.*, 1995; Coynault *et al.*, 1996). Not only could such vaccines reduce the incidence of serious disease such as typhoid fever, but immunisation of animals could be used to combat the contamination and spread of *S. enterica* through the food chain. The development of vaccines therefore has an important role in the elimination of diseases caused by both typhoidal and non-typhoidal serovars of *S. enterica*. However, to date, the vaccine design strategies targeted at preventing diseases caused by *S. enterica* have

had variable success (Ivanoff *et al.*, 1994; Pang *et al.*, 1995).

Much of the current research into developing effective prophylactic therapies to disease caused by *S. enterica* has focused on the use of live attenuated organisms. This angle of research has profited from examining the interaction of the bacterium with the host. To successfully establish an infection, *S. enterica* must withstand or circumvent the host's non-specific and specific arms of the immune system. However, *S. enterica* is a facultative intracellular pathogen and has developed a complex array of virulence mechanisms to promote its survival (Finlay and Falkow, 1989b; Galan and Sansonetti, 1996). Of particular importance to the survival of this bacterium is its capability to invade and survive within the hosts' cells, particularly macrophage cells, which are equipped with an arsenal of anti-microbial effector mechanisms (Jones and Falkow, 1996; Gulig, 1996). Thus, by identifying genes and their products which are important for virulence we may be able to target these factors and develop immunological strategies to eliminate diseases caused by *S. enterica*. The role of such virulence factors and their immunological properties is the basis of the present study.

The introduction to the present study is split into three sections to give an overview of the major factors involved in host-infection by *S. enterica* and the development of vaccines. The first section deals with the immune response and features involved in eliminating *S. enterica* from the host. The following section then examines the cellular and molecular bases by which *S. enterica* is able to accomplish a successful infection. Finally, a brief summary will be given of the types of vaccine strategies that have been employed, or that have potential for development, in combating diseases caused by *S. enterica*.

1.2 THE IMMUNE RESPONSE TO INFECTION BY *S. ENTERICA*

Immunity to microbial infection is very complex and the type and degree of response is as much dependent upon the bacterium as the host (Finlay and Falkow, 1989b). In order to discuss the immune responses to infection by *S. enterica*, it is first necessary to give a brief, but comprehensive, overview of the immune system. However, for relevance to *S. enterica*, this account will be biased towards the immune responses generated to bacterial infection.

1.2.1 Immunity to infection is dependent upon both innate and specific immunity

Essentially, immunity can be split into two types; innate (natural) immunity and specific (acquired immunity) (reviewed in Abbas *et al.*, 1991; Weir and Stewart, 1993). Innate immunity consists of factors which are present prior to any contact with a particular microbe and which are not enhanced by such exposure. On the other hand, specific immunity is stimulated by the identification of specific macromolecules (antigens) and responses are enhanced, and defensive capabilities increased, with each successive exposure to that antigen. Furthermore, the specific immune system "remembers" past encounters with a particular microbe (which is the basis of protective vaccination). It is important to note that both elements of immunity, although distinct, interact with each other. For example, specific immunity can involve recruiting components of innate immunity and some aspects of innate immunity also play a role in stimulating specific immunity (Abbas *et al.*, 1991; Gulig, 1996).

1.2.2 Innate immunity

Innate immunity provides an important anti-microbial defence to infection. The presence of intact physiochemical barriers such as the skin or mucous membranes prevent organisms gaining direct entry into the underlying tissues of the body (Finlay and Falkow, 1989b). In addition, there are humoral factors present in the blood and tissue fluid that have anti-microbial activity. This includes the complement system, a family of serum proteins which are activated in a proteolytic cascade, and which can

be triggered by the surface of the microorganism (called the alternative pathway of activation) (Joiner *et al.*, 1984). Complement mediates a number of effector functions (Joiner *et al.*, 1984). Firstly, complement proteins (C5-C9) can form a hydrophobic complex in the cell membrane of the bacterium, called a membrane attack complex, which results in the lysis of the bacterium by osmotic swelling. In addition, various components of the complement system which are generated in the enzymatic cascade, can themselves stimulate inflammatory processes (*e.g.* C5a and C3a) or act as chemoattractants (*e.g.* C5a) to draw phagocytic cells, which are designed to engulf and destroy bacteria, to the site of infection (Adams and Hamilton, 1984). Moreover, phagocytes have specific cell surface receptors for components of complement (*e.g.* C3b), therefore the deposition of complement proteins onto the surface of the microorganism acts as a mechanism to opsonise the bacterium and facilitate its uptake into the phagocyte (Joiner *et al.*, 1984; Adams and Hamilton, 1984).

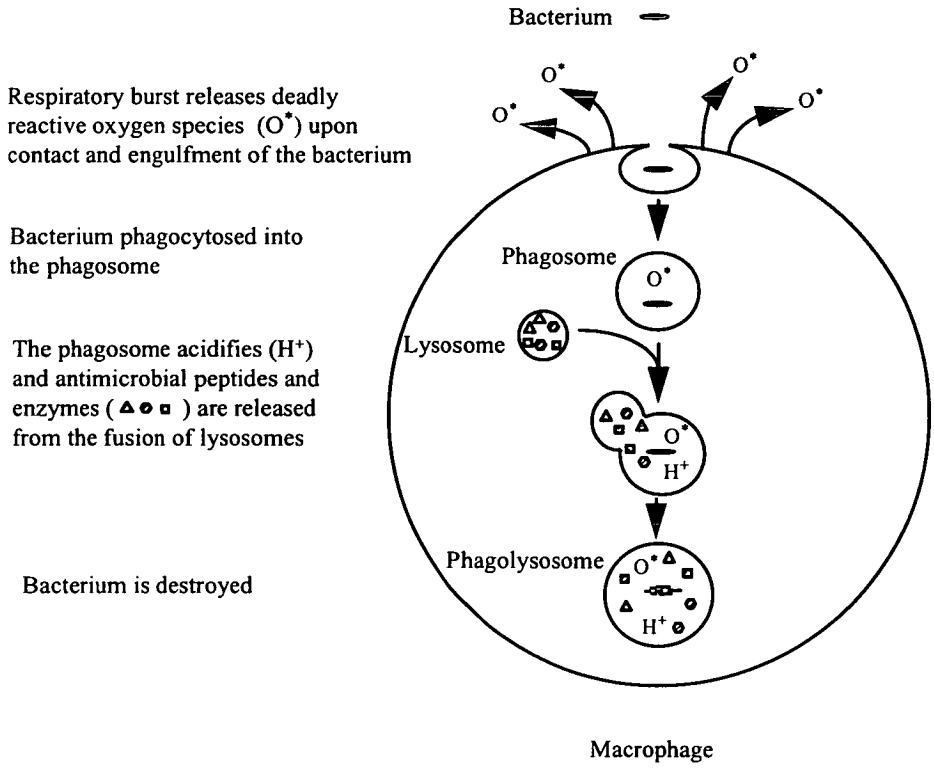
The anti-microbial role of phagocytic cells

The involvement of professional phagocytes, namely mononuclear phagocytes (monocytes and macrophages) and polymorphonuclear leucocytes (such as neutrophils), is a major factor in preventing microorganisms entering the body (Adams and Hamilton, 1984; Moulder *et al.*, 1985). Neutrophils mature in the bone marrow and are then released into the blood where they function for around 4-5 days (Mims, 1987; Gulig *et al.*, 1996). In contrast, mononuclear phagocytes have a life span of many months. After extensive development in the bone marrow, mononuclear phagocytes migrate via the blood as monocytes and enter into the various tissues and the organs of the body, including the liver, spleen and draining lymph nodes, where they develop into tissue-resident macrophages (Mims, 1987). Such macrophages constitute the reticuloendothelial system (RES) and the liver macrophages (Kuppfer cells) are quantitatively the most important component of this system (Adams and Hamilton, 1984; Mims, 1987; Kaufmann, 1993). Importantly, although being part of the innate immune system, macrophages play a vital role in generating, and acting as the effectors of specific immunity (Abbas *et al.*, 1991, 1996).

Phagocytic killing of bacteria

Both macrophages and neutrophils respond rapidly to invading microorganisms in the following sequence: chemotaxis through stimulation by host related substances (such as inflammatory products [*e.g.* C5a] or components derived from bacteria), target recognition, ingestion and degradation (Weir and Stewart, 1993). Phagocytosis involves the internalisation of the bacterial cell into a membrane-bound vesicle, the phagosome, and represents the first step in an endocytic pathway. In this pathway, the contents of the phagosome are destined for destruction by fusion with azurophillic lysosomes (Figure 1.1) The anti-microbial activity of phagocytic cells occurs before and after engulfment of the bacteria and involves two mechanisms, oxygen-dependent and oxygen-independent killing (Adams and Hamilton, 1984; Hasset and Cohen, 1989) (Figure 1.1 and as described in the following text).

Figure 1.1 Summary overview of phagocytosis and the bacterial killing mechanisms in phagocytic cells



Killing of bacteria by oxygen-dependent mechanisms

The oxygen-dependent killing mechanisms are initiated upon contact and engulfment of microbes into the phagocyte and is mediated via a respiratory burst associated with the hexose monophosphate shunt system. The shunt yields a high quantity of NADPH which is required for the action of a NADPH-oxidase, the principal enzyme involved in bringing about oxidative killing (Adams and Hamilton, 1984; Morel, *et al.*, 1991). In unstimulated macrophages, there is no detectable activity of the NADPH-oxidase. However, immunological evidence suggests that the enzyme is present in the cells (Morel *et al.*, 1991). It is therefore believed that external stimuli are required for activation of the enzyme, probably via receptor-mediated events (Forman and Thomas, 1986). As a result of the activity of this enzyme, toxic superoxide radicals (O_2^-) are generated which in turn can undergo further reactions to yield a spectrum of reactive oxygen species (ROS) including singlet oxygen ($^1O_2$), hydroxyl radicals (OH*) and hydrogen peroxide (H_2O_2).

Nitric oxide (NO) has also been shown to be an important component in the oxidative killing mechanism produced by macrophages (Vidal *et al.*, 1993; Pacelli *et al.*, 1995). NO is formed by the biotransformation of L-arginine to L-citrulline by a nitric oxide synthase. NO is itself toxic but can also potentiate the activity of the other ROS in the oxidative killing of microorganisms. For example, nitric oxide reacts with H_2O_2 to produce peroxynitryl radicals and other toxic nitric oxide derived intermediates (Pacelli *et al.*, 1995). In neutrophils, H_2O_2 may also be halogenated to form highly bactericidal halides via the action of a granule-associated myloperoxidase (MPO). Bacterial cells subjected to such a barrage of all these different ROS can be damaged at all fundamental levels (nucleic acids, lipids and proteins) resulting in the death of the microorganism (Goldman and Raz, 1975; Adams and Hamilton, 1984; Imlay and Linn, 1986).

Killing of bacteria by oxygen-independent mechanisms

The factors involved in oxygen-independent killing are extremely complex. Nevertheless, a number of important anti-bacterial mechanisms have been identified. One feature of the phagolysosome is the low pH (pH 3.5-4.0) which is created by the acidification of the phagolysosome using a Na^+/H^+ antiporter (Ohkuma and Poole, 1978; Seguin *et al.*, 1990, 1991; Foster, 1992). Moreover, these low pH conditions are inhibitory to a vast range of bacteria (Slonczewski and Foster, 1996). However, many of the degradative enzymes present in the phagolysosome, required to destroy the endocytosed bacterium, act optimally under these conditions.

A large number of proteins and peptides are associated with the anti-microbial environment of the phagolysosome and some of their functions have been determined (Nathan, 1987; Weir and Stewart, 1993; Gulig, 1996). For example, lysozyme is capable of breaking the peptidoglycan component of the bacterial cell wall and acid-hydrolases are well characterised in their degradative abilities. In addition, lactoferrin sequesters iron (Fe) and deprives the bacterium of this essential element (Foster and Spector, 1995). However, there are a much wider range of proteins with cytotoxic activity whose mode of action are not understood. These include azurocidin, cathepsin G, bactenecins, major basic protein (MBP), and bactericidal permeability increasing protein (BPI) (Lehrer *et al.*, 1990; Gulig *et al.*, 1996).

Another feature associated with the phagolysosome are defensins, a family of low molecular weight peptides (29-34 amino acids) that have anti-bacterial, anti-viral, anti-fungal and cytotoxic activities *in vitro* (Ganz *et al.*, 1990). These anti-microbial peptides form α-helical amphiphillic structures which are capable of introducing anion-specific channels in lipid bilayers and killing the bacterium by depolarising the cytoplasmic membrane (Lehrer *et al.*, 1990). In humans, defensins are abundant in both macrophages and neutrophils, making up to 5-7% of the total cell protein (Groisman, 1994). In contrast, murine macrophages and polymorphonuclear leukocytes appear not have defensins (Groisman, 1994). However, *S. typhimurium* mutants which were identified by their susceptibility to purified defensins or crude

neutrophil granule components, were found to survive poorly in murine macrophages (Fields *et al.*, 1986). Moreover, using the DNA sequence of the gene that encodes cryptdin, a type of defensin-like anti-microbial protein, genes with homology to defensin-like peptides were identified in murine leucocytes (Oullette and Lualdi, 1990). It is likely therefore that equivalent anti-microbial peptides exist in murine phagocytic cells.

The importance of both the oxygen-dependent and -independent mechanisms in controlling the infection of invading microorganisms is highlighted by a variety of human genetic disorders. For example, in Chronic Granulomatous Disease macrophages lack a respiratory burst (Ganz *et al.*, 1990) and in Chediak-Higashi syndrome and Specific Granule Deficiency phagocytic cells lack anti-microbial granule components (Finlay and Falkow, 1989b). These conditions result in the host being more susceptible to recurrent and severe infections by Gram-negative bacteria including *S. enterica*. This suggests that both arms of the killing mechanism are required for optimal phagocytic destruction of invading pathogens.

1.2.3 Specific immunity

Specific immunity is regulated by T lymphocytes (T cells) which are capable of identifying antigens, through a membrane-bound receptor protein (the T cell receptor), and which dictate the appropriate defensive measure to be taken (Abbas *et al.*, 1991, 1996). However, in order to identify antigens, T cells require accessory cells to process and present (display) the antigen. Hence these cells are called antigen presenting cells (APC's). Many types of cells are capable of performing this role including mononuclear phagocytes (monocytes, macrophages), Langerhans cells (specialised epithelial cells) and B cells (Abbas *et al.*, 1991). In addition, a whole range of cells can be stimulated to perform the function of antigen presentation during bacterial infection, including endothelial and epithelial cells (Abbas *et al.*, 1991). This allows for activation and regulation of immune responses in almost any part of the host.

T cells are generally considered to co-ordinate the immune system and mediate their

effects by producing soluble messengers called cytokines (Abbas *et al.*, 1996; Mosmann and Sad, 1996). These molecules not only modulate the activity of other cells, such as B cells or macrophages, but can also enhance the activity of T cells. However, cells that are regulated by T cells can themselves produce cytokines which in turn stimulate T cells and other cells. Moreover, there is also evidence that early in bacterial infection, cells of the natural immune system help bias or dictate which type of specific immune response develops (Nauciel and Espinasse-Maes, 1992; Kelso, 1995; Abbas *et al.*, 1996). Thus, during the course of an immune response there is a great deal of cross-talk between cell types and this helps to shape the manner in which the response develops as well as to dampen it down again after the infection has been dealt with.

The role of the Major Histocompatibility Complex (MHC) in determining immune responses

There are a number of cell surface molecules which are essential in determining the type of immune response generated during infection. These molecules include cluster of differentiation molecules CD4 and CD8, which are found on T cells, and the Major Histocompatibility molecules I and II (MHCI and MHCII), which are found on APC's (reviewed in Abbas *et al.*, 1991; Weir and Stewart, 1993).

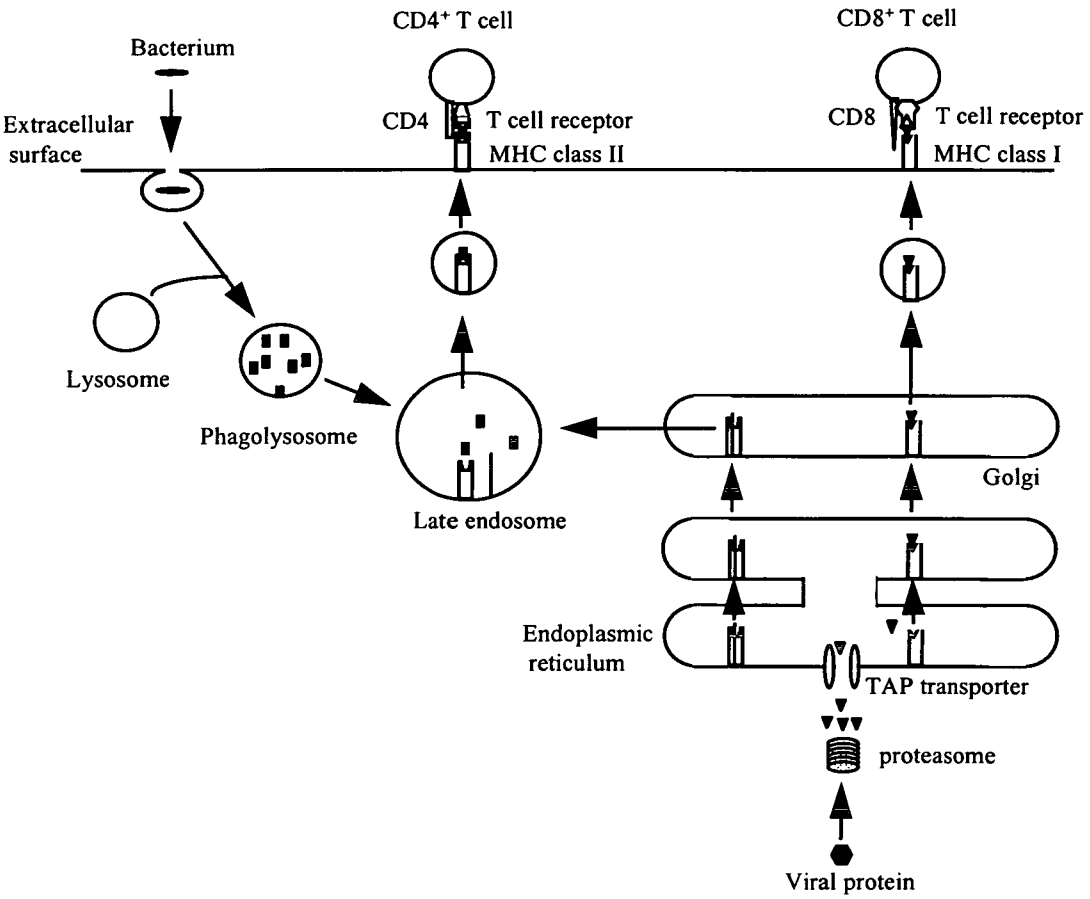
T cells can only identify a processed antigen when it is associated with the products of MHC (Weir and Stewart, 1993). In addition, the ability of a T cell to recognise antigen in association with MHCI and MHCII is restricted to T cells expressing CD8 or CD4, respectively. Since T cells only express only CD4 or CD8, they can be split into two mutually exclusive subsets, $CD4^+CD8^-$ T cells, which recognise antigen only in association with MHCII and $CD4^-CD8^+$ T cells, which only recognise antigen in association with MHC I.

Antigen processing and presentation

The $CD4^+CD8^-$ and $CD4^-CD8^+$ T cell subsets produce very different immune responses and this is largely dependent upon the source and type of antigen. To understand this, it is necessary to give examples of how different antigens are

processed and presented to T cells (reviewed in Brodsky and Guagliardi, 1991; Weir and Stewart, 1993). (This information is summarised in Figure 1.2 and described in the following text).

Figure 1.2 Summary diagram of the antigen processing and presentation pathways



Bacteria are engulfed (exogenous antigen pathway) and proteins degraded into peptides (▮). MHCII is synthesised in the ER and prevented from binding to endogenous (▼) antigen by the presence of an invariant chain (|). MHCII then travels to the late endosome where the invariant chain is removed making MHCII accessible for binding exogenous antigen and the complex transported to the cell surface where it is recognised by $CD4^+$ T cells. In contrast, viral proteins (endogenous pathway) are processed in the cytoplasm and the resulting peptides transported into the ER where they associate with MHCI. The MHCI/antigen complex is then directed to the cell surface where it is recognised by $CD8^+$ T cells. (Adapted from Weir and Stewart, 1993).

When bacteria are engulfed by phagocytes, such as macrophages, they start on an endocytic pathway that results in their degradation in the phagolysosome. As a result, protein antigens are processed to form small peptides, although it is unclear why complete degradation does not occur. Vesicles containing MHCII molecules, which

are synthesised at the endoplasmic reticulum (ER) and that are delivered via the golgi, fuse to this phagolysosome to form the late endosome. Here, the small peptide fragments associate with MHCII and, in an undefined manner, the complex is directed to the cell surface. This surface-presented MHCII/antigen complex is then recognised only by the T cell receptors on $CD4^+$ T cells.

An alternative scenario may ensue when a virus infects a host cell. The virus does not remain within a lipid-bound vesicle but normally escapes to the cytoplasm or nucleus. Here, the virus is capable of synthesising proteins using the machinery of the host cell. These proteins can be processed by a cytosolic enzyme complex, called the proteasome, to generate small peptide fragments and a special transporter system, TAP (transporter for antigen presentation), is then believed to feed these fragments into the endoplasmic reticulum where each peptide associates with MHCI. The resulting complex is then transported to the cell surface and can only be recognised by the T cells receptor on $CD8^+$ T cells. Importantly, the MHCI and MHCII processing pathways are kept distinct by preventing endogenous antigen from binding to the MHCII molecules in the ER. This is achieved by the association of a polypeptide chain (the invariant chain) with the MHCII molecule. However, upon entry into the late endosome the invariant chain is lost and MHCII becomes accessible to exogenous antigen.

The association of antigens with class I or class II MHC molecules is therefore due to the trafficking of the antigens through different intracellular compartments (Brodsky and Guagliardi, 1991). This in turn is dependent upon whether the antigen is from an exogenous or endogenous source. In general, the recognition of exogenous and endogenous antigens is associated with distinct responses ideally tailored to dealing with particular types of infection. For example, the recognition of exogenous antigen by $CD4^+$ T cells results in cytokines being secreted that enhance the ability of phagocytes to kill bacteria or stimulate B cells to produce antibody (See later). These types of T cell are called helper T cells (T_H), because they help direct the immune system to deal with the particular infection (Abbas *et al.*, 1996). In contrast, host cells expressing endogenous antigen associated with MHCI, are destroyed by $CD8^+$ T cells that recognise this complex. These T cells are called cytolytic T cells (CTL)

and are, in general, required to destroy virally infected cells (Abbas *et al.*, 1991). However, experimental evidence has suggested that endogenous antigen can be associated with MHCII molecules and exogenous antigen can be associated with MHCI molecules and this may be an important factor in the development of an efficient immune response (Malnati *et al.*, 1992; Pfeifer *et al.*, 1993).

1.2.4 The anti-microbial role of humoral and cell-mediated immunity

There are two key types of response to consider in specific immunity to bacterial infection, humoral and cell-mediated immunity. Humoral immunity involves the production of antibody from B cells, and the effector phase is mediated by binding of secreted antibody to antigen. In contrast, cell-mediated immunity to bacterial infection involves T cells acting to influence and enhance the cells of natural immunity, such as the macrophages, to act as agents of specific immunity.

Humoral immunity

The production of antibody

Antibodies (or immunoglobulins [Ig]) are antigen-specific glycoproteins that are derived from activated B lymphocytes (B cells). B cells are formed in the bone marrow and possess membrane-bound antibody molecules which act as antigen-specific receptors (reviewed in Parker, 1993). Although B cells are not professional phagocytes, they can act as APC's because antigen bound by the surface antibody is internalised, processed, and the antigen expressed in conjunction with MHCII molecules on the surface of the cell. In turn, T_H cells activated by this secrete cytokines such as interleukin-4 (IL-4), IL-5 and IL-6 to bring about the activation and proliferation of the B cell, as well as the generation of memory B cells (Parker, 1993). As a result of B cell stimulation, antibody molecules with the same antigen specificity as the membrane-bound molecule are expressed in a secreted form. Naive B cells which are exposed to antigen generally produce IgM, which has multiple antigen-binding sites and is therefore good at complexing antigens. This is called the primary response. However, during the course of antigen stimulation or upon re-exposure to antigen, the type as well as the specificity of antibody changes

(processes termed isotype switching and affinity maturation, respectively). This is called the secondary response and generally results in the production of high affinity IgG. In general, a primary response to antigen can be seen within 5-10 days after exposure to that antigen. However, in the secondary response, the lag time in appearance of antibody is greatly reduced and specific antibody can be observed 1-3 days post-antigen challenge (Weir and Stewart, 1993).

An important feature of microbial infection by Gram negative bacteria, such as *S. enterica*, is that humoral immunity can also be developed independently of T cell help. The basis for this is associated with the Gram negative bacterial cell wall component, lipopolysaccharide (LPS). T cells are unable to recognise non-protein antigens, and in an unknown manner LPS can induce B cell proliferation and antibody production (IgM and IgG_3 in mice). Nevertheless, because these responses are independent of T cell help there is no affinity maturation of B cells nor is there development of immunological memory (Weir and Stewart, 1993).

The role of antibody

Antibody has a number of roles in mediating protective immunity. Firstly, it binds bacterial toxins and receptors to prevent interaction with their respective targets. Secondly, antibody (IgG and IgM) bound on the surface of the microorganism, can activate the complement system via the so-called Classical pathway (Joiner *et al.*, 1984). A major function of antibody, like complement, is opsonisation of microbes. Macrophages and PMNs, in addition to having receptors for complement proteins (*e.g.* C1R and C3R), possess receptors for the C-terminal region (Fc) of immunoglobulin, especially IgG_1 and IgG_3 (humans). Target recognition and uptake of bacteria via these receptors is extremely efficient (Adams and Hamilton, 1984). Moreover, the binding of antibody or complement to these receptors stimulates the respiratory burst thus boosting the phagocytes anti-microbial activity (Joiner *et al.*, 1984; Gulig, 1996).

Cell-Mediated Immunity

In bacterial infection, especially to intracellular bacterial pathogens, the major effector in cell-mediated immunity (CMI) is the macrophage and these cells are regulated by $CD4^+$ T cells to become increased in their anti-microbial activity. (Collins, 1974; Finlay and Falkow, 1989b; Kaufmann, 1993).

During infection, macrophages kill endocytosed microorganisms and present their antigens in association with MHCII molecules to T_H cells. In turn, T_H cells secrete a number of cytokines, including interferon-γ (IFN-γ), which change the macrophage's morphology and functional characteristics, a phenomenon called activation. For example, IFN-γ enhances the microbial killing mechanisms of the macrophage by elevating the rate of phagolysosomal fusion and by increasing the expression of the NADPH oxidase (Adams and Hamilton, 1984; Ishibashi and Arai, 1990). Moreover, activated macrophages act as the effectors of inflammation by releasing cytokines (IL-1 and TNF-α) and other molecules such as prostaglandins that cause an acute inflammatory response (Larsen and Hanson, 1983). Inflammation results in fluid and leucocyte accumulation at the site of infection and is an important factor in containing the spread of microorganisms. Finally, activated macrophages become enhanced in their antigen presenting capabilities. For example, IFN-γ stimulates an increase in the expression of MHCII molecules (Abbas *et al.*, 1991). In addition, some of the cytokines released by activated macrophages activate T cells (IL-1) and stimulate their proliferation (IL-6), thus cross-talk between cells increases the efficiency of the immune response (Weir and Stewart, 1993). Activation of macrophages is particularly important in bacterial infections involving intracellular bacterial pathogens such as *S. enterica*, *Yersinia enterocolitica* and *Mycobacterium tuberculosis* (Moulder, 1984; Finlay and Falkow, 1989b; Kaufmann, 1993). However, this intracellular survival is greatly reduced when macrophages are fully activated.

Delayed-type hypersensitivity

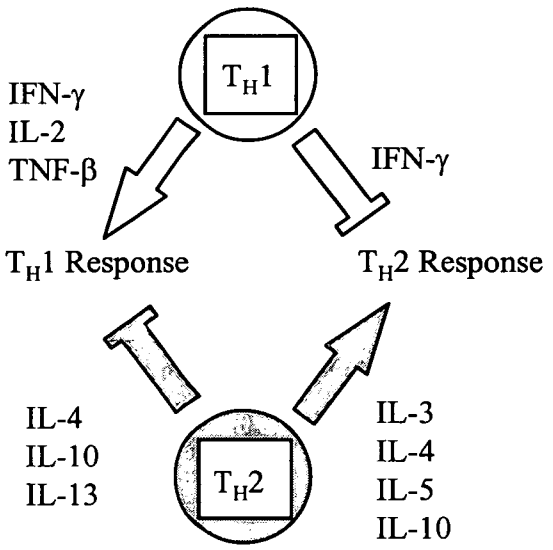
One important method of investigating CMI is through a delayed-type hypersensitivity reaction (DTH), as DTH is an *in vivo* manifestation of a cell-mediated response (Abbas *et al.*, 1991). If an experimental animal is injected with an antigen, the antigen will be processed and presented by APC's in the context of MHCII carrier molecules to T_H cells (Kagaya *et al.*, 1992). As a result, a subpopulation of the T cell repertoire becomes primed to recognise the foreign agent. Upon secondary exposure to the same antigen, typically injected subcutaneously (*e.g.* into the footpads of mice), a DTH reaction evolves over a period of 24-72 hours (hence the reason for being called delayed). In this scenario, neutrophils accumulate at the site of administration within 4 hours post-injection, followed by substantial increases in both T cells and macrophages by 12 hours. Over this period inflammation starts to occur with deposition of fibrin and, by 24-72 hours, a sizeable swelling can be observed in the injected footpad. This is called induration, the hallmark of DTH. DTH reactions have been used by investigators to examine whether certain antigens can stimulate CMI responses and have important value in the development of vaccines (Kagaya *et al.*, 1992; Horwitz *et al.*, 1995; Gupta *et al.*, 1996).

Functional diversity of T cell help

One interesting phenomenon of help by $CD4^+$ T cells in immune responses is that these cells can produce different types of responses, through varying the selection of the cytokines they secrete (Kelso, 1995; Mosmann and Sad, 1996; Abbas *et al.*, 1996). All $CD4^+$T cells come from a common origin and upon interaction with antigen these cells produce IL-2 (a T cell growth factor) and develop into a population called T_H0. T_H0 cells produce multiple cytokines and, depending upon the antigen, the T cell population subsequently develops into either a T_H1 or T_H2 subset (reviewed in Abbas *et al.*, 1996; Mosmann and Sad, 1996). T_H1 cells secrete IL-2, IFN-γ and tumour necrosis factor-β (TNF-β) and the immune responses generated are principally effected by CMI, especially by macrophages (Figure 1.3). Therefore, T_H1 cells produce responses which are particularly effective at eliminating intracellular

bacteria, such as *S. enterica*. Moreover, IFN-γ stimulates activated B cells to secrete IgG which can bind to high affinity receptors of phagocytic cells. Therefore, the antibody produced in a T_H1 response generally acts to assist CMI (Abbas *et al.*, 1996). In contrast, T_H2 cells produce IL-3, IL-4, IL-5, IL-10 and IL-13 (Figure 1.3). These cytokines are particularly effective at suppressing parasite infections and mediating allergic reactions through the action of antibody (IgE) and the degranulation of mast cells (Mosmann and Sad, 1996). Therefore, T_H2 responses tend to be phagocyte-independent and are characterised by the lack of an ability to induce CMI reactions like DTH. Interestingly, the cytokines released in T_H1 or T_H2 responses act to suppress the effector functions of the alternative response (Figure 1.3). For example, IFN-γ promotes a T_H1 response but acts to down-regulate a T_H2 response (Abbas *et al.*, 1996). In contrast, IL-4 *per se* or in conjunction with IL-10 and IL-13 helps to down-regulate the T_H1 response and promote a T_H2 response (Figure 1.3) (Denich *et al.*, 1993; Abbas *et al.*, 1996). The role of such an antagonistic activity is believed to polarise the immune response towards the most appropriate method for dealing with the particular infection.

Figure 1.3 Cytokine production by T_H1 and T_H2 populations



The cytokines produced by T_H 1 and T_H2 cells result in characteristic types of response (described in the text) but also act to inhibit the generation of each other. Positive and negative regulatory roles for T cells and their cytokines are indicated by pointed or blunt arrowheads respectively.

This idea however, that the $CD4^+$ T cell population consists of two functional types of T cell has been challenged (Kelso, 1995) since T cells have been found that produce mixtures of the characteristic cytokines and therefore cannot be specifically categorised as T_H1 or T_H2 cells. Therefore, as an alternative explanation, it has been postulated that what are considered as the two distinct subsets are in fact the end result of polarisation in the immune response. The new theory suggests that during the course of an infection, the T cell population initially produces a mixture of both T_H1 and T_H2 cytokines, but as the response progresses it is shaped to pursue a predominant form of response, which is characterised by what was previously considered a T_H1 or T_H2 regulated phenomenon. The conditions that result in this polarisation are dependent upon a combination of features including antigen stimulation, the cytokines produced and the type of APC which present the antigen (Kelso, 1995; Abbas *et al.*, 1996). For the purpose of this thesis, immune responses will be described as either of a T_H1 or a T_H2 type, to identify the characteristics of the immune mechanisms involved.

One important finding in determining which type of response develops is the composition of the cytokines produced early on in the response. For example, both IL-12 and IFN-γ have been shown to promote a T_H1 type response (Lamont and Adorini, 1996; Abbas *et al.*, 1996). Macrophages that come into contact with bacteria secrete IL-12, which in turn causes T cells and natural killer (NK) cells (large granular lymphocytes) to produce IFN-γ. This cytokine, as described previously, activates macrophages and down-regulates T_H2 responses thereby helping to polarise the immune response. In contrast, during helminth infection for example, IL-4, IL-10 and IL-13 are released and these help to down-regulate a T_H1 response (Denich *et al.*, 1993; Abbas *et al.*, 1996). Interestingly, despite the clear role for T cells in co-ordinating CMI, it has been suggested that the natural immune responses, which are activated in the early stages of immunity, may play a substantial role in shaping the nature of the specific immune response that follows (Nauciel and Espinasse-Maes, 1992; Kaufmann, 1993; Mastroeni *et al.*, 1996).

Organisation of the immune system

In order for an immune response to develop, antigen must come into contact with APC's which in turn require cell to cell contact with T cells. These requirements are eased by two factors. Firstly, the immune system is organised into specialised lymphoid tissues into which many different types of cell, including B and T cells and APC's, can enter and reside (Abbas *et al.*, 1991; Weir and Stewart, 1993). This facilitates the close contact required for displaying antigens and for developing the required immune response. These lymphoid tissues include the spleen, liver, draining lymph nodes and the Peyer's patches of the gut, and are connected by the blood and the lymph system. Antigen entering into any part of the body, such as the skin or the intestine, is therefore likely to migrate to these tissues.

Secondly, antigens are structurally diverse and only a small population of B and T cells are likely to recognise specific portions of any particular antigen. These cells therefore migrate between tissues, a process called lymphocyte recirculation, such that the chances of antigen-specific lymphocytes coming into contact with the antigen are increased (Abbas *et al.*, 1991; Weir and Stewart, 1993; Siebers and Finlay, 1996). Thus, the organisation of the immune system and the behaviour of the lymphoid cells act to produce a highly efficient monitoring system against infection.

1.2.5 Immune responses to *S. enterica* infection

The use of a murine model to study *S. enterica* infection

Much of the research into the immune responses to *S. enterica* infection has been stimulated by the need to develop effective vaccines to the causative agent of typhoid fever, *S. typhi*. Unfortunately, this organism only infects and causes disease in humans and this hinders experimentation. Most experiments into *S. enterica* infection therefore employ *S. typhimurium* which causes an invasive systemic disease in mice that resembles human typhoid fever (Collins, 1974). In particular, inbred mouse strains that are genetically susceptible to *S. typhimurium* infection are used for study (see later) and these are infected with low virulence derivatives of *S. typhimurium*. The reason for this approach is that genetically resistant mice can eliminate

S. typhimurium through natural immune responses, but susceptible mice require both cell-mediated and humoral immunity, which is characteristic of the responses generated by humans to typhoid fever (Collins, 1974; Hormaeche, 1979; Hormaeche *et al.*, 1985; Mastroeni *et al.*, 1993).

The role of humoral immunity in *S. enterica* infection

S. enterica is an intracellular pathogen and immune responses to infection depend upon a mainly phagocyte-dependent killing system (Collins, 1974). By residing in the macrophage, *S. enterica* is protected from the actions of extracellular immunological components such as antibody or complement (Moulder, 1985; Finlay and Falkow, 1989b). However, during parts of the infection cycle, such as after crossing the epithelial barrier in the gut, *S. enterica* must be exposed to these factors. Complement aids lysis and phagocytosis of bacteria. Mutants of *S. typhimurium* that do not produce a complete LPS molecule are attenuated (reduced in virulence) and are readily lysed by complement (Collins *et al.*, 1991; Gulig, 1996). This indicates the importance of LPS in protecting the bacterium but suggests that complement normally plays a minor role in protecting against infection (Gulig, 1996).

The efficacy of antibody in mediating protection to *S. enterica* is also questionable. However, Mastroeni *et al.* (1993) demonstrated that naive BALB/c mice were only optimally protected (against challenge with a virulent strain of *S. typhimurium*) when both T cells and serum were donated from a susceptible mouse immunised with an attenuated strain of *S. typhimurium.* In addition, it is known that people infected with *S. typhi*, or vaccine derivatives that have been attenuated, produce significant levels of IgM and IgG (Forrest *et al.*, 1991; Tacket *et al.*, 1992), but most of this is likely to be due to the polyclonal stimulation of B cells with LPS. Moreover, the presence of antibody to an antigen does not necessarily correlate with immunity to that antigen (Weir and Stewart, 1993). However, at least one effective vaccine to typhoid fever relies solely on humoral immunity (the Vi polysaccharide vaccine) (Ivanoff *et al.*, 1994). Furthermore, some investigators have achieved some degree of protection in mice against *S. typhimurium* infection through the use of antibodies to LPS (Michetti *et al.*, 1992). IgA is associated with secretory immunity and is produced at mucous

membranes such as those of the gastrointestinal tract. By using an IgA monoclonal antibody-producing hybridoma line (implanted into a mouse's back) with specificity for the O-antigen of LPS, it was shown that mice can be protected from *S. typhimurium* infection. (Michetti *et al.*, 1992).

The role of cell-mediated immunity in *S. enterica* infection

Infection by *S. enterica* results in a T_H1 type of response

Most research indicates that the major effector of protective immunity to *S. enterica* is cell-mediated immunity and is particularly dependent on the activities of macrophages. Thus, if antibody does have a role, it is likely to augment the ability of the macrophages to eliminate bacteria by opsonisation (Collins, 1974; Mastroeni *et al.*, 1993; Hassan and Curtiss, 1994; Gulig; 1996). This is supported by the observation that immunity to *S. enterica* infection appears to show the characteristics of a T_H1 type of response. For example, cytokines associated with a T_H1 type of response, including IL-12 and IFN-γ, are induced in the Peyer's patches, mesenteric lymph nodes and spleens of *S. typhimurium*-infected mice (Nakano *et al.*, 1990; Nauciel and Espinasse-Maes, 1992; Ramarthrinam *et al.*, 1993; Berbenou *et al.*, 1994; Morrissey and Charrier, 1994; George, 1996). In addition, injection of resistant mice (CBA or A/J) with monoclonal antibody (MAb) specific to either IFN-γ or IL-12 (anti-IFN-γ MAb and anti-IL-12 MAb, respectively) resulted in these mice being overcome by the disease much more rapidly than a control group (Nauciel and Espinasse-Maes, 1992; Mastroeni *et al.*, 1996). In contrast, addition of IFN-γ or IL-12 to susceptible mice (BALB/c) infected with *S. typhimurium* greatly enhanced their resistance to the infection (Nauciel and Espinasse-Maes, 1992; Mastroeni *et al.*, 1996). It is believed therefore, that IFN-γ and IL-12 play major roles in suppressing the growth of *S. enterica* in the host.

Another important cytokine which increases in response to *S. typhimurium* infection in mice is tumour necrosis factor (TNF-α) and this is believed to act synergistically with IFN-γ (Nakano *et al.*, 1990; Nauciel and Espinasse-Maes, 1992; Berbenou *et al.*, 1994). Addition of TNF-α to infected mice results in an increased rate of clearance of

S. typhimurium cells from the peritoneal cavity, and this effect can be abolished by the injection of anti-TNF-α MAb (Nakano *et al.*, 1990). Interestingly, during the early stages of infection by *S. typhimurium* the immune responses appear to be independent of T cells as T cell-depleted mice still show resistance early in infection (Mastroeni *et al.*, 1993; Nauciel and Espinasse-Maes, 1992). Instead this early resistance is believed to be mediated by macrophages and NK cells which release cytokines and exhibit cross-talk between these two cell types and other cells (Nauciel and Espinasse-Maes, 1992; Mastroeni *et al.*, 1996). As mentioned above these cytokines are fundamental to the subsequent development of a protective immune response by stimulating the development of T_H1 cells. Moreover, when *S. typhimurium* cells expressing recombinant IL-4 (a cytokine which promotes a T_H2 response) were injected into mice, the mice displayed poor clearance of the bacteria and this was attributed to a reduction in the anti-microbial activity of the macrophages (Denich *et al.*, 1993). This highlights the importance of innate immunity, particularly that of the macrophages, in mediating immunity to *S. enterica* infection.

A genetic locus in mice correlates early resistance with macrophages

A genetic locus has been found in mice which correlates early resistance to *S. typhimurium* infection with innate immunity. This particularly applies to the involvement and effectiveness of the macrophages. It has been demonstrated that mice can show substantial differences in their resistance or susceptibility to *S. typhimurium* infection. The genetic basis of this has been linked to chromosome one, to a locus called *ity* (immunity to typhimurium). This locus is also believed to be allelic to the *lsh* and *bcg* loci, which modulate murine susceptibility to *Leishmania donovani* and *Mycobacterium bovis* (BCG), respectively (Hormaeche, 1979). Homozygous mice either display resistance (ity^R; for example CBA or A/J mice) or susceptibility (ity^S; for example BALB/c and B10 mice) to *S. typhimurium* on the basis of function or lack of function of this locus, respectively. For example, the ity^S genotype has been shown to result in decreased resistance to *S. typhimurium* early (1-3 weeks) in the infection period (Hormaeche, 1979; Hormaeche *et al.*, 1985).

Originally, the *ity* gene was believed to affect cytokine production. When ity^R and ity^S mice were examined for cytokine production after *S. typhimurium* infection, it was shown that resistant mice produced more IFN-γ and TNF-α, two cytokines essential for early resistance to infection (Ramarathinam *et al.*, 1990). This was substantiated by injecting resistant CBA mice with anti-IFN-γ MAb and showing that they became susceptible to an *S. typhimurium* infection that would normally be controlled (Nauciel and Espinasse-Maes, 1992). However, in conflict with these findings, a separate study has suggested that the *ity* phenotypes are not due to differences in cytokine production (Eckmann *et al.*, 1996). When both types of mice were examined for the production of IFN-γ, TNF-α and IL-12 (as well as other cytokines), no difference was observed in the quantity or time of appearance of these cytokines for equivalent infections.

This latter view has been supported by the cloning and expression of a gene at the *bcg* locus called *nramp* (natural resistance-associated macrophage protein) (Vidal *et al.*, 1993). This gene is believed to encode a transmembrane protein which transfers nitric oxide (and its derivatives) through membranes. When the *nramp* genes from resistant and susceptible mice were compared, a single amino acid substitution in the predicted amino acid sequence was found in one of the transmembrane portions of the protein. The effect of this was believed to alter the insertion and stability of NRAMP within the phagolysomal membrane and thereby cause severe impairment of function. Nitric oxide is an important anti-microbial agent (Pacelli *et al.*, 1995) and therefore it is currently believed that the differences in resistance and susceptibility of mice to *S. typhimurium* infection is due to the effectiveness of microbial killing by nitric oxide (Vidal *et al.*, 1993).

The role of T cells in *S. enterica* infection

As stated above, T cells are believed not to play a major role in events early in the host response to *S. enterica* infection, especially in naive mice, but they are required later to suppress the infection and to develop protective immunity (Mastroeni *et al.*, 1993; Gulig, 1996). In fact, genetically engineered (*nu*) mice which do not develop a thymus (the organ in which T cells develop) survive for up to three weeks

post-infection before succumbing to systemic disease (Gulig, 1996).

Although, $CD4^+$ T cells appear critical in the co-ordination of immune responses to *S. enterica* infection, studies have also suggested a role for $CD8^+$ T cells. *S. enterica* is believed to survive and remain within the phagosome whilst within the host cell and therefore its antigens would not be expected to be associated with the MHCI complex (endogenous antigen) pathway. However, it has recently been shown that an alternative MHCI pathway exists such that phagosomal antigens can, using an unknown mechanism, be displayed with MHCI molecules (Pfeifer *et al.*, 1993; Wick and Pfeifer, 1994). Thus it is likely that CTL cells play some role in *S. enterica* infection and this has been supported by the identification of $CD8^+$ MHCI restricted CTL's in effector responses of human volunteers injected with an attenuated *S. typhi* vaccine strain (Sztein *et al.*, 1995).

One further type of T cell that may also have a role to play in *S. enterica* infection, are the γδ T cells, which do not display either CD8 or CD4 molecules. These cells appear to have cytolytic activity and are especially prominent in *M. tuberculosis* infection where they lyse bacterially infected cells (Kaufmann, 1993). How they are involved is unknown but they appear to be activated particularly in response to bacterially-derived heat shock proteins. Such cytolytic activity has also been shown in *S. typhimurium* infection of synovial joints, where the γδ T cells provide important anti-microbial immunity (Hermann *et al.*, 1995)

Host immunosuppression mediated by *S. enterica* infection

When susceptible mice are injected with an attenuated strain of *S. typhimurium* they elicit excellent protection against challenge with the virulent parental strain. However, it has also been recognised that over the period during which resistance develops, mice show poor responses to heterologous antigens (Al-Ramandi *et al.*, 1991; Al-Ramandi *et al.*, 1992). The cells responsible for this resistance as well as the apparent unresponsiveness have been demonstrated to be the macrophages. IFN-γ is a potent inducer of nitric oxide synthesis in macrophages and if anti-IFN-γ MAb is injected into *S. typhimurium*-immunised mice during the development of resistance,

it is found to alleviate unresponsiveness. Therefore, it has been proposed that NO has an immunosuppressive effect during this period (Al-Ramandi *et al.*, 1992). This view has been substantiated further by showing that suppression could be blocked by the addition of N^G-monomethyl-L-arginine, a competitive inhibitor of nitric oxide synthase (Al-Ramandi *et al.*, 1992). The cells mediating protection in the early stages of infection may therefore affect the development of protective immunity, especially to heterologous antigens. These observations have particular relevance to the use of attenuated *S. enterica* strains in vaccine development because immunisation of an individual with such vaccines may lead to immunosuppression and provide a window of opportunity for other pathogens (expressing heterologous antigens) to capitalise on the weaknesses of the host defence system (Al-Ramandi *et al.*, 1992).

1.3 THE INTERACTIONS OF *S. ENTERICA* WITH HOST CELLS DURING INFECTION

1.3.1 Introduction

In the previous section, the perspective of the host in *S. enterica* infection was discussed in terms of the characteristics of the immune response. In the following section, the perspective of the bacteria will be given to demonstrate how *S. enterica*, faced with a hostile anti-microbial host environment, has evolved mechanisms to succeed in infection. Microbial pathogenicity is a complex phenomenon however, and successful pathogens must be able to enter the host and establish one or more unique niches in which they can persist and multiply. As such, at least a proportion of the infecting microorganisms must be able to withstand, circumvent or avoid the defence mechanisms of the host (reviewed in Finlay and Falkow, 1989b).

S. enterica generally enters the host via oral ingestion of contaminated food and water. Once ingested the bacteria breach the cells of intestinal epithelial lining and, with invasive serovars such as *S. typhi*, may proceed into deeper tissues where they come into contact with phagocytic cells such as the macrophages (Finlay and Falkow, 1989a). Other types of cell have also been implicated in the strategy used by

S. enterica for infection. For example, *S. typhimurium* cells have been shown to invade a transformed EBV B-cell line and a T-cell leukaemic cell line, suggesting potentially that these lymphocytes may be used, like macrophages, to provide a safe haven and a vehicle for dissemination around the body (Verjans *et al.*, 1994). It should be noted however, that a contradictory proposal suggested that the intracellular survival of *S. typhimurium* in macrophages is unlikely to be the centre for propagating such infections, based on a controversial view that the spleens and livers of infected mice show considerable extracellular bacteria (Hsu, 1989). Nevertheless, the outcome, and many of the features of *S. enterica* infection are dependent upon interaction with epithelial and phagocytic cells, particularly macrophages. The following sections will therefore focus on these two important cell types and their interaction with *S. enterica.*

During infection of the host, bacteria encounter many changes in the environment, some of which are potentially hazardous. For example, upon entry into the host, *S. enterica* cells are suddenly exposed to a higher temperature, extremes or rapid changes in pH (acid in the stomach and neutral to alkaline in the small intestine), limited availability of nutrients and iron, high concentrations of bile salts, and also, to the immune responses of the host (Foster and Spector, 1995). In addition, *S. enterica* is an intracellular pathogen and the environments of the cells it enters are likely to be very different (*e.g.* epithelial cells versus professional phagocytes) (Fields *et al.*, 1986). The importance of the ability of bacterial cells to sense and respond to environmental change or stress has recently been recognised (Miller *et al.*, 1989c; Mekalanos, 1992; Mahan *et al.*, 1996). In fact, the sensing of environmental change within the host may act as a signal to activate expression of specialised virulence determinants and regulatory mechanisms which influence the progression of the infection process (*e.g.* attachment or invasion of host cells) (Miller *et al.*, 1989c; Galan and Sansonetti 1996; Mahan *et al.*, 1996). However, for most bacteria, the molecular basis of microbial pathogenesis is, as yet, poorly understood.

1.3.2 Experimental methods for investigating the interaction of *S. enterica* with the host

Our understanding of the events that occur during host-infection by *S. enterica*, and the genetic basis of virulence have been greatly assisted by the following approaches:

A murine model for salmonellosis

Determining the factors involved in the interaction of *S. enterica* with its hosts is difficult. Much of the evidence about the factors involved during interaction has been facilitated through the use of a murine model, involving the infection of susceptible mice with *S. typhimurium*. This produces a comparable systemic infection to typhoid fever in humans (Collins, 1974). In humans, *S. typhimurium* mainly produces a localised infection of the gastrointestinal tract. Nevertheless, *S. typhimurium* and a number of other non-typhoidal serovars contain virulence plasmids that give the potential to cause serious systemic disease and the murine model therefore has relevance to general salmonellosis in humans (Falkow, 1996; Gulig, 1996).

Perhaps one of the most useful properties of the mouse model is the ability to examine the importance of *S. typhimurium* genes in the overall virulence process. This is achieved by performing an LD_{50} test (50% lethal dose) which involves administering mice with a range of bacterial inocula and determining the number of organisms required to kill half the mice in the study, over a suitable time interval. Strains of *S. typhimurium* carrying mutations in the genes of interest can be compared against an otherwise isogenic wild type strain, using the LD_{50} test, to determine whether loss of a particular gene function causes a decrease in the virulence of the bacterium. This type of experimental analysis has been particularly useful in the development of vaccines to prevent typhoid fever (Chatfield *et al.*, 1992a).

Tissue culture systems

Simplified systems have been developed to assay how *S. enterica* is likely to interact with host cells (Gulig, 1996). These systems include tissue culture where polarised epithelial cells such as Madin Darby Canine Kidney cells have been used to simulate intestinal epithelial cell situations (Finlay and Falkow, 1989a). In addition, tissue culture has likewise provided a means for examining interactions between *S. enterica* and macrophages. Finally, the value of a ligated intestinal loop model has also been explored and this has the advantage over other tissue culture systems, in that it is more realistic, by allowing the interplay of a number of different cells types. This type of model, in particular, has provided insights into tissue destruction and the inflammatory responses induced by *S. enterica* (Galan and Sansonetti, 1996).

Molecular biological and genetic experiments

One advantage of using *S. typhimurium* in models of host infection is that the genetics of this bacterium have been extensively investigated and it is amenable to genetic manipulation. Indeed, the use of genetic approaches have proved invaluable in determining the functions of *S. typhimurium* genes and also in the construction of attenuated bacterial strains for vaccine development.

Generally, approaches to studying the genetic factors involved in *S. enterica* virulence (normally using *S. typhimurium*) have taken into account the influence of the host environment upon the expression of bacterial genes. Most studies therefore attempt to mimic, *in vitro*, possible host environmental conditions (*e.g.* acidity) or use cell culture or animal models to select and identify genes that may be involved in virulence. For example, polyacrylamide gel electrophoresis (Buchmeier and Heffron, 1990; Abshire and Neidhardt, 1993a) or gene-reporter systems such as *phoA* or *lux* (Galan and Curtiss, 1989; Miller *et al.*, 1989b; Francis, PhD Thesis, 1993) have been used to examine if genes are influenced by a particular environment. Alternatively, *S. typhimurium* has been mutagenised using transposable elements (*e.g.* Tn*10*) and then screened in tissue culture experiments to determine if the insertion has had any

effect upon the virulence of the bacterium in comparison to the parental strain (Fields *et al.*, 1986; Garcia-del Portillo *et al.*, 1993a; Shea *et al.*, 1996).

One of the problems in investigating the genetic response of *S. enterica* to the host environment is that it is relatively uncharacterised and therefore many of the experiments performed are dependent on, and limited by, the knowledge of host conditions that are being mimicked. However, Mahan *et al.* (1993, 1994) have circumvented this problem by developing a strategy that uses the mouse model to select for genes that are wholly required for the survival of *S. enterica* during infection. The strategy called *in vivo* expression technology (IVET) employs a *pur* mutant of *S. typhimurium* which grows poorly in mice because of the auxotrophic requirement for purine. Random fragments of wild type *S. typhimurium* chromosomal DNA are placed in front of promoterless *purA* and *lacZY* genes situated in tandem on a plasmid and the plasmid is integrated onto the *S. typhimurium* chromosome. When these bacteria are injected into mice, only those microorganisms which can express the *pur* gene will be able to survive and multiply. This in turn indicates that the random chromosomal fragment that has been in front of the promoterless *pur* gene contains a promoter region from a gene that is capable of switching on the expression of the *purA lacZY* genes during the course of infection. *In vivo* expressed genes (*ivi*) can then be identified by extracting the bacteria from the spleens of the mice, plating onto MacConkey lactose indicator medium, and selecting for those bacterial colonies which are Lac^-. This serves to eliminate the Lac^+ bacteria which contain fragments of chromosome carrying constitutively expressed promoters.

As an alternative to IVET, Shea *et al.* (1995) have developed a novel transposon-based strategy, termed signature-tag mutagenesis, to identify genes required for the survival of *S. typhimurium* within the mouse. The strategy results in every transposon mutant being individually tagged with a unique DNA sequence. The advantages of this system are that a large number of transposon mutants can be screened simultaneously, and that those insertions that do alter the gross virulence of *S. typhimurium* can be rapidly and specifically identified from the original inoculum.

1.3.3 The cellular basis of the entry of *S. enterica* into epithelial cells

Portals of entry into the intestinal epithelium

The primary site of entry of *S. enterica* into the intestinal mucosa is not certain but recent evidence from (host) studies points to the role of specialised microfold cells (M cells) in the Peyer's patches (Finlay and Falkow, 1989a; Siebers and Finlay, 1996). Peyer's patches form part of the gut-associated lymphoid tissue (GALT) and essentially consist of lymphoid follicles. In mice, the follicle-associated epithelial layer consists of around 5-10% M cells which form tight junctions with neighbouring enterocytes. M cells have a shrunken cytoplasm that forms a thin bridge between the intestinal lumen and subepithelial space. The basolateral surface of the M cell is invaginated to form a pocket into and out of which lymphocytes and macrophages can migrate. M cells contain few lysosomes and produce only low levels of digestive enzymes, indicating that their main function is not to digest and absorb food from the intestinal lumen. However, these cells show active endocytosis, and material taken up in this way is rapidly transported, virtually intact, and exocytosed at the basolateral surface within 10 minutes (Siebers and Finlay, 1996).

The role of M cells in promoting intestinal epithelial cell invasion

It is has been suggested that M cells sample the gut for antigens, as the cells rapidly transport intestinally located antigens to the antigen presenting cells in the basolateral pocket (Siebers and Finlay, 1996). However, although M cells act as a surveillance point for antigens it appears that many types of enteric pathogens including *Vibrio cholerae, Yersinia enterocolitica, Shigella flexneri* and also *S. typhimurium* and *S. typhi* specifically target these cells as a means to gain entry into the epithelial lining (Siebers and Finlay, 1996). Nevertheless, although M cells may be targeted in this way, it is quite clear that *S. enterica* can also invade the columnar epithelial cells that line the intestine and this may serve as an additional pathway for tissue invasion (Jones *et al.*, 1994).

The entry of *S. enterica* into M cells and the ability to move through epithelial cell membranes

Using murine intestinal loops, it has been shown that *S. typhimurium* cells invade M cells within 30 minutes of introduction. *S. typhimurium* enters these cells by rearrangement of the apical cell membrane ('ruffling'), a phenomenon also seen in tissue culture epithelial cells (MDCK and CaCo-2) (Jones *et al.*, 1994; Finlay and Falkow, 1990). However, in contrast to other cell types, M cell internalisation of *S. typhimurium* is destructive to the cytoskeletal architecture. Moreover, 60 minutes after applying *S. typhimurium* to intestinal loops it is observed that M cells are dying and detaching from the epithelial lining (Jones *et al.*, 1994). The invading bacteria do not seem to replicate to any extent within the M cell but move through the cell to interact with cells in the follicle dome as well as adjacent enterocytes. Interestingly, *S. typhi* also enters M cells by ruffling, but the cells do not die to the same extent as seen with *S. typhimurium* and there is less invasion of adjacent enterocytes (Jones and Falkow, 1996).

One problem in investigating the role of M cells is that they have not been successfully cultured therefore polarised epithelial cells have been used as a model. When *S. typhimurium* infects polarised MDCK cells the bacteria do not appear to be specifically transported through the host cell to any significant level (Finlay *et al.*, 1988). Most of the bacteria remain within the cells (90%), whilst a small number (8.7%) exit from the apical surface (the surface they are added to). Only a tiny proportion (1.3%) pass through the monolayer and this passage requires around 3-4 hours. In contrast to this, the ability to pass through epithelial barriers is essential to *S. enterica* serovar Choleraesuis (*S. choleraesuis*) (Finlay *et al.*, 1988). Indeed, using Tn*phoA* disruptions, several mutants of the latter serovar were identified that could not transcytose through epithelial monolayers. The differences observed with these individual serovars of *S. enterica* may well reflect on the way they infect the host. For example, in the majority of cases, *S. typhimurium* causes a focal infection in the small intestinal epithelium and is not usually thought to proceed into deeper tissue (in man) and so results in acute gastro-enteritis (Gulig, 1996). However, both *S. typhi*

and *S. choleraesuis* are invasive and their ability to cause disease correlates with efficient movement through the intestinal epithelial barrier (Gulig, 1996).

Host factors involved in bacterial uptake into epithelial cells

Epithelial cells are not usually phagocytic, however *S. enterica* is believed to subvert the cell signalling pathways of the host to induce it to engulf the bacterium. This results in the characteristic ruffling seen in M cells and tissue culture epithelial cells and occurs within one minute after attachment of the bacteria to the cell (Jones *et al.*, 1994). The nature of this pathway however, is elusive and it appears that *S. typhimurium* can evoke different signalling pathways in different cell lines (reviewed in Galan, 1994). Nevertheless, entry requires increases in intracellular Ca^{2+} and inositol phosphates and rearrangements in the host cell cytoskeletal proteins, including actin and tubulin (Finlay *et al.*, 1991).

1.3.4 The molecular and genetic basis for the entry of *S. enterica* into host epithelial cells

The role of fimbriae and flagella

In order to enter epithelial cells *S. enterica* must first make contact. Surface components including fimbriae and flagella have been investigated for their role in mediating entry into eucaryotic cells. Although motility requires flagella it is also linked with the chemotactic responses of the bacterium. Mutations in the chemotaxis genes such as *cheA*, *cheR*, *cheW*, and *cheY* which cause the bacterium to have a smooth-swimming phenotype appeared to result in a more invasive phenotype than observed with the wild type parent cells (Jones *et al.*, 1992). Equally, a *cheB* mutant, which displays a tumbling phenotype, was less able to enter cells. Therefore, chemotaxis may have a role to play in entry into cells (Jones *et al.*, 1992; Khoramican-Falsafi *et al.*, 1990) and chemotactic signals from host cells may enhance the ability of bacteria to target them, particularly if damaged (Uhlman and Jones, 1982). This is supported by the fact that low pH-treated HeLa cells appeared

to cause an increased frequency of collision from salmonellae as if some attractant was being released (Uhlman and Jones, 1982). It is noteworthy however, that the invasiveness of non-flagellate or non-motile flagellate *S. typhimurium* cells was not significantly different, suggesting that the flagellum itself provides little advantage, and that motility is therefore the more important feature for invasion (Khoramican-Falsafi *et al.*, 1990). Nevertheless, in murine model studies, *S. typhimurium* mutants lacking flagella or motility remained as virulent as the wild type strain (Lockman and Curtiss, 1990). Thus, the contribution of motility *per se* to the process of virulence cannot be considered highly significant.

S. typhimurium expresses type I fimbriae which mediate attachment to mannosyl receptors, and these were shown to enhance the binding of *S. typhimurium* to rat enterocytes (Lindquist *et al.*, 1987). In enteropathogenic *E. coli* (EPEC), type I fimbriae have been shown to help binding of the microorganism to the urinary tract and also, to have some role in preventing phagocytic killing. However, a *fimA* mutation, which resulted in the loss of expression of the major subunit for fimbrial formation, did not attenuate *S. typhimurium* in BALB/c mice (Lockman and Curtiss, 1992). However, it is of interest that when a *fimA* mutation was combined with a mutation in flagellum synthesis, the resultant bacteria were found to be 1000-fold more attenuated than a wild type strain after oral challenge in BALB/c mice (Lockman and Curtiss, 1992). Nevertheless, although flagella *per se* do not appear to be important in *S. typhimurium*, a regulator of flagella synthesis (*flgM*) is believed to modulate the expression of other unknown loci which are thought to have a role in virulence (Schmitt *et al.*, 1994).

The role of flagella and fimbriae in infection therefore remains controversial and is probably dependent upon the serovar of *S. enterica* examined and possibly also, the nature of the experimental model used. For example, the ability of *S. typhi* to enter epithelial cells has been shown to be dependent on intact flagella, as well as motility functions (Liu *et al.*, 1988). Furthermore, virulent *S. enterica* serovar Gallinarum (*S. gallinarum*), the causative agent of fowl cholera, is naturally non-flagellate highlighting the fact that different salmonellae may have optimised different

strategies for entry into host cells (Lockman and Curtiss, 1992).

The role of lipopolysaccharide

Lipopolysaccharide may also have a role in mediating entry into epithelial cells but this also appears to depend on the serovar of the *S. enterica* being investigated. *S. typhimurium* mutants which are unable to form a complete LPS molecule, rough mutants, are no different to wild type strains in their ability to invade cultured HeLa cells (Kihlstrom and Ebedo, 1976). In contrast, both *S. typhi* (Mroczenski-Wildey *et al.*, 1989) and *S. choleraesuis* (Finlay *et al.*, 1988) rough mutants are deficient for entry. Furthermore, the deficiency in LPS was shown to affect the ability of *S. choleraesuis* to transcytose through the cells (Finlay *et al.*, 1988). It could be that differences in LPS trigger different signalling pathways to stimulate entry into host cells. Alternatively, there may be differences in the ability of LPS from different serovars to facilitate the attachment of the bacteria to the outside of the host cell.

The role of an invasion-protein-export system for *S. enterica* invasion

S. enterica produces a specialised invasion structure

Early work by Jones and colleagues (1981) showed that the interaction of *S. typhimurium* cells with HeLa cells consisted of two phases, a reversible phase in which the bacteria can be readily washed off the target cells, and an irreversible phase where bacteria are resistant to washing. This suggested that the bacteria were becoming firmly attached to the cells with which they were interacting, and that *S. typhimurium* could only be internalised in the irreversible phase. In a separate study, treatment of MDCK cells with neuraminidase and trypsin lowered the ability of *S. typhimurium* and *S. choleraesuis* to adhere to and invade such cells suggesting that eucaryotic surface components, possibly receptors, were important for binding (Finlay and Falkow, 1989). Moreover, pulse labelling studies with radiolabelled methionine in *S. choleraesuis* and *S. typhimurium* demonstrated that several novel bacterial proteins were induced upon interaction of the bacteria and MDCK cell surfaces, indicating that *S. enterica* senses and responds to this interaction (Finlay

and Falkow, 1989a).

Interestingly, these observations have been supported by electron microscope studies which reveal that *S. typhimurium* cells form appendages with epithelial cells. Moreover, these appendages are produced in a transient manner but are never seen when the host cell membrane undergoes ruffling (Gulig, 1996). These structures were called invasomes (or invasion organelles). It appears then, that upon host cell contact, possibly in response to some form of receptor, *S. enterica* produces a transient structure, the invasome, that mediates the cell signalling events that result in the internalisation of the bacterium.

The genetic basis of the invasion apparatus

Many of the above findings have been confirmed by the identification of a region at centisome 63 of the *S. typhimurium* chromosome which is responsible for cellular internalisation and which, unlike flagella, fimbriae or LPS, is absolutely required for invasion of epithelial cells. This region spans 35-40 kb of the *S. typhimurium* chromosome, encompassing approximately 29 genes, and is believed to encode a specialised invasion-protein-export system for the delivery of virulence factors which functions whilst the bacteria are in contact with the host cell (reviewed in Galan, 1996).

Polypeptide secretion in Gram negative bacteria, such as *S. enterica*, requires that the protein passes through an inner membrane, the periplasmic space and an outer membrane (reviewed in Wandersman, 1996). The specialised protein secretion apparatus encoded by the invasion locus has been termed a Type III system, or an invasion-protein-export system, to distinguish it from other known systems (Galan *et al.*, 1996). Type I systems transport the protein across both membranes in a single step, such as in the secretion of the *E. coli* haemolysin. In contrast, Type II systems transport the protein in two steps, firstly across the inner membrane to the periplasm and then from the periplasm across the outer membrane, such as occurs in pullulanase secretion by *Klebsiella oxytoca* (Wandersman, 1996). Both Type I and Type III secretion systems are similar in that they involve a protein complex that spans the inner and outer membrane and that they mediate secretion directly across

both membranes. However, the Type III system is believed to be very distinct because of the number and type of components involved and because the system appears to be activated in a contact dependent manner upon interaction with host cells (Galan, 1996).

The majority of the genes encoded at centisome 63, organised into the *inv* (invasin), *spa* (suface presentation of antigens) and *prg* (PhoP repressed genes) operons, are believed to encode polypeptides which form the structural components of the invasion-protein-export system. *S. typhimurium* entry has also been shown to be an energy-requiring process since dead or inactivated bacteria are unable to enter cultured epithelial cells (MacBeth and Lee, 1993). Moreover, one of the components, InvC, is believed to provide the energy for the secretory process as it shows 50% amino acid similarity to the α and β subunits of the F_0F_1ATPase (Eichelberg *et al.*, 1994) and InvC has been shown to possess ATPase activity *in vitro*, confirming its ability to use ATP as a substrate (Eichelberg *et al.*, 1994).

The genes that encode the invasion apparatus are highly conserved amongst bacterial pathogens

Surprisingly, the organisation of the genes or the products of the genes encoded at centisome 63 appear to be conserved in a number of other bacterial pathogens including *Shigella* spp. and *Yersinia* spp. and also in the plant pathogens *Erwinia amylovora* and *Pseudomonas* spp. (reviewed in Galan, 1996; Galan and Sansonetti, 1996). For example, the amino acid sequences of InvC and InvG are 68% and 67% similar (57% and 46% identical) to the Spa47 and MxiD proteins of *Shigella*, respectively, and 65% and 55% similar (46% and 31% identical) to the YscN and YscC proteins of *Yersinia*, respectively (Eichelberg *et al.*, 1994; Kaniga *et al.*, 1994). Moreover, the SipB and SipC proteins, also encoded at centisome 63, show 63% and 42% similarity to the *Shigella* IpaB and IpaC proteins, and when expressed on a plasmid were able to restore a full invasive capacity to a non-invasive *ipaB ipaC* double mutant of *Shigella* (Galan, 1996).

Interestingly, features of the DNA sequence of these loci differ from the bulk of the

genome present in the bacteria, suggesting that they have been acquired from another organism. For example, the typical cytosine and guanine composition of *S. typhimurium* DNA is 52% yet the chromosomal region containing the invasion genes is only 42%. Such blocks of apparently foreign genes which are required for virulence have been called pathogenicity islands and the genes at centisome 63 have been designated SPI1 (*Salmonella* pathogenicity island 1).

Secretion of proteins by the invasion-protein-export system

A number of genes have been identified which appear not to form part of the secretory apparatus and are therefore believed to be components specifically transported by the Type III system. These include the proteins encoded by the *sip* locus (*Salmonella* invasion proteins), which is composed of *sipABCD*, the *sop* locus (*Salmonella* outer proteins), which is composed of *sopABCDE*, and the *invJ*, *spaO* and *sptP* genes (Galan, 1996; Hueck *et al.*, 1995; Wood *et al.*, 1996; Kaniga *et al.*, 1996). In agreement with this view, the Sip and Sop proteins were isolated from culture media after growth of *S. typhimurium* and *S. dublin*, respectively, indicating that they had been secreted (Hueck *et al.*, 1995; Wood *et al.*, 1996).

S. typhimurium has been shown to form an invasome, which is believed to mediate the signalling events required to trigger the host cell to internalise the bacterium (Galan, 1996). Mutations in some of the genes that form the structural component of the Type III secretion system, such as *invA*, *invC* and *invG*, result in cells which do not produce this invasion structure or secrete Sip proteins (Galan and Sansonetti, 1996; Chen *et al.*, 1996b). Although the actual structure of the invasome is unknown, it has been postulated that a number of the proteins secreted by the Type III system may form part of this structure (Galan, 1996). Moreover, it is also believed that part of the signalling mechanism for bacterial internalisation involves the transfer of effector molecules into the cell to subvert host cell signalling processes and this has been supported by comparisons with other bacterial pathogens. For example, the *Yersinia* specialised invasion-protein-export system has been shown to translocate YopE and YopH (*Yersinia* outer proteins) into host cells in a YopB-dependent fashion (Hakansson *et al.*, 1996). YopE and YopH function as a cytotoxin and a

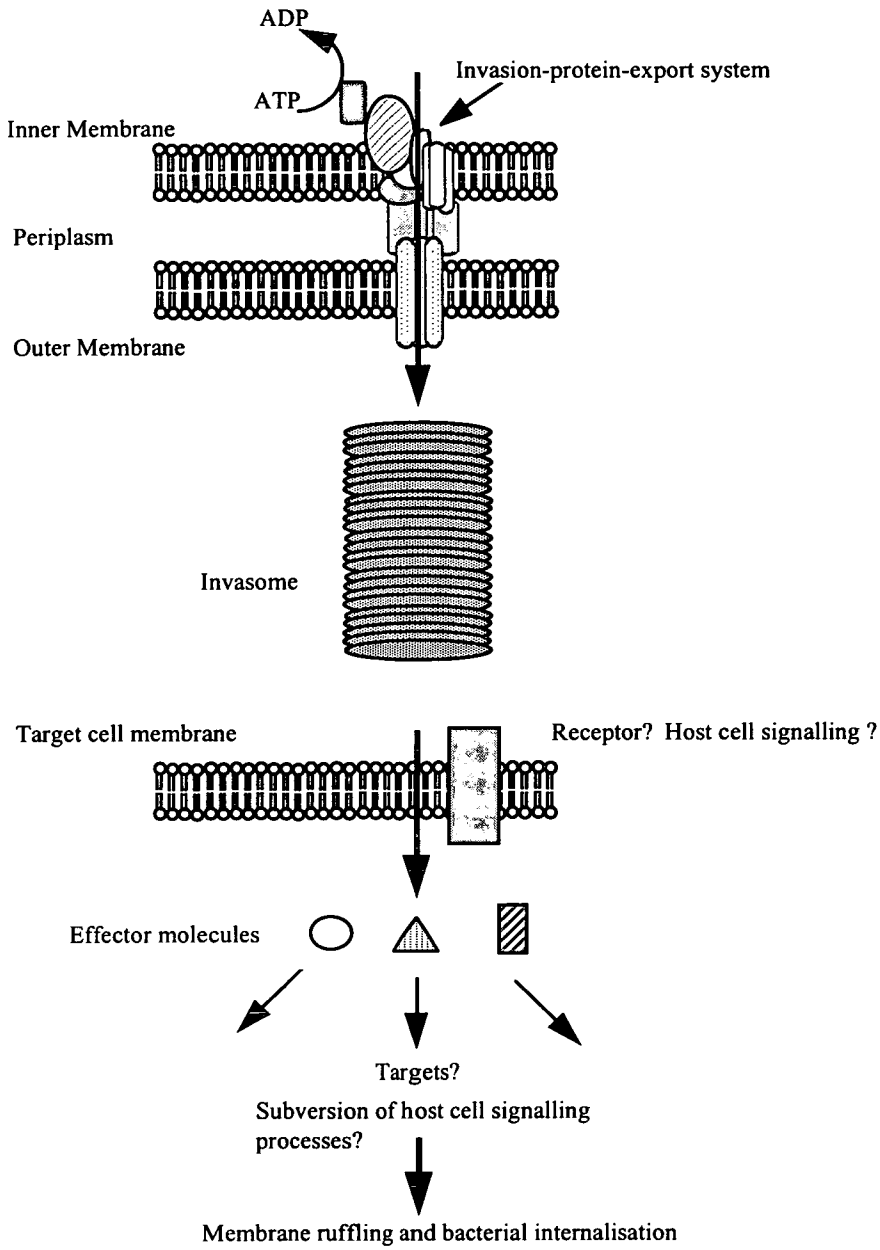
tyrosine phosphatase, respectively, with the latter possibly having a role in preventing tyrosine phosphorylation of important components of the host cell's signalling processes. Interestingly, SipB has been implicated in transporting *S. typhimurium* effector molecules into host cells as it displays 58% amino acid similarity to YopB (Kaniga *et al.*, 1995). Moreover, supporting evidence for this has been provided by demonstrating that SopE was translocated into HeLa cells by a SipB-dependent mechanism (Wood *et al.*, 1996). Thus, it appears that *S. enterica* cells have developed a complex system to promote entry into epithelial cells and this is summarised in Figure 1.4.

Regulation of the *S. enterica* invasion system

Regulation of expression of the invasion system, including the Type III secretion machinery and the invasome, appears complex. A number of different environmental factors, especially those associated with the intestine, have been shown to influence the invasion phenotype and the expression of the invasion genes in *S. typhimurium*. For example, the *invA* gene (and therefore the *inv* locus) was demonstrated to be induced by conditions of high osmolarity (Galan and Curtiss, 1989) and the *orgA* (Jones and Falkow, 1994) and *hilA* (Lee *et al.*, 1992) genes, also identified at centisome 63, have been shown to respond to low oxygen tension

One problem in characterising the regulation of the invasion genes is that at least four different regulatory proteins including HilA, InvF, PhoP and SirA, have been identified that influence their expression (Lee *et al.*, 1992; Bajaj *et al.*, 1995, 1996; Galan, 1996; Johnston *et al.*, 1996). HilA and InvF encode proteins of 56% and 49% similarity (32% and 24% identity) to the OmpR and AraC regulatory proteins, respectively (Lee *et al.*, 1992; Galan, 1996). Little is known about the regulatory role of InvF, but HilA has been shown, using β-galactosidase fusions, to influence *invF*, *orgA* and *prg* expression. However, HilA expression is itself influenced by the PhoP regulatory protein (Bajaj *et al.*, 1995; 1996) and by a recently identified regulator, SirA (*Salmonella* invasion regulator; Johnston *et al.*, 1996), suggesting that there may be a hierarchy of control. Nevertheless, how all these signals and regulators interact to affect the secretion apparatus and their substrates is unknown, but it has

Figure 1.4 Hypothetical representation of the components involved in the invasion of epithelial cells by *S. enterica*



Upon contact with the epithelial cell, *S. enterica* exports proteins through the bacterial cell membrane using a specialised invasion-protein-export system. Some of these proteins form an invasion organelle, the invasome, which subverts the epithelial cell signalling processes directly at the cell surface, by binding to a receptor or by transporting effector molecules into the cell. For a description of the proteins involved in the formation of these structures see the text. (Note: Not to Scale)

been proposed that the induction of *S. enterica* invasion proteins in response to multiple environmental cues ensures that bacterial entry is limited to the specific sites where an invasion phenotype is required (Galan *et al.*, 1992; Bajaj *et al.*, 1995).

The importance of the invasion-protein-export system for *S. enterica* infection

Mutants in the *inv* loci of *S. typhimurium* are defective for entry into cultured epithelial cells. Furthermore, investigations have shown that the *inv* locus (and presumably the other genes) is present in over 100 serovars of virulent *S. enterica* including *S. typhi* (Galan and Curtiss, 1991). However, in *S. typhimurium*, mutation of *invA*, an essential component of the invasion-protein-export apparatus, resulted in only moderate (50-fold) attenuation after oral administration to BALB/c mice but was not attenuated by the intraperitoneal route (Galan and Curtiss, 1989). Therefore, it would seem that the genes encoded at centisome 63 are only required during infection of the intestinal epithelium, and are unlikely to play a further role after invasion.

Interestingly, using mini-Tn*5* mutagenesis, a second invasion-protein-export apparatus was recently identified at approximately 40 minutes on the *S. typhimurium* chromosome and was designated SPI2 (Shea *et al.*, 1996). Moreover, a number of the genes were sequenced and shown to display a high degree of similarity to components of the system encoded in SPI1 at centisome 63. For example, the protein sequences of orf3 and orf9 (open reading frames) showed 50% and 40% identity to InvA and SpaQ. When mutants of *S. typhimurium* carrying Tn*5* insertions in genes from this SPI2 were investigated for attenuation, it was found that such strains were attenuated approximately 100- and 10, 000-fold by the intraperitoneal and oral routes of administration, respectively, in BALB/c mice (Shea *et al.*, 1996). This raises the intriguing possibility that this invasion-protein-export system has a wider role in promoting the ability of *S. enterica* to cause a systemic infection.

1.3.5 The cellular and molecular basis of the replication of *S. enterica* within epithelial cells

After *S. enterica* enters the host cell, it must exploit the resources of that intracellular environment for successful growth in order to increase the chances of a successful infection (Leung and Finlay, 1991). Electron microscope studies have demonstrated that *S. typhimurium* enters both M cells and cultured epithelial cells, such as MDCK or CaCo-2 cells, within 30 minutes of contact (Finlay and Falkow, 1990; Jones *et al.*, 1994). Host cell entry by *S. typhimurium* involves the bacteria being internalised individually in small vesicles which subsequently coalesce to form a large vacuole (Finlay and Falkow, 1989a). *S. typhimurium* then replicates in these structures, after a lag of 5-6 hours, with a generation time of approximately 40-50 minutes. How these bacteria meet their nutritional requirements in such an intracellular environment is unknown as the vacuole they survive in has not been well characterised.

Intracellular replication in epithelial cells is essential

Leung and Finlay (1991) have shown that the ability of *S. typhimurium* to replicate in epithelial cells is essential for a successful infection. After screening 45, 000 independent mini-Mu*dJ* transposon mutants of *S. typhimurium*, three *rep* mutants (for replication deficient) were identified which were unable to replicate in MDCK, HeLa and CaCo-2 epithelial cells. Moreover, these mutants showed no difference to their virulent parent in aerobic or anaerobic growth, motility, serum resistance or macrophage survival. However, even when the oral infection dose of these *rep* mutants was increased 1000-fold above the LD_{50} of the parental *S. typhimurium* strain ($1x10^6$ organisms), approximately 80% of the BALB/c mice survived, indicating that the genetic information disrupted in these mutants was essential for the growth of *S. typhimurium* in epithelial cells and also for virulence.

Intracellular *S. typhimurium* cells induce filament formation in epithelial cells

Recently, filamentous structures containing lysosomal glycoproteins (lgp) were shown to emanate from *S. typhimurium*-filled vacuoles, and these have been termed *Salmonella*-induced filaments (sif) (Garcia-del Portillo *et al.*, 1993b). The

appearance of sif requires the presence of viable intracellular bacteria and vacuolar acidification. Moreover, sif formation correlates well with the start of intracellular replication for *S. typhimurium* (5-6 hours post entry). The importance of sif has not been determined, although it has been suggested that these filaments somehow help deliver nutrients to the vacuole-bound *S. typhimurium* (Garcia-del Portillo *et al.*, 1993b). Interestingly, these structures were never seen in *rep* mutants suggesting that *S. typhimurium* must trigger sif formation. Subsequently, a genetic locus, *sifA*, was identified whose disruption produces the phenotype of a *rep* mutant (Stein *et al.*, 1996). SifA is a 38 kDa protein with no similarity to presently known polypeptides. However, the DNA sequence of the *sifA* gene has a G+C content of 41%, diverging significantly from the genomic average (approximately 52%-54%), suggesting that this gene, like the genes that encode the invasion-protein-export system at centisome 63, has been acquired from another organism (Stein *et al.*, 1996).

1.3.6 The interaction of *S. enterica* with the macrophage

After breaching the intestinal mucosal membrane, invasive *S. enterica* serovars encounter the phagocytic defence system including the polymorphonuclear leucocytes (PMN), such as neutrophils, and the mononuclear phagocytes, such as monocytes and macrophages (Jones and Falkow, 1996). These cells are specifically designed to destroy invading microorganisms and are equipped with an arsenal of oxygen-dependent and -independent mechanisms to effect killing (Adams and Hamilton, 1984; Lehrer *et al.*, 1990). Nevertheless, intracellular pathogens such as *S. enterica* are capable of surviving within phagocytic cells such as macrophages. These cells then provide a safe haven for the bacteria, from the components of the immune system in the extracellular environment such as antibody or complement. In addition, macrophages act as vehicles for dissemination around the host. Nevertheless, the basis by which *S. enterica* cells are capable of overcoming the anti-microbial environment of phagocytes is still relatively uncharacterised.

During infection by invasive serovars of *S. enterica*, the main sites of bacterial growth are the spleen and liver, where the macrophages of the reticuloendothelial

system deal with the infecting organisms (Buchmeier and Heffron, 1989). The role of PMNs in suppressing infection is less certain, in fact, the majority of research into the interaction of *S. enterica* cells with the phagocytes has focused on the macrophage. However, the following observations are of interest. Firstly, if mice are injected with factors that occupy or kill macrophages, such as dextran sulphate, silica or liposome encapsulated dichloromethylene diphosphate, then they become more susceptible to infection by *S. typhimurium* in the early phase (within a week) of the infection (Gulig, 1996). Secondly and in contrast to the latter finding, mice injected with cyclophosphamide (which depletes circulating granulocytes by approximately 90%) show no increase in susceptibility to *S. typhimurium* (Gulig, 1996), confirming that macrophages are likely to have the predominant role in phagocytic defence. The following sections therefore relate to the interaction of *S. enterica* with macrophages, but do not exclude that some of the findings relate to PMNs.

1.3.7 The cellular basis of the interaction of *S. enterica* with macrophages

Entry into the macrophage

Macrophages readily ingest particles, including bacteria, by phagocytosis. Macrophages have receptors for the Fc portion of antibody (IgG and IgM) and components of the complement system (C1R and C3R). As a result, the process of bacterial uptake is enhanced when antibody or complement proteins (*e.g.* C3b), deposited on the surface of the microorganism, are recognised by such receptors (Joiner *et al.*, 1984; Weir and Stewart, 1993). Therefore, during the interaction of *S. enterica* cells with macrophages, entry would not appear to be a difficulty. However, it appears phagocytosis may not be the only mode of entry into the intracellular environment. *S. typhimurium* can enter a wide variety of cell types and therefore by comparison it is believed that an alternative portal of entry may also exist in macrophages (Finlay and Falkow, 1989a; Gulig, 1996; Chen *et al.*, 1996b; Monack *et al.*, 1996).

Early, evidence for a non-phagocytic route of entry into macrophages came from the

identification of a Tn*10* mutant of *S. typhimurium* (SL3792) which entered macrophages less well than the parent strain, even when opsonised (Finlay and Falkow, 1989a). Recently, it has been shown that entry of epithelial cells by *S. typhimurium* requires an invasion-protein-export system, and mutants in some of the genes required for expressing this system are defective for entry into the host cell (Galan and Curtiss, 1990; Galan, 1996). Subsequently, a role for this invasion apparatus for entry into macrophages has also been implied from the recent finding that *S. enterica* is cytotoxic to macrophages and this phenomenon has been correlated with the expression of the specialised export machinery (Chen *et al.*, 1996b; Monack *et al.*, 1996). It therefore appears that *S. enterica* may actively target the macrophage for entry. The importance of such a mode of entry is unclear, however, a non-phagocytic route may be less likely to stimulate the macrophage respiratory burst and also possibly places the bacterium in a unique intracellular compartment, safe from the anti-microbial contents of the lysosome. Such a process would constitute an important survival strategy (Chen *et al.*, 1996b; Lindgren *et al.*, 1996; Monack *et al.*, 1996).

Intracellular location and replication of *S. enterica*

There is a great deal of conflicting evidence as to the nature of the intracellular macrophage compartment within which *S. enterica* survives. In general, phagocytosed material enters the cells in a membrane-bound vesicle called a phagosome. This phagosome then follows the endocytic pathway and lysosomes, containing anti-microbial factors and degradative enzymes fuse to it, to form a phagolysosome. There are a variety of strategies used by intracellular pathogens to avoid the anti-microbial activities present within the macrophage (reviewed in Moulder, 1985). Some pathogens escape from the phagosome or fused phagolysosome compartment and replicate in the safe environment of the host cell cytoplasm (*e.g. Listeria monocytogenes*). Others block phagosome-lysosome fusion, thereby preventing the delivery of the deadly cocktail of anti-microbial effector molecules into the intracellular compartment (*e.g. Chlamydia*). Finally, intracellular pathogens may simply reside in the phagolysosome and resist, or neutralise, the

action of the anti-microbial mechanisms directed against them (*e.g. Coxiella burnetti*). These latter two strategies have been described for the intracellular survival of *S. enterica* within the macrophage.

Early studies (Carrol *et al.*, 1979) demonstrated that formaldehyde-killed *S. typhimurium* cells were rapidly destroyed in phagolysosomes, whereas viable bacteria were resistant to destruction over the same time period. Furthermore, there were no apparent differences in the level of phagosome-lysosome fusion when macrophages treated with live and dead bacteria were compared. This was interpreted to indicate that *S. typhimurium* simply resisted all the anti-microbial factors whilst residing in the phagolysosome. In contrast, using electron microscopy, Buchmeier and Heffron (1991) suggested that macrophage-internalised *S. typhimurium* inhibited phagolysosomal fusion. When live and dead *S. typhimurium* cells were added to murine macrophages (J774), and observed after 14 hours, the live bacteria were found in half as many fused vacuoles as the dead bacteria. This indicated that an important part of the *S. enterica* survival mechanism was to avoid the delivery of the lysosome contents and this was subsequently supported by the identification of a *S. typhimurium* mutant, S5635, which lacked this ability (Ishibashi *et al.*, 1992).

S. enterica may enter into a unique intracellular compartment

Using time-lapse light microscopic techniques, Alpuche-Aranda *et al.* (1994) demonstrated that *S. typhimurium* cells do not always enter J774 macrophages by conventional phagocytosis. Instead, a process reminiscent of *S. typhimurium*-induced entry into epithelial cells was observed, in which membrane ruffling occurred followed by internalisation of the bacteria into small vesicles. These vesicles then coalesced to form a large vacuole and which was termed a spacious phagosome to distinguish it from the phagosomes generated by classical phagocytosis. Indeed, in early studies, *S. typhimurium* was shown to replicate after a lag of approximately 4 hours, in a membrane-bound compartment formed from the coalescence of small vesicles, in a manner similar to that in epithelial cells (Finlay and Falkow, 1989a). These observations therefore suggest that *S. enterica* may enter and replicate in a

unique intracellular compartment which may not undergo phagolysosomal fusion.

Evidence to support that *S. enterica* enters and replicates in a unique intracellular compartment is implied from examining the endocytic route that *S. typhimurium* follows upon entry into the host cell (Garcia-del Portillo and Finlay, 1995). *S. typhimurium* cells were observed to enter into compartments which contained lysosomal glycoproteins (lgps). However, these vesicles containing the live bacteria were never associated with the mannose 6-phosphate receptor or cathepsin D which are normally associated with the phagolysosome. This indicates that *S. typhimurium* subverts the intracellular trafficking system such that it never meets up with the lysosome contents. Furthermore, an examination of *S. typhimurium* cells within the macrophages suggested that there were two populations of bacteria present, one capable of rapid multiplication and the other static (Abshire and Neidhart, 1993b). It has therefore been suggested that *S. typhimurium* either enters into a safe site where it may multiply or, it enters the classical phagosome pathway where it is destined to be destroyed (Lindgren *et al.,* 1996). However, these studies do not exclude the possibility that bacterium engulfed by phagocytosis can prevent phagolysosomal fusion as a survival strategy and that a post-phagocytic entry escape route exists.

S. enterica is cytotoxic to macrophages

S. enterica serovars including *S. typhimurium* and *S. typhi* have recently been shown to be cytotoxic to murine bone marrow-derived and cultured macrophages (Lindgren *et al.*, 1996; Chen *et al.*, 1996b). In some studies, this phenomenon manifested itself rapidly (within 20 minutes) upon interaction of the bacterium with the macrophage and by 8 hours post-infection 80% of the macrophages were dead (Chen *et al.*, 1996b; Monack *et al.*, 1996). In contrast, a separate study did not observe the phenomenon until approximately 14 hours (Lindgren *et al.*, 1996). Cytotoxicity is believed to be the result of the induction of apoptosis in the macrophage, as apoptosis-associated phenomena such as chromosome condensation, membrane blebbing and apoptotic bodies are observed in *S. typhimurium*-infected cells (Chen *et al.*, 1996b).

The role of the invasion-protein-export system in cytotoxicity

Many of the features of the entry and replication of *S. enterica* within the macrophages are analogous to the events that occur in epithelial cells and, as has been described previously, are dependent upon the expression of an invasion-protein-export system. Interestingly, two studies recently demonstrated that the *S. enterica*/macrophage interaction phenomena, including the membrane ruffling upon contact of the bacterium, and cytotoxicity, correlated with the expression of this specialised invasion apparatus (Chen *et al.*, 1996b; Monack *et al.*, 1996). *S. typhimurium* mutants defective in components required for the invasion apparatus, such as HilA or OrgA (Monack *et al.*, 1996) or the secreted products such as InvJ, SpaO, SipB, SipC and SipD (Chen *et al.*, 1996b) were found not to cause membrane ruffling and were non-cytotoxic, although bacteria were still observed within the macrophage. These observations suggested that the invasion-protein-export system is important for killing the macrophage. In addition, the entry mechanism of *S. enterica* into a safe site within the macrophage, is likely to result from the subversion of the cell signalling pathway by the specialised bacterial secretory apparatus to bring about the internalisation of the bacterium (and also, cytotoxicity).

It remains to be seen whether the secreted proteins described above mediate the cytotoxicity or whether other secreted proteins such as the recently identified Sop proteins are involved, especially since SopE has been shown to be translocated into host (epithelial) cells (Wood *et al.*, 1996). However, in an independent study, the regulatory protein OmpR, which is not encoded at centisome 63, was implicated in mediating the macrophage cytotoxicity phenomenon (Lindgren *et al.*, 1996). OmpR regulates the expression of two outer membrane porins, OmpF and OmpC, and a cytoplasmic membrane permease (TppB), in response to osmotic changes (Dorman *et al.*, 1989). Studies into the role of OmpR in the virulence of *S. typhimurium* have shown that *ompR* mutants are attenuated approximately 10^4- and 10^5-fold by the oral and intravenous routes of administration, respectively (Dorman *et al.*, 1989; Chatfield *et al.*, 1991). However, *tppB* mutants are not attenuated and an *ompCompF* double mutant was shown to be highly attenuated by the oral route (approximately

300-fold) but only mildly attenuated by the intravenous route (<10-fold) (Chatfield *et al.*, 1991). Therefore, the additional level of attenuation in the *ompR* mutant, especially by the intravenous route, appears independent of the known OmpR-regulated genes and may reflect the lack of expression of an (unidentified) OmpR-dependent factor involved in the killing of macrophages.

The role of such cytotoxicity is unclear, but it has been proposed that by inactivating the macrophage the bacteria may establish a safe haven in which to multiply and also, may possibly prevent the macrophage from signalling to the immune system that the host is undergoing a bacterial infection (Chen *et al.*, 1996b). However, it should be remembered that *inv* mutants, which are deficient for producing the invasion-export system, are only attenuated by the oral route but not by intraperitoneal injection, suggesting that the relative role of such cytotoxicity may not be essential (Galan and Curtiss, 1989). It should be noted however, that mutants of a second invasion-protein-export system (SPI2), identified at approximately 40 minutes on the *S. typhimurium* chromosome, were attenuated by both oral and intraperitoneal routes, although whether this system mediates cytotoxicity is unknown. Nevertheless, macrophages are critical in the immunological defence mechanism to *S. enterica* (Collins, 1974; Fields *et al.*, 1986; Buchmeier and Heffron, 1989) and this would be unexpected if they were so readily killed. Thus, it is believed that other mechanisms are involved in the ability of *S. enterica* to survive within the macrophage and some of these will be described in the following text.

1.3.8 The genetic basis of the survival of *S. enterica* within the macrophage

The basis of intracellular survival of *S. enterica* within the macrophage is complex and poorly characterised. Unlike epithelial cells, macrophages are specifically adapted to defending against infection. In fact, the ability of *S. enterica* to survive in the harsh anti-microbial environment of the phagocyte has been shown to require additional adaptive mechanisms to those necessary for survival in epithelial cells (Fields *et al.*, 1986; Groisman and Ochman, 1990). For example, Fields *et al.* (1986) isolated 83 Tn*10* mutants of *S. typhimurium* which were unable to survive or

replicate in the macrophage and were attenuated in BALB/c mice when compared to the virulent parental strain. However, 22 of the least virulent of these mutants were found to survive and replicate in epithelial cells.

The identification of genes which are required for the survival of *S. enterica* within the macrophages is slowly being unravelled. Studies have indicated that upon infection of macrophages approximately 30-40 proteins are elevated in expression and 100 are repressed (Buchmeier and Heffron, 1990; Abshire and Neidhart, 1993a). Moreover, the ability of bacteria to sense and respond to the environment during infection has received a great deal of interest recently (Miller *et al.*, 1989c; Mekalanos, 1992; Mahan *et al.*, 1996). Environmental signals within the host may act as signals to induce specific virulence traits, such as protective mechanisms, required for successful infection. However, the patterns of proteins observed after 2D-PAGE analysis from intracellular *S. typhimurium* cells do not simply reflect the sum of all the responses of bacteria to all the stimuli thought to exist in the macrophage (such as oxidative stress or acidity) (Buchmeier and Heffron, 1990; Abshire and Neidhart, 1993a). This suggests that intracellular survival in macrophages (and probably also, epithelial cells) is a hugely complex and co-ordinated event with the appropriate modulation of all relevant responses (or parts of them) to optimise survival. The following section will briefly examine some of the stresses within the macrophage and outline the mechanisms which *S. enterica* employs to promote its survival.

Defence against anti-microbial peptides

Several *S. typhimurium* loci have been identified which express proteins that mediate defence to the bactericidal actions of anti-microbial peptides and defensin-like molecules produced by neutrophils and macrophage (Fields *et al.*, 1986; Miller *et al*, 1990; Groisman *et al.*, 1992). Originally, mutations in *phoP* were shown to result in sensitivity to defensins or defensin-like anti-microbial peptides (*e.g.* magainins, cecropins, cryptdins) and resulted in an LD_{50} 10^5-fold greater than the parental strain (Fields *et al.*, 1986; Miller *et al.*, 1989a). PhoP forms part of a two component

regulatory system. Its partner PhoQ, encodes a transmembrane protein and is believed to be the sensory component which activates PhoP, the transcriptional activator, by phosphorylation (Miller *et al.*, 1989a, 1989b; Miller, 1991). The signal to activate PhoPQ is thought to be low Mg^{2+} concentrations, but activation may also be modulated by low pH, conditions believed to exist within the macrophage phagolysosome (Seguin *et al.*, 1990; Soncini *et al.*, 1996).

The PhoP/Q system regulates approximately 40 different genes, as judged by 2D-PAGE, which can be split into two sets, *pag* (PhoP activated genes) and *prg* (PhoP repressed genes) (Soncini *et al.*, 1996). As described previously, the *prg* genes encode components of the invasion-protein-export system (Galan, 1996). However, it is the *pag* genes which are believed to confer resistance to anti-microbial peptides because a PhoP constitutive mutant is resistant to these peptides (Miller, 1990). However, to date, mutational analysis followed by sensitivity studies has failed to identify the *pag* gene(s) responsible for conferring resistance to defensins. Moreover, it was recently shown that PhoP regulates the expression of a separate system for defence to anti-microbial peptides, encoded by *pmrA* (initially identified because it provided *S. typhimurium* with polymixin resistance). The nature of the resistance is relatively uncharacterised, but is believed to involve the modification of the outer membrane and in fact, PmrA may regulate at least two genes capable of altering the lipid A component of the outer membrane (Gunn and Miller, 1996). PhoP therefore regulates at least two systems capable of combating the action of anti-microbial peptides.

The importance of mechanisms for defence to anti-microbial peptides in the survival of *S. enterica* within macrophages is further emphasised by the identification of a third system. A locus called *sap* (sensitivity to anti-microbial peptides) was identified after screening 20, 000 mutants of *S. typhimurium*, containing Mu*dJ* fusions, for sensitivity to defensin-like compounds (Groisman *et al.*, 1992). This system, encoded by *sapABCDF*, produces an active uptake system to bring anti-microbial peptides into the cell for destruction (before they insert into the cell envelope). Disruption of the *sap* genes results in an approximately 1, 000-fold attenuation of *S. typhimurium*

cells when administered by the oral route (between 10^2-10^5-fold by the intraperitoneal route) to BALB/c mice, indicating the importance of this locus for virulence.

The survival of *S. enterica* during nutrient deprivation

When *S. typhimurium* and *E. coli* are starved of nutrients, including carbon, phosphorous and nitrogen, they induce a starvation stress response (reviewed in Foster and Spector, 1995; Hengge-Aronis, 1996a). This can be induced deliberately by growing cells in defined medium or alternatively, by growing cells in rich medium to the stationary phase of growth (where the growth of cells naturally depletes the nutrients). The stationary phase response of *S. typhimurium* is regulated in part by an alternative sigma factor called RpoS or σ^S (reviewed in Hengge-Aronis, 1996a). Sigma factors provide the specificity for the genes that the RNA polymerase can recognise and transcribe (Lonetto *et al.*, 1992; Record *et al.*, 1996). Moreover, alternative sigma factors provide a way of regulating developmental pathways or stress responses by reprogramming the core RNA polymerase (E) to transcribe a set of genes which are recognised selectively by the new sigma factor (Lonetto *et al.*, 1992; Record *et al.*, 1996). For example, the sigma factor used under normal growth conditions is RpoD, or σ^{70}, but during starvation this is replaced by σ^S which regulates genes more appropriate for growth under nutrient limitation. Importantly, an *rpoS::lacZ* fusion has been demonstrated to be induced within J774 murine macrophages (Chen *et al.*, 1996a), indicating that *S. enterica* resides within a vacuole low in nutrients and also, that RpoS plays a role in mediating intramacrophage survival. In fact, *rpoS* mutants are highly attenuated in BALB/c mice, when administered by either oral or intraperitoneal injection (10^4- and 10^5-fold, respectively) in comparison to the parental strain, suggesting that RpoS plays a major role in *S. enterica* virulence (Coynault *et al.*, 1996).

The role of RpoS in the survival of *S. enterica* within macrophages

Starvation results in the expression of high affinity nutrient uptake systems to scavenge all the possible nutrients (Hengge-Aronis, 1996a). However, characteristically, starvation stressed cells also display defence mechanisms to a wide range of environmental stresses (*in vitro*), including heat, osmolarity, pH and oxidants, and this is likely to play the predominant role in providing general defence to the anti-microbial environment of the macrophage and survival of the bacteria (Fang *et al.*, 1992; Hengge-Aronis, 1996a). Many of these defensive measures appear to result from RpoS bringing about the expression of multiple stimulons, groups of genes induced by a particular stress stimulus (*e.g.* such as those to acidity or oxidative stress), even in the absence of the inducing stimulus for that particular stimulon (Hengge-Aronis, 1996a).

The role of the *Salmonella* virulence plasmid

Invasive serovars of *S. enterica*, excluding *S. typhi*, carry a large plasmid (approximately 90 kb) which is associated with virulence and, which is believed to promote the survival of the bacteria in the reticuloendothelial system (Gulig, 1990). However, an independent study has suggested that the plasmid somehow prevents the recruitment of an unusual T cell subset, the γδ T cells, to the site of infection, as plasmid-cured strains exhibited greater accumulation of these cells during the course of infection (Emoto *et al.*, 1992).

S. enterica strains cured of this plasmid display between 10^3-10^6-fold increases in LD_{50} compared to strains bearing the plasmid, however its exact function is unknown (Fang *et al.*, 1992). Strains carrying the plasmid have been demonstrated to have increased growth rates in the liver and spleens of the host (which are the foci in invasive *S. enterica* infection) but the plasmid does not promote invasion or transfer of the bacteria to the reticuloendothelial system (Gulig and Doyle, 1993). In addition, investigations to date have indicated that only an approximately 8 kb portion of the plasmid is required to restore wild type levels of virulence to plasmid-cured strains

(Gulig *et al.*, 1992, 1993). This region contains five genes *spvRABCD* (Caldwell *et al.*, 1991; Gulig *et al.*, 1992, 1993; Coynault *et al.*, 1992) however, very little is known about the functions of the products encoded by these genes. The *spvR* gene is believed to produce a regulatory protein that mediates control over the expression of *spvABCD*. Interestingly, an *spvB*::*lacZ* fusion was demonstrated to be induced within the macrophage, indicating that the functions of the *spv* locus may promote intramacrophage survival (Fierer *et al.*, 1993; Chen *et al.*, 1996a). Expression of *spvR* is regulated by the alternative sigma factor RpoS, with maximal induction occurring during stationary phase (Fang *et al.*, 1992). Moreover, it has been postulated that one of the most influential factors responsible for attenuating non-typhoidal *S. enterica* *rpoS* mutants is the loss of *spv* induction (Fang *et al.*, 1992; Coynault *et al.*, 1996).

Defence against acidity

Part of the oxygen-independent anti-microbial defence system involves decreasing the pH of the phagolysosomal compartment in which *S. enterica* enters (Seguin *et al.*, 1991). This acidification is believed to occur within 30 minutes of bacterial internalisation (Rathman *et al.*, 1996) and can drop as low as pH 3.5-4.0 (Foster, 1992). This pH is inhibitory to many microorganisms as it disrupts the proton gradient, which is required to drive transport of substrates, motility and the respiratory chain (Slonczewski and Foster, 1996). However, *S. typhimurium* has been demonstrated to induce approximately 50 proteins in response to acid stress, called the acid tolerance response (ATR) (Foster and Hall, 1990; Foster, 1992).

A number of these proteins have been identified but mutations in *atp* and *fur*, produce the greatest sensitivity to low acid conditions (pH <3.3) and are therefore critical to the development of the ATR (Garcia-del Portillo *et al.*, 1993a). These genes encode a Mg^{2+}-dependent proton translocating ATPase, which pumps protons out of the bacterial cell, and an Fe^{2+}-binding regulatory protein, which regulates changes to the components of the respiratory chain, respectively (Garcia-del Portillo *et al.*, 1993a; Slonczewski and Foster, 1996). In addition, Fur also plays a major role in regulating the synthesis of enterochelins, which are iron-chelating compounds, and

fur is therefore important for obtaining iron in the iron-limited environment of the phagolysosome (Spector and Foster, 1995). However, *fur* and *atp* mutations have no significant affect upon *S. typhimurium* survival within the macrophage, indicating that these genes are not required for phagolysosomal survival (Garcia-del Portillo *et al.*, 1993a). Whether the other genes induced in response to acidity are important for macrophage survival is unknown. However, the PhoPQ locus has been shown to be regulated by low pH, demonstrating that acidity may be an important signal to induce other forms of defensive measures (*e.g.* against anti-microbial peptides).

The role of a heat shock response

When bacteria such as *S. typhimurium* and *E. coli* are shifted from their optimal temperature for growth (37°C) to a higher temperature they synthesise heat-shock proteins (reviewed in Gross, 1996). This heat-shock response is predominantly regulated, like stationary phase, by an alternative sigma factor, RpoH or σ^{32}, which reprograms the RNA polymerase to identify and transcribe heat shock-inducible genes. In *E. coli*, this response is believed to involve approximately 30 genes. These genes encode proteins such as GroEL, GroES, DnaK and DnaJ, which are believed to assist in the refolding of aberrant proteins, or proteases such as Lon and Clp, which degrade damaged proteins (Lindquist, 1986; Gross, 1996). Importantly, the heat shock response is also induced, in part, by a wide variety of environmental insults such as acidity or oxidative stress (Kogoma and Yura, 1992; Hengge-Aronis, 1996a). For example, DnaK and GroEL are abundant proteins on 2D-PAGE gels after exposing *S. typhimurium* and *E. coli* to hydrogen peroxide or superoxide, respectively (Morgan *et al.*, 1986; VanBogelen *et al.*, 1987; Greenberg and Demple, 1989). In addition, it appears that some components of the heat shock response, such as GroEL and GroES, are essential for cellular processes at normal or optimal growth temperatures (Fayet *et al.*, 1989). It is likely therefore, that the heat shock response plays an important role in mediating survival within the macrophage.

In addition to RpoH, heat shock is also regulated by another alternative sigma factor, RpoE or σ^{24} (Gross, 1996). RpoE is capable of binding to the promoter of *rpoH* and

under extreme heat stress (50°C) is believed to augment the transcriptional activation of this gene (Gross, 1996). Importantly, RpoE has also been shown to regulate *htrA* (high temperature resistance), a gene which is likely to play a role in the survival of *S. typhimurium* within the macrophage (Johnson *et al.*, 1991; Gross *et al.*, 1996). Surprisingly, *htrA* mutants of *S. typhimurium*, are not sensitive to heat stress but are extremely sensitive to oxidative killing (Johnson *et al.*, 1991). HtrA shares homology with DegP from *E. coli*, which degrades periplasmic proteins, and therefore suggests that degradation of damaged proteins is an important feature of intracellular survival. This is further supported by the fact that *S. typhimurium htrA* mutants survive poorly in macrophages (Fields *et al.*, 1986; Baulmer *et al.*, 1994) and are attenuated approximately 10^4-fold, compared to the virulent parental strain, after oral inoculation into BALB/c mice (Chatfield *et al.*, 1992b).

Defence against oxidative stress

Upon exposure to, and when within macrophages, *S. enterica* cells are subjected to the toxic reactive oxygen species generated by the respiratory burst. These molecules include superoxide, hydrogen peroxide, the hydroxyl radical and singlet oxygen. In addition, toxic nitric oxide whose anti-microbial effect is potentiated by the other radicals is also generated by a nitric oxide synthase (Pacelli *et al.*, 1995). These toxic molecules readily diffuse across the bacterial cell membrane and are capable of causing extensive cellular damage at all fundamental levels (Imlay and Linn, 1986; Farr and Kogoma, 1991; Demple, 1991). For example, DNA is deaminated and depurinated or even subject to strand breakage. Amino acids may be decarboxylated or oxidised, peptide bonds within polypeptides may be cleaved, and whole protein complexes involved in respiration or uptake of substrates may be inactivated (Farr *et al.*, 1988). Moreover, lipids can be peroxidised and this not only increases the permeability of the membrane but lipid peroxides themselves are capable of damaging DNA (Storz *et al.*, 1987).

S. typhimurium and *E. coli* have been shown to induce two distinct stimulons in response to hydrogen peroxide and superoxide, respectively, which each comprise of

approximately 30-40 genes and which mediate protection to these stresses (Christman *et al.*, 1985; Greenberg and Demple, 1989; Walkup and Kogoma, 1989). Most of these genes have not been identified but two regulons (groups of genes under the control of a single regulatory element), regulated by OxyR and SoxRS, have been identified which are largely responsible for the protection mediated to these stresses (Christman *et al.*, 1985; Tsavena and Weiss, 1990). These two regulons are also thought to play an important role in eliminating nitric oxide derivatives, as both SoxRS and OxyR regulons have recently been demonstrated to be induced by exposure to NO (Nunoshiba *et al.*, 1993; Hausladen *et al.*, 1996).

The importance of such defences against oxidative stress in *S. typhimurium* was demonstrated by Fields *et al.* (1986) who identified three transposon mutants which were deficient for survival within the macrophage and which were attenuated in BALB/c mice. This indicates that the ability to prevent oxidative damage to the bacterial cell may have an important role in *S. enterica* virulence.

Defence against superoxide stress

SoxRS regulates approximately 10 genes which are involved in the response to superoxide stress (Greenberg and Demple, 1989; Walkup and Kogoma 1989; Tsavena and Weiss, 1990). The functions of some of these genes have been identified and include a superoxide dismutase (*sodA*) which breaks down superoxide to hydrogen peroxide, a DNA repair enzyme (*nfo*) and an enzyme, glucose-6 phosphate dehydrogenase, capable of producing large quantities of NADPH (*zwf*) required in the reduction of oxidised molecules (Greenberg and Demple, 1989; Walkup and Kogoma, 1989). Mutations that inactivate either of the *soxRS* genes results in cells that are particularly sensitive to superoxide. Unfortunately, very little information exist about the protective role of the SoxRS regulon in the survival of *S. enterica* within the macrophage. However, an *E. coli soxRS* mutant was shown to be extremely susceptible to macrophage killing suggesting that this regulon may be important for mediating resistance to oxidative stress (Nunoshiba *et al.*, 1993). Nevertheless, an *S. typhimurium SodA* mutant was shown to be only slightly

attenuated (approximately 10-fold) when injected into BALB/c, suggesting that defence against superoxide is not a critical feature for macrophage survival (Tsolis *et al.*, 1995). Loss of all the functions which are regulated by SoxRS however, may well result in a more severe phenotype.

1.3.9 Defence against hydrogen peroxide stress

Of particular relevance to the studies undertaken in this thesis is the ability of *S. typhimurium* to mount a defensive response to hydrogen peroxide. In previous studies, Francis and Gallagher (1993) identified a hydrogen peroxide inducible locus, *ahpCF* (alkyl hydroperoxide reductase), which was induced upon interaction of *S. typhimurium* cells with macrophages. Subsequently, an independent study demonstrated that another hydrogen peroxide inducible gene, *dps* (DNA binding protein from starved cells), was also induced by *S. typhimurium* within the macrophage (Valdiva *et al.*, 1996). Interestingly, both of these genes are part of the OxyR regulon, perhaps indicating that a small subset of genes are critical to the defence of *S. enterica* cells against hydrogen peroxide which is generated in the respiratory burst of phagocytes. Such *in vivo* expressed genes may also have important applications in vaccine development, as disruption may well lead to attenuation of *S. enterica* virulence. In relation to this feature, the following sections will focus on the hydrogen peroxide-inducible response of *S. enterica*, with particular emphasis on the OxyR regulon.

The hydrogen peroxide-inducible response and the role of OxyR

As a result of applying hydrogen peroxide (H_2O_2) or organic peroxides, such as cumene hydroperoxide, *S. typhimurium* and *E. coli* cells have been shown by 2D-PAGE analysis to respond by increasing the expression of approximately 30 different proteins above a basal level (Christman *et al*, 1985; Van Bogelen *et* al., 1987). 12 of these proteins were maximally synthesised in the first 10 minutes of being exposed to the stress and were termed early proteins, whereas 10-30 minutes into the stress time a further 18 proteins became elevated and were termed late

proteins. This has been termed the peroxide stress response.

The precise nature of the substrate which mediates stimulation of induction is not entirely clear. Nevertheless, by screening diethyl sulphate-mutagenised *S. typhimurium* cells for enhanced survival to peroxide stress, a genetic locus called *oxyR* was identified (Christman *et al.*, 1985). Such mutants (*oxyR1* [*oxyR2*, in *E. coli*]) constitutively expressed a subset of approximately 8-9 proteins, even in the absence of H_2O_2, and these cells were found to be significantly more resistant to hydrogen peroxide than wild type cells. Moreover, *S. typhimurium* mutants deleted for the *oxyR* locus (designated *oxyR2* [*oxyR1*, in *E. coli*]) failed to induce this subset of proteins and were extremely sensitive to concentrations of hydrogen peroxide which the wild type cells could resist (Christman *et al.*, 1985). This suggested that the role of *oxyR* was critical for mediating hydrogen peroxide resistance to *S. typhimurium* and therefore, the *oxyR* gene was proposed to encode a positive transcriptional activator (OxyR) of a subset of hydrogen peroxide inducible genes (the OxyR regulon).

These studies and further work have characterised the nature and function of some of the genes of the OxyR regulon in *S. typhimurium* and *E. coli* (Table 1.1). However, a number of OxyR targets such as *dps* have been identified that remain relatively uncharacterised. For example, approximately 23 proteins are absent from the 2D-PAGE gels of *E. coli dps* mutants examined in the stationary phase of growth (Almiron *et al.*, 1992). This suggests that Dps may have a regulatory role to play and may not protect cells from oxidative stress by simply binding to the DNA (Almiron *et al.*, 1992; Altuvia *et al.*, 1994). In addition, it appears that OxyR may act as both a positive and negative transcriptional regulator, as several genes, including *oxyR* itself, can be repressed by OxyR (Tao *et al.*, 1991; Seymour *et al.*, 1996).

Importantly, a number of OxyR-independent genes have been identified which are involved in mediating protection towards hydrogen peroxide. These include a second catalase (*katE*) and two DNA protective enzymes including exonuclease 3 and an enzyme involved in DNA recombination and repair, RecA (Farr and Kogoma, 1991).

Table 1.1 Genetic loci regulated by the OxyR regulatory protein

Gene or locus	Organism	Gene product	OxyR regulation	Reference
oxyR	*S. typhimurium*	OxyR regulatory protein of the OxyR regulon	yes-autoregulated	Christman *et al.* (1985)
	E. coli		as above	Tao *et al.* (1989)
ahpCF	*S. typhimurium*	C22 and F52 subunits of alkyl hydroperoxide reductase	yes-induced	Christman *et al.* (1985)
	E. coli		yes-induced	Van Bogelen *et al.* (1987)
katG	*S. typhimurium*	HPI catalase	yes[(a)]-induced	Christman *et al.* (1985)
	E. coli			Ivanova *et al.* (1994)
dps	*S. typhimurium*	DNA binding protein from starved cells	-	
	E. coli		yes-induced	Altuvia *et al.* (1994)
gorA	*S. typhimurium*	Glutathione oxidoreductase	yes[(a)]-induced	Christman *et al.* (1985)
	E. coli			Tartaglia *et al.* (1992)
oxyS	*S. typhimurium*	Untranslated regulatory RNA	-	
	E. coli		yes-induced	Tartaglia *et al.* (1992)
orfO	*S. typhimurium*	Unknown	yes-induced	Tartaglia *et al.* (1992)
	E. coli		-	
D64a	*S. typhimurium*	Heat shock protein	yes[(a)]-induced	Christman *et al.* (1985)
	E. coli		-	
E89	*S. typhimurium*	Heat shock protein	yes[(a)]-induced	Christman *et al.* (1985)
	E. coli		-	
sodA	*S. typhimurium*	Manganese-containing superoxide dismutase (MnSOD)	yes[(a)]-induced	Christman *et al.* (1985)
	E. coli		no	Touati (1988)
mom	*S. typhimurium*	DNA modification enzyme from phage Mu	-	
	E. coli		yes-repressed	Bolker and Kahmann (1989)
stiA	*S. typhimurium*	Starvation inducible protein	yes-repressed	Seymour *et al.* (1996)
	E. coli		-	

(a) Gene or locus has not been proven to be OxyR-regulated directly but has been shown to be constitutively expressed or not expressed in an *S. typhimurium oxyR1* or *oxyR2* genetic background, respectively.

The latter protein has a major role in protecting *S. typhimurium* from intracellular oxidative stress as *recA* mutants were extremely susceptible to the respiratory burst of J774 macrophages and were highly attenuated (approximately 10^4-fold) when compared to the parental strain, after intraperitoneal injection into BALB/c mice (Buchmeier *et al.*, 1993).

OxyR is a member of the LysR family of transcriptional regulators

The *oxyR* gene was mapped to approximately 89.5 minutes on both the *S. typhimurium* and *E. coli* chromosomes and the gene was subsequently sequenced and cloned (Christman *et al.*, 1989; Tao *et al.*, 1989). Using the *E. coli* sequence, it was established that OxyR is a 34.4 kDa protein (305 aa) with homology to the LysR family of transcriptional regulatory proteins (25% protein sequence identity to LysR) (Tao *et al.*, 1989). These proteins are between 30-35 kDa in size and include LysR from *E. coli*, the NodD gene of *Rhizobium meliloti* and TrpI from *Pseudomonas aeruginosa* (Henikoff *et al.*, 1988). In addition, almost all these members are autoregulatory and regulate a transcript from an overlapping divergent promoter on the complementary strand. In the case of OxyR, this is *oxyS*, an untranslated regulatory RNA (Tartaglia *et al.*, 1992) (Table 1.1).

Regulation of genes by OxyR occurs at the transcriptional level

Regulation of the genes in the OxyR regulon appears to occur at the transcriptional level. Using *S. typhimurium oxyR*1 constitutive mutants it was shown that the *katG* transcript was elevated approximately 50-fold and in turn, resulted in a 50-fold increase in catalase activity in these cells (Christman *et al.*, 1985; Morgan *et al.*, 1986). In contrast, a *katG*::*lacZ* operon fusion was found to show no β-galactosidase activity in an *oxyR* deletion mutant of *E. coli* upon addition of H_2O_2. Regulation at the transcriptional level has also been implied for *ahpCF* based on correlating the level of *ahpCF* transcript and amount of AhpCF activity in the cells of *S. typhimurium oxyR1* mutants (Christman *et al.*, 1985; Tartaglia *et al.*, 1989).

In line with many LysR homologues, OxyR appears to negatively regulate its own

expression (Christman *et al.*, 1989; Tao *et al.*, 1989, 1991). When a plasmid borne *oxyR*::*lacZ* gene fusion was transferred to an *E. coli oxyR*$^+$ strain, no increase in the level of β-galactosidase activity was observed when the cells were exposed to concentrations of H_2O_2 shown to induce the OxyR regulon (Tao *et al.*, 1989). This suggested that the induction of the OxyR regulon required no elevation of OxyR protein, but rather, was likely to be due to modification or activation of the existing cellular OxyR. Moreover, when a similar plasmid was transferred to an *oxyR* deletion mutant, a five-fold greater increase in the level of β-galactosidase activity was observed than when the plasmid was examined in the parental (*oxyR*$^+$) strain in the absence of hydrogen peroxide (Tao *et al.*, 1991). Furthermore, when OxyR was constitutively expressed *in trans* from a separate plasmid this increase in β-galactosidase activity was abolished (Tao *et al.*, 1991). Therefore, OxyR acts both as a transcriptional activator of the genes in the regulon and as a repressor of its own synthesis.

OxyR recognises a specific target sequence of DNA in the genes it regulates

OxyR mediates its regulatory effects upon the genes of the OxyR regulon by binding to a specific region of DNA (the OxyR operator) which partially overlaps with the -35-σ^{70} promoter consensus sequence (Christman *et al.*, 1989; Tartaglia *et al.*, 1989; Tartaglia *et al.*, 1992). In fact, Tao and colleagues (1993) have shown that this overlap with the σ^{70} promoter site results in contact between OxyR and the C-terminus of the RNA polymerase α-subunit and is essential for transcriptional activation. One exception to this is the *oxyRS* locus where the *oxyR* and *oxyS* genes are divergently transcribed from a shared OxyR operator region. In this arrangement the OxyR operator region acts to regulate both the transcriptional activation and repression of the *oxyS* and *oxyR* genes, respectively. This is because like the other OxyR regulon genes, the operator of the *oxyS* gene is positioned such that the OxyR-binding site overlaps with the -35-σ^{70} promoter and the binding of OxyR facilitates transcriptional activation. However, for the *oxyR* gene, this OxyR target sequence not only overlaps with the -35-σ^{70} consensus hexamer but also extends into the -10-σ^{70} hexamer, and binding of OxyR within this region facilitates repression of

transcriptional activation from *oxyR*. Such an overlap is an arrangement common to transcriptional repressors (reviewed in Gralla and Collado-Vides, 1996), where binding of a transcriptional regulator within the -35 and -10-σ^{70} sequences reduces the access of the RNA polymerase, thus preventing transcriptional activation, and accounts for the role of OxyR as a transcriptional repressor.

The OxyR operator of all the OxyR-regulated genes examined to date, is approximately 45 bp in length (Toledano *et al.*, 1994). However, inspection of these apparently similar sites indicated that there was no common highly conserved consensus sequence (Tartaglia *et al.*, 1989; Tartaglia *et al.*, 1992). Nevertheless, degenerate homologies were observed in which only two of the four possible base pairs are represented and this led to the suggestion that OxyR may have a "degenerate recognition code". By analysing 54 synthetic binding sites, selected from pools of random oligonucleotides, Toledano and colleagues (1994) postulated that OxyR bound to a degenerate consensus motif (ATAGntnnnanCTATnnnnnnnATAGntnnnanCTAT). The postulated consensus sequence shows 2-fold dyad symmetry, and the spacing of the ATAGnt elements suggests that OxyR binds on one face of the DNA helix in four adjacent major grooves to bring about transcriptional activation. This binding is believed to be through the formation of an OxyR tetramer, as each OxyR subunit (like other LysR members) only has one HTH domain, which would be unlikely to span two helical turns of the DNA (Kullik *et al.*, 1995a, 1995b).

OxyR undergoes a conformational change upon oxidation

In vitro transcription assays using purified OxyR have demonstrated that binding, and transcription from, the *ahpC* and *katG* promoters only occurred when the OxyR was purified in the absence of dithiothreitol (DTT, a reducing agent), suggesting that OxyR was sensitive to oxidation in air-saturated buffers and, that only oxidised OxyR mediated transcriptional activation (Storz *et al.*, 1990; Toledano *et al.*, 1994). However, both the oxidised and reduced (*i.e.* prepared in the presence of DTT) forms of OxyR, were found to be capable of binding to the *oxyRS* promoter and repressing transcription of the *oxyR* gene. This was explained by identifying an

additional unique target recognition sequence for OxyR which was only present in the *oxyRS* promoter and not in the promoters of the other OxyR-regulated genes (Toledano *et al.*, 1994). Moreover, it was shown using treatment with DNase, that the regions of DNA protected differed when the oxidised or reduced forms of OxyR were bound at the *oxyRS* promoter. This indicated that OxyR was capable of repositioning along the *oxyRS* promoter (Toledano *et al.*, 1994) and suggested that upon oxidation the OxyR protein undergoes a conformational change.

Model for OxyR regulation

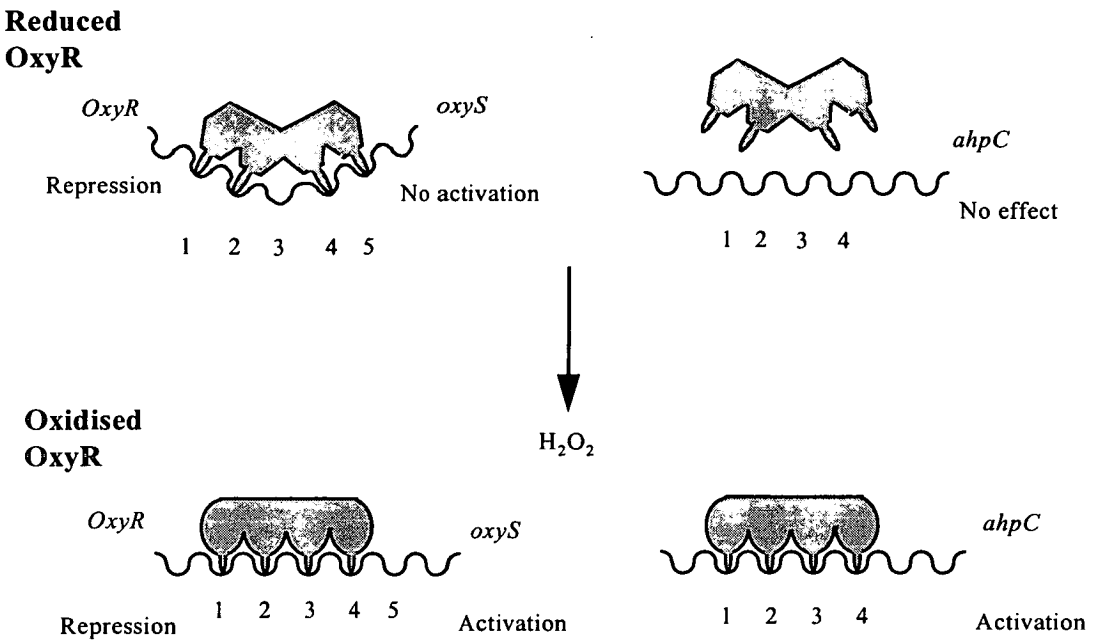


Figure 1.5 Model for reduced and oxidised OxyR binding and activity at the *oxyRS* and *ahpC* promoters. For simplicity only one strand of DNA is shown. Numbers refer to the OxyR target sequences described in the text. (Adapted from Toledano *et al.*, 1994).

From the studies outlined above, Toledano and colleagues (1994) have proposed a model for the OxyR-mediated regulation (Figure 1.5). For genes such as *ahpC* and *katG*, which are transcriptionally activated under hydrogen peroxide stress, the OxyR tetramer is unable to bind to the target promoter regions because of its conformation in the reduced state. Upon oxidation, the conformation alters and OxyR binds to its target sites in four adjacent major grooves (1, 2, 3 and 4) bringing about transcriptional activation of the genes via interaction with RNA polymerase. For the

oxyRS promoter, however, the conformation of the reduced OxyR allows it to bind two pairs of adjacent major grooves (1 and 2, 4 and 5) separated by one helical turn. This results in repression of *oxyR* and prevention of *oxyS* activation; whether the repression of *stiA* and *mom* is achieved in a similar manner is unknown at present. Upon oxidation the conformational change occurs and OxyR repositions its contacts upon the DNA, unable to bind the fifth unique site but able to bind to the other four. This maintains the repression of *oxyR* as well as stimulating the transcription of *oxyS*. Although this model explains the results obtained during experimentation, it is still unclear why reduced OxyR, binding at the *oxyRS* promoter does not activate transcription from *oxyS*. However, it is possible that the conformation of reduced OxyR does not permit a productive interaction with RNA polymerase.

Alternative regulation of the OxyR regulon genes

Aside from oxidative stress, *S. enterica* cells are exposed to a number of different environmental stresses during the course of infection. For example they experience temperature upshift upon entering the host, acidity in the stomach and high osmolarity in the intestine (Foster and Spector, 1995). Moreover, within the macrophage *S. enterica* must then also cope with the hostile environment of the phagolysosome including the low pH, anti-microbial peptides and nutrient deprivation. It is apparent however, that considerable overlaps occur between the responses to specific stresses (Farr and Kogoma, 1991; Hengge-Aronis, 1996a; Mahan *et al.*, 1996) and this suggests that specific functions can be recruited to mediate effects under a number of conditions. This co-ordinated expression of genes would seem to allow efficient use of the bacterial genome. In support of this, components of the OxyR regulon have been shown to be induced under a number of other stress conditions.

Overlap of the hydrogen peroxide-inducible response with heat shock

Exposure of *S. typhimurium* cells to 60 μM hydrogen peroxide results in the induction of five heat shock proteins (HSP), three of which belong to the OxyR regulon (Christman *et al.*, 1985; Morgan *et al.*, 1986). Amongst these proteins is AhpF, which in *E. coli* is not heat shock-inducible and suggests that different

regulation mechanisms exist in these bacteria (Christman *et al.*, 1985). Moreover, the promoter lies upstream of the *ahpCF* locus and *ahpC* is not heat shock-inducible therefore, the mechanism by which *ahpF* is induced by heat shock, in an OxyR-dependent manner, or the possible role of AhpF, is unclear. The identities and functions of the other two OxyR-regulated heat shock proteins, D64a and E89, have not yet been established.

Overlap of the hydrogen peroxide-inducible response with starvation stress

The expression of four genes (to date) have been shown to be critical for the long term survival of starvation-stressed cultures. These genes are *rpoS* and three RpoS-regulated starvation-inducible genes, *stiA*, *stiB* and *stiC* (Seymour *et al.*, 1996). Interestingly, each of these genes are required for the development of hydrogen peroxide resistance in the stationary phase of growth. In addition, *stiA* and *stiB* can be induced in the exponential phase by exposure of *S. typhimurium* to 60 μM H_2O_2 and *stiA* is regulated (repressed) by OxyR. However, the nature of the *sti* genes and the reason why OxyR should be involved in the regulation of *stiA* have not been reported yet.

At least three other OxyR-regulated genes have been demonstrated to be regulated by RpoS in the stationary phase of growth, and their involvement is believed to contribute to the substantial resistance of stationary phase cells to oxidative stress. These include *katG* (Ivanova *et al.*, 1994), *dps* (Altuvia *et al.*, 1994), *gorA* (Becker-Hapak and Eisenstark, 1995). Interestingly, the induction of *katG* and *dps* in stationary phase has been demonstrated to be independent of OxyR (Ivanova *et al.*, 1994; Altuvia *et al.*, 1994). How induction is achieved in the presence of RpoS and the absence of OxyR is not fully understood, but it may involve alternative DNA binding factors, as in the case of *dps* where integration host factor (IHF) has been shown to be involved (Altuvia *et al.*, 1994).

Overlap of the hydrogen peroxide-inducible response with osmotic stress

There is also a very close link between regulation by RpoS and osmotic stress as osmotic-induction of many genes in the exponential phase is dependent upon RpoS

(Hengge-Aronis *et al.*, 1993; Hengge-Aronis, 1996b). Osmotic upshift has been shown to increase the level of RpoS in the cell and extend the half life of the *rpoS* transcript. Furthermore, exponential phase cells exposed to osmotic stress display increased resistance to oxidants in an *rpoS*-dependent manner (Hengge-Aronis *et al.*, 1993). However, only one OxyR-regulated gene (*dps*) has been shown to be induced under osmotic stress (to date). The *dps* gene was independently identified as *pexB*, a stationary phase-inducible gene that could be induced in exponential phase by osmotic stress (Lomovskaya *et al.*, 1994). The RpoS-dependent osmotic-induction of *dps* is not clear as investigations into the role of OxyR have not been performed. However, when two different sized fragments of the *dps* promoter region, regions -240 to +440 bp or -82 to +440 bp, were cloned in front of a promoterless *lacZ* gene, it was observed that osmotic stress resulted in stimulation of β-galactosidase activity from both constructs whilst peroxide stress only increased expression from the larger construct (Lomovskaya *et al.*, 1994). This suggests that the mechanism required for osmotic stimulation differs from that of oxidative stress.

Indirect evidence for the role of another gene of the OxyR regulon in osmotic stress has come from studies on a commensal skin organism, *Staphylococcus aureus* (Armstrong-Buisseret *et al.*, 1995). When *S. aureus* was osmotically challenged it responded by inducing a number of proteins to a high level and one of these proteins was determined to be 50% similar at the amino acid sequence level to the *S. typhimurium* AhpC protein. Further evidence for a role of the *ahp* locus in osmotic stress has also been recently inferred from a separate study in which the *Bacillus subtilis ahpC* gene was shown to be osmoregulated (Antelmann *et al.*, 1996). Thus, it may also be possible that the *S. typhimurium ahp* locus is induced under osmotic stress, and this feature will be explored as part of the present study. Why oxidative stress genes may be required for osmotic stress is unknown however, it may well be that these functions are required outside the macrophage.

It is clear that some of the OxyR-regulated genes have roles in several response mechanisms for example, *dps* is induced by oxidants, starvation, osmolarity and, as recently shown, by acidity (Valdiva *et al.*, 1996). This may suggest that the genes of the OxyR regulon may be required or modulated in different locations during the

course of infection to promote the survival of *S. enterica* within the host.

1.3.10 Summary of the factors involved in the infection of the host by *S. enterica*

Infection of the host by *S. enterica* is a complex phenomenon in which the bacterium must enter the host and establish one or more unique niches in order replicate and ultimately be transmitted to another individual (Finlay and Falkow, 1989b). Nevertheless, the host is extensively equipped, via the immune system, for detecting and dealing with invading bacteria and as a result, *S. enterica* must avoid, withstand or circumvent these anti-microbial mechanisms in order to survive and propagate. As has been described in this introduction, *S. enterica* is an intracellular pathogen and is capable of invading and surviving within numerous types of cells, including macrophages which are ideally equipped for bringing about the destruction of microorganisms, and this appears fundamental to the virulence of *S. enterica* (Collins, 1974; Fields *et al.*, 1986; Finlay and Falkow, 1989a; Garcia-del Portillo *et al.*, 1993b; Galan, 1996). This ability is dependent on the presence of a complex array of virulence traits encoded by genes on disparate parts of the genome (some of which are summarised in Table 1.2). Several of these appear to regulate multiple factors important for successful infection. For example, the two component regulatory system PhoPQ is required for invasion of epithelial cells in the gut (and possibly cytotoxicity) by regulating the *prg* genes of the invasion-protein-export system (Galan, 1996), and mediates resistance to the anti-microbial peptides within the macrophage (Fields *et al.*, 1986; Miller *et al*, 1989a).

Disruption of many genes involved in the *S. enterica* infection process results in a decrease in virulence (Table 1.2), and as will be discussed in the following section, such genes have potential in the development of prophylactic therapies to diseases caused by *S. enterica*.

Table 1.2 Summary of some of the genetic determinants involved in *S. enterica* virulence

Gene or Region	Function	Role in Infection	Cs[a]	Attenuation [b]	Reference
galE	O-antigen (LPS) formation	Protection from complement, entry into epithelial cells	18	>100, 000-fold (o)[c]	Collins *et al.* (1991)
SPI1	Invasion-protein-export system	Entry into epithelial cells (macrophages?), cytotoxicity to macrophages	63	>50-fold (o)	Galan and Curtiss (1989)
SPI2	Invasion-protein-export system	Entry into epithelial cells? Cytotoxicity to macrophages?	40	1, 00-fold (o) 1, 000-fold (i.p.)[d]	Shea *et al.* (1996)
sifA	*Salmonella* induced filaments	Replication in epithelial cells	42	>1, 000-fold (o)	Stein *et al.* (1996)
phoPQ	Regulates genes in response to acidity or low Mg^{2+}	Regulates genes of the invasion-export system, regulates defence against anti-microbial peptides in macrophage	27	>10, 000-fold (o)	Miller *et al.* (1989a)
sapA	Component of an anti-microbial peptide uptake system	Defence against anti-microbial peptides in the macrophage	37	1, 000-fold (o) 10, 000-fold (i.p.)	Groisman *et al.* (1992)
ompR	Regulates genes in response to osmolarity	Protects bacteria from gut environment, cytotoxicity to macrophages	76	10, 000-fold (o) 100, 000-fold (i.v.)[e]	Chatfield *et al.* (1991)
fur	Regulates genes in response to iron-limitation or low pH	Defence against pH and scavenging for iron within the macrophage	17	<50-fold (i.p.)	Garcia-del Portillo *et al.* (1993a)
oxyR	Regulates genes in response to hydrogen peroxide	Defence against oxidative stress	90	-	Christman *et al.* (1985)
recA	DNA recombination repair enzyme	Protects DNA from oxidative stress	62	>10, 000-fold (o)	Buchmeier *et al.* (1993)
htrA	Degrades heat shock damaged proteins	Protects cells from oxidative stress	5	>10, 000-fold (o)	Chatfield *et al.* (1992b)
rpoS	Alternative sigma factor that regulates genes under starvation stress	Scavenging of nutrients, resistance to multiple stresses, regulation of the *Salmonella* virulence plasmid	64	10, 000-fold (o) 100, 000-fold (i.p.)	Coynault *et al.* (1996)

(a) Chromosomal map position in centisomes (Cs), (Chromosome split into 100 centisomes)
(b) Level of attenuation of the bacteria in a murine model, after disruption or mutation of the gene, compared to the virulent parental strain of *S. enterica* serovar Typhimurium administered orally (c), intraperitoneally (d) or intravenously (e).

1.4 THE DEVELOPMENT OF VACCINES TO *S. ENTERICA*

Diseases caused by *S. enterica* infection are a global health problem. On a global scale, typhoid fever, caused by *S. typhi*, remains the most serious *S. enterica*-related illness. This is especially true in the developing world, where it results in 600, 000 deaths annually (Ivanoff *et al.*, 1994). In addition, non-typhoidal *S. enterica*-related disease accounts for 1.3 billion incidences of human acute gastro-enteritis in the world each year (Ivanoff *et al.*, 1994). The likelihood of disease is worsened by the poor sanitation and health care conditions many people face, especially in developing countries (Bloom, 1989). In addition, infection of animals by *S. enterica* is also common and not only has economic consequences, but is believed to be a major factor in the spread of disease through the food chain (Maurice, 1994). For example, it has been estimated that 57% of cattle and 70% of chickens in the USA are carriers of *S. enterica* (Jay, 1992). Unfortunately, although antibiotics have proved useful in combating *S. enterica* infection in both humans and animals (especially in the developed world), there is a growing concern over the development of antibiotic resistance which, in addition to the cost factor, makes antibiotic therapy impractical (Cohen and Tauxe, 1986; Pang *et al.*, 1995). There is a major need therefore, to develop effective prophylactic therapies to minimise or eliminate *S. enterica*-related disease. This view has stimulated attempts to develop vaccination strategies, especially against typhoid fever, to combat infection by *S. enterica.*

1.4.1 Vaccines composed of inactivated whole cells

The earliest vaccines used to combat typhoid fever consisted of heat and phenol inactivated *S. typhi* cells. Although moderately efficacious, these vaccines caused adverse reactions including malaise and fever in around 25-40% of all subjects and required multiple doses for effective protection (Ivanoff *et al.*, 1994). However, effective immunity to *S. enterica* requires the development of a strong cell-mediated response, and one disadvantage of this latter type of vaccine is that dead cells are poor inducers of cell-mediated immunity (Collins, 1974). As a result, protection is often short-lived (Collins, 1974; Chatfield *et al.*, 1992a).

1.4.2 Vaccines composed of live attenuated bacteria

Recent vaccine development has focused on attenuating live bacteria, as these persist in the host long enough to stimulate protective immunity yet which are attenuated to the level where they do not produce overt disease (Collins, 1974; Chatfield *et al.*, 1992a; Ivanoff *et al.*, 1994). Although most of the developments in live, attenuated *S. enterica*-related vaccines have addressed the problem of typhoid fever, they also have a wider potential in controlling diseases caused by non-typhoidal *S. enterica*, especially those which relate to animals where antibiotic resistance is particularly rife (Cohen and Tauxe, 1986; Hassan and Curtiss, 1994).

One of the earliest live attenuated vaccine strains to be tried was a *galE* mutant of the *S. typhi* strain, Ty21a, which was produced by nitrosoguanidine mutagenesis (Germanier and Furer, 1975). This strain lacks an essential enzyme for the production of a complete LPS molecule and such a mutation attenuates *S. typhimurium* in mice (approximately 10^5-fold) (Collins *et al.*, 1991). However, subsequent investigations indicated that the *galE* mutation was not the attenuating factor in Ty21a (Hone *et al.*, 1988). Ty21a probably contains multiple lesions (because of the way it was generated), which largely remain undefined. Importantly, it appears to be defective in the expression of RpoS and such a mutation has been shown to have an attenuating influence in *S. typhimurium* (Robbe-Saule *et al.*, 1995; Coynault *et al.*, 1996). Nevertheless, Ty21a remains the most widely used vaccine at present and it has been shown to provide protection in 60% of individuals for up to 7 years, with very little adverse reaction (Ivanoff *et al.*, 1993). However, this vaccine normally requires up to four doses to ensure protective immunity and in some cases it has been shown to be ineffective (Plotkin and Bouveretlecam, 1995).

Direct use of *S. typhi* for vaccine design has been hampered by the fact that humans are its only host, therefore most research has focused on *S. typhimurium* because of its amenability to genetic manipulation and, because it produces a comparable typhoid-like disease in susceptible mice (although they lack symptoms of diarrhoea). Indeed, several genetic lesions that attenuate *S. typhimurium* in the murine model have been shown to be effective in attenuating *S. typhi* in humans. However, because

of the nature of undefined genetic lesions in Ty21a, attempts have been made to construct strains of various *S. enterica* serovars with defined attenuating lesions. Multiple lesions are also more desirable than a single lesions as additional mutations reduce the likelihood that the vaccine strain can revert to full virulence (Curtiss and Kelly, 1987; Miller, 1993).

The design of attenuated bacterial vaccine strains has particularly profited from studies which investigate factors required for the virulence and propagation of *S. typhimurium* in the infection of mice. For example, studies on auxotrophic mutants such as *aro* (Hosieth and Stocker, 1981), or mutants of global regulators, such as *cya* and *crp* (which regulate many genes involved in the metabolism of carbohydrates (Curtiss and Kelly, 1987)), *phoPQ* (Miller *et al.*, 1990), *ompR* (Dorman *et al.*, 1989; Chatfield *et al.*, 1991), and *rpoS* (Coynault *et al.*, 1996) have identified attenuating lesions of potential use for vaccine development.

Most of the recent developments in the use of live attenuated bacteria, have focused on *S. typhimurium aro* mutants which were shown by Hosieth and Stocker (1981) to be attenuated approximately 10^6-fold, in comparison to a parental strain, when injected intraperitoneally into mice. These mutants are unable to produce para-amino benzoic acid (PABA) or 2,3-Dihydroxybenzoic acid, essential compounds for producing folates, ubiquitin, aromatic amino acids and enterochelin. Furthermore, these precursor compounds are not present in mammalian tissues and this results in a reduction in the rate of growth within the mammalian host (Hosieth and Stocker, 1981; Ivanoff *et al.*, 1994). Nevertheless, although these bacteria are attenuated, they are still capable of reaching the reticuloendothelial system and persist in the host long enough to induce protective immunity.

An *S. typhi* vaccine (CVD908) has been produced which carries defined lesions in both the *aroC* and *aroD* genes (Tacket *et al.*, 1992). Clinical trials have indicated it is 100-fold more immunogenic than Ty21a and the number of doses required is less, with good protection occurring even after a single dose (Ivanoff *et al.*, 1994; Pang *et al.*, 1995). However, in a number of cases this vaccine resulted in symptoms of typhoid fever and this has prompted the introduction of additional lesions in order to

try and reduce the reactogenicity (Miller *et al.*, 1993; Ivanoff *et al.*, 1994). These additional mutations have included *phoPQ* (Hohmann *et al.*, 1996), *purA* (needed for purine metabolism)(Levine *et al.*, 1987), *htrA* (Chatfield *et al.*, 1992b) and *rpoS* (Coynault *et al.*, 1996). However, addition of either the *phoPQ* or *pur* mutations in the *aro* strain resulted in over-attenuation and highlighted the need to maintain a careful balance between attenuation and virulence when constructing genetically defined vaccines.

Vaccines that use live attenuated *S. enterica* cells as carriers of heterologous antigens

Live, attenuated *S. enterica* strains have been shown to persist within their hosts and to induce good immune responses. This makes them ideal carriers for foreign antigens, providing a means for inducing protective immune responses to both *S. enterica* and the expressed antigen. This strategy has particular appeal because it may be possible to generate immunological protection to multiple pathogens with a single vaccine. *S. typhimurium aro* mutants have been used to present antigens to the immune system to provide varying degrees of protection against *Leishmania* and malarial antigens, portions of cholera and diphtheria toxins and viral coat proteins (Chatfield *et al.*, 1992a; Gonzalez *et al.*, 1994; Verma *et al.*, 1995; Chabalgoity *et al.*, 1996; Karem *et al.*, 1997).

One problem however, is the stability of the system for antigen expression in the host. Plasmids have been used to express the foreign antigen but these can be rapidly lost in the absence of selection by the dividing bacteria. For example, Cardenas and Clements (1993) demonstrated that over 99% of *S. typhimurium* cells injected into mice lost their expression plasmid within 24 hours. Attempts have therefore been made to increase the maintenance of the plasmid (Galan *et al.*, 1990). One study stabilised the maintenance of the expression vector by co-expressing the product of the *asd* gene from the plasmid together with the antigen of interest in an attenuated *S. typhimurium asd* mutant (Galan *et al.*, 1990). Mutants of *asd* are unable to make the cell wall component diaminopimelic acid, which is involved in cross-linking the peptidoglycan of the bacterial cell wall, and therefore there is strong selection for the

maintenance of the plasmid. An alternative method to stabilise the expression of the foreign antigen has been to incorporate it into the chromosome. However, this has the disadvantage of lowering the copy number which in turn reduces the ability of the antigen to stimulate the immune response (Andersen *et al.*, 1996; Verma *et al.*, 1995).

1.4.3 Vaccines composed of chemically defined subunits

The Vi polysaccharide of *S. typhi*

Although cell-mediated immunity has been suggested as essential to protection against infection (Collins, 1974; Mastroeni *et al.*, 1993) at least one vaccine currently tested depends solely on humoral immunity. Virulent isolates of *S. typhi* are surrounded by a polysaccharide capsule, consisting of a polymer of galacturonic acid, and loss of this capsule has been associated with loss of virulence (Plotkin and Bouveretlecam, 1995). The purified capsular polysaccharide (Vi polysaccharide) has been tried in vaccine trials and as little as a single dose of 25 μg was found to result in 65% protection for up to 3 years. Moreover, it produced only mild adverse reactions (Ivanoff *et al.*, 1994; Plotkin and Bouveretlecam, 1995). However, since this vaccine is specific for the Vi antigen of *S. typhi* it is unlikely to have a broader use for general protection against serovars of *S. enterica.*

Polypeptide subunit vaccines

Perhaps the ideal vaccine would consist of defined immunogenic polypeptides because these would be less likely than whole cells to induce adverse reaction (Horwitz *et al.*, 1995). Moreover, there is concern that injection of live attenuated *S. enterica* cells may result in temporary immunological unresponsiveness to heterologous antigens and this could provide a window of opportunity for infection by other pathogens (Al-Ramandi *et al.*, 1992). However, there is a paucity of data relating to the effectiveness of polypeptides in inducing protective immunity, possibly as a consequence of the pursuit of vaccines involving attenuated bacterial strains. Nevertheless, the reasons why live bacterial vaccines are believed to be so

effective in inducing cell-mediated immunity has been attributed largely to the production of proteins by *S. enterica*, in response to the host environment, which are identified by T_H cells (Kagaya *et al.*, 1992). It is likely then, that a combination of proteins capable of stimulating T_H cells could be used as the basis of a protective vaccine. In support of this, a number of *S. typhimurium* polypeptides have been shown to elicit DTH and antibody responses, when appropriately administered, indicating that specific proteins may be able to generate some level of protective immunity. Indeed, the injection of *S. typhimurium*-derived porins or a hydrogen peroxide-inducible catalase (KatG) into mice was shown to provide some degree of protective immunity against subsequent challenge with a virulent strain of *S. typhimurium* (Udhayakumar and Muthukkaruppan, 1987; Kagaya *et al.*, 1992). Such an approach obviously warrants further investigation.

1.5 AIMS OF THE PRESENT STUDY

1.5.1 Background

Macrophages play a critical role in defence against infection of the host by *S. enterica* and the ability of *S. enterica* to survive within macrophages is essential for virulence (Fields *et al.*, 1986). Macrophages are equipped with an arsenal of oxygen-dependent and -independent anti-microbial effector mechanisms (Adams and Hamilton, 1984; Foster and Spector, 1995). Thus, genes which are expressed within the macrophage may promote the survival of *S. enterica* and have a role in virulence.

Using a Mu*dlux* transcriptional reporter system, Francis and Gallagher (1993) identified a *S. typhimurium* hydrogen peroxide-inducible locus which was expressed upon contact and within macrophages. Further studies demonstrated that this was the OxyR-regulated locus *ahpCF* (alkyl hydroperoxide reductase) (Francis, PhD Thesis, 1993). AhpCF prevents lipid peroxidation of the cell membrane by converting alkyl hydroperoxides to their corresponding non-toxic alcohols (Jacobson *et al.*, 1989; Tartaglia *et al.*, 1990). Subsequently, in an independent study, a second OxyR regulated gene, *dps* (DNA-binding protein of starved cells), was shown to be induced within the macrophage environment indicating *in vivo* expressed genes that defend

against hydrogen peroxide, particularly the OxyR regulon, may have an important role in the virulence of *S. enterica*.

1.5.2 Project aims

Regulation of the *ahp* locus

The genes of the OxyR regulon are induced after exposure of *S. typhimurium* to hydrogen peroxide. However, a number of OxyR-regulated genes including *dps*, *katG* and *gorA* have been demonstrated to show OxyR-independent regulation via the stationary phase sigma factor, RpoS (Altuvia *et al.*, 1994; Ivanova *et al.*, 1994; Becker-Hapak and Eisenstark, 1995). Moreover, *S. aureus* and *B. subtilis* homologues of *ahpC* were recently demonstrated to be induced under osmotic stress (Armstrong-Buisseret *et al.*, 1995; Antelmann *et al.*, 1996). The ability of OxyR-regulated genes to be regulated under different conditions might indicate that their functions may be required for additional roles other than simply defence against the respiratory burst of macrophages. However, the role of the stationary phase sigma factor and osmotic stress in the regulation of the *S. typhimurium ahpCF* had not been reported previously, and so constituted part of the present study.

The role of the *ahp* and *oxyR* loci in the virulence of *S. enterica*

Vaccine development for controlling *S. enterica* infection has prospered from investigations into the molecular genetic basis of *S. enterica* virulence (Chatfield *et al.*, 1992a). Since the *ahp* locus is expressed during interaction with macrophages it was perceived that it may play a vital role in the virulence of *S. typhimurium*. In addition, the demonstration that a second OxyR-regulated gene, *dps* (Valdiva *et al.*, 1996), was induced within the macrophage may implicate OxyR as an important regulator of a number of virulence genes. The role of these loci upon the virulence of *S. enterica* was also assessed as part of this study.

The immunological properties of the AhpC protein

The effectiveness of vaccines composed of live attenuated *S. enterica* cells to stimulate strong protective immunity has been attributed to the presence of immunogenic proteins expressed in response to the host environment *i.e. in vivo*-expressed antigens (Kagaya *et al.*, 1992). However, very little information currently exists about the immunological properties of the majority of *S. enterica* polypeptides. Because the *ahp* locus had previously been shown to be expressed during macrophage interaction (Francis and Gallagher, 1993), it was perceived that, potentially, the Ahp proteins could be recognised by the host immune system. The immunological properties of the AhpC protein were therefore investigated as part of this study.

CHAPTER 2

Materials and Methods

2.1 MATERIALS

2.1.1 Enzymes, isotopes and chemicals

Enzymes were obtained from Boehringer Mannheim (Sussex, UK). Agarose was purchased from FMC BioProducts (Rockland, USA); antibiotics were supplied by Sigma Chemical Company Ltd. (Dorset, UK); Isopropyl-β-D-thiogalactoside (IPTG) and 5-bromo-4-chloro-3-indoyl-β-D-galactoside (X-gal) were obtained from Boehringer Mannheim. Rabbit anti-mouse antibodies were supplied by Sigma Chemical company Ltd. Other standard laboratory chemicals were supplied from Sigma Chemical company Ltd., BDH Chemicals Ltd. (Dorset, UK), and Fisons Scientific Equipment (Leicestershire, UK). Hybond-N nylon membrane was purchased from Amersham International plc. (Buckinghamshire, UK) and nitro-cellulose was obtained from Schleicher and Schuell (Dassel, Germany). X-ray film (AGFA Curix RP1, 100 NIF) was supplied by H. A. West (Edinburgh, UK). Stabilised solutions of [α-^{32}P]-dCTP (3, 000 Ci mmol^{-1}) and [^{35}S]-methionine (800 Ci mmol^{-1}) were purchased from Amersham International plc., and NEN Du Pont Ltd. (Hertfordshire, UK), respectively.

Synthetic oligonucleotides were purchased from Oswel DNA Service (Edinburgh, UK) or Perkin and Elmer Ltd. (Cheshire, UK). The oligonucleotides used in this study are listed in Table 2.1.

2.1.2 Bacterial strains and plasmids

Bacterial strains and plasmids and their sources are shown in Tables 2.2 and 2.3.

Primer Name	Primer Sequence	Comments and usage
G7858 (198-214)	5'- GCGGATCCCAAAAACCAGGCGTTCA -3' *Bam*HI	PCR of the *S. typhimurium ahpCF* locus (5'-3')
G7859 (2524-2508)	5'- CGAAGCTTGGTGCGAATCAGATAAT -3' *Hin*dIII	PCR of the *S typhimurium ahpCF* locus (3'-5')
N5138 (156-179)	5'- GGAATTCCATATGTCCTTAATTAA -3' *Nde*I	PCR of the *S typhimurium ahpC* gene (5'-3')
N5139 (761-748)	5'- GCGGATCCAACGCAGCTATGGC -3' *Bam*HI	PCR of the *S. typhimurium ahpC* gene(3'-5')
G8493 (5270-5245)	5'-GCGATATC AAGCTT ACGCGTCGTAGC *Eco*RV *Hin*dIII *Mlu*I ACCAGGCGTTTAAGGGCAC -3'	PCR of the pBR325 *cml* cassette (3'-5')
H1286 (2288-2316)	5'- GGGATATC AAGCTT GTTAACCGTCTA *Eco*RV *Hin*dIII *Hpa*I AGAAACCATTATTATCATG -3'	PCR of the pBR325 *cml* cassette (5'-3')
M6184 (1353-1335)	5'-CCGGATCCTTACTCGCGGAACAGCG-3' *Bam*HI	PCR of the *S. typhimurium rpoS* gene (3'-5')
MO917 (1-18)	5'-CCAAGCTTATGTTCCGTCAAGGGAT-3' *Hin*dIII	PCR of the *S typhimurium rpoS* gene (5'-3')
OXYR1 (167-183)	5'-CCGAATTCATCGCCATGACTATCG-3' *Eco*RI	PCR of the *E. coli* or *S. typhimurium oxyR* gene (5'-3')
OXYR2 (1238-1211)	5'- CCAAGCTTATATCGGTCAGGCGATT -3' *Hin*dIII	PCR of the *E. coli oxyR* gene (3'-5')
OXYR3 (1238-1211)	5'- CCGAATTCATATCGGTCAGGCGATT -3' *Eco*RI	PCR of the *E. coli* or *S. typhimurium oxyR* gene (3'-5')
T7 (736-720)	5'-AATACGACTCACTATAG-3'	Sequencing of the *S. typhimurium oxyR gene*

Table 2.1 Primers used during the course of the present study

Shown is a list of primers (oligonucleotides) that were used during the course of this investigation. The numbers shown in parentheses refer to the position at which each of the primers lay with respect to the published sequence from which they are derived; *ahpCF* (Tartaglia *et al.*, 1990); *oxyR* (Christman *et al.*, 1989); *rpoS* (Prince *et al.*, 1994); *cml* (Bolivar *et al.*, 1988).

Table 2.2 Table showing the *E. coli* and *S. typhimurium* strains, and their derivatives, which were used during the course of this study

Bacterial Strain	Genotype	Source/Reference
Escherichia coli		
BL21(DE3)	*hsdS gal* (λ*c*Its857 *ind*1 *S*am7 *nin*5 *lacUV5*-T7 gene 1), pLysS	Studier *et al.* (1990)
JM101	*supE thi-1* Δ(*lac-pro*AB) F'[*tra*D36 *proAB*$^+$ *lac*I^q ZΔ*M15*]	Yanisch-Perron (1985)
DH5α	*supE*44 Δ*lacU169* (ϕ80 *lacZ*Δ*M15*) *hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	Hanahan (1985)
K090	*hsdR2* P_{BAD}-*groE*, *kan*	N. McClennan
MPG480	BL21(DE3), pET-19b	This thesis
MPG481	BL21(DE3), pPDT14	This thesis
Salmonella typhimurium		
CH23	*recA*1	C. Higgins
CH1350	LT2 *opp250 ppB84*::Mu d1-8 *ompR1009*::Tn*10*	Dorman *et al.* (1989)
CH1701	LT2 *osmZ6*::Tn*10*	Hulton *et al.* (1990)
SL1344	*his*	Hosieth and Stocker (1981)
SF1005	ATCC 14028s *rpoS*::*bla*	Fang *et al.* (1992)
TA4100	LT2 *oxyR1*	Christman *et al.* (1985)
TA4108	LT2 *oxy*Δ2[oxyΔ(*oxyR argH*) 2]	Christman *et al.* (1985)
TT15276	MS1868 *melAB396*::Mu*d*P	Benson and Goldman (1992)

Table 2.2 (continued)

Bacterial Strain	Genotype	Source/Reference
MPG203	SL1344 *ahp*::Mu*dlux*	Francis and Gallagher (1993)
MPG350	LT2 *ahp*::Mu*dlux*	This thesis
MPG352	TA4108 *ahp*::Mu*dlux*	This thesis
MPG470	SL1344 *rpoS*::*bla*	This thesis
MPG471	MPG203 *rpoS*::*bla*	This thesis
MPG473	SL1344 *ahp*::*cml*	This thesis
MPG474	MPG203 *ompR1009*::Tn*10*	This thesis
MPG475	MPG203 *osmZ6*::Tn*10*	This thesis
MPG477	MPG352 pPDT3	This thesis
MPG478	MPG352 pBAD18	This thesis
MPG479	SL1344 *aroA554*::Tn*10*	This thesis
MPG482	CH23 pPDT15	This thesis
MPG484	SL1344 *oxyR*::*kan*	This thesis
MPG485	MPG484 pPDT3	This thesis
MPG486	MPG484 pPDT4	This thesis
MPG487	MPG484 pBAD18	This thesis
MPG488	SL1344 pBADLAC	This thesis

Table 2.3 Plasmids

Table showing all the plasmids and their derivatives mentioned in this thesis

Plasmid	Relevant Features	Source Reference
pBR322	pMB1 replicon, Amp^R, Tet^R	Bolivar *et al.*(1977)
pBR325	pMB1 replicon, Amp^R,Tet^R, Cml^R	Bolivar *et al.* (1978)
pBAD18	pMB1 replicon, Amp^R, contains the arabinose inducible P_{BAD} promoter	Guzman *et al.* (1995)
pBADLAC	pBAD18 derivative containing the *E. coli lacZ* gene on a 4.0 kb *Sma*I-*Sna*BI fragment (from pRS551)	N. McClennan
pGEM-T	ColE1 replicon, Amp^R, *lacZ*α	Promega
pLysS	Cml^R, pACYC184 derivative containing T7 lysozyme gene	Studier *et al.* (1990)
pET-19b	ColE1, Amp^R, P_{T7}	Novagen
pUC4-K	pUC19 derivative carrying a *kan* cassette	Pharmacia
pGT3270	Amp^R pJF118EH derivative carrying 2.1 kb *Eco*R1-*Hin*dIII *groESL* fragment from *E coli*	McClennan *et al.* (1993)
pPDT3	pBAD18 derivative containing a 1.1 kb *Eco*RI-*Hin*dIII fragment of the *E. coli oxyR* gene, amplified using primers OXYR1 and OXYR2	This thesis
pPDT4	pBAD18 derivative containing a 1.1 kb *Eco*RI-*Hin*dIII fragment of the *oxyR* gene from *S. typhimurium* strain TA4100, amplified using primers OXYR1 and OXYR3	This thesis
pPDT5	pBR322 derivative containing a 2.3 kb *Bam*HI-*Hin*dIII fragment carrying the *S. typhimurium ahpCF* locus, amplified using primers G7858 and G7859	This thesis

Table 2.3 (continued)

Plasmid	Relevant Features	Source Reference
pPDT6	pPDT5 derivative containing a 0.98 kb *Mlu*I-*Hpa*I fragment carring the *cml* cassette from pBR325, amplified using primers G8493 and H1286	This thesis
pPDT7	pGEM-T derivative containing a 1.1 kb *Eco*RI fragment of the *S. typhimurium* SL1344 *oxyR* gene, amplified using primers OXYR1 and OXYR3	This thesis
pPDT8	pPDT7 derivative containing the 1.3 kb *Hinc*II fragment from pUC4K, carrying a *kan* cassette, subcloned into the *sma*I site of *oxyR*	This thesis
pPDT14	pET-19b derivative containing a 0.6 kb *Nde*I-*Bam*HI fragment carrying the *ahpC* gene from *S. typhimurium*, amplified using primers N5138 and N5139	This thesis
pPDT15	pBR325 derivative containing an *Eco*RI fragment of approximately 6.5 kb, subcloned from Mu*d*-P22 DNA, obtained from *S. typhimurium* strain TT15276	This thesis

2.1.3 Solutions

All solutions were made up in dH_2O and sterilised by autoclaving at 15 pounds per square inch for 20 minutes prior to use. Heat labile components were separately filter sterilised and added to the main solution after the latter were autoclaved.

Tris. Cl:
Tris base (tris [hydroxymethyl] aminomethane) was dissolved to the desired molarity in dH_2O and the pH was adjusted to the required value by addition of concentrated HCl.

EDTA:
EDTA (ethylenediaminetetraacetic acid, di-sodium salt) was dissolved in dH_2O to a concentration of 0.5 M and was adjusted to pH 8.0 by addition of NaOH.

TE buffer:
1 litre of buffer contained 10 mM Tris. Cl (pH 8.0) and 1 mM EDTA dissolved in dH_2O.

10x TBE buffer:
1 litre of buffer contained 0.9 M Tris. Cl (pH 7.5), 0.9 M Boric acid and 0.02 M EDTA dissolved in dH_2O.

20x SSC:
1 litre of 20x SSC contained 3 M NaCl and 0.3 M tri-sodium citrate dissolved in dH_2O.

Sodium Acetate:
Sodium acetate was dissolved in dH_2O to a final concentration of 3 M and the pH adjusted to 5.0 with acetic acid.

Ethidium Bromide:

Ethidium bromide was dissolved as a stock solution of 10 mg ml^{-1} in dH_2O and stored at room temperature in the dark.

6x DNA loading buffer:

6x loading buffer for nucleic acid gel electrophoresis consisted of a 40% (w/v) sucrose solution with 0.25% (w/v) bromophenol blue.

PBS:

Dulbecco's phosphate buffered saline (PBS), pH 7.4, was prepared by dissolving 1 tablet of PBS (800 mg NaCl, 20 mg KCl, 144 mg Na_2HPO_4, 24 mg KH_2PO_4 in 100 ml.

Antibiotic Solutions:

All antibiotic solutions were used as indicated in Table 2.4. Aqueous solutions were filter sterilised prior to use, and all were stored at -20°C.

Table 2.4 Antibiotics used during this investigation

ANTIBIOTIC	SOLVENT	STOCK CONCENTRATION	FINAL CONCENTRATION
Ampicillin	H_2O	10 mg ml^{-1}	50 μg ml^{-1}
Chloramphenicol	Ethanol	34 mg ml^{-1}	5 μg ml^{-1}
Kanamycin	H_2O	10 mg ml^{-1}	50 μg ml^{-1}
Tetracycline	70% Ethanol	5 mg ml^{-1}	10 μg ml^{-1}

Protein Standard	Approximate Molecular Weight (Da)
α-Lactalbumin	14, 200
Trypsin Inhibitor	20, 100
Trypsinogen	24, 000
Carbonic Anhydrase	29, 000
Albumin (egg)	45, 000
Albumin (bovine)	66, 000

Table 2.5a Molecular weight markers

Markers (MW-SDS-70L, Sigma) were used as a mixture of standards for SDS-PAGE.

Protein Standard	Native Molecular Weight (Da)	Apparent Molecular Weight (Da)
β-Galactosidase	116, 000	120, 000
Pyruvate Kinase	58, 000	75, 200
Trisosephosphate Isomerase	26, 600	35, 200

Table 2.5b Pre-stained molecular weight markers

Markers (SDS-7B, Sigma) were used as a mixture of standards for SDS-PAGE.

2.1.4 Molecular weight standard markers

Molecular weight standard markers (normal and pre-stained) for SDS-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of proteins are indicated in Tables 2.5a and b.

2.1.5 Media

Luria-Bertani (LB) medium and agar:
LB media consisted of Difco bacto-tryptone (10 g), Difco yeast extract (5 g) and NaCl (10 g), unless otherwise stated, dissolved in 1 litre of dH_2O. The pH was adjusted to pH 7.2 with 5 N NaOH and the solution sterilised by autoclaving. LB agar was formed by adding 15 g l^{-1} Difco bacto-agar.

LC top agar:
LC top agar consisted of Difco bacto-trptone (10 g), Difco yeast extract (5 g), NaCl (5 g) and Difco bacto-agar (7 g) dissolved in 1 litre of dH_2O. The pH was adjusted to pH 7.0 and the solution sterilised by autoclaving.

SOC medium:
SOC media was made from Difco bacto-tryptone (20 g), Difco yeast extract (5 g), NaCl (10 mM), KCl (2.5 mM), $MgCl_2$ (10 mM), $MgSO_4$ (10 mM) and glucose (20 mM) dissolved in 1 litre of dH_2O. The solution was sterilised by autoclaving.

Spitzizen Minimal Medium:
Spitzizen minimal medium (Spitzizen, 1958) consisted of 300 ml dH_2O, 80 ml (4x) Spitzizen Salts (7 g K_2HPO_4, 10 g $(NH_4)SO_4$, 3 g KH_2PO_4, 5 g sodium citrate and 1 g $MgSO_4.7\ H_2O$ dissolved in 1 litre of water), 10 ml 20% (w/v) glucose and 0.5 ml thiamine B1 (1 mg ml^{-1}).

2.2 METHODS

2.2.1 Manipulations of bacteria and phage

Growth of bacterial cultures

Liquid cultures of *S. typhimurium* and *E. coli* were prepared by inoculating a 5 ml volume of LB broth (plus a suitable antibiotic if required) with a single bacterial colony. Cultures were grown at 37°C with shaking (300 rpm) unless otherwise stated. Larger cultures were prepared by diluting an overnight culture 100-fold in conical flasks, with an overall capacity 5-10 fold greater than that of the culture volume, under the growth conditions stated above.

Storage of bacterial cultures

For long term storage of bacterial cultures, a 1 ml volume of an overnight culture was mixed with 75 µl of DMSO and stored in a sterile vile at -80°C. A culture was recovered by streaking onto an agar plate, containing antibiotic if necessary. After overnight incubation at the correct temperature a single colony was picked and a fresh culture propagated. For short term storage (4-6 weeks) bacteria were stored as streaks on agar plates at 4°C.

Measurement of bioluminescence from strains bearing the Mu*dlux* element following exposure to hydrogen peroxide stress

S. typhimurium strains carrying the Mu*dlux* bioluminescent reporter system in the *ahp* locus were tested for light emission in response to exposure of cells to hydrogen peroxide. Overnight cultures were grown in LB medium containing the appropriate antibiotic(s) at 37°C with shaking. Cultures were then diluted into fresh media, typically at a cell density of 10^5 cells ml^{-1}, and grown for two hours at 30°C to re-establish exponential growth (for modifications see results section). Samples were then treated with 100 µM hydrogen peroxide (Sigma) and light production was

recorded at appropriate intervals in a 96-well plate-reading luminometer (Labsystems Luminoskan), as relative light units per second (RLU s^{-1}). Samples were monitored at 30°C for the duration of the experiment.

Measurement of bioluminescence from strains bearing the Mu*dlux* element following exposure to osmotic stress

S. typhimurium strains bearing the Mu*dlux* light reporter system in the *ahp* locus were examined for light emission in response to osmotic upshift. Cultures to be tested were grown at 37°C with shaking in LB medium containing the appropriate antibiotic. In order to test the osmotic inducibility of the *ahp*::Mu*dlux* strain and its derivatives, an LB medium was used containing 1 g l^{-1} NaCl, instead of 10 g l^{-1}, so that the osmolarity of the medium could be raised to the required level. Overnight cultures were diluted to a density of 10^5 cells ml^{-1} into this LB medium and pre-incubated for a further 2 hours at 30°C to re-establish exponential growth, prior to osmotic upshift. Osmotic stress was then brought about by adding NaCl to a final concentration of 0.3 M, unless otherwise stated. Cells were then recorded for light emission in a 96-well plate-reading luminometer, as relative light units per second (RLU s^{-1}), at 30°C for the duration of the experiment. For cells bearing the plasmid pPDT3, this protocol was modified such that after 30 minutes of pre-incubation, arabinose was added to a final concentration of 0.5 % (w/v), and samples were incubated a further 1.5 hour before being subjected to the osmotic stress.

β-galactosidase enzyme assay

The expression of the *lacZ* gene was measured using a β-galactosidase enzyme assay, as essentially described by Miller (1972b). The strain of interest was diluted to approximately 10^5 c.f.u. ml^{-1} and grown at 37°C until the OD_{600} had reached 0.1-0.2. Assays were performed in 1 ml volumes in 1/4 ounce Bijou bottles. 0.5 ml of the culture was added to 0.5 ml of enzyme buffer (per litre: 0.06 M $Na_2HP0_4.7H_20$, 0.04 M $NaH_2P0_4.H_20$, 0.01 M KCl, 0.01 M $MgS0_4.7H_20$ and 0.05 M β-mercaptoethanol), 50 µl 0.1% SDS and 100 µl chloroform. The sample was then

vortexed for 30 seconds to permeabilise the cells. (If the culture density was greater than OD_{600} 0.1-0.2, the sample was diluted appropriately with LB immediately prior to proceeding with the assay). 200 µl of ONPG (o-nitrophenyl-β-D-galactoside; Sigma) (4 mg ml^{-1} in 0.1 M Mops, pH 7.0) was added to the sample and mixed by inversion. The sample was then incubated at 28°C until a visible yellow colour was observed. 0.5 ml of 0.5 M Na_2CO_3 was added to stop the reaction and the tube centrifuged at 20, 000 *g* for 30 seconds to remove cell debris (this measure would prevent any effect of light scattering due to suspended debris in subsequent spectrophotometric determinations). 1 ml of the cleared sample was then measured in a spectrophotometer at OD_{420} and β-galactosidase activity was calculated using the following equation:

$$1,000 \times (OD_{420} / t \times d \times OD_{600})$$

where t = the time (minutes) of incubation of the samples until a yellow colour appeared, d = the dilution factor (value is normally 1 unless sample was diluted prior to assaying). All tests were performed in triplicate and the values were expressed as Miller Units *i.e.* Enzyme units per cell mass (Miller, 1972b).

Determination of the sensitivity of *S. typhimurium* strains to peroxide stress using a disc inhibition assay

Disc inhibition assays were performed essentially as described by Christman *et al.* (1985). Cultures to be tested were grown overnight at 37°C with shaking in LB medium containing the appropriate antibiotic. For each strain, 100 µl volumes of culture were mixed with 3 ml of LC top agar (50°C) and poured onto an LB agar plate. Once the top agar had set, a 5 mm Whatman No. 4 cellulose disc was placed carefully onto the centre of the plate and 10 µl of 3% hydrogen peroxide (v/v in dissolved dH_2O) or 10 µl of 3% cumene hydroperoxide (v/v dissolved in DMSO) was dispensed ono the disc. Plates were then incubated at 37°C overnight and the zone of killing measured. Tests always included a virulent strain of *S. typhimurium*, SL1344, as a control.

Preparation of P22 phage lysates

S. typhimurium strains carrying the desired chromosomal alteration were cultured overnight in the presence of an appropriate antibiotic. A culture was diluted 100-fold into 10 ml of fresh LB medium and incubated until the OD_{600} had reached 0.2-0.3 10 µl of a P22 stock (titre 10^9 phage ml^{-1}) was added to the culture and the incubation was continued for at least 6 hours. 200 µl of chloroform ($CHCl_3$) was then mixed with the culture, by inversion, several times and the mixture was left for 2 hours at 4°C. The culture was then centrifuged at 3, 000 *g* for 15 minutes in a bench-top MSE Centaur 1 centrifuge to remove cell debris. The supernatant was transferred to a fresh universal bottle and a further 200 µl of $CHCl_3$ was added and the sample was mixed as before. The sample was then left at 4°C overnight and centrifuged to remove any further debris. The supernatant from this final spin, containing the P22 phage, was stored in a fresh universal bottle at 4°C with 100 µl $CHCl_3$ until further use.

Phage P22 titration

An appropriate host strain was cultured overnight in LB. 100 µl volumes of culture were dispensed into a set of 9 sterile micro-centrifuge tubes, and 100 µl volumes of a serially diluted phage lysate (typically diluted from 10^8-10^5 phage ml^{-1}) were added. Tubes were then incubated at 30°C for 30 minutes. 100 µl volumes of each tube were added to 3 ml of LC top agar (50°C) and poured onto LB plates. Controls containing P22 phage or cells only were also included. Plates were incubated overnight at 37°C and titres were calculated by enumerating the number of plaques formed at each dilution.

Phage P22 transduction

Phage P22 transduction was performed by the method of Roth (1970). Strains to be transduced were grown as overnight cultures in LB medium. 100 µl aliquots of the recipient strains were dispensed into 5 sterile micro-centrifuge tubes, and 100 µl of a

serially diluted P22 phage lysate (dilution range 10^9-10^5 phage ml^{-1}) was added to each. Tubes were mixed by inversion and incubated at 30°C for 30 minutes. The contents of each tube was then plated onto LB agar plates containing the appropriate antibiotic, and incubated overnight at 37°C. Controls containing P22 phage or cells only were also included. Following overnight incubation transductants were streaked several times on selective LB agar plates to remove residual P22 phage.

Preparation of a Mu*d*-P22 lysate

Mu*d*-P22 lysates used for mapping and isolating specific portions of the *S. typhimurium* chromosome, were prepared as outlined by Benson and Goldman (1992). The required Mu*d*-P22 lysogen was grown overnight in 5 ml LB medium containing chloramphenicol (40 μg ml^{-1}). The complete overnight culture was added to 25 ml of LB broth containing chloramphenicol and mitomycin C (final concentration 2 μg ml^{-1}) and was incubated overnight at 37°C with aeration. 3 ml of $CHCl_3$ was added and the flask was vigorously shaken for 5 minutes. The supernatant was then decanted off from the $CHCl_3$ and centrifuged in a Beckman J2-21 (JA-20 rotor) for 10 minutes at 8, 000 *g*, to remove cell debris. The pellet of phage particles was then resuspended in 1 ml of TE and phenol/chloroformed (see later) to extract the bacterial DNA.

2.2.2 Nucleic acid manipulation and detection methods

Agarose gel electrophoresis

DNA was resolved by agarose gel electrophoresis. The gel consisted of electrophoresis grade agarose (0.8% w/v in 1x TBE unless otherwise stated) containing 0.5 μg ml^{-1} ethidium bromide. DNA was prepared for loading with 1/6th volume of 6x loading buffer and electrophoresed at 100 V, in a 110 mm x 150 mm horizontal gel unless otherwise indicated, until suitable resolution had been obtained. Nucleic acid size was estimated in relation to the position of pre-digested *Hin*dIII phage Lambda DNA fragments (from Boehringer Mannheim) in the agarose gel.

DNA was visualised under UV light (λ=313 nm) and photographed using a Mitsubishi video copy processor.

Equilibration of phenol

Phenol (Rathburn Chemicals) was prepared by the addition of 0.1% (w/v) 8-hydroxyquinoline, followed by an equal volume 0.5 M Tris. Cl, pH 8.0. This solution was mixed by stirring for 15 minutes and the phenol and Tris. Cl phases were allowed to separate. The upper (Tris. Cl phase) was removed and an equal volume of 0.1 M Tris. Cl pH 8.0 was added to the phenol, and mixed as before. The upper phase was removed and the equilibration process was continued until the phenolic phase reached a pH above 7.8. The phenol was then dispensed in 10 ml volumes and stored with 1 ml 0.1 M Tris. Cl pH 8.0 in a light tight bottle at -20°C.

Phenol/Chloroform extraction of DNA

Phenol/chloroform extractions were performed to remove cell debris and proteins associated with DNA. To one volume of a DNA solution, an equal volume of equilibrated phenol was added and the solution was mixed vigorously with vortexing. The aqueous phase (containing the DNA) and the phenolic phase were separated by centrifugation at 20, 000 *g* for two minutes. The aqueous layer was then transferred to a fresh receptacle and one volume of $CHCl_3$ was added. The two phases were mixed and separated as before and the process was repeated. Finally, the aqueous layer was transferred to a fresh tube, leaving a small amount to prevent carry-over of the chloroform phase.

Ethanol precipitation of nucleic acid

In a micro-centrifuge tube, one tenth volume of sodium acetate (3 M, pH 5.0) was added per volume of DNA, followed by 2.5x this total volume of absolute ethanol. The solution was mixed thoroughly and left at -80°C for 15 minutes, after which the DNA was pelleted by centrifugation at 20, 000 *g* for 15 minutes. The pellet was then

washed with 1 volume of 70% ethanol, to remove salt, and centrifuged for a further 5 minutes at 20, 000 *g*. The pellet was then dried under vacuum and resuspended in dH_2O.

Restriction endonuclease digestion of DNA

0.1-20 μg of DNA was digested in dH_2O containing 1/10th volume of a 10x enzyme buffer (appropriate to the enzyme) and enzyme was added according to the manufacturers guidelines (normally 1 unit of enzyme per 1 μl). Digestion was allowed to proceed for 1-2 hours at 37°C (unless the manufacturer suggested otherwise) and overnight for chromosomal DNA. For double digests involving enzymes with different recommended buffers, DNA was first cleaved with the enzyme requiring the lowest salt buffer, after which the digest was optimised for the higher salt buffered enzyme.

Dephoshorylation of vector DNA

Plasmid vectors digested with restriction enzymes were treated with shrimp alkaline phosphatase (SAP) to aid cloning of inserts into the vector. The cut vector DNA was incubated at 37°C for 1 hour in dH_2O containing 1/10th volume of a 10x SAP buffer and 1 unit of SAP. The SAP was then inactivated by incubation at 65°C for 20 minutes, and the DNA was purified by phenol/chloroform extraction, followed by ethanol precipitation.

Recovery of DNA from agarose gels

The DNA fragment of interest was resolved on an agarose gel as described previously. A slice of gel containing the DNA was removed, weighed and purified using "Geneclean II" (BIO101). Briefly, 0.5 volumes of TBE modifier was added with 4-5 volumes of sodium iodide to the gel slice (where 1 g of gel is assumed to be equivalent to 1 ml). The gel slice was then melted at 45-55°C, after which 5 μl of a silica binding-matrix ("GLASSMILK") was added to the sample. The sample was

then mixed thoroughly and incubated on ice for 5 minutes, with further mixing every 1-2 minutes to ensure the "GLASSMILK" stayed in suspension. The silica, with bound DNA, was pelleted by spinning at 20, 000 *g* for 5 seconds and the supernatant was removed. The pellet was resuspended in 0.5 ml of "NEWWASH" and spun at 20, 000 *g* for 5 seconds. The supernatant was again discarded and the pellet was treated with "NEWWASH" twice more, as before. Finally, the pellet was resuspended in 10 µl of dH_2O and the sample was incubated at 50°C for 3 minutes, to release the DNA from the silica matrix. The sample was then spun at 20, 000 *g* for 30 seconds to pellet the "GLASSMILK" and the supernatant, containing the DNA, was transferred to a micro-centrifuge tube.

Ligation of DNA

Ligations were carried out in dH_2O containing 1/10th volume of 10x ligase buffer, using 10 units of T4 DNA ligase (Boehringer Mannheim) in a final volume of 10 µl. In this reaction mixture, DNA vector (50-100 ng) was incubated overnight at 15°C with 5-10 times excess DNA insert.

Polymerase Chain Reaction

The polymerase chain reaction (PCR) was used for amplification of DNA fragments. Primers specific for the region of DNA of interest were designed as required (Table 2.1 lists the primers used in this study). Reactions were performed in 100 µl volumes containing template DNA (10 ng), 2.5 µl of each primer (100 pM), 10 µl of a 10x PCR reaction buffer (Boehringer Mannheim), 8 µl of dNTP mix (containing 2.5 mM of each dNTP), 1 µl Taq DNA polymerase (2 U μl^{-1}) and dH_2O. 100 µl of sterile mineral oil was added to the surface of the reaction mixture to prevent evaporation during the PCR process. The PCR was carried out in a Techne "Gene E" thermal cycler dry-block.

Amplification of the DNA was performed over 30 cycles, each cycle consisted of the following three steps: Denaturation at 94°C for 1 minute, annealing at 55°C for

1 minute and elongation at 72°C for 2 minutes. At the end of the 30 cycles, a final single cycle of 72°C for 10 minutes was performed to complete the process. PCR reaction mixtures were then examined under agarose gel electrophoresis.

Small-scale preparation of *E. coli* plasmid DNA

E. coli cells carrying the plasmid of interest were grown overnight, with the appropriate antibiotic, and normally were incubated at 37°C with shaking. The plasmid DNA then was prepared by the "TELT" method of He *et al.* (1990). The culture was dispensed into a micro-centrifuge tube (1.5 ml volume) and centrifuged at 20, 000 *g* for 30 seconds to pellet the cells. The supernatant was discarded and the pellet was resuspended in 800 µl of TELT solution (50 mM Tris. Cl, pH 7.5; 62.5 mM EDTA, 2.5 M LiCl, 0.4 % [v/v] Triton X-100). 50 µl of a 100 mg ml^{-1} solution of lysozyme, made up in TELT, was also added and the tube was vigorously mixed and allowed to stand for 2 minutes at room temperature. The tube was incubated in a boiling water bath for 2 minutes and then was transferred to ice for 10 minutes, before being spun for 15 minutes at 20, 000 *g* to pellet cell debris. The supernatant was transferred to a fresh tube and 0.6 volumes of isopropanol was added. The sample was mixed by inversion and was centrifuged at 20, 000 *g* for 30 minutes to pellet the plasmid DNA. The supernatant was discarded and the pellet was washed in 70 % (v/v) ethanol, desiccated and resuspended in 50 µl of dH_2O.

Large-scale preparation of *E. coli* and *S. typhimurium* plasmid DNA

To obtain large amounts of very pure plasmid DNA, the protocols of Birnboim and Doly (1979) and Ish-Horowicz and Burke (1981) were employed.

Bacterial cultures carrying the plasmid of interest were grown overnight in 500 ml of LB media, with the appropriate antibiotic(s), at 37°C with shaking. Cells were pelleted at 4, 000 *g* in a Beckman J2-21 (JA-14 rotor) centrifuge and excess liquid was removed by inversion of the centrifuge tube for 5 minutes. The pellet was then resuspended in 18 ml of Solution I (50 mM glucose, 25 mM Tris. Cl, pH 8.0 and

10 mM EDTA) and was then treated with 1 ml of a 10 mg ml^{-1} lysozyme solution for 5 minutes at room temperature. 40 ml of Solution II (0.2 N NaOH and 1% [w/v] SDS) was then added, the solution was mixed by inversion several times, and the sample was left on ice for 5 minutes. After this time, 20 ml of ice-cold Solution III (5 M potassium acetate buffer, pH 4.8) was mixed thoroughly with the sample. The sample was left on ice for 15 minutes and was then centrifuged at 6, 000 *g* for 10 minutes at 4°C to pellet the cell debris. The supernatant was filtered through non-absorbent cotton wool, to remove unpelleted debris, into a fresh centrifuge pot and 48 ml isopropanol was added. The plasmid DNA was harvested by centrifugation at 6, 000 *g* for 10 minutes, the pellet was rinsed with 70% (v/v) ethanol and was air dried by inversion of the centrifuge pot, to remove excess liquid. The dried pellet was then resuspended in 9 ml of TE and 10 g of CsCl was added, followed by 0.25 ml of a 10 mg ml^{-1} ethidium bromide solution. The sample was then vortexed and centrifuged in a MSE Centaur I centrifuge at 4, 000 *g* to remove precipitates. The cleared supernatant was then transferred to Sorvall TV865 quickseal ultracentrifuge tubes and centrifuged at 200, 000 *g* for 18 hours at room temperature in a Sorvall OTD55B ultracentrifuge. The plasmid band was visualised on the CsCl gradient under UV light, and was extracted using a sterile needle and syringe. Ethidium bromide was removed by mixing the sample (three times) with TE saturated butan-1-ol, discarding the upper (butanol/ethidium bromide) layer each time. The aqueous phase was then dialysed against dH_2O, several times to remove the CsCl. Subsequently, the DNA solution was examined by agarose gel electrophoresis, and if necessary, concentrated by extraction with butan-2-ol.

Small-scale preparation of *S. typhimurium* plasmid DNA

Use of the TELT method of plasmid preparation proved unsatisfactory for *S. typhimurium* therefore, plasmid was prepared using a similar procedure to that described for the large scale preparatory method.

S. typhimurium cells carrying the plasmid of interest were grown overnight in 5 ml of LB medium, with the appropriate antibiotic(s), at 37°C with shaking. Cells were

pelleted in a micro-centrifuge tubes at 20, 000 *g* for 30 seconds. The supernatant was removed and 100 µl of Solution I was added, as well as 10 µl of a 100 mg ml^{-1} lysozyme solution. After vortexing, the tube was left to stand for 2 minutes before 200 µl of Solution II was added and the sample was mixed thoroughly by inversion. 150 µl of Solution III was then added, mixed as before and left on ice. After 15 minutes, the cell debris was removed by centrifuging the sample at 20, 000 *g* for 15 minutes and the supernatant was transferred to a fresh tube. The supernatant was then extracted with a mixture of phenol/chloroform and the DNA was precipitated with ethanol. The pellet was rinsed with 70 % (v/v) ethanol, dried and resuspended in dH_2O.

Preparation of genomic DNA from *E. coli* and *S. typhimurium*

Preparation of genomic DNA from bacteria was accomplished using the selective precipitation properties of hexadecyltrimethyl ammonium bromide (CTAB) (Murray and Thompson, 1980). Cell debris, polysaccharides and proteins can be removed from a bacterial lysate using CTAB and high-molecular weight DNA can be recovered by isopropanol precipitation.

The bacterial strain of interest was inoculated into 5 ml LB broth, with the appropriate antibiotic(s), and was grown overnight at 37°C with shaking. 1.5 ml of culture was transferred to a micro-centrifuge tube and cells were pelleted at 20, 000 *g*. The supernatant was discarded and the cells were resuspended in 567 µl of TE buffer. 30 µl of a 10% (w/v) SDS solution and 3 µl of proteinase K (100 µg ml^{-1}) were added and the sample was mixed thoroughly. The tube was then incubated for 1 hour at 37°C with occasional mixing. 100 µl of 5 M NaCl was added and mixed in by inversion, prior to the addition of 80 µl of a CTAB/NaCl solution (10% [w/v] CTAB in 0.7 M NaCl). The sample was incubated at 65°C for 10 minutes and was then centrifuged at 20, 000 *g* for 5 minutes to spin out the CTAB-protein/polysaccharide complexes. The upper aqueous layer of viscous supernatant was then transferred to a new tube and an equal volume of phenol/chloroform (1:1) was added and mixed in thoroughly. The tube was again

spun at 20, 000 *g* for 5 minutes and the upper aqueous layer was transferred to a fresh tube, where 0.6 volumes of isopropanol was added. The tube was gently inverted several times until a stringy white precipitate of the DNA appeared. The DNA was spooled out of the sample using a capillary tube and was transferred to a new tube containing 0.5 ml of 70% (v/v) ethanol, to remove the residual CTAB and NaCl. The DNA was then pelleted by centrifugation at 20, 000 *g* for 5 minutes, was dried and was resuspended in 100 µl of dH_2O.

Southern blotting and hybridisation

Identification of specific sequences of DNA was established by the transfer technique of Southern (1975). In brief, DNA is separated by gel electrophoresis and transferred to a solid support *e.g.* nylon membrane. A radiolabelled DNA fragment is then hybridised to the DNA attached to the membrane, and autoradiography is used to identify the position of the band complementary to the probe.

DNA samples of interest were digested with restriction enzymes and fragments were separated according to size by agarose gel electrophoresis for 12-15 hours at 1 V cm^{-1}. The gel was photographed, and the distance each molecular weight marker had migrated was recorded to allow size determination once the DNA had been transferred from the gel. The gel was soaked in 0.25 M HCl for 30 minutes, to depurinate the DNA, and then was rinsed in dH_2O to remove the excess HCl. Subsequently, the gel was soaked in 0.5 N NaOH/1.5 M NaCl, for 40 minutes and was gently rocked on a moving platform, to denature the DNA. Finally, the gel was rinsed with dH_2O and treated with 0.5 M Tris. Cl/1.5 M NaCl for a further 40 minutes with gentle agitation. Two strips of blotting paper were soaked in 20x SSC and placed on the upturned agarose gel casting tray, with the ends of the blotting paper resting into a reservoir of 20x SSC. The gel was turned upside down and was transferred onto this support, ensuring no bubbles were trapped underneath the gel. One corner of the gel was removed to allow the orientation to be determined and all the sides of the gel were then overlaid with Clingfilm, to ensure the buffer could only move through the gel. Nylon membrane (Hybond^{TM}-N, Amersham) was cut to the

size of the gel and was laid directly on top of the gel, again ensuring no bubbles were trapped under the membrane. Three pieces of blotting paper were cut to the size of the gel, were soaked in dH_2O and laid over the nylon membrane. A stack (6 cm) of paper towels which were cut to the size of the gel, was then placed on top of the blotting paper producing a wick to draw liquid through the gel, thereby transferring the DNA to the nylon. Finally, a 500 g weight was placed on top of the stack to ensure good contact between the layers and the system was left overnight at room temperature. The following day the nylon membrane was retrieved and rinsed in dH_2O to remove excess SSC and then, was baked at 80°C for 2 hours. Immediately prior to use, the nylon membrane was subjected to UV irradiation (1200 joules) for 2 minutes in a UV Stratalinker™ 1800 (Stratagene) to cross-link the DNA to the membrane before hybridisation was performed with a radiolabelled DNA fragment.

Random primed end-labelling of DNA

Random prime labelling was performed as essentially described by Feinberg and Vodelstein (1983, 1984). Random hexanucleotides bind to the DNA fragment to be labelled and in the presence of nucleotides and the Klenow fragment of DNA polymerase I, the spaces in between the hexanucleotides are filled in using the parental strand as a template. One of the deoxynucleotides used is radiolabelled, hence the newly synthesised strand becomes radiolabelled.

DNA was labelled using the Boehringer Mannheim Random Primed DNA labelling kit. Approximately, 50-100 ng of the DNA (0.2 kb upwards) to be labelled was made up to a volume of 11 µl with dH_2O in a micro-centrifuge tube and was boiled for 10 minutes. The sample was then instantly placed on ice, to maintain the DNA in a single-stranded form. To this sample, 1 µl of dATP, dTTP and dGTP were added as well as 2 µl of Reaction buffer (containing the hexanucleotides and buffer for the enzyme). 3 µl of [α-^{32}P]-dCTP (10 µCi µl^{-1}) and 1 µl of Klenow enzyme were then added to make a final volume of 20 µl and the whole reaction mixture was incubated at 30°C for 1-1.5 hours. The total volume was then made up to 200 µl and was separated through 1 ml of a TE-equilibrated pre-spun Sephadex G-50 column by

centrifuging at 2, 000 *g* for four minutes. Unincorporated radio-isotope was retained in the column and the labelled DNA was eluted into a fresh micro-centrifuge tube.

Hybridisation protocol for DNA probes

DNA hybridisations were performed at high-stringency (65°C) in a "Techne Hybridiser HB-1D" oven. A pre-hybridisation buffer containing 7% SDS (w/v), 0.5 M NaH_2PO_4 (pH 7.2) and 1 mM EDTA, and a hybridisation roller were incubated in the oven at 65°C. The nylon membrane, used to blot the DNA, was placed inside the roller and 20 ml of the pre-hybridisation solution was added to block the membrane. Meanwhile, the labelled DNA probe was denatured by boiling for 10 minutes. After 10 minutes the roller was removed from the oven and the original pre-hybridisation mixture was replaced with an equal volume of fresh buffer, along with the denatured probe. Hybridisation was allowed to proceed overnight after which the membrane was washed twice with a solution containing 5% SDS (w/v), 40 mM NaH_2PO_4 (pH 7.2) and 1 mM EDTA, followed by two washes of 1% SDS (w/v), 40 mM NaH_2PO_4 (pH 7.2) and 1 mM EDTA. All washes were performed at 65°C and after completion the membrane was wrapped in Clingfilm and autoradiographed at -80°C.

Sequencing of double-stranded DNA

DNA sequencing was performed using a modification of the dideoxynucleotide method of Sanger *et al.* (1977). DNA was sequenced using T7 DNA polymerase in conjunction with a Sequenase Version 2.0 DNA sequencing kit (USB) according to the manufacturers instructions. The method is dependent upon base-specific termination by dideoxynucleotides, of enzyme catalysed primer extension reactions.

Annealing Reaction:

Approximately 2-5 µg of CsCl purified plasmid DNA was made up to 100 µl in dH_2O. DNA was denatured by the addition of 25 µl of a solution containing 1 M NaOH and 20 mM EDTA. The sample was then incubated at 37°C for 30 minutes before being ethanol precipitated, dried and resuspended in 7 µl of dH_2O. 2 µl of

reaction buffer and 1 μl of the relevant primer (approximately 1 μg) were added and the sample mixed with a pipette. This mixture was then incubated at 37°C for 30 minutes, to allow the primer to anneal to the DNA sequence of interest.

During this time 6 microfuge tubes were labelled "G", "A", "T", "C", "LM" and "E", and were filled with:

"G" 2.5 μl of termination mix G (ddGTP)

"A" 2.5 μl of termination mix A (ddATP)

"T" 2.5 μl of termination mix T (ddTTP)

"C" 2.5 μl of termination mix C (ddCTP)

"LM" 4 μl of dH_2O and 1 μl of labelling mix

"E" 7 μl of enzyme dilution buffer and 1 μl of T7 polymerase

The four tubes containing the termination mixes were then incubated at 37°C ready for further use.

Labelling reaction:

Once the annealing reaction was complete, the annealed DNA was labelled by mixing the following:

10 μl of annealed DNA mix

1 μl of 0.1M DTT

2 μl of labelling mix ("LM")

1 μl of [α-^{35}S]-dATP (10 μCi)

2 μl of enzyme ("E")

This mixture was then spun for 2 seconds at 20, 000 *g* and incubated at 37°C for 3 minutes.

Termination reaction:

Immediately after labelling, the reaction was terminated by pipetting 3.5 μl of labelling reaction mix into each of the tubes "G", "A" ," T" and "C". The tubes were spun at 20, 000 *g* for 2 seconds to mix the labelling solutions with each of the

termination mixes and were incubated at 37°C for 3-5 minutes. 4 µl of stop solution was then added, and the samples were used immediately or were frozen at -20°C until required.

Electrophoresis of DNA sequencing reaction mixtures:
Electrophoresis of sequencing reactions was performed on a BIORAD sequi-Gen nucleic acid sequencing gel electrophoresis system. Denaturing polyacrylamide gels were made in this apparatus by mixing 71.4 g Urea, 17 ml 10x TBE, and 40 ml of a 30% (w/v) acrylamide: bisacrylamide (19:1) solution, and adjusting the final volume to 170 ml with dH_2O. 40 ml of the gel mixture was removed and polymerised to form a seal at the bottom of the apparatus, in the casting tray, by adding 200 µl of 20% (w/v) ammonium persulphate solution and 200 µl of TEMED. The sequencing gel was then polymerised, between two glass plates, by the addition of 120 µl of 20% (w/v) ammonium persulphate and 120 µl of TEMED. Gels were then used immediately or were stored for up to 24 hours at 4°C before use.

The sequencing reactions were denatured prior to loading by heating samples to 75-80°C for two minutes, and were loaded onto a pre-heated polyacrylamide gel (50°C). Samples were then run at 50 mA in 1x TBE buffer for 2-6 hours. Gels were then dried under vacuum at 80°C for two hours before being autoradiographed overnight at room temperature.

<u>One-step transformation of *E. coli*</u>

E. coli cells were transformed using a similar protocol to that of Chung *et al.* (1989). The *E. coli* strain to be transformed was grown overnight with shaking at 37°C. The overnight culture was then diluted 1/100 into fresh LB medium and grown under the same conditions until the OD_{600} had reached 0.4-0.6. The cells were then pelleted in a MSE Centaur I bench-top centrifuge, at 4, 000 *g* for 10 minutes. The supernatant was discarded and the cells resuspended in 300 µl of TSS solution (1 ml of TSS contains 0.5 ml LB medium, 0.5 ml 20% [w/v] PEG 6000, 50 µl DMSO and 45 µl of 1 M $MgSO_2$). Cells were transferred to a micro-centrifuge tube and were incubated

on ice for 10 minutes. The DNA to be transformed was then added to the tube and was mixed into the cells by pipette and the sample left for 1-2 hours on ice. After this time, 0.5 ml of LB broth was added to the tube and the cells were incubated for 1 hr at 37°C prior to plating on selective LB plates.

Transformation of *E. coli* and *S. typhimurium* by electroporation

Cells were made electro-competent using the following protocol. The bacterial strain to be transformed was grown overnight in 5 ml LB broth, with the appropriate antibiotic(s), at 37°C with shaking. 2 ml of this overnight culture was dispensed into a conical flask containing 200 ml LB broth and grown under the same conditions until the OD_{600} had reached 0.5-0.6. The flask was then chilled on ice for 20 minutes. During this period sterile dH_2O (500 ml) and sterile centrifuge pots were also chilled. Cells were then pelleted at 4, 000 *g* for 10 minutes in a Beckman JA-21 (JA-14 rotor) at 4°C. The supernatant was discarded and the cells were resuspended in 200 ml ice-cold sterile dH_2O and left on ice a further 15 minutes. The cells were then centrifuged and resuspended in the same way once more. After the final spin, the bulk of the dH_2O was removed such that the cells (originally from 200 ml of culture) were resuspended in 1 ml of dH_2O. Cells were then dispensed as 100 µl aliquots into pre-chilled micro-centrifuge tubes. Between 5 pg and 0.5 µg of the DNA to be transformed was added to each tube and the samples were mixed by pipette. The cells and DNA mixture were then transferred to pre-chilled electroporation cuvettes, ensuring no bubbles were formed when dispensed. Electroporation was performed using the BIORAD "Gene-Pulser" apparatus, with the voltage set at 2.5 kV and the Pulse controller set to 200 ohms. Cuvettes were wiped to remove excess moisture and pulsed under the conditions described. After the pulse, 1 ml of SOC medium was added to the cuvette, and the whole contents were transferred to a fresh micro-centrifuge tube. The cells were then incubated at 37°C for 1 hour, prior to plating on selective agar.

Blue/white screen for pGEM-T derived phagemids

The vector pGEM-T (Promega) is specifically designed for the direct cloning of PCR products. PCR-generated DNA fragments contain 3' deoxyadenosine residues which are added in a non-template dependent fashion by the thermostable polymerase. The vector has been treated such that it contains 3'-end thymidine overhangs, thus allowing direct ligation between the vector and the PCR fragment without the need for restriction digestion of DNA prior to ligation. The cloning site lies within the coding sequence for the β-galactosidase α-peptide, such that when a fragment of DNA is inserted, the *lacZα* gene is inactivated. Such recombinant clones, when transformed into JM101 (an *E. coli* derivative lacking *lacZ*), can be selected for by a blue white screen which directly tests for β-galactosidase activity (Short *et al.*, 1988). The screen involves selection of transformant JM101 cells on LB medium containing 50 μg ml^{-1} ampicillin combined with the blue white screen provided by the addition of 40 μl of a 2% X-gal solution (w/v, dissolved in dimethylformamide) and 60 μl of a 2% IPTG solution (w/v). After overnight incubation of the plates at 37°C, JM101 colonies harbouring pGEM-T appear as blue colonies, whilst JM101 colonies bearing recombinant pGEM-T plasmids appear as white colonies.

2.2.3 Protein detection procedures

SDS-polyacrylamide gel electrophoresis of proteins

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed essentially as described by Laemmli (1970). The procedure dissociates proteins into their individual sub-units and subsequently separates them according to their size.

Proteins were resolved on either a 16 x 20 cm or 7 x 8 cm SDS-polyacrylamide gel, using a PROTEAN II xi Slab Gel or MINI-PROTEAN II Dual Slab Gel apparatus respectively (BIORAD). The composition of the gel was dependent upon the protein to be investigated, either a 12.5% or 10% (v/v) resolving gel was routinely used and the stacking gel was always used at a 4% (v/v) polyacrylamide concentration.

The composition of the protein gels is shown below (the quantity of solutions indicated were designed for the formation of a 7 x 8 cm gel; the values in parentheses reflect the quantities of components required to make a 16 x 20 cm gel).

Stacking Gel (4% [v/v] polyacrylamide):

30% (w/v) acrylamide/ bisacrylamide(19:1):	1.4 ml (3.9 ml)
Tris. Cl pH 6.8, 0.5 M	2.3 ml (7.5 ml)
20% (w/v) SDS	50 µl (150 µl)
dH_2O	6.2 ml (18.3 ml)
10% APS	100 µl (300 µl)
TEMED	10 µl (30 µl)

Resolving Gel (10% [v/v] polyacrylamide):

30% (w/v) acrylamide/ bisacrylamide(19:1):	3.4 ml (16.7 ml)
Tris. Cl pH 8.8, 1.5 M	2.5 ml (12.5 ml)
20% (w/v) SDS	50 µl (250 µl)
dH_2O	4.1 ml (20.3 ml)
10% APS	50 µl (250 µl)
TEMED	5 µl (25 µl)

Resolving Gel (12.5% [v/v] polyacrylamide):

30% (w/v) acrylamide/ bisacrylamide(19:1):	4.0 ml (20.0 ml)
Tris. Cl pH 8.8, 1.5 M	2.5 ml (12.5 ml)
20% (w/v) SDS	50 µl (250 µl)
dH_2O	3.4 ml (17.0 ml)
10% APS	50 µl (250 µl)
TEMED	5 µl (25 µl)

APS - Ammonium persulphate

TEMED-N,N,N'-tetramethylethylethylenediamine

Laemmli (Loading) Sample Buffer (10x) for SDS-polyacrylamide gels:

25 mM Tris. Cl, pH 6.8

2% (w/v) SDS

10% (v/v) Glycerol

0.2 M β-Mercaptoethanol

0.002% (w/v) Bromophenol Blue

5x Running buffer for SDS-polyacrylamide gels:
144 g Glycine, 30 g Tris-base, 5 g SDS in 1 litre dH_2O

The resolving gel was poured between two glass plates and allowed to polymerise by overlaying the acrylamide solution with dH_2O. Once the resolving gel was set, the layer of water was removed and the stacking gel poured. A comb was then immediately inserted into the stacking gel, ensuring no bubbles were trapped around the teeth of the comb. Once the stacking gel had polymerised, it was used immediately or stored at 4°C for up to 24 hours before use.

Samples to be loaded were mixed with 2x Laemmli (or Loading) sample buffer (LSB) and boiled for five minutes, unless otherwise stated, prior to loading. Samples were loaded (15-50 µl, depending upon the gel used) into individual wells and the proteins electrophoresed in 1x running buffer at 25-50 mA for 2 hours (MINI-PROTEAN II) or 100 V for 18 hours (PROTEAN II). Molecular weight standard markers were also added to allow molecular weight determinations of polypeptides seen on the gel.

Staining SDS-polyacrylamide gels with Coomassie Brilliant Blue

Polypeptides were visualised by staining with Coomassie Brilliant Blue. Once the polypeptides had been separated by electrophoresis, the gels were immersed in a fixing/staining solution (0.1% [w/v] Coomassie Brilliant Blue R250 (BIORAD), 45% [v/v] Methanol, 10% [v/v] glacial acetic acid) and 45% dH_2O). Gels were left to stain on a shaking platform for 30-60 minutes at room-temperature. Excess stain was then removed by soaking the gel in a destaining solution (45% [v/v] methanol, 10% [v/v] glacial acetic acid, 45% dH_2O) for 4-8 hours at room temperature, until polypeptide bands could be seen clearly.

Estimation of protein concentration

The concentration of a protein in a solution was estimated using the modified method of Bradford (1976) by following a BIORAD standard assay procedure. BSA solutions of 0, 2, 4, 6, 8, 10 and 12 μg ml^{-1} were prepared in 1 ml of dH_2O, and 800 μl of each standard was added to 200 μl of BIORAD protein assay dye-reagent. Each sample was mixed by gentle inversion, taking care not to cause foaming, and after 5 minutes (one hour maximum) the optical density of the solution was read at 595 nm (OD_{595}) in a spectrophotometer. For each BSA standard solution, triplicate measurements of the OD_{595} were obtained and the average reading plotted against concentration to give a standard curve. The protein concentration of the solution of interest was then determined by appropriately diluting the sample in 1 ml of dH_2O and adding 800 μl of this solution to 200 μl of the dye-reagent. The optical density was then determined as outlined above. This reading was used in conjunction with the standard curve, to determine the concentration of protein in the solution of interest.

2.2.4 Protein overexpression and purification

[^{35}S]-methionine labelling of proteins using pET-19b vectors in *E. coli*

BL21 (DE3) pLysS and two derivatives containing either pET-19b or pPDT14 (the plasmid containing the *ahpC* gene to be overexpressed) were grown in LB medium supplemented with antibiotics. All strains required 5 μg ml^{-1} chloramphenicol (which maintains the pLysS plasmid) but only the strains carrying pET-19b plasmids required 100 μg ml^{-1} ampicillin. Strains were then cultured overnight with shaking at 37°C. The cells were spun down in an MSE Centaur 1 bench-top centrifuge at 4, 000 *g* for 10 minutes and resuspended in 1 ml of fresh LB. 0.5 ml of each culture was transferred into 24.5 ml of Spitzizen minimal medium with the appropriate antibiotics, and incubated at 37°C with shaking until the OD_{600} had reached 0.6. For each culture, four 0.5 ml aliquots were then transferred to micro-centrifuge tubes. In each of these set of four tubes, two of the tubes had IPTG (I) added to a final

concentration of 0.5 mM. Incubation for all tubes was continued at 37°C for a further 30 minutes. After this time rifamycin (R) was added to a final concentration of 200 μg ml^{-1} to one of the IPTG treated tubes and one of the untreated tubes, in each batch of four tubes. Thus, the tubes from each culture contained -I/-R, -I/+R, +I/-R, +I/+R, respectively. The incubation was then continued for a further 45 minutes before each tube was pulse labelled with 5 μCi of [^{35}S]-methionine for 5 minutes. The tubes were then cooled on ice, spun at 20, 000 *g* for 30 seconds and the supernatant (containing unincorporated label) was safely discarded. Cells were then resuspended in 100 μl of 2x LSB, immediately boiled for 5 minutes and then loaded onto a 16 x 20 cm 12.5% (v/v) polyacrylamide gel. The gel was then run at 100 V for 18 hours and was then dried and autoradiographed at room temperature overnight.

Large-scale overproduction of AhpC from pET-19b vectors in *E. coli*

Overexpression using the pET vectors followed methods described by Sambrook *et al.* (1989) and the manufacturers recommendations (Novagen). 50 ml of LB containing 5 μg ml^{-1} chloramphenicol and 100 μg ml^{-1} ampicillin was inoculated with a single colony of the *E. coli* BL21 (DE3) pLysS strain carrying pPDT14. The bacteria were then grown overnight at 37°C with shaking. The culture was spun at 4, 000 *g* in a Beckman JA-20 (JA-14 rotor) centrifuge at room temperature for 10 minutes. The supernatant was discarded and the cells were resuspended in 10 ml of fresh LB. This was used to inoculate 500 ml of minimal Spitzizen medium, containing the appropriate antibiotic and the sample was incubated at 37°C with shaking until the OD_{600} reached 0.6. IPTG was then added to a final concentration of 0.5 mM and incubation was continued for a further 3 hours. Cells were then pelleted in a Beckman JA-20 (JA-14 rotor) centrifuge at 4, 000 *g* for 10 minutes and then were resuspended in 20 ml ice-cold Buffer A. The bacteria were then lysed by sonication using a Lucas Dawes Ultrasonics Soniprobe, with probe size 60 mm x 12 mm diameter. Sonication was carried out on output setting 5, for 40% of each second over a 4 minute period in an ice-cold water bath. Cell debris was then pelleted at 20, 000 *g* for 30 minutes at 4°C in a Beckman JA-20 (JA-21 rotor) centrifuge and the

supernatant was retained and stored at -80°C. This procedure could be scaled up easily, but typically, each run was limited to a 1.5 l final culture volume.

Purification of histidine-tagged AhpC using a nickel-nitrolotriacetic acid (NTA) column

The nickel (Ni^{2+}) groups in Ni^{2+}-NTA resin (Qiagen) are capable of binding the imizadole ring of histidine amino acids. Proteins containing a His-tag can therefore be selectively bound and separated from non-His-tagged proteins with relative ease.

All the subsequent steps were performed at 4°C. 1.5 ml of resin was transferred to a column and allowed to settle (forming a 1 ml column). The column was washed with 10 ml of dH_2O and equilibrated using a further 10 ml of Buffer A (see below). 10 ml of cell extract was defrosted, imizadole added to a final concentration of 1 mM and the sample was loaded onto the column. The column was then washed with 20 ml of Buffer B, containing 20 mM imizadole, to remove any non-His-tagged proteins. The His-tagged protein was eluted by the addition of 6 ml of Buffer B containing 80 mM imizadole. The eluted fraction was diluted 4-fold in Buffer B (so that the concentration of imizadole was 20 mM) and the whole procedure repeated. All column fractions were kept at -80°C until further use. The purification of the his-tagged protein was monitored by examining the composition of column fractions by SDS-PAGE. Typically 20 µl of each column fraction was mixed with 10 µl 2x LSB, incubated at 37°C for 10 minutes and loaded onto 7 x 8 cm 12.5% (v/v) polyacrylamide gels. Gels were then run at 25-50 mA for 2 hours, stained with Coomassie Brilliant Blue stain and destained. Purification was stopped when the protein was considered to be >95% pure. Eluates containing the purified AhpC protein were then pooled, dialysed against several changes of PBS and the protein concentrated (see later).

The Ni^{2+}-NTA columns could be regenerated, after elution of the protein had occurred, by adding buffer R. In this way a column could be used many times. Columns were stored at 4°C in either dH_2O or Buffer R. The columns were not

stored for long periods of time in the presence of Buffers A or B as these appeared to have a detrimental effect upon the column.

Buffer A:	*Buffer B*:	*Buffer R*:
10 mM Tris. Cl (pH 7.9)	20 mM Tris. Cl (pH 7.9)	6 M guanidine HCl
10% (v/v) glycerol	20% (v/v) glycerol	0.2 M acetic acid
0.5 M NaCl	100 mM KCl	
0.1% (v/v) NP40	5 mM DTT	
5 mM DTT	0.5 mM PMSF	
0.5 mM PMSF		

(All buffers were made up in dH_2O and Buffers A and B also contained the following protease inhibitors (Sigma): 1 μM pepstatin A, 1 μM chymostatin and 5 μM leupeptin)

Overexpression of GroEL from pPDT15 in *S. typhimurium*

CH23 containing pPDT15 was grown in 5 ml of LB containing ampicillin (50 $\mu g\ ml^{-1}$) overnight with shaking at 30°C. The culture was then diluted into 500 ml of LB medium containing ampicillin (as before) and grown at 30°C with shaking until the OD_{600} had reached 0.6. The culture was then rapidly shifted to a water bath set at 42°C and incubated for 10 minutes, with occasional hand shaking. After ten minutes the cells were pelleted in a Beckman JA-20 (JA-14 rotor) by centrifugation at 4, 000 *g* for 10 minutes at 4°C. Cells were resuspended in 20 ml of ice-cold Buffer Z and were sonicated on ice using a Lucas Dawes Ultrasonics Soniprobe with probe size 60 x 12 mm. Sonication was performed for 40% of each second for 4 minutes on an ouput setting of 5. After sonication, cell debris was removed by centrifugation in a pre-cooled (4°C) Beckman JA-20 (JA-21 rotor) at 20, 000 *g* for 30 minutes. The supernatant was then stored at 4°C until further use.

Buffer Z:

10 mM Tris. Cl pH 7.5

1 mM EDTA

1 mM DTT

50 mM NaCl

0.5 mM PMSF

1 μM pepstatin A, 1 μM chymostatin and 5 μM leupeptin (unless otherwise stated).

Sucrose density gradient centrifugation

Sucrose gradient centrifugation was performed using a Sorvall AH-627 swinging bucket rotor in a Sorvall OTD55B ultracentrifuge. Sucrose was dissolved in ice-cold Buffer Z to a concentration of 10% or 30% (w/v). To form one gradient, 15 ml of each solution was mixed and poured in a gradient mixer, such that the gradient formed from 30% to 10% in a 36 ml centrifuge tube. 4 ml of cell supernatant was then carefully laid on the top of the gradient. Tubes were then spun at an average relative centrifugal force of 86, 000 *g* at 4°C for 18 hours. 1 ml fractions of the gradient were collected using a BIORAD Econo pump, pumping from the bottom of the gradient. The fractions were stored at 4°C and examined by SDS-PAGE to determine the composition of proteins present. Those fractions containing the GroEL protein were then pooled, dialysed against several changes of PBS, to remove the sucrose, and the protein concentrated (see later).

Ion exchange column chromatography

Ion exchange column chromatography was performed using a Whatman DE52 diethylaminoethyl cellulose (DEAE) column (80 x 15 mm [approximately 8 ml]). Proteins were bound to the column and were purified by differential elution in a step-wise gradient of NaCl. The columns could also be used to concentrate purified protein. 2 g of pre-swollen DE52 was dissolved in 50 ml Buffer Z and stirred for 10 minutes at room temperature. The resin was allowed to sediment and was then resuspended in 50 ml of fresh Buffer Z and the process repeated. After sedimentation

the supernatant was decanted off such that most of the resin was concentrated in a small volume. The resin was then poured into a column and allowed to settle. The column was extensively washed with Buffer Z until the eluent had the same pH as this buffer. Columns were then stored in Buffer Z at 4°C until use.

For purification, 20 ml of cell supernatant was applied to the DE52 column via a BIORAD Econo pump, pumping at 1 ml min^{-1} (all subsequent washes and elutions were performed using this apparatus set at the same rate). The column was then washed by with 100 ml of Buffer Z. Elution of proteins was performed by addition of a succession of modified Buffer Z solutions. 10 ml of Buffer Z containing 0.15 M NaCl was pumped through the column, followed by 10 ml of 0.5 M NaCl buffer and finally, 10 ml of Buffer Z containing 1 M NaCl. Flow-through and wash fractions were collected in 10 ml aliquots and eluted fractions were collected in 3-4 ml fractions. All samples were then stored at 4°C until further use. Fractions were examined for protein composition by SDS-PAGE on 10% (v/v) polyacrylamide gels. Those fractions containing GroEL were pooled, dialysed against several changes of PBS and the protein concentrated (see later).

2.2.5 Immunodetection of polypeptides

Immunological detection of proteins was performed using a Western blot procedure. Proteins were firstly transferred (blotted) to a solid support (Towbin *et al.*, 1979) *e.g.* nitro-cellulose membrane and the blot was exposed to sera (antibody) specific to that protein. Bound sera was then detected using antisera conjugated to an alkaline phosphatase, allowing visualisation of the protein band by chromogenic staining when the enzyme was exposed to its substrate (Knecht and Diamond, 1984).

Transfer of proteins to nitro-cellulose membranes by electrophoresis

The procedure for transferring proteins to nitro-cellulose membranes from polyacrylamide gels was essentially that of Towbin and colleagues (1979), with modifications suggested by the manufacturers of the apparatus used. A BIORAD

"TRANS-BLOT CELL" was utilised for the transfer of polypeptides from the polyacrylamide gel to a nitro-cellulose membrane

Once SDS-PAGE had been performed, a piece of 3MM Whatman paper was cut to the size of the gel and was soaked in 1x transfer buffer (1 litre of 10x Transfer Buffer contained 250 mM Tris. Cl, pH 8.3, 1.5 M Glycine and 20 % [v/v] methanol). The gel was then carefully transferred to the filter paper, ensuring no bubbles were trapped between the gel and the paper, and was laid onto a transfer buffer soaked pad. An appropriately sized piece of nitro-cellulose (Schleicher and Schuell) was then soaked in transfer buffer and was carefully placed over the gel, again taking care to avoid bubbles. A second piece of buffer-soaked filter paper was placed over the membrane and the sandwich was completed by a second soaked filter pad. The entire sandwich was placed into a holder and immersed into a gel tank containing 1x transfer buffer, making sure that the membrane "side" of the sandwich was closest to the anode. Polypeptides were then transferred from the gel to the membrane by electrophoresing at 10-15 V overnight at 4°C. After blotting the polypeptides, the membrane was used immediately for immunodetection experiments.

Immunodetection of *Salmonella typhimurium* polypeptides

The detection of polypeptides followed the method of Knecht and Diamond (1984). A blotted membrane, carrying *S. typhimurium*-derived proteins, was shaken for 1 hour at room temperature in 100 ml of a 20% (w/v) milk powder solution. The milk proteins blocked sites on the membrane not already filled by proteins from the gel sample. The membrane was then rinsed twice in 100 ml washes of Tween wash I, and the membrane was shaken in 20 ml of the same buffer with 100 µl of mouse sera (polyclonal anti-*S. typhimurium* protein antisera) for one hour at room temperature. After this time, the membrane was washed six times with Tween Buffer I, each wash involving shaking at room temperature with 100 ml of buffer for 10 minutes. Following washing, the membrane was shaken at room temperature, in Tween Wash I containing a 1:1, 000 dilution of alkaline phosphate-conjugated rabbit anti-mouse antibody (Sigma). After a further hours agitation, this solution was discarded and

unbound sera was removed by washing six times as described above, except using Tween wash II. Bound antisera was detected by the addition of 10 ml of a developing solution. The reaction was allowed to proceed until bands were clearly visible, and the reaction was terminated by extensive rinsing of the membrane with dH_2O.

Tween wash I and II:
Both Tween washes consisted of 9 g NaCl, 0.5 ml of polyoxyethylene sorbitan monolaurate (Tween 20) and 10 ml Tris. Cl, pH 7.4 (Tween wash I) or 10 ml Tris. Cl, pH 8.8 (Tween wash II) dissolved in a final volume of 1 l dH_2O.

Developing Solution:
1 ml of 0.1% (w/v) nitroblue tetrazolium in 10 mM Tris. Cl, pH 8.8, was mixed with 40 µl $MgCl_2$, 0.1 ml of a solution of 5-bromo-4-chloro-3-indoyl phosphate (p-toluidine salt) in dimethyl formamide (5 mg ml^{-1}) and 9 ml of 0.5 M Tris. Cl, pH 8.8.

2.2.6 Murine model studies

Innately susceptible (*ity*^{s}) female BALB/c mice (8-10 weeks of age) were used for testing the attenuation of *S. typhimurium* strains and the immunological properties of *S. typhimurium*-derived proteins. Mice were obtained from Edinburgh University and segregated into cages, never more than three to a cage, and were supplied with a commercial pellet diet and water *ad libitum*.

Preparation of bacterial cells for injection into mice

The *S. typhimurium* strain of interest was grown overnight in 5 ml LB medium, containing the appropriate antibiotic, with shaking at 37°C. Bacteria were pelleted in an MSE Centaur I bench-top centrifuge at 4, 000 *g* for 10 minutes. The cells were then washed and resuspended in 5 ml sterile phosphate-buffered saline (twice). Serial dilutions were then performed in PBS until the required bacterial concentration had been obtained. The bacterial inoculum was determined by plating aliqouts from the

serial dilution series. Mice were injected by the intra-peritoneal (i.p.) route with 100 µl aliquots of the PBS-diluted bacteria.

Test for bacterial virulence using a mouse model

S. typhimurium strains were tested for attenuation by the use of a 50% lethal dose (LD_{50}) test (Reed and Muench, 1938) *i.e.* the number of bacteria it takes to kill half the mice in the sample. This LD_{50} value could then be compared to that of the virulent parent strain. Typically 6 groups of 6 mice were used, and were injected by the i.p. route with a range of bacteria from approximately 10^5-10^6 organisms to 10^0-10^1 organisms. The survival of mice in each group was then monitored for at least 28 days and the LD_{50} subsequently established.

Preparation of *S. typhimurium* proteins for injection into mice

Proteins which had been purified were first of all dialysed in PBS to remove the buffers which they were purified in. Dialysis membrane (Medicell International Ltd.) was prepared by boiling in a solution containing 2% (w/v) sodium bicarbonate and 0.05% (w/v) EDTA for 10 minutes, ensuring that the membrane remained submerged. This solution was then discarded and the membrane then boiled twice in dH_2O. After this final step, the membrane was allowed to cool, submerged in dH_2O, and was transferred to a solution containing 20% (v/v) ethanol and stored at 4°C until further use. Prior to dialysis, the membrane was rinsed with sterile dH_2O to remove the ethanol and the protein solution then placed into an appropriately sized membrane bag. Proteins were dialysed in several changes of 2.5 l PBS at 4°C. The dialysed solution was then concentrated using a Millipore immersible filter unit (CX-10), with a nominal molecular weight cut off of 10, 000 kDa, attached to a vacuum pump. Under a vacuum, the protein is trapped by the filter but excess liquid easily passes through. Protein concentration was then determined using a Bradford assay (Bradford, 1976) using a BIORAD standard protein assay procedure. The concentrated protein was then stored at -80°C until use.

Delayed-type hypersensitivity testing

S. typhimurium proteins were examined for their ability to induce a delayed-type hypersensitivity (DTH) reaction in mice, using a footpad swelling assay. Female BALB/c mice were injected by the i.p. route with 100 µl of PBS (control group) or a 100 µl of PBS containing approximately 2.0 x 10^5 c.f.u. of an attenuated *S. typhimurium* strain, MPG479. On days 33 and 104 post-infection, uninfected mice and mice infected with *S. typhimurium* were challenged with the purified *S. typhimurium* proteins, AhpC or GroEL. One day before the challenge, the proteins were diluted to 0.8 mg ml^{-1} in PBS and heat aggregated at 70°C for one hour. The proteins were then stored at 4°C overnight until needed. Prior to injection, the footpad thickness of the right and left hind footpads (RHFP and LHFP, respectively) of each mouse in every group was measured using dial-type callipers, taking three measurements per footpad. Mice were subsequently injected subcutaneously with 40 µg of protein in 50 µl of PBS, into the LHFP and 50 µl of PBS (only) was injected into the RHFP. As a further control, to show that neither the physical injection nor the PBS could elicit a DTH response, one group of bacterially-infected mice were injected with 50 µl of PBS in the LHFP and nothing in the RHFP. After 24 and 48 hours, the thickness of the LHFP and RHFP was measured as described above and compared to establish whether a DTH reaction had occurred.

Obtaining *S. typhimurium*-specific serum from mice

Mice (six per group) and were injected by the i.p. route with 100 µl of PBS containing approximately 2 x 10^5 c.f.u. of an attenuated *S. typhimurium* strain, MPG479. A professional animal handler then lightly anaesthetised the mice and bled them, by retro-orbital bleeding, prior to infection (day 0) and on 14, 28, 43 and 110 days post-infection. Approximately 100 µl of blood was obtained per mouse, and bleeds from the same group of mice were pooled. The blood was allowed to clot at room temperature for 5-6 hours and the cell-free portion of the blood, the serum, was then obtained by spinning the samples at 1, 800 *g* in an MSE Centaur bench-top

centrifuge for 15 minutes. Serum was then aliquoted and stored at -80°C until further use.

Preparation of anti-AhpC antisera, obtained from mice

Purified his-tagged AhpC, which had been dialysed in PBS and concentrated, was diluted to 0.5 mg ml^{-1} in sterile PBS. 700 µl of this solution was then aliquoted into a 1/4 ounce bijou bottle and an equal volume of Alum (Pierce Rockford, Illinois) was added dropwise, with stirring, at room temperature for approximately 30 minutes. A group of 5 CBA/Ca female mice (Edinburgh University) was injected with 200 µl (50 µg ml^{-1} of protein) per mouse of the AhpC-Alum mixture, with each dose being distributed equally between two separate intraperitoneal sites. After an interval of 6 weeks, mice were challenged with 200 µl of the protein, prepared and injected as above. Samples of blood were collected under light halothane anaesthesia on days 7, 10, 14, 20 and 25 post-challenge and the blood from each group of mice was pooled. The serum was obtained as described above and samples were stored in 100 µl aliquots at -70°C until required.

CHAPTER 3

The effect of growth phase and RpoS upon the expression of *ahp*

3.1 INTRODUCTION

When *S. typhimurium* cells become starved of nutrients they enter into a stationary phase where the culture displays no net increase in cell number (reviewed in Hengge-Aronis, 1996a). The transition from rapid growth to stationary phase represents an adaptation of the bacteria to new conditions, and is sometimes called a starvation stress response (Foster and Spector, 1995). Associated with this phenomenon is an extensive change to the bacterial cell morphology including a rounding and shortening of the cell, cytoplasmic contraction and expansion of the periplasm (Huisman *et al.*, 1996). In addition, the bacterial genome experiences increased negative supercoiling, the nucleoid becomes compacted and the spectrum of genes which are expressed and their level of expression dramatically alters in the cell (Huisman *et al.*, 1996; Hengge-Aronis, 1996a).

Characteristically, stationary phase cells display a high level of resistance to a wide range of environmental stresses, including heat, ethanol, osmolarity, pH and oxidants (McCann *et al.*, 1991; Hengge-Aronis, 1996a). Interestingly, many of the mechanisms which underpin resistance form part of regulated and inducible responses to stress in growing cells yet, at the onset of stationary phase, they are induced independently of any particular stress being present. This is perhaps because stationary phase cultures are less able to support an inducible response, due to nutrient depletion and limited energy generation, whilst actively growing cells do not have such limitations. Cells appear therefore to employ a definite strategy to put in place a wide range of protective measures, prior to the complete cessation of growth.

Major aspects of the stationary phase response are regulated through the activity of an alternative sigma factor RpoS (σ^S), which allows the core RNA polymerase (E) to facilitate expression of genes required for the survival of the cell at the onset of starvation (Hengge-Aronis, 1996a; Espinosa-Urgel *et al.*, 1996). However, it should also be noted that RpoS is present in exponentially growing cells, but is much less abundant than the vegetative sigma factor RpoD (σ^D) (Hengge-Aronis, 1996a). Nevertheless, RpoS has a significant role to play in the exponential phase regulation

of a number of genes, especially those that are responsive to osmotic shock (Hengge-Aronis *et al.*, 1993, Hengge-Aronis, 1996b).

RpoS mutants are extremely susceptible to a wide range of environmental stresses, indicating the essential nature of RpoS in generating the stationary phase response (McCann *et al.*, 1991). RpoS has also been shown to be a prime factor in regulating the virulence of *S. typhimurium* (Fang *et al.*, 1992, Coynault *et al.*, 1996). The ability of *S. typhimurium* cells to survive within macrophages appears to be partly linked to the capacity for survival in stationary phase, as the *rpoS* gene and RpoS-dependent genes are induced within the macrophage environment (Chen *et al.*, 1996a). In addition, *rpoS* mutants of *S. typhimurium* are attenuated in mice, and may be useful in the generation of vaccines to *S. enterica*-related disease (Fang *et al.*, 1992; Coynault *et al.*, 1996).

Of interest to this thesis, is the involvement of RpoS in providing cells with stationary phase resistance to oxidative stress, in particular its influence on hydrogen peroxide resistance and the OxyR regulon. In previous studies, it was shown that the *ahp* locus of *S. typhimurium* was induced during macrophage interaction and specifically in response to hydrogen peroxide generated from the macrophage respiratory burst (Francis and Gallagher, 1993; Francis PhD Thesis, 1993). The *ahp* locus encodes a heterodimeric enzyme, alkyl hydroperoxide reductase, which is essential in the reduction of organic hydroperoxides generated when the membrane is attacked with reactive oxygen species (Jacobson *et al.*, 1989; Storz *et al.*, 1989). Therefore, this locus may aid the survival of *S. typhimurium* during the course of infection and could encode a virulence determinant. Identification of virulence loci has importance for the development of vaccines, as the construction of defined mutants in such loci may lead to attenuation of the virulence of *S. enterica* (Chatfield *et al.*, 1992a).

Importantly, in *E. coli*, RpoS has been shown, to date, to regulate the expression of three OxyR-induced genes including *katG* (Ivanova *et al.*, 1994), *dps* (Altuvia *et al.*, 1994) and *gorA* (Becker-Hapak and Eisenstark, 1995). Moreover, the expression of

dps and *katG* in the stationary phase of growth was shown to be independent of the OxyR regulatory protein (Altuvia *et al.*, 1994; Ivanova *et al.*, 1994). The situation with regards to the role of growth phase and RpoS in the expression of the *S. typhimurium ahp* locus had not been reported previously and it was perceived that these factors may have important implications for the role of *ahp* in infection and the host immune response. The possible role of these factors was therefore addressed in the present study by using *S. typhimurium* strains in which the *ahp* locus had been tagged with a bioluminescent reporter system (*ahp*::Mu*dlux*) or by immunoblotting with anti-AhpC antisera.

3.2 RESULTS

3.2.1 Construction and characterisation of *S. typhimurium rpoS* mutants

The *rpoS* gene from *S. typhimurium* had previously been identified and inactivated by an insertion of an ampicillin gene in strain SF1005 (Fang *et al.*, 1992). This mutation was transduced via phage P22 into the virulent *S. typhimurium* strain, SL1344, and its derivative MPG203 (*ahp*::Mu*dlux*), to generate MPG470 and MPG471, respectively.

Inactivation of *rpoS* by the insertion of an ampicillin gene introduced an additional *Bam*HI site into the chromosomal DNA which could be detected by Southern blotting (Fang *et al.*, 1992). Thus, when genomic DNA from the parental *S. typhimurium* strain and SF1005 were digested with *Bam*HI, one fragment of 10 kb and two fragments of 8.5 kb and 6.5 kb were identified, respectively, when probed with the *rpoS* gene (Fang *et al.*, 1992). This distinction was used in a similar manner to confirm the presence of the *rpoS* mutation in MPG470 and MPG471. Genomic DNA isolated from these strains and from SF1005 and SL1344, was digested with *Bam*HI, electrophoresed and blotted onto nylon membrane. The filter was then probed with the *rpoS* gene, which was amplified from the *S. typhimurium* (SL1344) chromosome using primers M0917 and M6184 (Table 2.1). The results are shown in Figure 3.1. Both MPG470 and MPG471 displayed the same pattern of bands (8.5

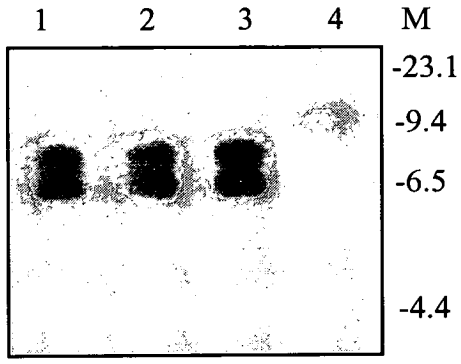


Figure 3.1 Southern blot analysis of *S. typhimurium* SL1344 derivatives to confirm the presence of an *rpoS* disruption

Genomic DNA was isolated from *S. typhimurium* strains SF1005 (*rpoS*), SL1344, MPG470 (*rpoS*) and MPG471 (*ahp*::Mu*dlux*, *rpoS*) and treated with *Bam*HI. The resulting digests were then electrophoresed in an agarose gel (0.8% (w/v)) and the DNA was transferred to a nylon membrane. The membrane was then probed with the *rpoS* gene amplified from the *S. typhimurium* SL1344 chromosome, using primers MO917 and M6184 (see Table 2.1). Key: Lane 1, SF1005; Lane 2, MPG470; Lane 3, MPG471; Lane 4, SL1344. The positions of phage λ *Hin*dIII molecular weight markers are shown (Lane M).

and 6.5 kb) as SF1005, whereas SL1344 only exhibited one band of approximately 10 kb, thus proving that the *rpoS* mutation had been transduced successfully into MPG470 and MPG471.

Phenotypic tests were also performed on MPG470 and MPG471 to confirm the result from the Southern blot analysis. Stationary phase *rpoS* mutants are known to exhibit extreme susceptibility to oxidative stress (Fang *et al.*, 1992). Stationary phase cultures of SL1344, SF1005, MPG203, MPG470 and MPG471 were therefore challenged with 20 or 50 mM hydrogen peroxide for one hour and cell numbers were determined by plating onto LB. The percentage (%) survival of the treated samples was then calculated in relation to the untreated samples. As can be seen from Table 3.1, the majority of SL1344 and MPG203 cells survived both challenge concentrations whereas MPG470, MPG471 and SF1005 displayed greatly reduced survival, confirming that they contained a defective *rpoS* gene.

Table 3. 1 Survival of *S. typhimurium rpoS* derivatives after treatment with hydrogen peroxide

Strain	**% Survival after treatment with hydrogen peroxide (HP)**		
	Untreated	**20 mM HP**	**50 mM HP**
SF1005	100.00	40.10	0.03
SL1344	100.00	96.98	87.43
MPG470	100.00	38.00	0.05
MPG203	100.00	95.01	86.23
MPG471	100.00	31.04	0.02

Overnight cultures of *S. typhimurium* SL1344, SF1005 (*rpoS*), MPG203 (*ahp*::Mu*dlux*), MPG470 (*rpoS*) and MPG471 (*ahp*::Mu*dlux*, *rpoS*) were split into three aliquots and treated with 20 mM or 50 mM hydrogen peroxide (HP) or left untreated. After incubation for 1 hour, 100 µl of each culture was appropriately diluted and plated onto LB. The percentage (%) survival of cells in the treated sample was calculated in relation to the untreated sample. The values in the table represent the average of two separate samples. Variation within samples was less than 6%.

3.2.2 Examination of the growth phase-dependent and hydrogen peroxide-dependent inducibility of the *ahp* locus, using a bioluminescent reporter system

The aim of this section was to examine how RpoS affects the expression of the *ahp* locus in growing (exponential phase) and growth arrested (stationary phase) cells. In order to examine these phenomena, it was necessary to define the culture conditions under which the 'exponential' and 'stationary' phases occurred for the strains to be tested. Since the *lux* reporter system encodes the *Vibrio fischeri* luciferase (Engebrecht *et al.*, 1983, 1985), which is inactivated above 34°C, the growth profile was established at 30°C.

Overnight cultures of MPG203 and MPG471 (*rpoS*), were diluted 1:10, 000 fold into 10 ml of LB and grown with shaking at 30°C. At hourly periods, 100 µl aliquots of these cultures were withdrawn and plated onto LB plates to determine the cell number. Figure 3.2 displays the growth curves of the two strains to be tested and indicates that MPG203 and MPG471 grow at equivalent rates, and to the same cell number, at 30°C, suggesting that the *rpoS* mutation does not substantially alter the growth of the cells. From these growth curves, it was determined that the window of time for examining cultures in the exponential phase was approximately 3-7 hours after dilution and incubation at 30°C, whilst the window for late log or early stationary phase was approximately 9-12 hours, under these conditions.

For the purpose of the present study, exponential phase expression of the *ahp* locus was tested at 4 hours, which appeared to be the mid-point of logarithmic growth. It is known that many genes are induced as cells reach growth arrest, despite the limited ability of cells to produce proteins as a result of nutrient depletion (Hengge-Aronis, 1996a). Thus, the expression of the *ahp* locus was examined at the time when *ahp* was most likely to be induced, early stationary phase. This was determined to be at

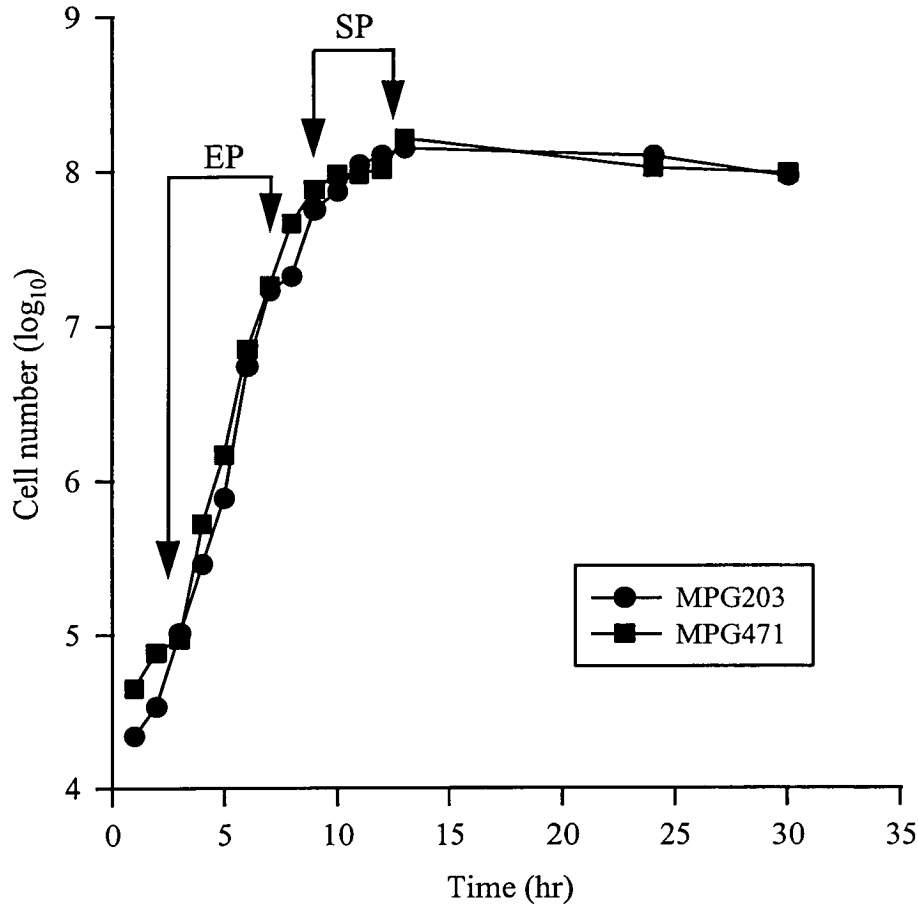


Figure 3.2 The growth profiles of MPG203 and MPG471

Overnight cultures of MPG203 and MPG471 (*rpoS*) were diluted 1: 10,000 into fresh LB and incubated at 30°C with shaking. At various time points 100 µl of each culture was appropriately diluted and plated to determine the cell number. Profiles were then plotted of the cell number (log_{10}) versus time (hr). The windows of time corresponding to the exponential (EP) and early stationary phases (SP) are indicated. (Experiments were repeated several times and found to be reproducible. The figure is a typical example).

the shoulder of the curve, approximately 9 hours after dilution and incubation at 30°C.

The role of RpoS in the growth phase-dependent and hydrogen peroxide-dependent inducibility of the *ahp* locus

Overnight cultures of MPG203 and MPG471 (*rpoS*) were diluted 1: 10, 000 into 20 ml of fresh LB. Cultures were then incubated at 30°C with shaking for 4 hours, the period deemed most representative of exponential phase (see Figure 3.2). The samples were then divided into two 10 ml aliquots, and one was treated with 100 μM hydrogen peroxide (final concentration) and the other was left untreated. At 30 minute intervals, 200 μl of each sample was withdrawn and the amount of bioluminescence measured in a 96-well plate-reading luminometer, pre-heated to 30°C. An equivalent aliquot was appropriately diluted and plated to determine the cell number. As is shown in Figure 3.3 Panel A, no light was induced from the untreated samples of MPG203 and MPG471. However, exposure to hydrogen peroxide resulted in substantial bioluminescence in both strains irrespective of the *rpoS* background. This suggests that RpoS does not substantially affect the basal or hydrogen peroxide-inducible expression of the *ahp* locus in the exponential phase of growth.

In order to determine whether *ahp* is expressed in early stationary phase, samples were also taken at 9 hours (Figure 3.2). At this time point cultures were divided into two 10 ml portions, and treated as before. (Previous studies (Ivanova *et al.*, 1994) had shown that 100 μM hydrogen peroxide was a suitable induction stimulus even in cultures of high cell density). 200 μl aliquots of the samples were withdrawn and examined for bioluminescence and cell number, as described previously. As shown in 3.3 Panel B, no light was induced in either MPG203 or MPG471 in the presence or absence of a stimulus indicating that *ahp* is not expressed at the onset of stationary phase irrespective of *rpoS* background.

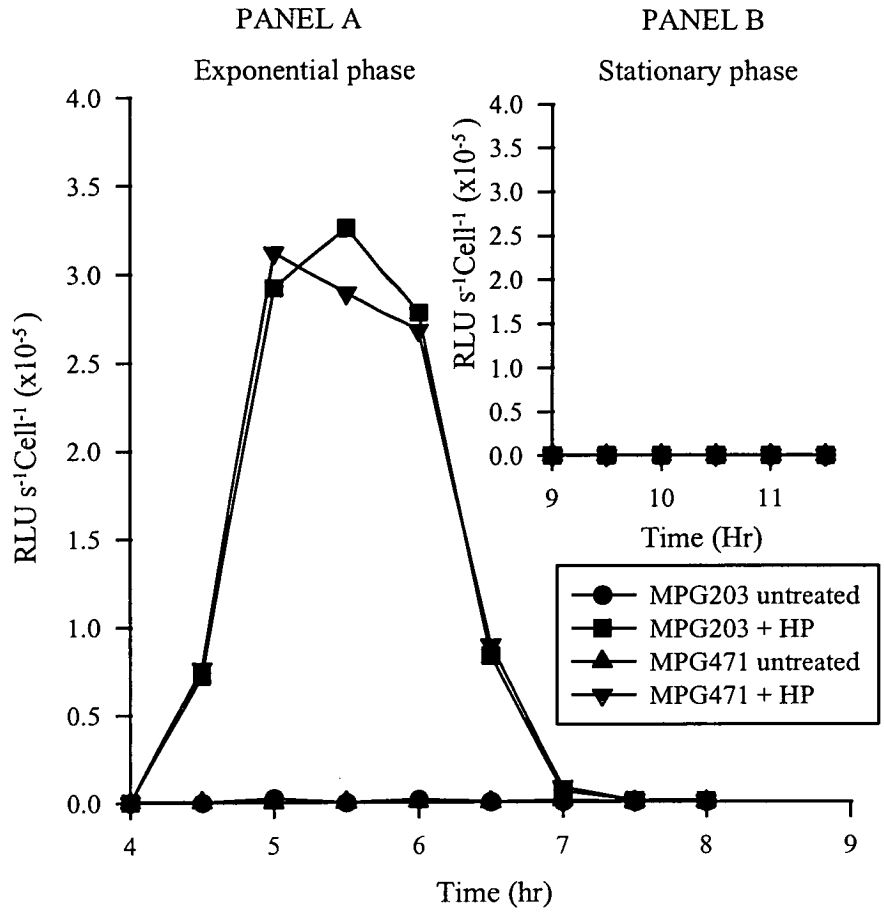


Figure 3.3 The effect of RpoS upon the expression of the *ahp* locus in exponential and stationary phases of growth

Overnight cultures of MPG203 and MPG471 (*rpoS*) were diluted 1: 10, 000 into fresh LB medium and grown with shaking at 30°C. Incubation was continued for 4 hours or 9 hours, the time points at which the cultures were deemed to be in exponential or early stationary phases, respectively (see Figure 3.2). At these time points each culture was split into two. One portion was treated with 100 μM hydrogen peroxide (HP), and the other portion was left untreated. At half-hourly time points after these treatments, 200 μl aliquots of each sample were extracted and examined for bioluminescence in a luminometer. An equivalent sample was also serially diluted and plated onto solid medium to determine the cell number. The level of induction of the *ahp* locus is expressed as relative light units per second produced by each cell (RLU s^{-1} $cell^{-1}$). Panels A and B represent the expression of the *ahp* locus in MPG203 and MPG471 during the exponential and stationary phases of growth respectively. The x-axis of each graph starts at the relevant time point for that growth phase. (Experiments were repeated several times and found to be reproducible. The graph is a typical example).

3.2.3 Examination of the growth phase-dependent and hydrogen peroxide-dependent inducibility of the *ahp* locus, using immunoblotting

The use of the bioluminescent reporter system suggested very substantial induction of *ahp* was possible with hydrogen peroxide in the exponential phase. However, it was unclear whether the reporter system exaggerated the true response. In addition, the stationary phase expression suggested that no induction of *ahp* was occurring. Thus, immunological procedures were employed to validate whether the reporter system provided a true reflection of *ahp* expression.

Immunological detection of AhpC production in exponential or stationary phase cultures of SL1344 and MPG470

Overnight cultures of SL1344 and MPG470 (*rpoS*) were diluted into 20 ml of fresh LB and grown for 4 hours at 30°C with shaking. These cultures were then split into two 10 ml aliquots and one was oxidatively stressed with 100 μM hydrogen peroxide whilst the other was left untreated. After a further hour of incubation at 30°C (OD_{600} of 0.1-0.2) the cells were pelleted, resuspended in 40 μl of LSB and subject to SDS-PAGE. Samples (15 μl) were loaded onto two parallel gels (12.5% v/v polyacrylamide) and electrophoresed. One gel was then stained with Coomassie Brilliant Blue to examine the profile of proteins in each sample. The other gel was used in Western blotting and the AhpC protein was subsequently detected using mouse anti-AhpC polyclonal anti-serum followed by rabbit anti-mouse alkaline phosphatase-conjugated antibody. A description of the purification of His-tagged AhpC protein for raising anti-sera in mice is given in chapter 6. Examination of the Coomassie Brilliant Blue stained gel showed that the lanes were evenly loaded. (Figure 3.4 Panel A). However, no distinct AhpC band was detected from the samples taken in the exponential phase even following exposure to hydrogen peroxide.

Two bands were detected after Western blotting (Figure 3.4 Panel B). The lower of these is believed to be the AhpC protein which has an approximate molecular mass

of 22 kDa (Jacobson *et al.*, 1989). This is supported by a control sample containing purified His-tagged AhpC protein (lane A). This purified AhpC is approximately 2.5 kDa larger than the native protein and as such, runs slightly above the native protein during SDS-PAGE. The upper band (labelled as X) is an unknown component, which may cross react with the AhpC-specific antibodies or the rabbit anti-mouse antibody conjugate. The presence of this band fortuitously provided a control to demonstrate that the amount of sample loaded into each lane was equivalent.

A basal level of AhpC was detected by immunoblotting untreated exponential phase samples (Figure 3.4 Panel B, lanes 1 and 3). Moreover, RpoS did not appear to affect this. In the presence of hydrogen peroxide, the level of AhpC increased but was equivalent for both *rpoS*$^{+}$ and *rpoS*$^{-}$ strains (Figure 3.4 Panel B, lanes 2 and 4). This is in agreement with the luminescence data (Figure 3.3 Panel A) and confirms that RpoS has little effect upon the hydrogen peroxide-dependent inducibility of *ahp* in the exponential phase. However, it is noteworthy that, from immunodetection, the level of induction of *ahp* above the uninduced samples appears only moderate suggesting that the light-based reporter system exaggerates the response.

Stationary phase cultures of SL1344 and MPG470 were similarly examined. Overnight cultures were diluted into 40 ml of fresh LB and grown for 9 hours at 30°C, the time point where cells should be entering early stationary phase (see Figure 3.2). The cultures were then split into 10 ml aliquots and oxidatively stressed with either 100 μM, 500 μM or 1000 μM hydrogen peroxide (final concentration in LB) or were left untreated. After a further hour of incubation at 30°C, 0.5 ml of cells were then pelleted, resuspended in 200 μl LSB and subject to SDS-PAGE. 15 μl of each sample was loaded onto identical polyacrylamide gels (12.5% [v/v]) and electophoresed. As before, gels were then stained or subject to Western blotting.

The results are shown in Figures 3.5 Panel A and Panel B. No specific AhpC band was seen in any of the samples from the Coomassie-stained gel (Figure 3.5 Panel A) indicating that it is not a prominant protein in the stationary phase. Examination of

Figure 3.4 The immunological detection of AhpC from exponential phase cultures of SL1344 and MPG470

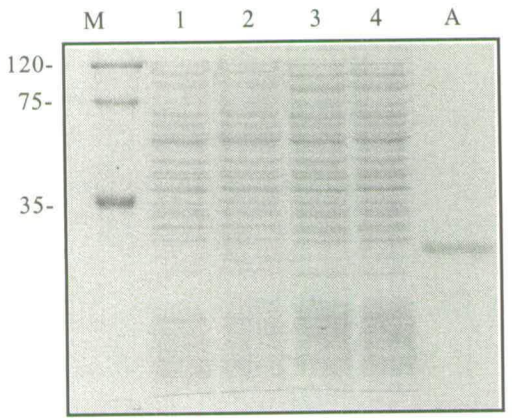
Overnight cultures of SL1344 (virulent parental strain) or MPG470 (*rpoS*) were diluted 1: 10, 000 into fresh LB, to give approximately 10^5 c.f.u. ml^{-1}, and grown with shaking at 30°C for 4 hours. The culture was then split into two 10 ml volumes and either treated with 100 μM hydrogen peroxide or left untreated. After incubation for a further hour, the samples were subject to SDS-PAGE on two identical gels. Following electrophoresis, one gel was stained using Coomassie Brilliant Blue to examine the protein profile of each sample (Figure 3.4 Panel A), and the other gel was subjected to immunoblotting, using an anti-AhpC antisera for detection (Figure 3.4 Panel B). Lanes 1 and 2, and lanes 3 and 4, represent SLI344 and MPG470, respectively. Lanes 1 and 3, and lanes 2 and 4, represent untreated samples and samples treated with hydrogen peroxide, respectively. Pre-stained molecular weight markers (120, 75 and 35 kDa) are shown in lane M and a purified sample of His-tagged AhpC protein (see chapter 6) is shown in lane A. Bands corresponding to the wild type AhpC protein and to an unidentified component, X, are indicated with arrowheads (Panel B).

Figure 3.5 The immunological detection of AhpC from early stationary phase cultures of SL1344 and MPG470

Overnight cultures of SL1344 (virulent parental strain) and MPG470 (*rpoS*) were diluted to give approximately 10^5 c.f.u. ml^{-1} and grown with shaking for 9 hours at 30°C. Cultures were then split into four and treated with 100 μM, 500 μM or 1000 μM hydrogen peroxide or left untreated. After a further hour of incubation, samples were loaded onto two identical gels and subjected to SDS-PAGE. Following electrophoresis, one gel was stained using Coomassie Brilliant Blue to examine the protein profile of each sample (Figure 3.5 Panel A), and the other subject to immunoblotting, using an anti-AhpC antisera for detection (Figure 3.5 Panel B). Lanes 1-4 and lanes 5-8 represent SL1344 and MPG470, respectively. Untreated samples or samples treated with 100 μM, 500 μM or 1000 μM hydrogen peroxide are represented by lanes 1 and 5, lanes 2 and 6, lanes 3 and 7, and lanes 4 and 8, respectively. Pre-stained molecular weight markers (120, 75 and 35 kDa) are shown in lane M and a purified sample of His-tagged AhpC protein (see chapter 6) is shown in lane A. Bands corresponding to the wild type AhpC protein and to an unidentified component, X, are indicated by arrows (Panel B).

Figure 3.4

PANEL A



PANEL B

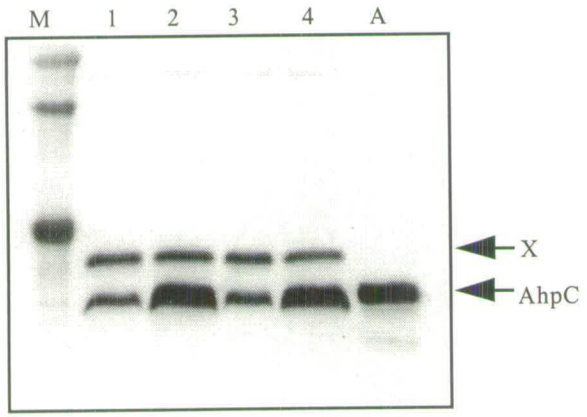
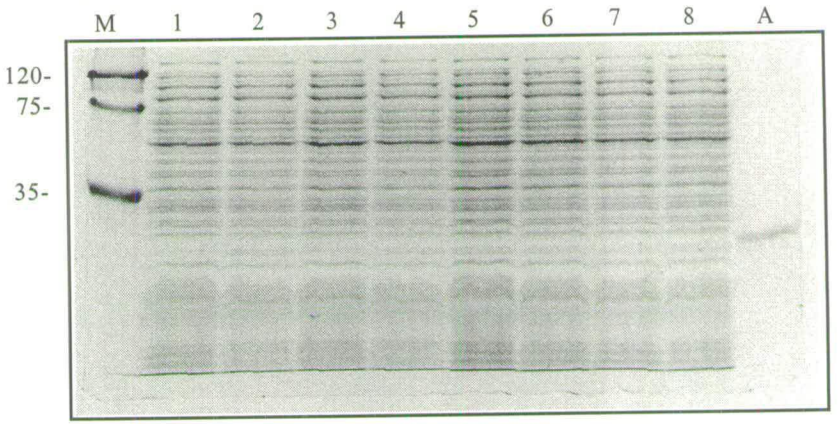
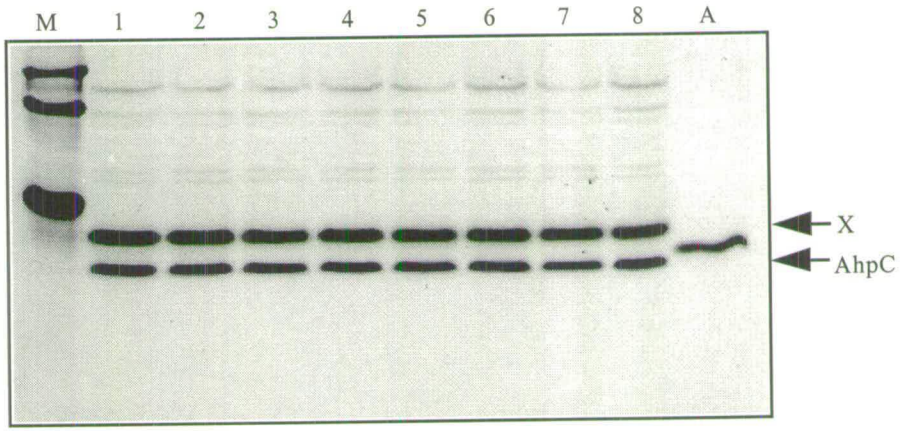


Figure 3.5

PANEL A



PANEL B



the Western blot (Figure 3.5 Panel B) showed that AhpC is not positively induced at the onset of stationary phase, either in the presence or absence of hydrogen peroxide, independently of the *rpoS* status, and this in agreement with the luminescent data from Figure 3.3 Panel B. This confirms that OxyR and RpoS have no effect upon the expression of *ahp* in the stationary phase. Interestingly, however an equivalent level of AhpC could be seen in all the cell extracts.

Native or activated OxyR, supplied *in trans*, is unable to bring about the induction of *ahp* in the stationary phase

The native OxyR protein is sensitive to oxidation and in order to bring about the induction of the OxyR regulon it must be converted into an activated form (Storz *et al.*, 1990). This activation occurs when cells are oxidatively stressed. Since the *ahp* locus appeared not to be induced in the stationary phase, it was unclear whether this reflected on a lack of OxyR protein, an inability of the protein to be activated, or an inability of the protein to function at its target site. These questions were addressed by cloning the native *oxyR* or a mutated form of *oxyR*, which mediates constitutive expression of genes of the OxyR regulon in the exponential phase of growth, into a controllable expression vector. These plasmids were then transferred into an *oxyR* deficient strain, in order that the effects of complementation could be examined. The vector pBAD18 which contains the arabinose-inducible promoter (P_{BAD}) from the *araBAD* operon of *E. coli*, was chosen as the recipient for these *oxyR* genes (Guzman *et al.*, 1995) (see Figure 3.6). The plasmid also contains the *araC* gene, which encodes a protein that can both repress and activate P_{BAD} (Figure 3.6). Repression and induction of the promoter occurs in the absence or presence of arabinose, respectively, and is mediated by a conformational change and alteration in the binding capacity of the AraC protein at P_{BAD}. Thus, in these constructs, expression of OxyR *in trans* could be regulated by the addition of arabinose to the media.

The sequence of the *oxyR* gene from *E. coli* had been determined previously (Christman *et al.*, 1989) but the equivalent gene from *S. typhimurium* had not. However, the two species appear to show highly conserved responses to hydrogen

peroxide stress. Furthermore, studies have shown that the *ahp* promoter of *S. typhimurium* can be regulated by the *E. coli* OxyR protein (Tartaglia *et al.*, 1990, 1992). On this basis, the *oxyR* gene from *E. coli* was used to address the role of native OxyR in the induction of *ahp* in the exponential and stationary phases of growth. Primers were devised to the 5' (OXYR1) and 3' (OXYR2) ends of the coding region of the *E. coli oxyR* gene (see Table 2.1), such that when amplified the *oxyR* gene would be promoterless. *Eco*RI and *Hin*dIII sites were included on the respective primers to allow directional cloning of the *oxyR* gene downstream of the P_{BAD} promoter in pBAD18. Using these primers, a single product of approximately 1.1 kb was amplified from the *E. coli* DH5α genome and was cloned into *Eco*RI-*Hin*dIII digested pBAD18 to form pPDT3 (Figure 3.6).

Because of the likely homology between the *oxyR* genes from *S. typhimurium* and *E. coli* an attempt was made to use the same primers to amplify the *oxyR* gene from the *S. typhimurium* strain TA4100. TA4100 was originally isolated following chemical mutagenesis of *S. typhimurium* and displays constitutive expression of the genes in the OxyR regulon (Christman *et al.*, 1985). This feature is a reflection of an unknown mutation in the *oxyR* gene which results in the OxyR protein being locked into an active configuration. Amplification, using the primers devised for the *E. coli* sequence, OXYR1 and OXYR2, resulted in a single band of the same size as that of the *E. coli oxyR* gene. This suggested that the DNA amplified from the *S. typhimurium* chromosome was the *oxyR* gene. The original primers to *oxyR* provided *Eco*RI and *Hin*dIII restriction sites. However, since the sequence of the *S. typhimurium* gene was unknown the restriction sites present in the gene were also uncharacterised. The PCR product was treated with *Eco*RI or *Hin*dIII to determine whether either of these sites were suitable to flank the gene for cloning purposes. The PCR product was found to contain internal sites for *Hin*dIII recognition but not for *Eco*RI (data not shown). Therefore, it was decided to flank the *oxyR* gene with *Eco*RI sites at either end.

With a change of primers, OXYR1 and OXYR3 (see Table 2.1), the *oxyR* gene was amplified from the *S. typhimurium* genome flanked by *Eco*RI sites. This

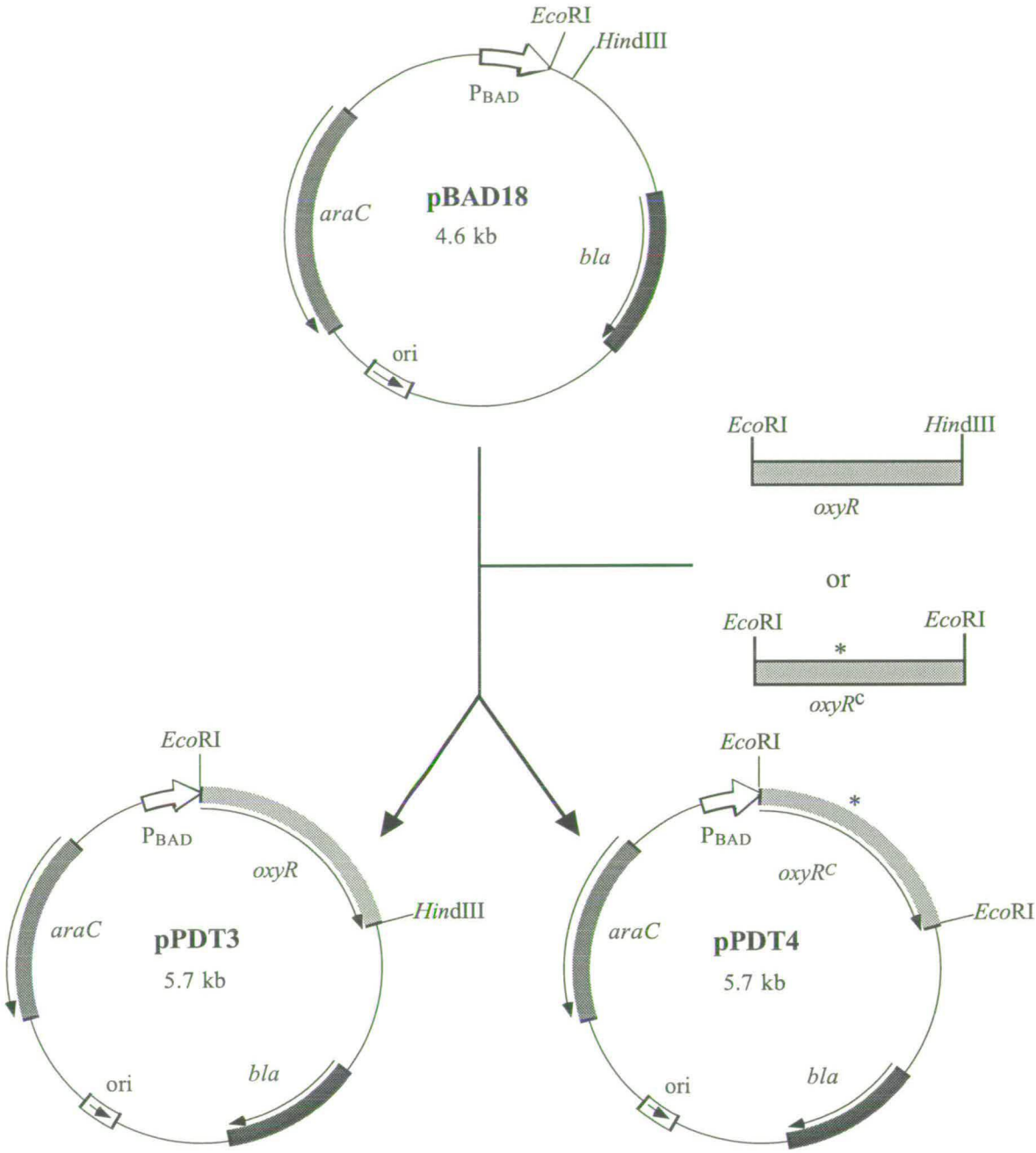


Figure 3.6 A summary of the cloning of the *oxyR* gene into a controllable expression vector

A promoterless copy of a wild type *oxyR* gene from *E. coli,* flanked by *Eco*RI and *Hin*dIII sites, and an *oxyR* gene, flanked by *Eco*RI sites, from an *S. typhimurium* strain TA4100 (Christman *et al.*, 1985) which produces a constitutively active form of OxyR, were obtained by PCR using primers OXYR1 and OXYR2 or OXYR1 and OXYR3, respectively (see Table 2.1). The resulting *oxyR* genes were cloned downstream of the arabinose inducible P_{BAD} promoter in pBAD18 to form pPDT3 and pPDT4, respectively. Key: *araC*, the gene encoding the AraC regulatory protein; P_{BAD}, the arabinose-inducible promoter from the *araBAD* operon of *E. coli*; *bla*, the ampicillin-resistance gene; ori, origin of replication; *oxyR*, the gene encoding the native OxyR regulatory protein; *oxyR* ^{c}, a mutant *oxyR* gene that produces constitutively active OxyR; *, denotes that the *oxyR* ^{C} gene carries a mutation.

S. typhimurium oxyR gene was cloned into the *Eco*RI site of pBAD18 to form pPDT4 (Figure 3.6). Plasmids pPDT3 and pPDT4 were subsequently transferred from *E. coli* strain DH5α into an *oxyR* mutant of *S. typhimurium*, MPG484, by electroporation to give MPG485 and MPG486, respectively. A full description of the construction of the SL1344 derivative MPG484 is outlined in chapter 5, but in brief the *oxyR* gene of this strain has been inactivated by the insertion of a kanamycin gene.

The native OxyR protein should only activate transcription of *ahp* in the presence of hydrogen peroxide. In contrast, the constitutively active protein should express high levels of *ahp* even in the absence of this oxidant. A preliminary test was performed to ensure that the cloned *oxyR* gene could be induced by arabinose in these *S. typhimurium* strains, and that expression of the genes resulted in a wild type or constitutive phenotype, as appropriate. This was confirmed by examining the sensitivity of MPG485 (*oxyR*::*kan*, pPDT3) and MPG486 (*oxyR*::*kan*, pPDT4) to hydrogen peroxide (3% v/v) or cumene hydroperoxide (3% v/v) using a disc inhibition test in the presence or absence of arabinose. The result of adding these peroxides to MPG485 and MPG486 are shown in Table 3.2. SL1344, MPG484 (*oxyR*::*kan*) and MPG487 (*oxyR*::*kan*, pBAD18) serve as a controls.

The addition of arabinose to the medium, to bring about the induction of *oxyR* from the plasmids, pPDT3 and pPDT4, resulted in enhanced survival compared to the *oxyR* deficient strain (MPG484). As expected the constitutively active form of OxyR in MPG486 provided the greatest protection to both oxidants, although the native gene also had a significant effect. Moreover, even in the absence of arabinose the presence of the plasmid-borne *oxyR* genes resulted in enhanced survival to the oxidant challenge, suggesting that the plasmids may be slightly leaky under the test conditions. This may reflect on the cyclic-AMP receptor protein regulator, which is capable of enhancing the expression of the arabinose operon under carbon starvation (Guzman *et al.*, 1995).

Table. 3.2 The effect of pPDT3 and pPDT4 on the sensitivity of an *oxyR* mutant to hydrogen peroxide and cumene hydroperoxide

Strain	Zone of Inhibition (mm)	
	3% (v/v) HP	3% (v/v)CHP
SL1344	16	22
" +Ara	16	22
MPG484	30	31
" +Ara	30	31
MPG485	20	27
" +Ara	17	23
MPG486	10	20
" +Ara	9	15
MPG487	30	31
" +Ara	30	31

Cultures of *S. typhimurium* SL1344, MPG484 (*oxyR*::*kan*), MPG485 (*oxyR*:: *kan*, pPDT3), MPG486 (*oxyR*::*kan*, pPDT4) and MPG487 (*oxyR*::*kan*, pBAD18) were grown overnight in LB containing the appropriate antibiotic. 100 µl of each sample was then added to 3 ml of LC top agar, at 50°C, containing 0.5% (v/v) arabinose or left untreated and poured onto LB plates. Once set, a 5 mm filter paper disc (Whatmann number 4) was gently placed onto the surface of the agar and 10 µl of either 3% (v/v) hydrogen peroxide (HP) or 3% (v/v) cumene hydroperoxide (CHP) was then administered to each disc. After overnight incubation at 37°C the zone of inhibitions were measured (mm). The values in the table represent the average of two separate experiments. Variation between experiments was no greater than 1 mm.

It can be concluded from Table 3.2 that addition of arabinose to MPG485 and MPG486 results in induction of the native or constitutively activated forms of OxyR, respectively. This knowledge would help to determine whether the lack of induction of *ahp* which was seen in stationary phase (Figure 3.5) was due to a deficiency of OxyR protein, or to some inability to convert the protein into an 'active' form or indeed, to a lack of ability of the OxyR protein to bind the target promoter. An

exponential phase culture was included to show that the *oxyR* genes in pPDT3 and pPDT4 behaved as expected.

Cultures of MPG485 and MPG486 were diluted 1: 10, 000 into 4 x 10 ml fresh LB and grown for 4 hours to establish exponential phase growth. After this time, samples were either treated with 0.5% arabinose (v/v), 100 μM hydrogen peroxide, both chemicals, or were left untreated. Each sample was then incubated for a further hour. The optical density was measured and the samples were subjected to SDS-PAGE and Western blotting as described previously. Early stationary phase cultures of MPG485 and MPG486 were treated similarly except that they were grown for 9 hours instead of 4 hours.

The results of these blots are shown in Figures 3.7 Panel A and 3.7 Panel B for the exponential and stationary phase samples, respectively. The unidentified component X acts a control to show that the loading is even. In the exponential phase (Figure 3.7 Panel A), induction of AhpC in MPG485 was observed in the dual presence of arabinose and hydrogen peroxide (lane 4) but not in their absence (lane 1). This indicates that the OxyR which was produced could be activated by exposure to hydrogen peroxide. However, enhanced AhpC expression was also observed in the sample treated with hydrogen peroxide only (lane 2) but not with arabinose only (lane 3). This suggested that even in the absence of arabinose, the plasmid provided sufficient OxyR to bring about the expression of AhpC. In contrast, all the samples of MPG486 from the exponential phase displayed essentially the same level of AhpC protein (Figure 3.7 Panel A, lanes 5 to 8), independently of the presence or absence of arabinose or hydrogen peroxide. These results also indicated that 'leaky' expression from the plasmid was sufficient to bring about induction of the *ahp* locus in the exponential phase but in addition, showed that hydrogen peroxide was not required for activation, as would be expected with the constitutively active mutant OxyR protein.

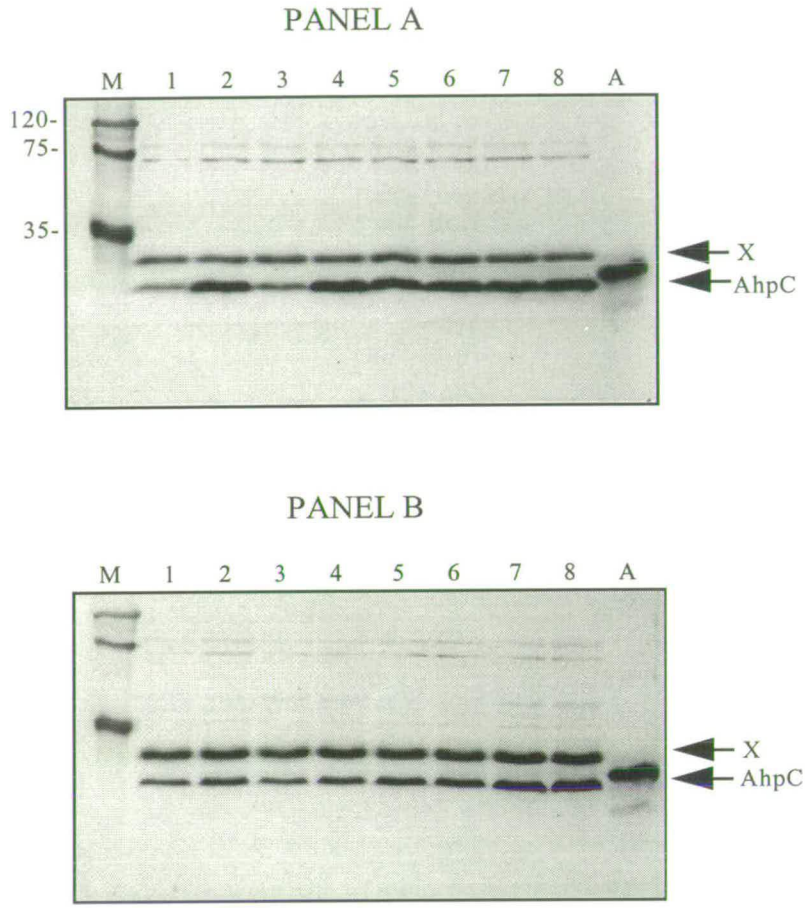


Figure 3.7 Detection of AhpC expression in MPG485 and MPG486 in the exponential and stationary phases of growth

MPG485 (*oxyR*::*kan*, pPDT3) and MPG486 (*oxyR*::*kan*, pPDT4) were diluted to approximately 10^5 c.f.u. ml^{-1} in LB medium and grown at 30°C with shaking for 4 or 9 hours. Each culture was then divided into four volumes and was left untreated or was treated with 0.5% (v/v) arabinose, or 100 μM hydrogen peroxide or a combination of these treatments. After incubation for a further hour, samples were subjected to SDS-PAGE and Western blot analysis. AhpC was detected using an anti-AhpC antisera. The results represent samples from exponential (Panel A) or early stationary phase (Panel B). Lanes 1-4 and 5-8 represent MPG485 and MPG486 respectively. Untreated samples (lanes 1 and 5) or samples treated with hydrogen peroxide (lanes 2 and 6), or arabinose (lanes 3 and 7), or a combination of these treatments (lanes 4 and 8) are shown. Pre-stained molecular weight markers (120, 75 and 35 kDa) were included in lane M and lane A contained a purified sample of His-tagged AhpC protein. Bands representing the wild type AhpC protein and the unidentified component X are indicated by arrows.

With the stationary phase samples, all the lanes displayed very similar levels of AhpC protein irrespective of the strain (Figure 3.7 Panel B). This indicates that the presence of OxyR, in either the native state or activated state, was unable to bring about the induction of AhpC, and that the protein detected solely represents basal gene expression independently of OxyR. This suggests that OxyR is unable to mediate any effect on the *ahp* promoter in the stationary phase of growth. It might be suggested that lanes 5 to 8 (MPG486) have slightly more AhpC. However, this is likely to reflect on the accumulation of the protein during the exponential phase, as a consequence of continuous induction by the permanently activated form of OxyR basally-expressed from pPDT4. This is unlikely to be seen in MPG485 because in the absence of hydrogen peroxide pPDT3 appears not to elicit *ahp* expression.

The above results could not exclude the possibility that the P_{BAD} promoter does not function in stationary phase and so is unable to express the *oxyR* gene. This situation was addressed by using an *S. typhimurium* SL1344 strain MPG488, containing a pBAD18 derivative, pBADLAC (a gift from Dr N. McLennan, Edinburgh University) in which the β-galactosidase gene (*lacZ*) has been cloned downstream of the P_{BAD} promoter. Using MPG488, the ability of arabinose to induce β-galactosidase activity in the exponential and early stationary phases of growth was therefore examined. As before, MPG488 was grown for 4 hours (exponential phase) or 9 hours (early stationary phase) and the samples were then treated with arabinose (final concentration 0.5% (v/v) or left untreated. After a further hours incubation at 30°C, β-galactosidase activity was assessed by the method of Miller (1972b) which takes into account cell number. The results of these tests are displayed below, together with the standard error of the mean (SEM).

The results displayed in Table 3.3 clearly show that P_{BAD} functions in both the exponential and stationary phases of growth. Moreover, from the data it can also be seen that the arabinose-independent expression of *lacZ* is higher in the stationary phase of growth. This strongly indicates that the lack of inducibility of *ahp* in the stationary phase (Figure 3.7 Panel B) does not result from a lack of ability to express OxyR in this phase of growth.

Table 3.3 The expression of *lacZ* from the P_{BAD} promoter during exponential and stationary phase

	Expression of β-galactosidase	
Strain	**Exponential phase**	**Stationary phase**
MPG488 untreated	442.4 ± 17.8	2800.0 ± 50.6
MPG488 + 0.5% (v/v) Ara	2345.0 ± 25.3	28692.0 ± 100.8

An overnight culture of MPG488 (pBADLAC) was diluted 1: 10, 000 fold into fresh LB medium and grown at 30°C for 4 or 9 hours with shaking. At these time points, samples were taken and treated with 0.5% arabinose (Ara) or left untreated. After a further hour of incubation at 30°C the β-galactosidase activity from each sample was assayed by the method of Miller (1972b). Values are expressed as Miller units and represent the mean and standard error of the mean of triplicate samples.

Examination of the role of OxyR in the basal level expression of the *ahp* locus in the exponential and stationary phases of growth

The results from Figures 3.3, 3.4 and 3.5 suggested that RpoS has no major influence upon *ahp* expression. In addition, Figure 3.7 Panel B suggested that even in the presence of activated OxyR, *ahp* expression could not be induced in the stationary phase. Nevertheless, AhpC is clearly visible in samples from stationary phase cultures. Although OxyR may not be able to induce expression of *ahp*, it was unclear whether OxyR was necessary or responsible for the basal level of expression seen in these experiments. The expression of *ahp* was therefore compared in SL1344 and in an *S. typhimurium* strain in which the *oxyR* gene had been insertionally inactivated, MPG484 (*oxyR*::*kan*).

Cultures of SL1344 and MPG484 were diluted to give approximately 10^5 c.f.u. ml^{-1} and were subsequently grown with shaking at 30 °C for 4 or 9 hours. Exponential phase cultures (4 hours growth) were then divided into two volumes and were treated

with 100 μM hydrogen peroxide or were left untreated and incubated for a further hour. Stationary phase cultures (9 hours growth) were split into four volumes and treated with 100 μM, 500 μM and 1000 μM hydrogen peroxide or were left untreated, and samples were then incubated for a further hour. After this time, samples were subjected to SDS-PAGE and Western blot analysis as described previously. The blots for the exponential and stationary phase cultures are shown in Figures 3.8 Panel A and 3.8 Panel B, respectively. Again, the presence of band X acted as a control to show that all lanes were evenly loaded.

Figure 3.8 Panel A shows that, as demonstrated previously (Figure 3.4 Panel A), the untreated sample of SL1344 (lane 1) displays no induction of *ahp* in the exponential phase. However, hydrogen peroxide induced a substantial response (lane 2). In contrast, the exponential phase samples of MPG484 (in which *oxyR* was disrupted) showed a basal level of AhpC in both tracks independently of the presence or absence of hydrogen peroxide (lanes 3 and 4). Since both the untreated samples of SL1344 and MPG484 (lanes 1 and 3) showed an equivalent level of AhpC protein it seemed that the OxyR protein is not responsible for basal level expression of *ahp* in the exponential phase but that, OxyR is required for the hydrogen peroxide inducibility of *ahp* in the exponential phase.

In the stationary phase cultures (Figure 3.8 Panel B), all the lanes displayed essentially the same level of AhpC protein, irrespective of the strain used or exposure to hydrogen peroxide. Thus, it seems that neither RpoS (Figure 3.5 Panel B) nor OxyR (Figure 3.8 Panel B) are required or responsible for the basal level of AhpC expression which occurs in *S. typhimurium* in the exponential and stationary phases of growth. Moreover, it seems that OxyR is only capable of inducing *ahp* expression in the exponential phase of growth and not in the stationary phase.

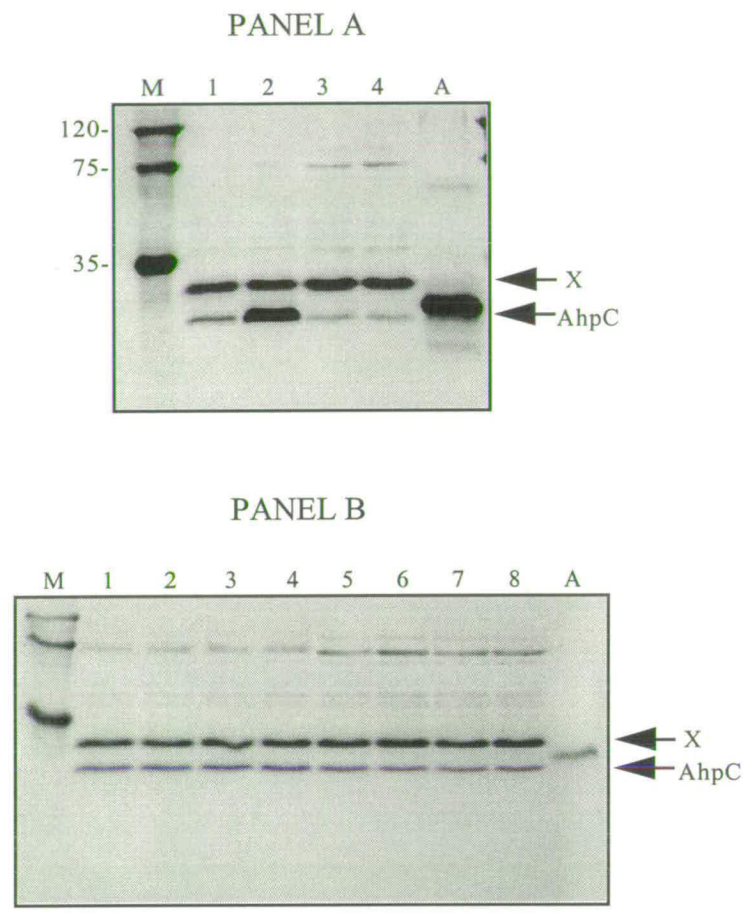


Figure 3. 8 Detection of AhpC in exponential and stationary phase cultures of SL1344 and MPG484

SL1344 and MPG484 (*oxyR*::*kan*) were diluted to give approximately 10^5 c.f.u. ml^{-1} and grown with shaking at 30°C for either 4 or 9 hours. For the exponential phase experiments, cultures were split into two volumes and were left untreated or were treated with 100 μM hydrogen peroxide and incubated for a further hour. For the stationary phase experiment, cultures were split into four volumes and were left untreated or treated with 100 μM, 500 μM or 1000 μM hydrogen peroxide and incubated for a further hour. After treatment, all samples were subject to SDS-PAGE and Western blot analysis. AhpC was detected using an anti-AhpC antisera. The blots for exponential phase and stationary phase samples are shown in Panel A and Panel B, respectively. For Panel A, lanes 1-2 and 3-4 represent SL1344 and MPG484 respectively. Untreated and treated samples are represented by lanes 1 and 3 and 2 and 4, respectively. For Panel B, lanes 1-4 and 4-8 represent SL1344 and MPG484, respectively. The untreated samples and samples treated with either 100 μM, 500 μM or 1000 μM are represented by lanes 1 and 5, lanes 2 and 6, lanes 3 and 7 and lanes 4 and 8, respectively. Pre-stained molecular weight markers (120, 75 and 35 kDa) were included in lane M and lane A contained a purified sample of His-tagged AhpC protein. Bands representing the wild type AhpC protein and the unidentified component X are indicated by arrows.

3.3 DISCUSSION

The *ahp* locus of *S. typhimurium* has been shown to be induced upon interaction with macrophages (Francis and Gallagher, 1993; Francis, PhD Thesis, 1993) and therefore may have a role in virulence. The alternative sigma factor RpoS (σ^S) regulates major aspects of the stationary phase response of *S. typhimurium* (Hengge-Aronis, 1996a) and, importantly, has been shown to be induced within the macrophage environment (Chen *et al.*, 1996a). Therefore, the role of growth phase and RpoS upon the expression of the *S. typhimurium ahp* locus was examined in the present study as these factors may have important implications in the expression of *ahp* during the course of infection.

The use of a bioluminescent reporter system tagged to the *ahp* locus and Western blotting, demonstrated that RpoS had no effect upon the hydrogen peroxide-dependent inducibility or the basal level of expression of *ahp* in the exponential phase of growth. Equally, cells in the stationary phase also showed no significant induction of the *ahp* locus in the presence or absence of hydrogen peroxide and independently of the RpoS status of the cells. This is in contrast to the *katG*, *dps*, and *gorA* genes of *E. coli* which are each regulated by OxyR in the exponential phase and which all display induction upon transition of cells into the stationary phase in an RpoS-dependent manner (Ivanova *et al.*, 1994; Altuvia *et al.*, 1994; Becker-Hapak *et al.*, 1995).

These findings might suggest that the functions expressed by the other OxyR-regulated genes have a greater importance than AhpCF in protecting the cell against oxidative stress in the stationary phase of growth. Equally, it might be that some alternative features of the cell make the function of AhpCF redundant in the stationary phase. For example, it is known that stationary phase cells become rounder and shorter and the periplasm expands and the cytoplasm contracts (Huisman *et al.*, 1996). These alterations may make it more difficult for hydrogen peroxide to penetrate the cell. The fact that the catalases KatE and KatG are induced on entry to the stationary phase and exist in the periplasm and cytoplasm, respectively (Kagaya

et al., 1992), might suggest that hydrogen peroxide is capable of entering each of these cellular compartments. However, that the cytoplasmic catalase is induced largely to protect the cell against reactive oxygen species, which form as by-products of the respiratory chain, cannot be excluded. Biochemical changes to the lipid membrane, including a 10% reduction in the level of unsaturated fatty acids and a 5-fold increase in cyclopropyl fatty acid derivatives, are also known to take place in stationary phase (Huisman *et al.*, 1996), and perhaps these may make the membrane less susceptible to lipid peroxidation.

In *E. coli*, RpoS regulates a number of genes with oxidative stress defence capabilities including *katE*, *xthA*, *katG*, *dps*, and *gorA* and these are likely to be regulated in a similar manner in *S. typhimurium* (Hengge-Aronis, 1996a; Ivanova *et al.*, 1994; Altuvia *et al.*, 1994; Becker-Hapak and Eisenstark, 1995). Furthermore, a group of starvation inducible genes, *stiA*, *stiB* and *stiC*, whose functions are unknown were recently shown to contribute to the hydrogen peroxide resistance of *S. typhimurium* in both the exponential and stationary phases of growth (Seymour *et al.*, 1996). These genes are regulated by RpoS and also appear to play a major role in the starvation stress response of *S. typhimurium*. Mutations in any of *stiA*, *stiB* and *stiC* reduce the long term survival of *S. typhimurium* to a level equivalent of an *rpoS* mutant (50-100 fold). Interestingly, *stiA* was also recently shown to be regulated by OxyR in a non-classical fashion in that it is repressed by reduced OxyR but it is neither activated or repressed by oxidised OxyR yet, its repression is alleviated under oxidative stress (Seymour *et al.*, 1996). RpoS therefore equips the cell with a wide range of oxidative stress defence measures and it could be that this circumvents the need for Ahp expression in the stationary phase of growth. In agreement with this explanation, stationary phase cultures of the *ahp*::Mu*dlux* strain (MPG203) displayed an almost equivalent level of sensitivity to that of *S. typhimurium* SL1344 after challenge with 20 mM or 50 mM hydrogen peroxide (see Table 3.1). In contrast, it has been shown that in stationary phase, that mutants of *dps*, *katG* (*E. coli*) and *stiA* (*S. typhimurium*) are hypersusceptible to similar peroxide challenges compared to parental strains (Almiron *et al.*, 1992; Ivanova *et al.*, 1994; Seymour *et al.*, 1996).

B. subtilis also displays an adaptive response to hydrogen peroxide, inducing several genes which are equivalent to those in the OxyR regulon, such as a catalase (*katA*), a Dps homologue (*mrgA*) and the small (*ahpC*) and large (*ahpF*) subunits of an alkyl hydroperoxide reductase (Hartford and Dowds *et al.*, 1994; Chen *et al.*, 1995). A regulator for these proteins has also been postulated called PerR. However, in contrast to OxyR, PerR is believed to act as a repressor of the hydrogen peroxide resistance genes in the absence of oxidative stress (Chen *et al.*, 1995). Interestingly, the *B. subtilis katA*, *mrgA* and *ahpC* and *ahpF* genes have also been shown to be induced by hydrogen peroxide in the stationary phase. However, it seems this regulation does not simply reflect on the level of oxidant in the cell but involves the level of metal ions present in the medium, particularly manganese (Mn), and to a lesser extent iron, cobalt or copper (Chen *et al.*, 1993, 1995). In fact, the stationary phase induction of the PerR regulon can be blocked if Mn is supplied to the medium. A role for iron in the regulation of the *Haemophilus influenzae* OxyR regulon and of an *ahpC* homologue from *Corynebacterium diptheriae* (35% identity to the *S. typhimurium* AhpC protein) has also been reported (Tai and Zhu, 1995; McIver and Hansen, 1996), although the role of growth phase was not determined. Whether the level of iron or other metal ions influences the expression of the *S. typhimurium ahp* locus has not been reported. Equally, the reason why defence against hydrogen peroxide should be regulated by the presence of such ions is unclear.

A diverse range of phenomena is seen in *B. subtilis* during stationary phase including expression of genes involved in the transition to growth arrest, development of motility and the initiation of sporulation processes (Hecker *et al.*, 1996). This stationary phase response in *B. subtilis* is largely regulated by an alternative sigma factor, SigB (σ^B) (Boylan *et al.*, 1993), and like σ^S, the level of σ^B increases towards the end of the exponential phase of growth and prepares the cell for periods of inactivity. Moreover, σ^B is responsible for the regulation of most of a core set of proteins, called general stress proteins (GSPs), which mediate resistance to a wide range of stress conditions including those to starvation, heat, acidity and oxidants (Hecker *et al.*, 1996).

Recently, the AhpC and AhpF proteins were identified as being members of a small number of GSPs which are independent of σ^B control, as the expression of *ahp* was not altered in a σ^B mutant (Antelmann *et al.*, 1996). Thus, the regulation of the *B. subtilis ahpCF* locus shows similarity to that of *S. typhimurium*, which seems to be independent of σ^S regulation. However, in contrast, the *B. subtilis ahp* locus can be induced in post-exponential growth and this has been attributed to a lack of specific metal ions in the medium or to activation via the production of endogenous hydrogen peroxide as a by-product of the electron transport chain as the growth rate diminishes (Chen *et al.*, 1995; Antelmann *et al.*, 1996; Bsat *et al.*, 1996). Whether or not the other genes of the PerR regulon display σ^B-independent expression is unknown at present. Interestingly, an *ahp* mutant of *B. subtilis* was found to be sensitive to cumene in the exponential phase of growth, but showed no difference to the parental strain in sensitivity to cumene in the stationary phase of growth (Antelmann *et al.*, 1996). This indicates that the cell may acquire some alternative factor that can deal with alkyl hydroperoxides in stationary phase. This possibility is supported by the observation that a *sigB* mutant is sensitive to cumene in the stationary phase of growth (Antelmann *et al.*, 1996).

Sigma factors control the genes that RNA polymerase can transcribe and provide a way of regulating developmental pathways (Lonetto *et al.*, 1992; Record *et al.*, 1996). Importantly, sigma factors are involved in protecting cells under stress conditions, for example RpoS (σ^S) and RpoH (σ^{32}) regulate genes that protect the cell from starvation and heat stress, respectively. In *S. enterica* cells, such sigma factors are likely to contribute to the virulence of the bacteria by protecting them from the stresses found within the host, particularly the macrophage (see chapter 1). σ^S is the closest relative of the σ family to σ^{70}, the primary sigma factor of vegetative bacterial cells. Sigma factors contain four regions (determined on the basis of predicted function), which in turn can be split into subregions (reviewed in Record *et al.*, 1996). Structural motifs, located in regions 2.4 (region 2 subregion 4) and 4.2 of σ^{70}, mediate binding to the -10 (Pribnow box) and -35 hexamer consensus sequences present in promoters of σ^{70}-dependent genes (Lonetto *et al.*, 1992; Record *et al.*,

1996). The difficulty in distinguishing between σ^S- and σ^{70}-regulated promoters is complicated because both sigma factors bind to very similar promoter sequences (Tanaka *et al.*, 1995; Espinosa-Urgel and Tormo, 1996). Moreover, *in vitro*, σ^S may bind to examples of genes which are regulated by σ^{70} yet, these genes are not expressed in the stationary phase *in vivo*. (Tanaka *et al.*, 1995; Kolb *et al.*, 1995).

A number of differences between the σ^S and σ^{70} promoters have nevertheless been postulated and these are displayed in Figure 3.9. σ^S appears to show little or no specificity for the σ^{70} -35 hexamer sequence (TTGACA) *in vivo*. However, -35 hexamers in which the two thymine residues were replaced by cytosine residues (CCGACA) were found to abolish *E. coli* σ^{70} recognition and introduced σ^S-dependent control (Wise *et al.*, 1996). Thus, a modified -35 sequence may have a role in σ^S recognition. In contrast to the -35 hexamer, the Pribnow box appears to be highly conserved in both σ^{70}- and σ^S-dependent promoters (Figure 3.9). Nevertheless, it is noteworthy that in approximately 70% of all σ^S-dependent genes thus far examined in *E. coli*, a cytosine residue occurs in front of the Pribnow box and at position 5 of this sequence (Kolb *et al.*, 1995; Espinosa-Urgel *et al.*, 1996).

Finally, it is of interest that a number of σ^S-regulated genes, including *dps* and *xthA*, have also been postulated to have intrinsic curvature in the promoter region 5' of the Pribnow box (Espinosa-Urgel and Tormo, 1993). Curvature is often characterised by adenine and thymine rich stretches of DNA. Such curvature can change under conditions of high osmolarity or upon association with DNA-binding proteins and is believed to be an important aspect of gene regulation (Owen-Hughes *et al.*, 1992; Dorman, 1996). DNA curvature may help to stabilise the interaction of RNA polymerase with the promoter, possibly compensating for a lack of a well defined -35 consensus sequence (Espinosa-Urgel and Tormo, 1993).

The *ahp* locus of *S. typhimurium* appears to lack many of the possible features of an RpoS-dependent gene (Figure 3.9), in agreement with the experimental observations made in this chapter. Firstly, it has a recognisable RpoD-type -35 consensus

Promoter sequence	Sequence		
	-35	intervening sequence	-10
RpoD consensus	TTGACA		TATAAT
RpoS consensus	**C**CCACA		**C**TATA**C**T
stahpCF	TTAGCCGAATCAGCAAAATTTCGTTTAACTT		
ecahpCF	TTACCGGAATCGGCAAAAATTGGTTACCTT		
eckatG	TTATAACTTCTCTCTAACGCTGTGTATCGT		
ecdps	GAATAGCGGAACACATAGCCGGTGCTATACT		
ecgorA	TTGCTGGCACCTATTACGTCTCGCGCTTACAAT		
SigA consensus	TTGACA		TATAAT
SigB consensus	GTTTAA		GGGTAT
bsahpCF	TTGACAAAAAATATATATTAATTAATAAT		

Figure 3.9 A comparison of sigma factor promoter recognition sequences involved in the regulation of the *ahp* locus in *S. typhimurium*, *E. coli* and *B. subtilis*

The *S. typhimurium* and *E. coli ahp* locus promoter sequence was compared to the known RpoD (σ^{70}) consensus sequence (Hawley and McClure, 1983), the postulated RpoS (σ^{S}) consensus sequence (Wise *et al.*, 1996; Espinosa-Urgel *et al.*, 1996) and also to promoter sequences from three OxyR regulated genes, *katG*, *dps* and *gorA*, known to be regulated by RpoS. Also included is a comparison of the *B. subtilis ahp* promoter and the consensus sequences for SigA (σ^{A}) (Moran *et al.*, 1982) and SigB (σ^{B}) (Hecker *et al.*, 1996). The relative positions of the -10 and -35 hexamers are indicated at the top of the table and are underlined within the displayed sequences. The cytosine residues in the -10 and -35 hexamers postulated to be important for the recognition of promoters by RpoS are highlighted in bold. Key: *stahpCF*, *S. typhimurium ahpCF* promoter (Tartaglia *et al.*, 1990); *ecahpCF*, *E. coli ahpCF* promoter (Smillie, 1994); *eckatG*, *E. coli katG* promoter (Toledano *et al.*, 1994); *ecdps*, *E. coli dps* promoter (Toledano *et al.*, 1994); *ecgorA*, *E. coli gorA* promoter (Toledano *et al.*, 1994); *bsahpCF*, *B. subtilis ahpCF* promoter (Antelmann *et al.*, 1996).

sequence. Secondly, the Pribnow box has more in common with a RpoD-type recognition sequence than an RpoS-type consensus because it does not carry cytosine residues at position five or one base upstream of the postulated hexamer. The level of intrinsic curvature in the promoter is uncertain, but an AT rich region overlaps with and extends 5' of the Pribnow box. The relevance of this or indeed the role of a cytosine residue at position four of the *ahp* -10 hexamer is unknown. Interestingly, the *ahp* locus promoter from *E. coli* is similar to that of *S. typhimurium* indicating that if this promoter sequence influences the expression of the gene during different growth phases, then the regulation of these genes is likely to be similar.

Figure 3.9 also shows the promoter sequence of the *B. subtilis ahp* locus. As stated previously, the *ahp* locus in this bacterium is regulated independently from σ^B, a sigma factor involved in the *B. subtilis* stationary phase response (Boylan *et al.*, 1993). In vegetative *B. subtilis* cells the major sigma factor is SigA (σ^A) and the -10 and -35 hexamers for this sigma factor are identical to that of σ^{70}. In support of the σ^B-independent expression of the *B. subtilis ahp* locus, the promoter sequence of this locus resembles that recognised by σ^A, an observation which compares favourably with the presence of a σ^{70}-type promoter sequence in the *ahp* locus of *S. typhimurium* and *E. coli.*

It should be noted that the promoter sequences of three genes of the OxyR regulon in *E. coli* known to be regulated by RpoS, *dps, katG* and *gorA* (Altuvia *et al.*, 1994; Ivanova *et al.*, 1994; Becker-Hapak and Eisenstark, 1995), do not appear to have the stereotypic characteristics of a σ^S-dependent promoter (Figure 3.9). Only *dps* contains the RpoS -10 consensus sequence. Moreover, although *dps* has been reported to have a curved region of DNA (Espinosa-Urgel and Tormo, 1993), the sequence of the promoter 5' of the -10 hexamer is not AT rich, nor are the promoter sequences of *katG* or *gorA*. In fact, compared to these genes, the *ahp* promoter sequence appears to be the most AT rich (Figure 3.9) yet, this locus is regulated in an RpoS-independent manner. It is likely therefore, that the promoters of OxyR-dependent genes which show σ^S-dependent regulation have other features, or

require other factors, to make them responsive to RpoS. In support of this, the stationary phase expression of the *dps* gene from *E. coli*, is known to require both σ^S and the DNA-binding protein Integration Host Factor (IHF) (Altuvia *et al.*, 1994).

The classical view of the transcriptional activation of the genes in the OxyR regulon is that activation occurs through the action of OxyR and the σ^{70}-containing RNA polymerase ($E\sigma^{70}$), after treatment of bacterial cells with hydrogen peroxide (Christman *et al.*, 1985; Tao *et al.*, 1993). Each of the genes of the OxyR regulon contains a conserved but degenerate consensus sequence stretching approximately 45 bp upstream of the σ^{70}-35 hexamer (Toledano *et al.*, 1994). Because of the size of the consensus sequence, it has been proposed that OxyR is capable of binding as a tetramer to the promoters of the genes it regulates and this view has been supported by purification studies (Toledano *et al.*, 1994; Kullik *et al.*, 1995a, 1995b). Indeed, it is thought that OxyR is a redox sensitive protein and will only promote transcription of the OxyR-regulated genes in an oxidised state where the protein is thought to have undergone a conformational change (Storz *et al.*, 1990). In this altered state, the OxyR polypeptide binds in four adjacent major grooves of the DNA at the OxyR promoter, thereby forming a tetramer and bringing about transcription through interaction with RNA polymerase (Toledano *et al.*, 1994). Studies with the *E. coli* *katG* promoter have also shown that $E\sigma^{70}$ is unable to bind and promote transcription efficiently in the absence of OxyR (Tao *et al.*, 1993). Furthermore, it has been shown that the C-terminus of the α polypeptide of RNA polymerase makes contact with OxyR suggesting that OxyR stabilises the binding of the polymerase to the promoter region and is essential for transcriptional activation (Tao *et al.*, 1993).

Interestingly both σ^{70} and OxyR have been reported to be present in stationary phase cells of *E. coli* (Hengge-Aronis, 1996a; Altuvia *et al.*, 1994) yet, the expression of at least two OxyR regulon genes, *katG* and *dps*, has been shown to be independent of both these factors (Ivanova *et al.*, 1994; Altuvia *et al.*, 1994). In this chapter, it was found that *ahp* did not show an OxyR-dependent stationary phase induction in the presence of hydrogen peroxide (Figure 3.5) and the reason for this is unclear.

However, this phenomenon has also been reported for the *dps* promoter of *E. coli* (Altuvia *et al.*, 1994). It could be that the expression of OxyR was insufficient to allow the formation of tetramers or that the protein is incapable of being activated. However, when expression of *ahp* was examined in *S. typhimurium* cells bearing plasmids expressing either native OxyR or a constitutively active form of OxyR, induction of *ahp* was still not observed (Figure 3.7). Moreover, studies with *lacZ* and the P_{BAD} promoter indicated that there was no lack of ability to induce the promoter under the experimental conditions (Table 3.3). This indicates that it is not the level or activation state of OxyR that leads to non-responsiveness of the *ahp* locus after exposure to hydrogen peroxide. However, this might simply reflect a lack of sufficient σ^{70} to drive expression of *ahp* in the stationary phase and also, on the abundance of the alternative sigma factor σ^{S} which might sequester RNA polymerase. The latter sigma factor would seem unable to activate transcription of *ahp* and moreover, does not seem to require OxyR for stationary phase activation of *katG* or *dps*.

As stated previously, other factors may play a role in the induction of OxyR-regulated genes by σ^{S} (Ivanova *et al.*, 1994). For example, the *dps* gene of *E. coli*, which requires OxyR and σ^{70} in the exponential phase of growth, is regulated by σ^{S} and Integration Host factor (IHF) in the stationary phase (Altuvia *et al.*, 1994). The *dps* promoter has also been shown to contain intrinsically curved DNA (Espinosa-Urgel and Tormo, 1993). Thus, DNA binding proteins, such as IHF, or curvature may serve to stabilise the RNA polymerase binding on the stationary phase promoter in the absence of OxyR. The lack of a hydrogen peroxide-inducible response of the OxyR-regulated genes in the stationary phase may therefore reflect on competitive exclusion of OxyR by other promoter binding factors or altered local topology.

Much of the work performed in this chapter has been dependent upon the detection of the AhpC polypeptide. It is important to note however, that the detection of the polypeptide does not necessarily correlate with the level of *ahpC* transcript. The amount of protein produced from a transcript can be influenced by a number of

factors including the quantity and the stability of the transcript. A more accurate method to assess transcription from the *ahpC* gene would be to perform quantitative reverse-transcription or Northern blotting, and this could be an angle of further studies. Alternatively, the half-life of the protein could be examined by pulse-chase labelling with [^{35}-S]-methionine. Such an approach may provide an answer to whether the AhpC detected in stationary phase (Figure 3.5 Panel B, Figure 3.7 Panel B and Figure 3.8 Panel B) is simply carry over from the exponential phase or reflects basal gene expression.

Previous studies have suggested that the *ahp* locus of *S. typhimurium* is expressed in the macrophage environment and is therefore likely to play a role in protecting the cell from oxidative killing (Francis and Gallagher, 1993; Francis PhD Thesis, 1993). The generation of a stationary phase response has also been implicated in the virulence of *S. typhimurium*, as *rpoS* and examples of RpoS-regulated genes have been shown to be expressed upon entry into the macrophage (Chen *et al.*, 1996a). This may appear to conflict with the observations made in this study, as *ahp* displays RpoS-independent expression. However, *rpoS* expression was shown to be maximal only after two hours post-infection of the macrophages by *S. typhimurium* (Chen *et al.*, 1996a). In addition, since the respiratory burst of the macrophage occurs upon contact and engulfment of bacteria, and since there is an apparent delay in maximal RpoS expression, it seems likely that a proportion of cells may be dependent upon an inducible (OxyR) response to deal with oxidative stress. Nevertheless, the results of this chapter would suggest that the major role for *ahp* is protection against oxidative damage during early macrophage interaction, and that *ahp* would play a lesser or no role in the long term survival of *S. typhimurium* in the macrophage, except perhaps when the cells were able to actively grow. This would also suggest that the importance of RpoS in the virulence of *S. typhimurium* is independent of *ahp*.

CHAPTER 4

Determination of the effect of osmolarity on the expression of *ahp* using a bioluminescent reporter system

4. 1 INTRODUCTION

Bacterial cells are surrounded by a cytoplasmic membrane which is freely permeable to water but not to most biological molecules (reviewed in Csonka, 1989; Csonka and Hanson, 1991). As a consequence, cells are susceptible to fluctuations in the osmotic environment within which they find themselves. Enteric bacteria, like *S. typhimurium*, normally maintain a cytosolic concentration of soluble substances of approximately 300 mOsm, a concentration greater than that of the external environment, to produce an outward pressure on the cell wall (turgor) (Ingraham, 1987). However, when bacterial cells are exposed to sudden increases in external osmolarity (hyperosmotic shock or osmotic upshift) dehydration occurs causing considerable shrinkage of the cytoplasm and inhibition of growth, as water moves out of the cell (Csonka and Hanson, 1991). The deleterious effects of this water efflux occur because the intracellular concentration of molecules increases as the cytoplasmic volume decreases. In general, an increase in the content of molecules, especially inorganic ions, can seriously alter the catalytic rate of enzyme reactions, disrupt non-covalent interactions of proteins with nucleic acids and drive the non-specific aggregation of proteins and biopolymers into non-functional complexes (Ingraham, 1987; Csonka and Hanson, 1991).

S. typhimurium and *E. coli* have been shown to respond to osmotic stress by inducing the synthesis and uptake of a number of substances including proline and glycine-betaine (Csonka and Hanson, 1991). This net rise in solutes raises the osmolarity of the cytoplasm and restores turgor. Unlike inorganic ions, these solutes can be accumulated to high levels in the cytoplasm without disturbing the functioning of cellular proteins, and are thus called compatible solutes (Csonka, 1989).

The ability of *S. enterica* to respond to environmental change is an important feature of virulence (Mekalonos, 1992; Mahan *et al.*, 1996). Environmental change is believed to act as a cue to alter gene expression in *S. enterica* such that the bacterial cell becomes equipped to deal with the new environment. These cues which occur

during the infection process may also act as signals to *S. enterica* cells to produce virulence determinants that increase the ability of the bacteria to invade and multiply in the tissues of the host. One such environmental cue is believed to be osmotic upshift, which is thought to be elicited in the intestine (Galan and Curtiss, 1990). For example, *S. typhimurium* has been shown to produce an invasion apparatus, or invasome, upon contact with intestinal epithelial cells (reviewed in Galan, 1996) and the expression of a number of genes involved in the production of this structure is stimulated under elevated osmolarity (Galan and Curtiss, 1989, 1990).

Recently, an investigation into the osmotic stress response of the commensal organism *Staphylococcus aureus* demonstrated that four proteins were highly expressed (Armstrong-Buisseret *et al.*, 1995). One of these proteins was 50% identical to the amino acid sequence of the AhpC protein from *S. typhimurium*. Moreover, upon subsequent cloning and sequencing of the *S. aureus ahpC* gene, a downstream open reading frame with homology to *ahpF* was also identified. Investigations into *Bacillus subtilis* have also shown that an *ahp* locus exists, and the expression of this operon is osmoregulated (Antelmann *et al.*, 1996). The above results therefore indicate that the expression of an alkyl hydroperoxide reductase activity is important in a number of bacteria during osmotic stress. These results suggest that as well as trying to prevent water loss from the cell, bacteria may also be experiencing oxidative stress during osmotic upshift. A similar response to osmotic stress is known to occur in plant cells, where an accumulation of reactive oxygen radical scavenging enzymes is observed as well as a cellular increase in compatible solutes (reviewed in Bohnert and Jensen, 1996).

After breaching the intestinal lining, invasive serovars of *S. enterica* pass into underlying tissues where they enter into macrophages (Finlay and Falkow, 1989a; Gulig, 1996). The latter cells destroy bacteria via a combination of a respiratory burst, which generates reactive oxygen species, and through an exposure to an arsenal of anti-microbial peptides and enzymes (Adams and Hamilton, 1984; Ganz *et al.*, 1990). However, *S. enterica* cells are capable of surviving within the macrophage, and part of this ability may be dependent upon inducible anti-oxidant

defence mechanisms that protect the bacterial cells from the respiratory burst (Fields *et al.*, 1986; Finlay and Falkow, 1989a; Francis and Gallagher, 1993). Interestingly, the OxyR-regulated *ahp* locus of *S. typhimurium* has been shown to be induced by interaction with macrophages and specifically, by macrophage-derived hydrogen peroxide (Francis and Gallagher, 1993; Francis PhD Thesis; 1993). This might suggest that *ahpCF* and possibly the other genes of the OxyR regulon have a role in virulence in which they protect the bacterial cell from the environment of the macrophage.

The finding that osmolarity may induce the expression of the *ahp* locus in other bacteria has important implications for the expression of *ahp* in *S. typhimurium*, were it to behave similarly. Indeed, the fact that *S. enterica* cells must pass through the high osmolarity of the intestine may mean that expression of *ahp*, and perhaps the rest of the OxyR regulon, occurs before *S. enterica* encounters the macrophages. Such expression may therefore pre-adapt *S. enterica* to the macrophage environment, especially against the products generated by the respiratory burst. In addition, the classical model of the regulation of the OxyR regulon is based on the activation of the target genes in response to hydrogen peroxide (Christman *et al.*, 1985; Toledano *et al.*, 1994). The induction of these genes via osmotic change would therefore represent an alternative and novel form of regulation. The effect of osmolarity upon the expression and regulation of *ahp* was therefore addressed using the *S. typhimurium* strain, MPG203, which carries the bioluminescent reporter system (Mu*dlux*) in the *ahp* locus.

4.2 RESULTS

4.2.1 Examination of the role of osmolarity in the regulation of the *ahp* locus, using a bioluminescent reporter system

In order to examine the effect of osmotic stress on *ahp* expression, it was first necessary to use a culture medium in which the osmolarity was initially low in order that osmotic upshift experiments could be performed. The commonly used medium

LB was unsuitable as it contains a high level of NaCl (10 g of NaCl per litre). A low salt derivative of LB containing only 1 g per litre (LSLB) was therefore used for the experiments described below since the osmolarity of the medium could be increased as required.

The effect of osmotic upshift on the expression of *ahp* in MPG203

In order to examine the effect of osmotic upshift in MPG203 an overnight culture (grown in normal LB) was diluted 1:10, 000 fold into LSLB, to give approximately 10^5 c.f.u. ml^{-1} and the culture was incubated with shaking at 30°C for 2 hours, to re-establish exponential phase growth (this preparative procedure was used standardly for all strains throughout these experiments, unless otherwise stated). After 2 hours, the culture was divided into four portions. One portion was left unaltered whilst for the other portions, the media was adjusted to give a constant physical osmotic pressure (West, 1990) by adding 0.3 M NaCl, 0.45 M sucrose or 5% glycerol (final concentrations). 200 µl of each sample was then withdrawn and the bioluminescence was measured over a 10 hour period (as relative light units per second (RLU s^{-1})) in a 96-well plate-reading luminometer, pre-incubated to 30°C. For comparison, the effect of exposure of MPG203 to hydrogen peroxide (the classical stimulus) was also examined. In the latter case, however, cells were prepared as above except that, normal LB was used and the culture was split into two volumes. The samples were then treated after the 2 hour incubation period with 100 µM hydrogen peroxide or were left untreated, and the bioluminescence was recorded.

Figure 4.1 shows the effect of the classical stimulus, hydrogen peroxide, on the expression of *ahp*. As can be seen, *ahp* was induced only in the presence of hydrogen peroxide. Figure 4.2 displays the effect of osmotic upshift upon the expression of *ahp*. Both 0.3 M NaCl and 0.45 M sucrose brought about a substantial induction of the *ahp* operon even in the absence of hydrogen peroxide. The use of both NaCl and sucrose also provided evidence that the response was not osmolyte specific. Furthermore, glycerol which freely enters the bacterial cell (and therefore does not elicit an osmotic stress), was not found to increase the expression of *ahp* even though

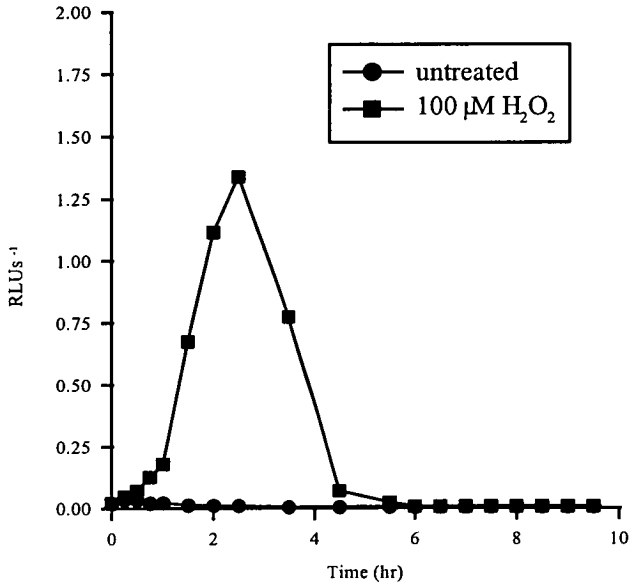


Figure 4.1 The effect of hydrogen peroxide upon the expression of the *ahp* locus in MPG203

An overnight culture of MPG203 (*ahp*::Mu*dlux*) was diluted 1: 10, 000 into LB, to give approximately 10^5 c.f.u. ml^{-1}, and was then grown for 2 hours at 30°C with shaking. After this period, the culture was split into two volumes and was either treated with 100 μM hydrogen peroxide (H_2O_2) or was left untreated. A 200 μl aliquot of each sample was then monitored for bioluminescence, over approximately 10 hours, in a 96-well plate-reading luminometer pre-heated to 30°C. Light induction is expressed as relative light units per second (RLU s^{-1}). The zero time point of the graph represents the time at which the cultures were treated. (Experiments were repeated several times and were found to be reproducible. The figure is a typical example).

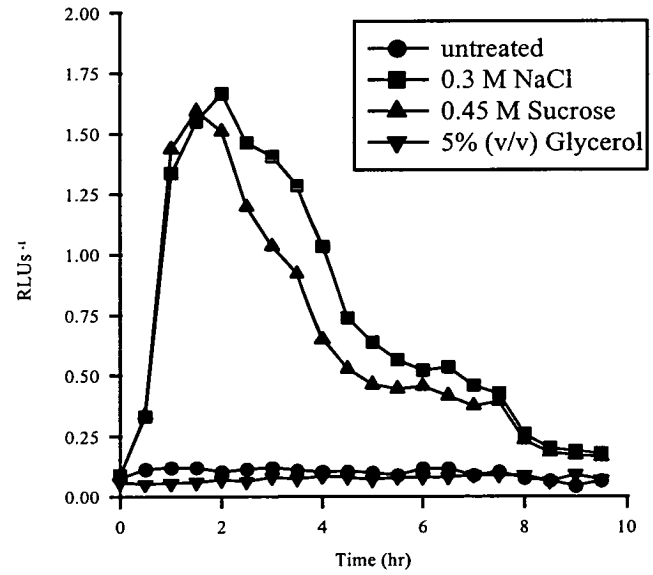


Figure 4.2 The effect of osmolarity upon the expression of the *ahp* locus in MPG203

Experiments were performed using a modified LB medium, LSLB. When unsupplemented this had a final NaCl concentration of 1g l^{-1} (0.017 M). A culture of MPG203 (*ahp*::Mu*dlux*) was grown overnight in LB and then diluted into fresh LSLB, to give approximately 10^5 c.f.u. ml^{-1}, and incubated at 30°C for 2 hours with shaking. The culture was then split into four volumes and was left untreated or was adjusted to a final concentration of either 0.3 M NaCl, 0.45 M sucrose or 5% (v/v) glycerol. Bioluminescence was determined as described in the legend for Figure 4.1. (Experiments were repeated several times and were found to be reproducible. The figure is a typical example).

it exerts the same physical osmotic pressure in solution. These results therefore suggest that the *ahp* operon is osmotically regulated.

The effect of osmolarity upon *ahp* expression is dose-dependent

The data from Figure 4.2 indicated that the expression of the *ahp* locus was osmotically sensitive. A test was therefore performed to see how sensitive the expression of *ahp* was to osmotic changes in the medium. Since the osmotic induction of *ahp* had been shown not to be specific to one compound, it was decided to use salt as the osmotic stress-inducer in all the further experiments.

A culture of MPG203 was prepared in LSLB as described previously before being split into four volumes and treated with 0.17 M, 0.3 M or 1 M NaCl. One sample was left untreated (which is equivalent to 0.017 M NaCl). 200 µl of each sample was then withdrawn and examined for bioluminescence as before.

From Figure 4.3 it can be seen that the expression of *ahp* appears to increase with NaCl concentration up to 0.3 M, suggesting it is a dose-dependent phenomenon. In addition, there appears to be an optimal concentration of osmolarity that influences the expression of *ahp* and above this point the response diminishes.

Osmotic upshift and hydrogen peroxide in combination enhance the expression of *ahp*

The data from Figure 4.1 and 4.2 showed that the *ahp* operon could be activated by hydrogen peroxide or osmotic upshift. To examine whether each of these stimuli operated via the same mechanism or through an alternative route of activation, the effect of these stimuli were therefore assessed separately or in combination.

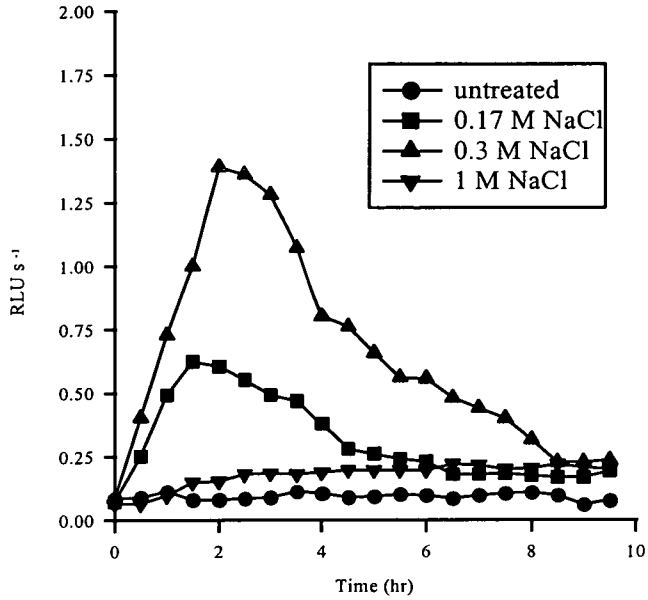


Figure 4.3 The osmotic induction of *ahp* in MPG203 is dose-dependent

A culture of MPG203 (*ahp*::Mu*dlux*) was prepared as described in the legend for Figure 4.2. Subsequently, the culture was divided into four volumes and was adjusted to a final concentration of 0.17 M, 0.3 M, or 1 M NaCl (final concentration) or was left untreated (0.017 M NaCl). Bioluminescence was recorded as described in the legend to Figure 4. 1. (Experiments were performed several times and found to be reproducible. The figure is a typical example).

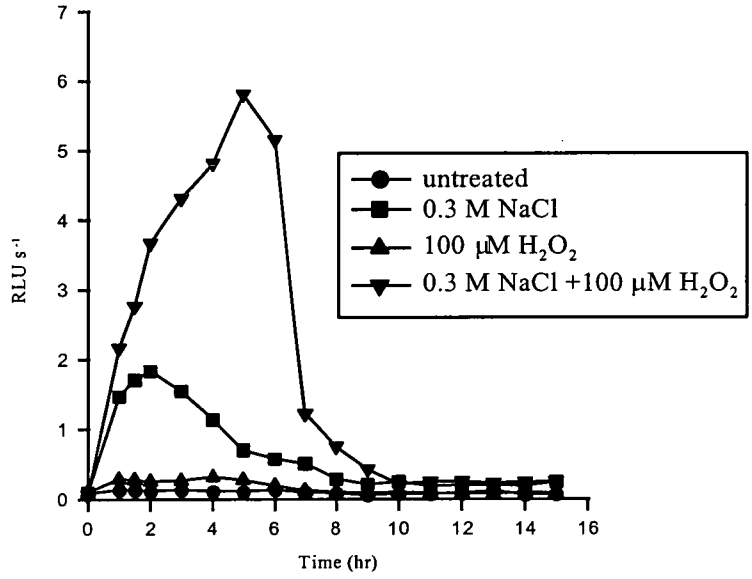


Figure 4.4 The effect of hydrogen peroxide, osmolarity or a combination of these stimuli upon the expression of the *ahp* locus in MPG203

A culture of MPG203 (*ahp*::Mu*dlux*) was prepared as described in the legend for Figure 4.2. Subsequently, the culture was divided into four aliquots and was treated with 100 μM hydrogen peroxide (H_2O_2), 0.3 M NaCl or a combination of these, or was left untreated. Bioluminescence was recorded as in the legend to Figure 4.1. (Experiments were performed several times and found to be reproducible. The figure is a typical example).

A culture of MPG203 was prepared in LSLB as described previously and was then split into four aliquots. To these samples either hydrogen peroxide or NaCl or both were added to final concentrations of 100 μM or 0.3 M respectively, whilst one sample was left untreated. 200 μl of each culture was withdrawn and examined for bioluminescence in a luminometer. From Figure 4.4 it can be seen that a combination of both stimuli potentiated the response far beyond that of the individual stimuli. In addition, the response to hydrogen peroxide in the low osmolarity medium was shown to be negligible when compared to the untreated sample. These results suggest that osmolarity is likely to be acting through a different regulatory pathway to that of peroxide stress, but that the ability of the *ahp* locus to be induced by hydrogen peroxide is dependent upon the osmotic environment of the cell.

OxyR but not OmpR, H-NS, or RpoS is required for the osmotic-inducibility of *ahp*

Classically, expression of the *ahp* locus occurs in response to hydrogen peroxide via the binding of the OxyR regulatory protein to the *ahp* promoter (Toledano *et al.*, 1994). The results from Figure 4.4 suggested that the mechanism for osmotic induction of *ahp* was different to that for hydrogen peroxide and raised the possibility that other regulatory factors may be involved in the osmotic-inducibility of *ahp*. A number of regulators have been shown to play important roles in the inducibility of a number of osmotically-regulated genes. For example, RpoS is the major regulator of the stationary phase response but has also been shown to play a role in the expression of osmotic stress resistance genes in exponential phase cells of *E. coli* (Hengge-Aronis *et al.*, 1993). Moreover, the osmotically-sensitive expression of the outer membrane porin proteins, OmpF and OmpC, and the glycine-betaine uptake system, which is encoded by *proU*, are known to be regulated by OmpR and H-NS, respectively (Csonka and Hanson, 1991). Therefore, the importance of the *oxyR*, *rpoS*, *ompR* or *hns* loci in the osmotic-inducibility of *ahp* was examined.

The *ahp* locus containing the bioluminescent reporter system (Mu*dlux*) from MPG203, was transduced, via phage P22, to an LT2-derived *S. typhimurium* strain which had been deleted for the *oxyR* locus (TA4108 (*oxyRΔ2*), Christman *et al.*,

1985) to form MPG352. Transposon insertion mutations, *ompR1006*::Tn*10* and *osmZ6*::Tn*10* (*osmZ* has been renamed *hns*), were transduced from CH1350 (Dorman *et al.*, 1989) and CH1701 (Hulton *et al.*, 1990), respectively, into MPG203 to form MPG474 and MPG475. In a similar way, an *rpoS* gene which had been inactivated by the insertion of a β-lactamase gene (*rpoS*::*bla*) in *S. typhimurium* strain SF1005 (Fang *et al.*, 1992), was transduced into MPG203 to form MPG471 (see chapter 3). Since *S. typhimurium* strains MPG203, MPG471, MPG474, and MPG475 were SL1344 derivatives but, MPG352 was an LT2 derivative, a wild type LT2 derivative containing the *ahp*::Mu*dlux* (MPG350) was also examined, as a control.

Cultures of the above strains were prepared in LSLB as described previously (the c.f.u. ml^{-1} of each culture was determined to ensure that each sample was composed of an equivalent number of cells). Each culture was then divided into two portions and these were left untreated or were adjusted to a final concentration of 0.3 M NaCl. Bioluminescence was then measured. Figures 4.5 Panels A-E display the results of examining the induction of the *ahp* locus in the mutant backgrounds. Panel A shows that the osmotic-inducibility of *ahp* in the *S. typhimurium* SL1344 (MPG203) and LT2 (MPG350) backgrounds was equivalent. Moreover, the osmotic induction of the *ahp* locus in either the *rpoS* (MPG471, Panel C), *ompR* (MPG474, Panel D) or *hns* (MPG475, Panel E) mutant backgrounds was similar to that seen in Figure 4.5 Panel A, suggesting that these regulatory loci did not significantly influence the osmotic-inducibility of *ahp*. In contrast, no *ahp* induction was detected in the response of the *oxyR* deleted derivative (MPG352, Panel B) to osmotic upshift thereby highlighting a requirement for OxyR in the osmoregulation of *ahp*.

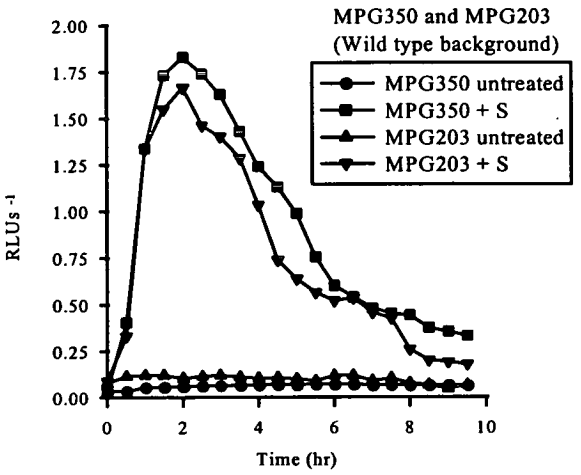
The effect of novobiocin upon the hydrogen peroxide-inducibility of the *ahp* locus

DNA supercoiling has been implicated in the regulation of a number of osmotically induced genes such as *proU* and the DNA gyrase genes, *gyrA* and *gyrB* (Jovanovich and Lebowitz, 1987; Higgins *et al.*, 1988; Dorman and Ni Bhriain, 1992). In addition, previous studies have shown that disruption or inhibition of genes which

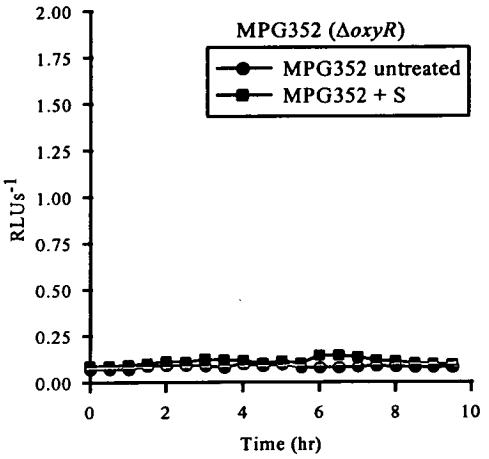
Figure 4.5 The effect of the *ompR*, *hns*, *oxyR* or *rpoS* loci upon the osmotic-inducibility of *ahp*

Cultures were prepared in LSLB as described in the legend to Figure 4.2. Each culture was then divided into two. One portion was left untreated whilst the other portion was adjusted to a final concentration of 0.3 M NaCl. Bioluminescence was measured as described in the legend to Figure 4.1. Figure 4.5 Panel A shows the effect of osmotic upshift upon the expression of the *ahp* locus within wild type LT2 (MPG350) and SL1344 (MPG203) backgrounds. Figure 4.5 Panels B-E show the effect of *oxyR* (MPG352), *rpoS* (MPG471), *ompR* (MPG474) or *hns* mutations (MPG475) upon the osmotic-inducibility of the *ahp* locus. Key to *S. typhimurium* strains: MPG203 (SL1344, *ahp*::Mu*dlux*); MPG350 (LT2, *ahp*::Mu*dlux*); MPG352 (LT2, *ahp*::Mu*dlux, oxyRΔ2*); MPG471, (SL1344, *ahp*::Mu*dlux*, *rpoS*::*bla*); MPG474 (SL1344, *ahp*::Mu*dlux*, *ompR1006*::Tn*10*); MPG475 (SL1344, *ahp*::Mu*dlux*, *osmZ6*::Tn*10* [*osmZ=hns*]). (Experiments were repeated several times and were found to be reproducible. The figure represents a typical example of the results).

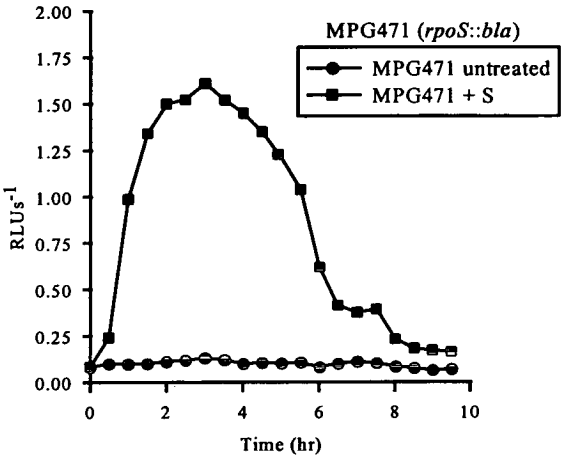
PANEL A



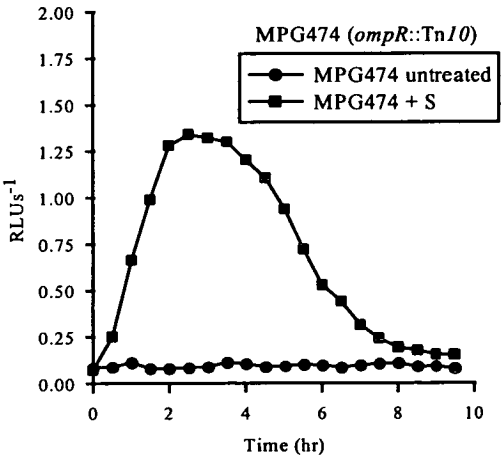
PANEL B



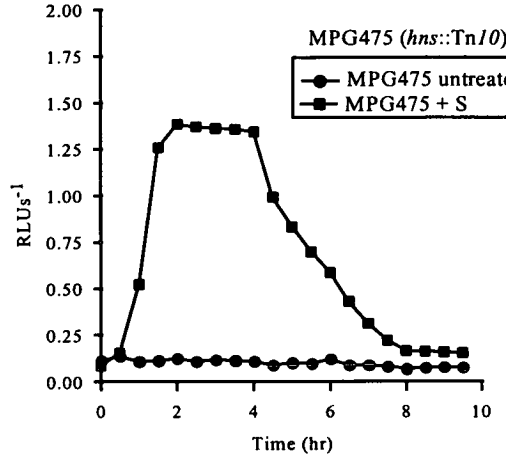
PANEL C



PANEL D



PANEL E



regulate supercoiling, such as topoisomerase and DNA gyrase, may alter the expression of osmotically regulated genes (Menzel and Gellert, 1983; Higgins *et al.*, 1988). Since it appeared that *ahp* could be induced by osmotic upshift, the effect of novobiocin (an inhibitor of the DNA gyrase subunit B) upon *ahp* expression was examined in conjunction with hydrogen peroxide or osmotic stimulation.

Cultures of MPG203 were prepared as described previously and were then split into six aliquots. Samples were then either left untreated or subjected to hydrogen peroxide, or novobiocin treatment or a combination of these chemicals, at final molarities or concentrations of 100 μM and 50 or 200 μg ml^{-1}, respectively. The bioluminescence from all the samples was then measured. The number of c.f.u. ml^{-1} of samples from parallel cultures was determined at suitable time points by plating onto LB media.

From Figure 4.6 it can be seen that hydrogen peroxide elicited no significant *ahp* induction in LSLB (in line with data from Figure 4.4). In contrast, exposure to novobiocin resulted in induction of *ahp* expression in a concentration-dependent manner. The fact that a combination of hydrogen peroxide and novobiocin did not elicit an enhanced response may suggest that they function via a common regulatory step. Nevertheless, it has to be remembered that hydrogen peroxide *per se* was incapable of eliciting any significant response at low osmolarity whilst *ahp* expression was observed in response to novobiocin under these conditions.

Closer examination of the cell number 5 hours after treatment revealed that a considerable difference existed between samples. Initially, the cell number of the culture, before being split and treated, was 1.1×10^5 c.f.u. ml^{-1}. However, after 5 hours the cell numbers (c.f.u. ml^{-1}) were as follows (where H represents hydrogen peroxide treated samples and N50 and N200 represent samples treated with 50 or 200 μg ml^{-1} novobiocin, respectively): untreated, 3.6×10^6; H, 1.1×10^6; N50, 3.8×10^5; N200, 6.1×10^4; N50+H, 2.5×10^5; N200+H, 5.7×10^4. Therefore, hydrogen peroxide appeared to retard cell growth slightly. However, novobiocin had a more dramatic and dose-dependent effect upon the growth of MPG203. Moreover, at

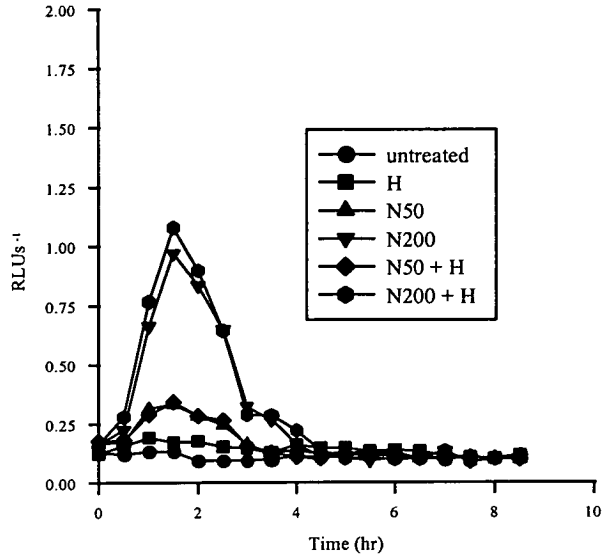


Figure 4.6 The effects of novobiocin and/or hydrogen peroxide upon *ahp* expression in MPG203

A culture of MPG203 (*ahp*::Mu*dlux*) was prepared in LSLB as described in the legend to Figure 4.2. The culture was then divided into six volumes and was treated with hydrogen peroxide (100 μM), novobiocin (50 or 200 μg ml^{-1}), or a combination of these stimuli, or was left untreated. Bioluminescence was recorded as described in the legend to Figure 4.1. Key: H, hydrogen peroxide; N50 and N200, novobiocin at either 50 or 200 μg ml^{-1}, respectively. (Experiments were performed several times and were found to be reproducible. The graph is a typical example of the results).

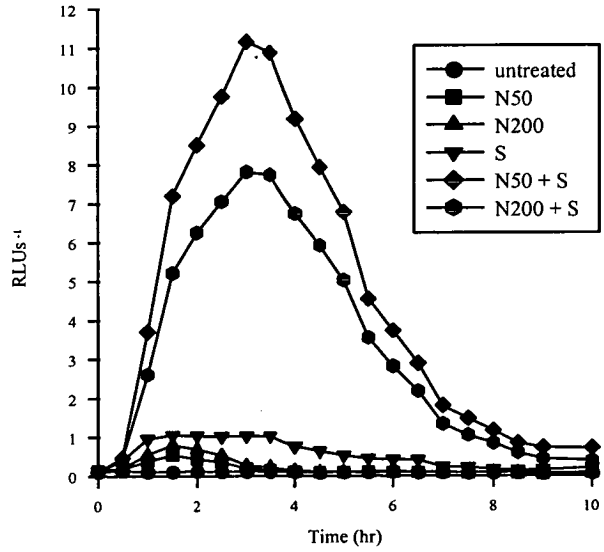


Figure 4.7 The effect of novobiocin and/or osmotic upshift upon *ahp* expression in MPG203

A culture of MPG203 (*ahp*::Mu*dlux*) was prepared in LSLB as described in the legend to Figure 4.2. The culture was then split into six samples and was adjusted to a final concentration of 0.3 M NaCl, 50 or 200 μg ml^{-1} novobiocin or a combination of these stimuli, or left untreated. Bioluminescence was recorded as described in the legend to Figure 4.1. Key: S, NaCl; N50 and N200, novobiocin at either 50 or 200 μg ml^{-1}, respectively. (Experiments were performed several times and were found to be reproducible. The graph is a typical example of the results).

200 μg ml^{-1} the novobiocin appeared to kill the cells, yet caused the greatest expression of *ahp* (Figure 4.6). This suggested that the effects of novobiocin may not have reflected an alteration in localised DNA supercoiling at a specific promoter, but could represent an indirect effect on DNA supercoiling in general, as a consequence of the inhibition of cell growth.

The effect of novobiocin and osmotic upshift upon the expression of *ahp*

The results from Figure 4.6 suggested that novobiocin can induce *ahp* expression in media of low osmolarity and, that hydrogen peroxide seemed not to influence this response. The possibility still existed that the novobiocin effect shared a common site of action with that for osmotic induction. To address this, MPG203 cells were prepared as before and then the culture was split into six aliquots. These were then treated with either NaCl (0.3 M), novobiocin (50 or 200 μg ml^{-1}), or a combination of these stimuli and the bioluminescence of the samples was measured (the cell numbers of parallel cultures which were treated similarly were determined by dilution and plating onto solid LB medium). The effects of the treatments upon the expression of *ahp* can be seen in Figure 4.7. Osmotic shock or exposure to novobiocin elicited a clear bioluminescent response. However, a combination of these stimuli produced a response that vastly exceeded the response from the individual stimuli. These results suggest that the induction of *ahp* by novobiocin occurs via a different regulatory mechanism or point from that of osmolarity but suggests that, as found with hydrogen peroxide, novobiocin is capable of augmenting the osmotic response. Unlike hydrogen peroxide however, novobiocin alone seems to elicit a small response.

Examination of cell number after treatment for 5 hours again suggested considerable differences existed between samples. The initial cell number of the culture prior to being split and treated was 9.3×10^4 c.f.u. ml^{-1}. After 5 hours, the cell numbers (c.f.u. ml^{-1}) were as follows (where S represents samples treated with NaCl, and N50 and N200 represent samples treated with 50 or 200 μg ml^{-1} novobiocin, respectively): untreated, 3.4×10^6; S, 1.3×10^6; N50, 3.5×10^5; N200, 5.9×10^4; N50+S, 2.5×10^5;

N200+S, 5.4 x 10^4. Thus, osmolarity, like hydrogen peroxide, appeared to have a mild growth retardation effect whilst, as before, novobiocin resulted in a deleterious effect upon cell survival, with the most severe decline in growth observed with 200 μg ml^{-1} novobiocin.

Plasmid-mediated expression of OxyR, in the presence of hydrogen peroxide, results in a high level of expression of *ahp* in media of low osmolarity

Previous work (Christman *et al.*, 1985) and the present studies (Figure 4.5 Panel B) have shown that the hydrogen peroxide-inducibility of *ahp* requires the OxyR transcriptional regulator but not the *rpoS*, *hns*, or *ompR* gene products. In order to dissect the regulatory system further and attribute sites of action to the various stimuli, plasmid pPDT3, which carries the *oxyR* gene under the controllable promoter (P_{BAD}) from the arabinose operon of *E. coli* (see chapter 3 for details of construction), was electroporated into an *oxyR* deletion strain, carrying the bioluminescent reporter system in the *ahp* locus (MPG352), to form MPG477. This would help determine whether the effects of osmolarity were acting upstream (*e.g. oxyR*) or downstream (*e.g. ahp*) in the regulatory circuit.

Using MPG477, the effect of hydrogen peroxide and osmotic upshift was examined in the presence or absence of OxyR as a result of adding or not adding arabinose to the medium (arabinose activates expression of OxyR from P_{BAD}). An overnight culture of MPG477 was diluted 1:10, 000 into LSLB and the culture was then divided into 8 volumes and grown for 2 hours. Samples that required the expression of OxyR, were treated with 0.5% (v/v) arabinose 30 minutes after dilution and then re-incubated for a further 1.5 hours. Cultures were then subjected to hydrogen peroxide (100 μM), osmotic stress (0.3 M NaCl) or a combination of these treatments or were left untreated. The results are shown in Figure 4.8. From Panel B it can be seen that the *ahp* locus is not induced by the presence of OxyR in the absence of a hydrogen peroxide or osmotic upshift stimulus. In contrast, in the presence of OxyR (expressed from the plasmid) and hydrogen peroxide, a substantial level of *ahp* expression was observed (Panel A), even in the medium of low osmolarity. The

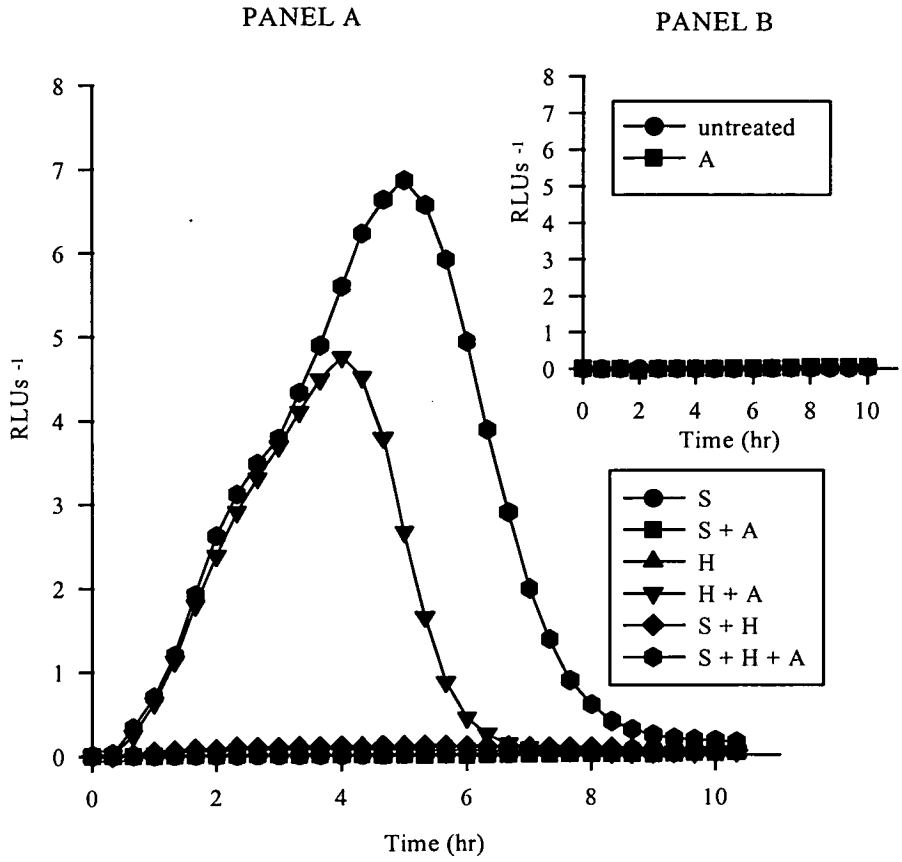


Figure 4.8 The effect of supplying OxyR *in trans* upon the expression of the *ahp* locus, in an Δ*oxyR* strain, under conditions of low osmolarity

Overnight cultures of MPG477 (*ahp*::Mu*dlux*, *oxyR*Δ*2*, pPDT3) were diluted into LSLB, containing the appropriate antibiotics, to give approximately 10^5 c.f.u ml^{-1}. Cultures were then incubated for 2 hours at 30°C, except for those which required the expression of OxyR from pPDT3, which were adjusted to 0.5% (v/v) arabinose 0.5 hours after dilution, before being incubated for the remaining 1.5 hours. Samples with or without arabinose were then exposed to 100 μM hydrogen peroxide, 0.3 M NaCl, a combination of these stimuli or none of these stimuli. Bioluminescence was measured as described in the legend to Figure 4.1. Panel A shows the effect of osmotic upshift and hydrogen peroxide upon the expression of *ahp* in the presence or absence of arabinose. Panel B shows the bioluminescence obtained from two control samples left untreated or treated with arabinose. Key: A, arabinose; S, NaCl; H, hydrogen peroxide. (Experiments were performed several times and were found to be reproducible. The figure represents a typical example of the results obtained).

requirement for high osmolarity in the hydrogen peroxide-inducibility of *ahp* therefore appeared unnecessary when OxyR was expressed from a plasmid. However, it is interesting that a combination of high osmolarity and hydrogen peroxide resulted in an increased response in the presence of OxyR compared with hydrogen peroxide alone. Surprisingly, however, under these conditions, 0.3 M NaCl alone apparently failed to elicit a significant response even in the presence of induced OxyR. The explanation for these points remain unclear at present. MPG477 is an LT2 derivative of *S. typhimurium*, and this is unlikely to account for the results obtained because the osmotic-inducibility of the *ahp* locus in MPG350 (LT2 *ahp*::Mu*dlux*) was shown to be equivalent to that of MPG203 (Figure 4.5 Panel A). Nevertheless, the *oxyR* mutation in MPG477 was generated by Tn*10*-mediated deletion, which can result in deletion of flanking genes. Thus, the possibility that some component which is encoded close to *oxyR*, and which influences the observed effect of osmolarity, may also have been lost, cannot be excluded. (It should also be noted that 0.5% arabinose is unlikely to have a significant osmotic effect upon the cultures because it is the equivalent of adding less than 0.5 g NaCl to a litre of media (West, 1990). In addition, pBAD18, the parental plasmid to pPDT3, had no effect upon the expression of *ahp* (data not shown)).

Osmotic stress does not cross-protect *S. typhimurium* against hydrogen peroxide or cumene hydroperoxide

The results outlined so far in this chapter suggested that *ahp*, or possibly the entire OxyR regulon, is influenced by osmolarity. The greatest and lowest levels of *ahp*::Mu*dlux* expression in response to hydrogen peroxide stress were observed under high and low osmolarity conditions, respectively. Tests were performed therefore, to determine if modulation of medium osmolarity correlated with differences in the protective responses to hydrogen peroxide elicited in SL1344 and MPG203.

Overnight cultures of SL1344 and MPG203 were diluted 1:10, 000 into 30 ml of LSLB, and were grown for 2 hours to establish exponential phase growth. After this

time the cultures were split into three 10 ml aliquots and one aliquot was left untreated (0.017 M NaCl) whilst the other two were adjusted to final concentrations of 0.17 M NaCl (the equivalent of normal LB) or 0.3 M NaCl. Samples were then incubated at 30°C for a further hour, to allow the cells to adapt. Each of the cultures was then split into two 5 ml portions and 20 mM hydrogen peroxide was added to one of the portions whilst the other was left untreated. The cultures were incubated for a further hour and subsequently, samples were plated to determine the number of viable colony forming units. The percentage survival of the treated sample was calculated relative to the untreated samples, and these results are shown in Table 4. 1.

Surprisingly, the results (Table 4.1) were found to be in complete contrast to those predicted from the luminometer studies. For both SL1344 and MPG203, those cells which were subjected to the lowest osmolarity showed the greatest survival when challenged with hydrogen peroxide. It appeared then, that high osmolarity did not help to cross-protect *S. typhimurium* against the effects of oxidative stress but, in fact, indicated that high osmolarity enhanced killing by hydrogen peroxide.

These results did not exclude the possibility however, that osmolarity could be acting solely to induce the *ahp* locus, in an OxyR-dependent fashion but independently of the other genes of the OxyR regulon. In order to address this possibility, the above test was repeated but using 20 mM cumene hydroperoxide, a chemical specifically used to test for Ahp activity (Christman *et al.*, 1985). The results displayed in Table 4.1 indicated that increased osmolarity did not enhance the survival of cells against exposure to cumene hydroperoxide, thus suggesting high osmolarity does not increase *ahp* expression. (The survival of MPG203 was not examined because it is hyper-susceptible to cumene).

	% Survival of Strain Tested					
	SL1344			MPG203		
	Salt Concentration			Salt Concentration		
Treatment	0.017 M	0.17 M	0.3 M	0.017 M	0.17 M	0.3 M
untreated	100.0	100.0	100.0	100.0	100.0	100.0
20 mM HP	20.0	2.3	1.0	19.2	2.0	0.9
20 mM CHP	22.0	2.3	1.6	-[(a)]	-	-

Table 4.1 Survival of SL1344 and MPG203, grown under different concentrations of salt, when treated with 20 mM hydrogen peroxide or cumene hydroperoxide.

Overnight cultures of SL1344 (virulent parental strain) and MPG203 (*ahp*::Mu*dlux*) were diluted to approximately 10^5 c.f.u. ml^{-1} in LSLB and grown with shaking at 30°C for 2 hours. The culture was then split into three portions and left untreated (0.017 M NaCl) or adjusted to 0.17 M NaCl or 0.3 M NaCl and incubated for 1 hour at 30°C. For SL1344, samples were again split and left untreated or were treated with 20 mM hydrogen peroxide (HP) or 20 mM cumene hydroperoxide (CHP) and incubated for a further hour at 30°C. For MPG203, samples were split and left untreated or were subjected to 20 mM HP (only) and incubated a further hour at 30°C. Samples were then appropriately diluted and plated onto LB to determine the cell number, and the percentage survival (%) was determined relative to the untreated sample. (a)- not determined because MPG203 is naturally hyper-sensitive to CHP. Values in the table represent the average of two separate experiments and variation between samples which were equivalently treated was less than 5%.

4.2.2 Examination of the osmotic-inducibility of the *ahp* locus, using immunoblotting

Experiments that use the luciferase genes from *Vibrio* species to monitor gene expression, have in some instances been reported to produce anomalous results, especially under conditions that may influence DNA supercoiling (*e.g.* osmolarity) (Forsberg *et al.*, 1994). The data obtained from the bioluminescence studies indicated that the *ahp* locus was induced by a high osmolarity medium, yet this did not result in enhanced protection against hydrogen peroxide or cumene hydroperoxide. The possibility that the bioluminescent reporter system had not accurately reflected *ahp*

expression was therefore explored using a Western blot procedure and an anti-AhpC antiserum.

Immunological detection procedures for identifying the AhpC protein in cell extracts were similar to those performed in chapter 3 of this thesis. Cultures of SL1344 and MPG203 (which encodes a 100 amino acid AhpC polypeptide, truncated by virtue of the Mu*dlux* insertion) were diluted 1:10, 000 fold into 40 ml LSLB and grown for 2 hours at 30°C before samples were split into four 10 ml aliquots. The culture media of each sample was then adjusted to contain a final concentration of either 0.3 M NaCl, 100 μM hydrogen peroxide, or both, and one sample was left untreated. After a further hour of incubation, an optical density measurement (OD_{600}) was taken and the cells were pelleted, resuspended in LSB and were subjected to SDS-PAGE (12.5% [v/v] polyacrylamide gel) and immunoblotting. Optical density comparisons were used to ensure each lane contained an equivalent amount of protein. Wild type AhpC was detected using mouse anti-AhpC serum, followed by rabbit anti-mouse antibody conjugated to alkaline phosphatase.

The results are shown in Figure 4.9. Lanes 1-4 and 5-8 represent the samples from SL1344 and MPG203 respectively. As a control, purified his-tagged AhpC, which was used to raise the antiserum, was included (lane A). This runs slightly higher than the native protein due to the presence of the poly-histidine tag. In the SL1344 samples, two prominent bands running close together were seen. The lower and upper bands are believed to represent the AhpC protein and an unidentified component (X), respectively (see chapter 3 for further discussion on component X). A basal level of AhpC was observed in samples from SL1344 at low osmolarity. However, AhpC was found to be induced substantially by hydrogen peroxide under both low (lane 2) and high (lane 4) osmolarity. Unfortunately, only the unidentified component X, but not the truncated AhpC protein, could be seen in samples from MPG203, perhaps suggesting that the truncated AhpC protein is rapidly targeted for degradation in the cell.

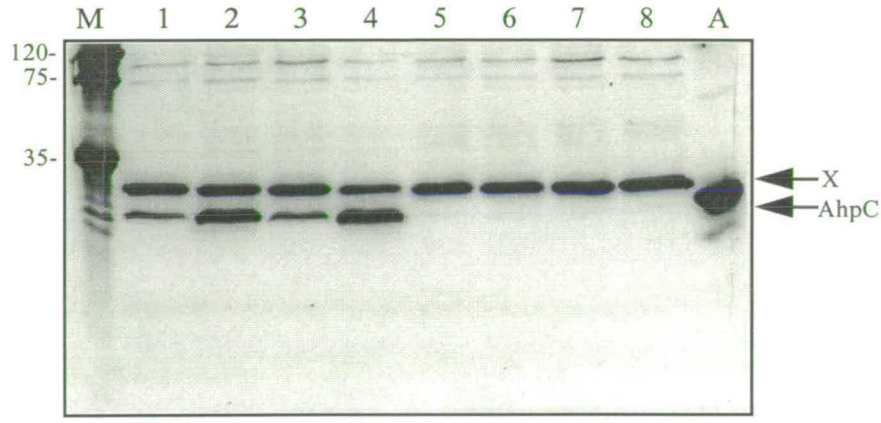


Figure 4.9 Immunological detection of AhpC in SL1344 and MPG203

Overnight cultures of SL1344 and MPG203 (*ahp*::Mu*dlux*) were diluted into LSLB to give approximately 10^5 c.f.u. ml^{-1} and were grown with shaking at 30°C for 2 hours. Each culture was then split into four volumes and treated with either 100 μM hydrogen peroxide, 0.3 M NaCl or a combination of these treatments whilst one sample was left untreated. Cultures were then incubated for a further hour. Equal amounts of each sample were subject to SDS-PAGE and Western blot analysis. AhpC was detected using a mouse polyclonal serum against AhpC. Pre-stained markers of 120, 75 and 35 kDa were added in lane M. Purified his-tagged AhpC was included as a control (lane A). Lanes 1-4 and 5-8 represent SL1344 and MPG203, respectively. The samples left untreated, or treated with hydrogen peroxide, NaCl or a combination of these stimuli are represented by lanes 1 and 5, lanes 2 and 6, lanes 3 and 7, and lanes 4 and 8, respectively. Bands corresponding to the wild type AhpC and an unidentified component (X) are indicated by appropriately labelled arrows.

These results, in addition to those of Tables 4.1, provide strong evidence that *ahp* expression is not influenced by the osmotic environment of *S. typhimurium* but rather, that the bioluminescent reporter system in *ahp* alters the natural behaviour of the *ahp* locus and converts it to an osmotically-regulated gene.

4. 3 DISCUSSION

The use of a bioluminescent reporter system indicated that the *ahp* locus of *S. typhimurium* was osmoregulated (Figure 4.2). However, peroxide survival studies (Table 4.1) and Western blot analysis of the AhpC protein (Figure 4.9) indicated that the Mu*dlux* fusion in *ahp* was producing anomalous results and, in fact, that the *ahp* locus was not osmotically sensitive. This suggests that the osmotic environment would have no significant effect upon the expression of the *ahp* locus in *S. typhimurium* during the infection of a host.

These results are in contrast to those observed with *S. aureus* and *B. subtilis* where the expression of the *ahp* locus is sensitive to osmotic change (Armstrong-Buisseret *et al.*, 1995, Antelmann *et al.*, 1996). For both *S. aureus* and *B. subtilis*, the ability to defend themselves against lipid peroxidation would seem to be an important requirement whilst under osmotic stress. Lipid peroxidation occurs as a direct result of reactive oxygen species interacting with the membrane. Evolution of genes co-regulated by oxidative stress and osmotic upshift would suggest therefore, that osmotic stress may lead to the generation of free radicals. A similar scenario is suggested from the study of plant cells (reviewed in Bohnert and Jensen, 1996). Like bacteria, plant cells maintain a positive turgor and are subject to osmotic stress. In fact, water deficit is the commonest environmental stress factor limiting plant productivity.

The ability of plants to tolerate water deficit shows some similarity to bacteria in that plants accumulate compatible solutes to prevent the loss of water from their cells. However, the most critical requirement under osmotic stress conditions appears to be the ability to detoxify oxygen free radicals (Bohnert and Jensen, 1996). Plants are

equipped with superoxide dismutases (SOD) and catalases and some of the compatible solutes that are accumulated, such as proline and mannitol, also show radical scavenging capabilities. The photosynthetic electron transport system in the chloroplast is the major source of oxygen free radicals in plant tissues. Water stress disrupts the cellular redox homeostasis and leads to the generation of free radicals in the chloroplast. Bacteria are also subject to these osmotic effects and it might be expected that disruptions would also occur to the electron transport chain, situated in the membrane, as the cell loses turgor. Alternatively, changes in the electrolyte balance of the bacterial cell, which are known to occur during osmotic stress (Csonka and Hanson, 1991), may disrupt respiratory chain processes to yield oxygen free radicals.

The studies in *S. aureus* and *B. subtilis* make the results obtained with the *S. typhimurium ahp* locus all the more surprising, as it might be expected that osmotic stress in *S. typhimurium* would also result in possible membrane lipid peroxidation or other oxidative stress damage. Differences do exist however, in the regulatory mechanisms of the *ahp* loci in *S. typhimurium*, *S. aureus* and *B. subtilis*. For example the *S. aureus* homologue has not been shown to be induced by hydrogen peroxide (Armstrong-Buisseret *et al.*, 1995) indicating that regulation is likely to be fundamentally different to that of *S. typhimurium.* In *B. subtilis*, the *ahpCF* locus belongs to a hydrogen peroxide-inducible regulon with many similarities to the OxyR regulon (Hartford and Dowds, 1994; Chen *et al.*, 1993, 1995). However, the *B. subtilis ahp* locus has been shown to be regulated by the level of metals, such as manganese or iron, in the medium which is a phenomenon not reported for *ahp* in *S. typhimurium* (Antelmann *et al.*, 1996).

A further observation that makes the lack of induction of the *ahp* locus of *S. typhimurium* look surprising is that the *E. coli dps* gene is induced under osmotic stress (Lomovskaya *et al.*, 1994). The *dps* gene is a member of the OxyR regulon (Altuvia *et al.*, 1994) and was independently identified as *pexB*, a gene which showed post-exponential phase induction (Lomovskaya *et al.*, 1994). A *pexB*::*lacZ* construct containing a truncated promoter region, importantly lacking the OxyR

promoter recognition sequence, was shown to be induced by osmotic and starvation stresses but not by hydrogen peroxide. This indicated that the osmotic-inducibility of *pexB* is independent of OxyR. In addition, the osmotic- and starvation-inducibility of the *pexB* gene was shown to be dependent upon RpoS regulation. RpoS acts as the master regulator of the stationary phase response (reviewed in Loewen and Hengge-Aronis, 1994) but has also been shown to have an important role in regulating osmotic stress resistance in growing cells (Hengge-Aronis *et al.*, 1993; Hengge-Aronis, 1996a). RpoS is found at a low concentration in growing cells where it is unstable ($t_{1/2}$=1.5 min) but after osmotic upshift the level of RpoS can increase dramatically by stabilisation of the mRNA ($t_{1/2}$=45 min).

The *S. typhimurium ahp* locus shows RpoS-independent expression in both its basal-and hydrogen peroxide-dependent inducibility (see Chapter 3). Furthermore, an *rpoS* mutation was also shown to have no effect upon the light induction of MPG203 when subjected to high osmolarity (Figure 4.5 Panel C), indicating that RpoS had no direct or indirect influence upon the expression of *ahp*. Hengge-Aronis and colleagues (1993) have shown that osmotically stressed *E. coli* cells develop a small level of resistance to hydrogen peroxide and this is an RpoS-dependent phenomenon. Therefore, one potential reason why the *ahp* locus is not induced under osmotic stress in *S. typhimurium* may be that it is not RpoS regulated. RpoS has been shown to regulate a number of other genes involved in oxidative stress including the *E. coli* loci *katE, xthA, katG, gorA* and the *S. typhimurium* loci *stiA, stiB* and *stiC* (Hengge-Aronis, 1996a; Ivanova *et al.*, 1994; Becker-Hapak and Eisenstark, 1995; Seymour *et al.*, 1996). The lack of induction of the *ahp* locus in stationary phase in *S. typhimurium* (see chapter 3) may suggest that these and other uncharacterised RpoS-regulated functions which may also be present under osmotic stress, circumvent the need for Ahp function. Nevertheless, it is noteworthy that substantial induction of AhpC could be elicited from wild type cells in response to hydrogen peroxide, under conditions of high osmolarity (Figure 4.9), which suggests that OxyR regulation is still operational, even when RpoS has been reported to be present at a substantial level. Whether this reflects on a more complex control of other compensating factors is unknown at present.

Osmolarity has been shown to have a profound effect upon the supercoiling of cellular DNA and many osmoregulated genes are known to have supercoiling sensitive promoters (Higgins *et al.*, 1988; Hulton *et al.*, 1990; Dorman and Ni Bhriain, 1992). An unusual influence of the *lux* bioluminescent reporter system has been observed previously, and was shown to occur when the *lux* genes were cloned downstream of the *S. typhimurium leu-500* promoter or the *proU* operon promoter (Owen-Hughes *et al.*, 1992; Forsberg *et al.*, 1994). One feature common to both these promoters is that they are known to be supercoiling sensitive, suggesting that a subset of promoters may be sensitive to the anomalous influence of *lux* (although it should be noted that the *leu-500* promoter is not osmotically-sensitive, indicating that this subset is not exclusive for osmotically-inducible promoters). Nevertheless, the promoter of the *gyrB* gene is supercoiling sensitive but the level and patterns of expression for a *gyrB*::*luxAB* fusion are similar to those obtained using other reporter systems (Forsberg *et al.*, 1994), therefore the situation is apparently more complex than this.

Perhaps the best characterised example of anomalous gene expression in the presence of a *lux* element is that for the *S. typhimurium proU* operon (Owen-Hughes *et al.*, 1992; Forsberg *et al.*, 1994). The *proU* operon encodes a high-affinity glycine betaine transport system, whose transcription is induced by growth under high osmolarity (Csonka and Hanson, 1991). The expression of *proU* is repressed in medium of low osmolarity by the presence of a downstream regulatory element (DRE), which exists in the coding sequence of the first gene of the operon, *proV* (Owen-Hughes *et al.*, 1992). Cloning of the *proU* promoter, excluding this DRE, upstream of a transcriptional reporter system would therefore be expected to result in expression of *proU* even under low osmolarity. Indeed, this is observed when a fusion is constructed between the relevant segment of the *proU* promoter and *lacZ*. In contrast, when *luxAB* was used as the reporter system, the *proU* promoter behaved as in the wild type situation and was repressed under low osmolarity (Owen-Hughes *et al.*, 1992; Forsberg *et al.*, 1994). Thus, *luxAB* appeared to substitute for the absence of a DRE and mediate repression in low osmolarity.

The way in which *luxAB* elicits unnatural behaviour from genes is not entirely clear, but this phenomenon has been reported using *lux* genes from *Vibrio harveyi* and *Vibrio fischeri* (Owen-Hughes *et al.*, 1992; Forsberg *et al.*, 1994). However, an examination of the first 200 nucleotides of *luxA* have shown that it contains tracts of adenine residues, which intrinsically curve the DNA (Owen-Hughes *et al.* 1992). Curved DNA has been shown to influence the promoter activity of genes and many of these affects are facilitated by the nucleoid-binding protein, H-NS (Hulton *et al.*, 1990; Owen-Hughes *et al.*, 1992). H-NS is thought to act as a scaffold, holding DNA in an appropriate configuration. H-NS appears not to have a sequence specific binding motif but has been shown to bind curved sequences of DNA (Yamada *et al.*, 1990), affecting the local topology and flexibility of the DNA (Jordi *et al.*, 1995). Interestingly, the DRE element in *proU* has also been shown to be curved and both DRE and *luxAB,* but not *lacZ,* fragments of DNA have been shown to bind H-NS efficiently (Owen-Hughes, 1992). Repression of *proU* expression in low osmolarity is believed to result from H-NS binding to the DRE (or equivalently to *luxAB*), making the *proU* promoter inflexible and unable to support a productive interaction between the promoter and RNA polymerase (Jordi *et al.*, 1995).

In the natural situation, *ahp* is expressed in the presence of hydrogen peroxide irrespective of the osmotic environment (Figure 4.9). However, in the presence of the Mu*dlux* element, the *ahp* locus becomes unresponsive to hydrogen peroxide in medium of low osmolarity (Figure 4.3), indicating that the *lux* element acts to repress the normal behaviour of the gene even though it is not osmoregulated naturally and is not known to contain a natural equivalent to the DRE. Moreover, in contrast to *proU*, H-NS was shown not to alter the observed osmotic effects (Figure 4.5 Panel E), therefore in this instance, the anomalous effects of the *lux* system upon the *ahp* locus appear to be H-NS-independent. However, it should be noted that the *hns* mutation used in the present study (*osmZ6*::Tn*10*) has been reported to have a mild phenotype compared to other *hns* mutations. This is due to the fact that the transposon lies within the *hns* promoter region and not within the coding sequence of the gene (Hulton *et al.*, 1990). Thus, the possibility that some functional H-NS was still present cannot be entirely excluded in this instance.

Another difference between this investigation and the others is the type of *lux* construct used. Previous studies have used plasmid-borne transcriptional fusions of the *luxAB* genes to the promoters of interest (Owen-Hughes *et al.*, 1992; Forsberg *et al.*, 1994), whereas this study involved a chromosome based Mu*dlux* system. A transcriptional fusion to the *luxAB* genes on a plasmid, places the *luxA* gene directly adjacent to the region of DNA of interest. However, the Mu*dlux* element consists of the *luxCDABE* operon from *V. fischeri* flanked by Mu phage sequence (Engebrecht *et al.*, 1985). Thus, in a Mu*dlux* fusion, *luxA* is approximately 4 kb downstream from the 5' end of Mu*dlux* element, and is therefore quite distal from the promoter of interest. This indicates that if curvature in *luxA* is responsible for the anomalous behaviour of the *ahp* locus, then it must be acting over a large distance in order to influence the *ahp* promoter. Whether other factors such as the DNA binding proteins IHF, HU or FIS, which are known to influence supercoiling or topology of DNA (Dorman and Ni Bhriain, 1992) play a role in the *ahp* phenomenon is unknown. Alternatively, it may be possible that the *lux* element somehow alters the activity of the *ahp* promoter directly, perhaps by introducing osmolarity-dependent changes in flexibility into the DNA helix. Equally, since changes in medium osmolarity have been shown to influence expression of a large number of genes by less than 2-3 fold (Jovanovich and Lebowitz, 1987), it may be possible that the *lux* genes act to magnify a very small osmotic change in the *ahp* promoter which is not detected by immunoblotting.

The addition of novobiocin to a culture of MPG203 was also shown to elicit *ahp* expression independently of hydrogen peroxide or osmolarity (Figure 4.6 and 4.7), suggesting that some component in the expression pathway is supercoiling sensitive. However, the response appeared to be concentration-dependent. In general, the cellular level of supercoiling is regulated predominantly by the action of two enzymes, DNA gyrase and topoisomerase (Dorman and Ni Bhriain, 1992). DNA gyrase consists of two subunits, GyrA and GyrB, which act together to introduce negative supercoils into the DNA. In contrast, topoisomerase I, is able to relax the DNA structure by reducing DNA supercoiling. Novobiocin inhibits DNA gyrase by blocking the activity of the gyrase B subunit (Menzel and Gellert, 1984). DNA from

cells lacking gyrase activity would therefore be expected to be more relaxed or less negatively supercoiled because topoisomerase is still active. In contrast however, high osmolarity is considered to increase the level of negative supercoiling. Perhaps the counter-balanced effects of these two agents resulted in the optimisation of *ahp* promoter activity. Alternatively, it may be that novobiocin is not affecting DNA supercoiling directly but produces some form of side-effect in the cell, especially at high novobiocin concentrations. The profound effect of novobiocin upon the growth rates of cells would support this interpretation.

The classical induction of the OxyR regulon involves transcriptional activation by the OxyR protein under hydrogen peroxide stress (Christman *et al*, 1985). OxyR is sensitive to oxidation, and only the oxidised form of the protein can activate transcription (Storz *et al.*, 1990). Activated OxyR binds as a tetramer, at a conserved but degenerate consensus sequence in the promoters of the genes it regulates (Tartaglia *et al.*, 1992; Toledano *et al.*, 1994). This conserved sequence results in OxyR binding to nucleotides within four adjacent major grooves. Control of binding to this consensus appears to lie in the conformation of the OxyR protein. In its reduced state OxyR is in the wrong conformation, and is unable to bind or 'fit' correctly into the DNA of the consensus sequence. However, upon oxidation the conformation changes and OxyR can bind to the consensus sequence, within the 4 grooves.

Any model of the osmotic induction of the *ahp* locus in association with the *lux* element has to take into account a requirement for OxyR (Figure 4.5 Panel B). In the wild type situation, the *ahp* promoter is not osmoregulated and the activated OxyR tetramer can bind and bring about Ahp expression irrespective of the osmotic environment (Figure 4.10 Panel A). In contrast, in the presence of the Mu*dlux* element the *ahp* promoter becomes osmoregulated and this is probably mediated through some effect of the *luxA* gene. Under low osmolarity the *lux* element acts to keep the *ahp* promoter in the wrong conformation, or inflexible, thus preventing access by activated OxyR and repressing the response (Figure 4.10 Panel B(1)). This repressive effect may be indirect and may well involve a DNA-binding protein. For

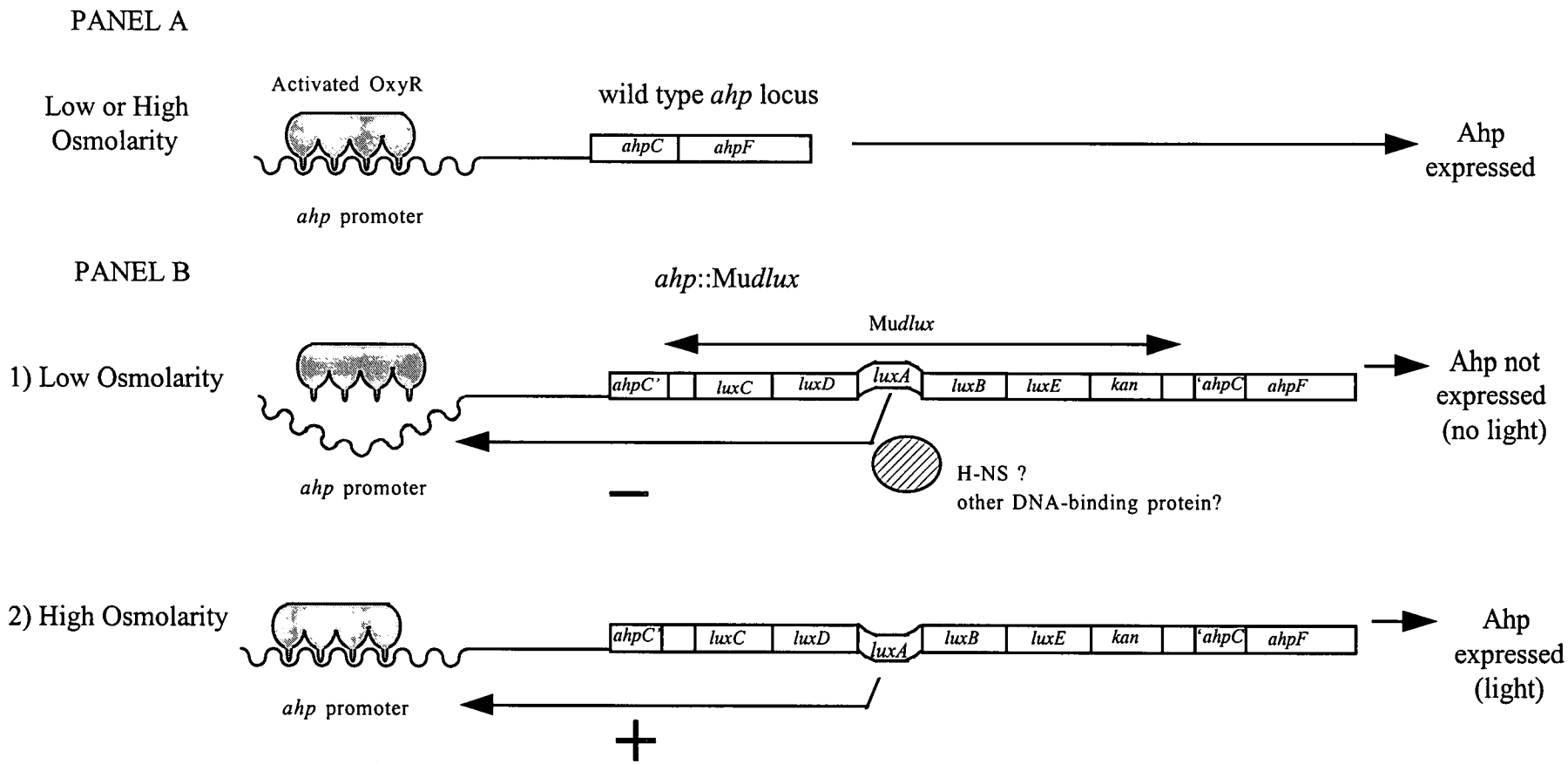


Figure 4.10 Summary diagram of the effect of the Mu*dlux* bioluminescent reporter system upon the *ahp* promoter under different osmotic environments. In the wild type situation (Panel A) the *ahp* promoter is not effected by osmolarity and in the presence of hydrogen peroxide the activated OxyR protein can bind as a tetramer and promote *ahp* transcription. In the presence of the Mu*dlux* reporter system (Panel B), the *ahp* promoter becomes osmoregulated as a result of some uncharacterised effect of the *luxA* gene. This effect may be indirect and involve a DNA-binding protein (shaded circle). H-NS can bind to *luxA* and was shown to be involved in the anomalous behaviour of a *proU*::*luxAB* construct (Forsberg *et al.*, 1994). However, a role for *hns* was less conclusive from the present study. Alternatively, the effect of *luxA* may act directly by somehow transmitting changes in the DNA helix to the promoter. Under low osmolarity, the effect of *luxA* is to alter the *ahp* promoter such that it is in an sub-optimal arrangement for the binding of the activated OxyR tetramer thus repressing (-) *ahp* expression (1). In contrast, in an high osmotic environment the repression is alleviated (+) and the *ahp* promoter adopts an optimal arrangement which is capable of binding OxyR, thus activating *ahp* transcription (2).

the purpose of this model, the role of H-NS is question marked but cannot be excluded because it is involved in the anomalous effect of *lux* at the *proU* promoter (Forsberg *et al.*, 1994). Equally, an as yet unidentified DNA-binding protein may be involved in interacting with the *luxA* sequence. On the other hand, the effect of *lux* may be direct and involve altering the flexibility of the DNA helix or restricting the accessibility of the *ahp* promoter, perhaps through an altered secondary or tertiary structure. Under high osmolarity, the repressive effect is alleviated, perhaps by altering the flexibility of the *ahp* promoter (by changing the DNA topology) and allowing OxyR to bind and stimulate a response (Figure 4.10 Panel B(2)).

When OxyR was placed on a multicopy plasmid under the regulation of a controllable promoter and expressed in an *oxyR* deletion strain, a significant response to hydrogen peroxide was obtained, even in a medium of low osmolarity (Figure 4.8). How this expression is obtained in low osmotic medium is unknown, perhaps the high level expression of OxyR from the plasmid, when activated by hydrogen peroxide, can overcome a sub-optimal promoter arrangement. This is supported by the enhanced level of response obtained in the same experiment in the presence of high osmolarity, where the promoter might adopt a more optimal configuration.

The results obtained in this chapter add to those from previous studies (Owen-Hughes *et al.*, 1992; Forsberg *et al.*, 1994) which indicate that caution should be taken when interpreting results from experiments involving the use of a *lux* transcriptional reporter system to monitor gene expression.

CHAPTER 5

The role of the *ahp* and *oxyR* loci in the virulence of *S. typhimurium*

5.1 INTRODUCTION

Typhoid fever, which results from infection by *S. typhi*, remains the most serious of the diseases caused by *S. enterica*. In developing countries an estimated 16.6 million cases of this disease occur per annum, with around 600, 000 deaths (Pang *et al.*, 1995). In addition, infection by non-typhoidal serovars of *S. enterica* is responsible for approximately 1.3 billion incidences of diarrhoeal disease per annum. Therefore, infection by both typhoidal and non-typhoidal serovars of *S. enterica* results in a considerable global health problem (Pang *et al.*, 1995; Maurice, 1994). Moreover, *S. enterica* infection of animals is also a common occurrence. Indeed, such infection of poultry, cattle, sheep and swine not only has economic importance in agriculture and animal husbandry (Coynault *et al.*, 1996), but is believed to be a major factor in the transmission of *S. enterica* to humans via the food chain (Maurice, 1994). Unfortunately, our ability to control *S. enterica* infection in humans and animals has become threatened by the spread of multi-drug resistance (Pang *et al.*, 1995). Thus, there is a growing need to develop effective vaccine strategies to eliminate reservoirs of this organism, especially within animals, and to reduce the level of *S. enterica*-related illness.

Several approaches have been followed in the development of vaccines to *S. enterica*, but one of the most promising is the development of live attenuated strains which carry defined genetic lesions in important genes and provide longer lasting and more effective immunity than dead cell vaccines (Collins, 1974; Chatfield *et al.*, 1992a; Ivanoff *et al.*, 1995). The development of live attenuated vaccines has promoted, and been aided by, studies into the virulence of *S. enterica*, through identifying genes that play roles in helping this bacterium to cause disease. It is very apparent that bacteria respond to the host environment and alter their gene expression accordingly. As a result, the bacteria enhance their survival under the new conditions and activate the appropriate invasion or defensive tactics for the particular stage of infection. This is an important facet of virulence (Mekalonos, 1992; Mahan *et al.*, 1996).

The development of vaccines to *S. enterica* has also been helped by the availability of an appropriate animal model, based on *S. typhimurium* which is capable of causing a typhoid-like illness in innately susceptible mice. Indeed, it would seem that mutations in genes that affect the ability of *S. typhimurium* to cause disease in the mouse model are also likely to influence the ability of *S. typhi* to cause disease in humans (Tacket *et al.*, 1992). Moreover, through the use of an LD_{50} test, the mouse model provides a means to quantitate attenuation thereby allowing the importance of a gene in virulence to be assessed.

A number of useful attenuating lesions have been identified, through such tests, that reduce the ability of *S. typhimurium* to cause disease in mice. These include mutations in genes encoding biosynthetic proteins such as *aro* (Hosieth and Stocker, 1981) or regulatory proteins like *cya crp* (Curtiss and Kelly, 1987) or PhoPQ (Miller *et al.*, 1993) and in some cases, the same lesions have been used in *S. typhi* as the basis for developing vaccines (Tacket *et al.*, 1992). However, in a number of cases where the genetic disruption attenuates *S. typhimurium*, the same disruption in strains of *S. typhi* has resulted in a vaccine which produces some symptoms of disease (Ivanoff *et al.*, 1994). Therefore, not only is their a need to identify new attenuating lesions, but also to identify possible lesions which can reduce further the virulence of present vaccines to a point where they are protective but do not produce significant symptoms of disease.

In general, invasive *S. enterica* serovars are capable of breaching the intestinal epithelium and penetrating the underlying tissue (Finlay and Falkow, 1989a; Gulig, 1996). Here they enter macrophages and shortly after, disseminate to the liver and spleen where they are capable of replicating and causing serious disease. The ability to follow this path has been attributed to their efficiency as intracellular pathogens, and survival in the macrophage has been shown to be the most important factor that decides the outcome of disease (Buchmeier and Heffron, 1989; Finlay and Falkow, 1989a). Normally, macrophages are capable of destroying bacteria through a combination of a respiratory burst, which produces high levels of reactive oxygen species such as superoxide, hydrogen peroxide and nitric oxide, and through

anti-microbial factors associated with the lysosome (Adams and Hamilton, 1984; Hasset and Cohen, 1989; Lehrer *et al.*, 1990, Pacelli *et al.*, 1995). Therefore, it would be expected that the ability of *S. enterica* to survive within macrophages would be closely linked to defensive strategies employed to deal with these anti-microbial mechanisms and this in turn would be important for virulence.

Of particular interest to the present study, is the ability of *S. enterica* to withstand the oxidative killing mechanisms of the macrophage. Oxidative stress is known to damage the bacterial cell at all fundamental macromolecular levels including that of proteins, lipids, DNA and RNA (reviewed in Farr and Kogoma, 1991) and *S. typhimurium* has been shown to respond to such oxidative stress by the derepression of multigenic responses (Demple, 1991). One such stress is the hydrogen peroxide stress response which results in the induction of around 30 proteins (Christman *et al.*, 1985). A small subset of the genes encoding these proteins are regulated by a protein called OxyR. Such genes which include *katG* (catalase), *ahpCF* (alkyl hydroperoxide reductase) and *dps* (DNA binding protein from starved cells), form the OxyR regulon which help to reduce the detrimental effects of hydrogen peroxide. In *S. typhimurium*, loss of the specific regulator, OxyR, or the genes it regulates, makes cells extremely susceptible to peroxide stress (Christman *et al.*, 1985; Buchmeier *et al.*, 1995; Francis, 1993; Almiron *et al.*, 1992), therefore it would be expected that such genes would be essential to the survival of *S. enterica* during the course of infection, especially during entry into the macrophages. These genes would also appear to be likely candidates for mediating attenuation in *S. enterica*, when disrupted, and therefore may be of interest for the on-going development of attenuated bacterial vaccines.

Indications that the genes of the OxyR regulon are important in virulence have been shown in a number of studies. Francis and Gallagher (1993) demonstrated, using the Mu*dlux* reporter system in the *ahpCF* locus (MPG203), that light was induced upon interaction with macrophages. Subsequently, in an independent study, the *dps* gene was also demonstrated to be induced within the macrophages environment (Valdiva *et al.*, 1996). Moreover, in studies by Fields *et al.* (1986), three transposon mutants

were identified which demonstrated hypersusceptibility to hydrogen peroxide (and other oxidants), exhibited reduced survival in macrophages and, were attenuated in mice.

The aim of the following study was to investigate the role of genes involved in oxidative stress responses in virulence. Specifically, those genes associated with the OxyR regulon, were to be examined and assessed for potential, when disrupted, for use in vaccine development.

5.2 RESULTS

5.2.1 The role of the *ahp* locus in the virulence of *S. typhimurium*

Testing the virulence of MPG203 using a mouse model

The availability of MPG203, which contains a Mu*dlux* element inserted within the *ahpC* gene, provided a good opportunity to test whether a disruption in the *ahp* locus had any effect upon the virulence of *S. typhimurium*. In MPG203, no functional alkyl hydroperoxide reductase (Ahp) is produced, as is clearly demonstrated using a cumene hydroperoxide disc inhibition test, a test which is specific for Ahp functionality (Francis, 1993).

Serial dilutions from overnight cultures of MPG203 (the *ahp* mutant) and SL1344 (the virulent parental strain) were made in phosphate-buffered saline (PBS) to give a range of bacterial concentrations of approximately 10^1-10^6 organisms per ml. Groups of six female BALB/c mice, aged 8-10 weeks, were then injected by the intraperitoneal route (i.p.) with 100 µl of one of the six concentrations of bacteria and their survival was subsequently followed over 28 days. From Table 5.1 (see page 211) it can be seen that the parental *S. typhimurium* strain (SL1344) killed almost all the mice in the study with a calculated 50% lethal dose (LD_{50}) of 1.0 ($\log_{10}$ cell number) (Reed and Muench, 1938). Therefore, it is clear that a bacterial inoculum of approximately 10 bacteria, administered by the i.p. route, is capable of killing a

susceptible mouse, and this is in line with other studies (Hosieth and Stocker, 1981; Coynault *et al.*, 1996).

In contrast, the LD_{50} for MPG203 was found to be 4.8 (log_{10} cell number). When compared to SL1344, this suggests that the *ahp* disruption may have an effect upon the survival of *S. typhimurium* during the course of infection. However, the attenuation could be attributed to factors other than that of disrupted gene function. With the bioluminescent reporter system, the luciferase enzyme converts an aldehyde substrate to a fatty acid, and requires $FMNH_2$ (Engebrecht and Silverman, 1984; Meighen, 1991). The Mu*dlux* element not only carries the ability to make the luciferase enzyme but also, the ability to synthesise the aldehyde substrate. It is possible then that when the *ahp* gene is induced, as has been shown upon interaction with macrophages (Francis and Gallagher, 1993), large amounts of aldehyde could be produced. This may impose a metabolic burden upon the cell or alternatively may result in some degree of intracellular toxicity, thereby affecting the ability of the bacterium to replicate and survive within the host. It was decided, therefore, to test whether the attenuation was due to the genetic disruption of *ahp* directly or resulted from some indirect action of the Mu*dlux*. A different type of *ahp* mutant was constructed in which the Mu*dlux* element was absent in order to address this issue.

Cloning of the *S. typhimurium ahp* locus

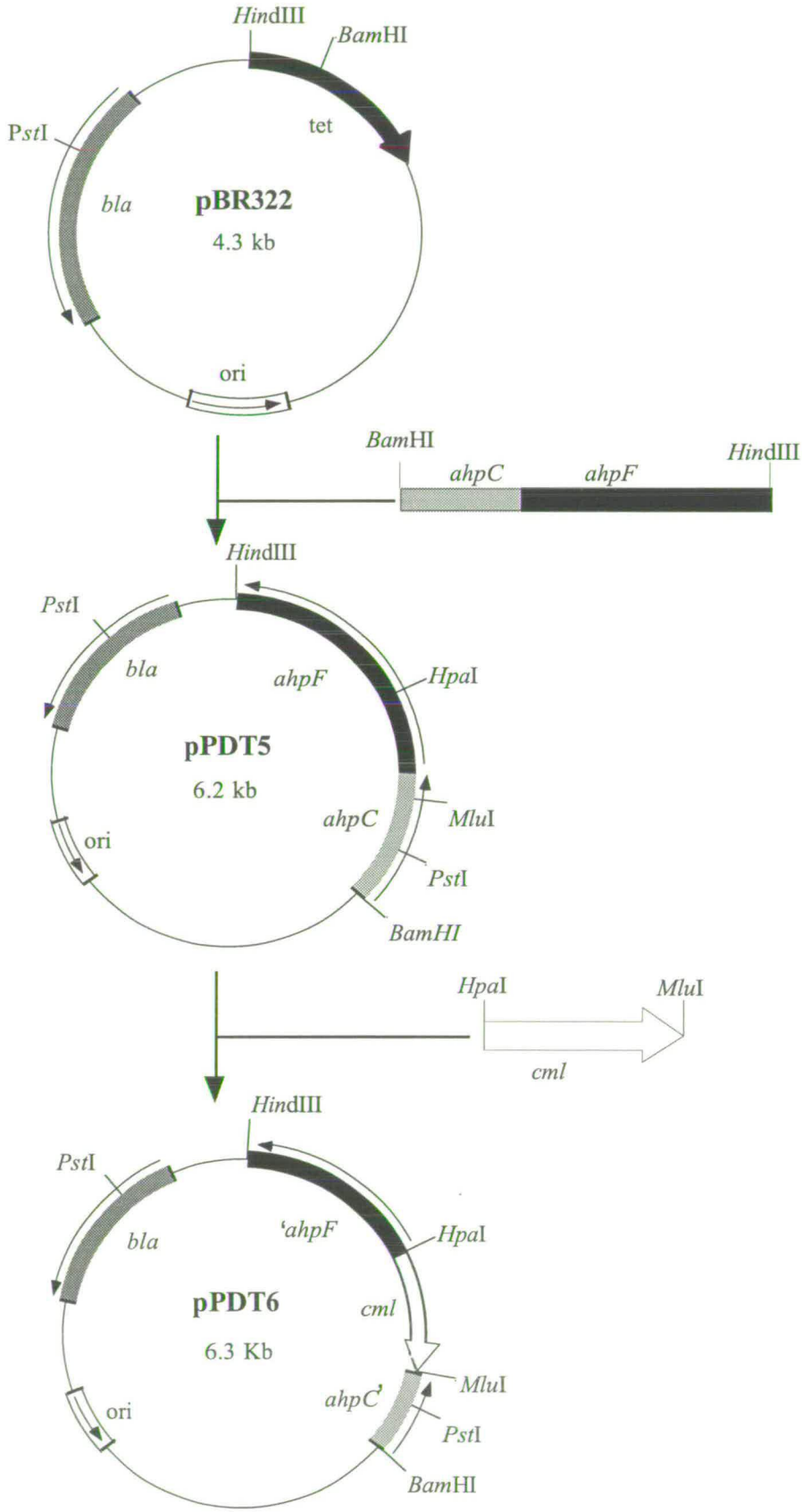
The *ahpCF* locus from *S. typhimurium* has been cloned and the sequence determined in previous studies (Tartaglia *et al.*, 1990). Using the known sequence, primers were devised to the 5'- and 3'-ends of *ahpCF* and incorporated *Bam*HI (G7858) and *Hin*dIII (G7859) sites respectively (see Table 2.1), to facilitate cloning into the vector pBR322 (Bolivar *et al.*, 1977). The location of primers and choice of restriction sites were chosen such that the *ahpCF* locus would be promoterless and cloned in an inverted orientation with respect to the P_{TET} promoter of pBR322, in case multicopy expression of *ahp* proved toxic.

Figure 5.1

A summary of the cloning and insertional inactivation of the *S. typhimurium ahpCF* locus

A promoterless *ahpCF* fragment was amplified from *S. typhimurium* genomic DNA by PCR using primers that incorporated *Bam*HI (G7859) and *Hin*dIII (G7859) restriction sites (see Table 2.1). After treatment with these enzymes, the 2.36 kb *Bam*HI-*Hin*dIII fragment bearing the *ahpCF* locus was cloned into similarly cut pBR322 to produce pPDT5. Insertional inactivation of the *ahp* genes was achieved by removing an internal 0.9 kb *Mlu*I - *Hpa*I fragment of DNA and replacing it with a 0.98 kb chloramphenicol cassette, amplified from pBR325 using primers G8493 (which carried an added *Mlu*I site) and H1486 (which carried an added *Hpa*I site) (see Table 2.1) to produce pPDT6. This plasmid was then used to generate the *pst*I fragment (approximately 3 kb) used to transform *S. typhimurium* SL1344. Key: *bla* - gene encoding β-lactamase; ori - origin of replication; *tet* - tetracycline resistance gene; *ahpC* - gene encoding alkyl hydroperoxide reductase subunit C; *ahpF* -gene encoding alkyl hydroperoxide reductase subunit F; *cml* - gene encoding chloramphenicol acetyl transferase from pBR325.

Figure 5.1


HindIII
BamHI
tet
PstI
bla
pBR322
4.3 kb
ori
BamHI
ahpC
ahpF
HindIII
HindIII
PstI
bla
ahpF
HpaI
pPDT5
6.2 kb
ahpC
MluI
ori
PstI
BamHI
HpaI
MluI
cml
HindIII
PstI
bla
'ahpF
HpaI
pPDT6
cml
6.3 Kb
MluI
ahpC'
ori
PstI
BamHI

The primers were then used in a polymerase chain reaction (PCR) to amplify a 2.36 kb *ahpCF* fragment from the *S. typhimurium* chromosome and the resulting DNA was digested with *Bam*HI and *Hin*dIII, ligated to similarly cut pBR322 and transformed into DH5α. The resulting plasmid carrying the *ahpCF* locus was designated pPDT5 (Figure 5.1).

The aim of the experiment was to produce an insertionally inactivated copy of the *ahpCF* locus. This was achieved in two ways, firstly a 0.9 kb *Mlu*I-*Hpa*I fragment, containing 0.13 kb of the 3' end of the *ahpC* gene, a 0.24 kb intragenic region and 0.53 kb of the 5' end of the *ahpF* gene, was removed from within the cloned *ahpCF* fragment and replaced with a 0.98 kb chloramphenicol cassette from pBR325 (Bolivar *et al.*, 1978). The latter cassette, which contains its own promoter, was amplified by PCR using primers that incorporated the *Mlu*I (G8493) and *Hpa*I (H1486) sites (see Table 2.1), to facilitate cloning into the corresponding sites of PDT5, to generate pPDT6 (Figure 5.1). In this construct, functional *ahpCF* cannot be regenerated even if the *cml* cassette is deleted within the bacterial cell. Moreover, the chloramphenicol cassette provides a selection for placing the modified *ahp* locus onto the chromosome by recombination.

In order to transfer the disrupted copy of *ahpCF* onto the *S. typhimurium* chromosome, it was first necessary to transfer pPDT6 into SL1344, such that the DNA was methylated in line with that of the final host. This was necessary as the plasmids had been constructed in an *E. coli* strain and the restriction modification systems of the *S. typhimurium* strain would lower the efficiency of incorporation of the modified *ahp* locus, if transferred directly.

Transfer of the insertionally inactivated *ahp* locus onto the *S. typhimurium* chromosome

The strategy for replacing the wild type *ahp* locus with that of the modified locus was based on the method of Nohmi *et al.* (1992). Their approach was to construct disrupted genes on pBR322, excise the disrupted gene using a single restriction

enzyme and then circularise this excised molecule by treating with DNA ligase. The circularised region of DNA therefore has no origin of replication so cannot self-propagate in the bacterial cell. When cells are transformed with this DNA it will either be destroyed through enzymatic degradation, lost as the bacteria replicate because the fragment does not have an origin of replication or, be integrated onto the chromosome through recombination at a low frequency. This latter option can be selected for if a resistance determinant is present in the fragment. Therefore this approach was attempted for transferring the disrupted *ahp* locus onto the chromosome. However, the construct pPDT6 was not ideal for attempting this procedure as the *ahpCF* locus had been cloned in on incompatible ends and so could not be circularised following excision. Nevertheless, *Pst*I sites were identified 0.17 kb downstream from the translation initiation codon of *ahpC* and in the *bla* gene of pBR322 and so, *Pst*I digestion of pPDT6 would generate a fragment of approximately 3 kb in length, containing 0.25 kb of *ahpC*, a 0.98 kb chloramphenicol cassette, 1.03 kb of *ahpF* and 0.77 kb of the pBR322 plasmid DNA. Importantly, this fragment would lack a replication of origin when recircularised.

Following digestion of pPDT6 with *Pst*I, this fragment was recircularised by ligation and electroporated into SL1344. Transformants were subsequently selected for on chloramphenicol plates and incubated overnight at 37°C. 320 chloramphenicol-resistant transformants were obtained. However, due to the efficiency of electroporation it was possible that undetectable traces of undigested plasmid had also been transformed. The transformants were therefore replica patched onto plates containing either ampicillin (the antibiotic marker of the plasmid) or chloramphenicol (the antibiotic marker in the disrupted *ahp* locus). These were then incubated overnight to identify and eliminate those cells containing the plasmid. Of the initial 320 transformants, only 75 were found to be chloramphenicol resistant and ampicillin sensitive.

Following transformation of the recircularised *Pst*I fragment from pPDT6 there are four possible outcomes (See Figure 5.2): (a) no recombination occurs (in which the wild type locus would remain intact); (b) a single cross-over event occurs in *ahpC*;

(c) a single cross-over event occurs in *ahpF*; (d) a double cross-over event occurs, one each in *ahpC* and *ahpF*. In the case of either of the single cross-over events occurring (options (b) and (c)), two copies of the hybrid *ahpCF* locus would exist, one wild type in function and the other insertionally inactivated. However, since the insertionally inactivated copy of the locus does not contain a promoter, then, depending on the point of recombination, intact *ahpCF* genes will be placed either proximal or distal to the chromosomal *ahp* promoter. In the latter case (Figure 5.2 (b)), the intact copy of *ahpCF* would effectively be promoterless and therefore such cells would be devoid of Ahp activity. Alternatively, if the recombination results in the former outcome (Figure 5.2 (c)), cells would retain Ahp activity and the insertional copy would have no determinable effect. Where recombination occurs in both *ahpC* and *ahpF* (Figure 5.2 (d)) the entire wild type locus would be replaced by the inactivated locus and cells would be deficient in Ahp activity. These different events could be screened for initially by examining the transformants for Ahp activity using a cumene hydroperoxide peroxide disc inhibition test (Christman *et al.*, 1985).

All 75 chloramphenicol resistant mutants were subject to a cumene hydroperoxide disc inhibition test and *S. typhimurium* SL1344 and MPG203 (the *ahp*::Mu*dlux* mutant) were included as controls. Plates were grown overnight at 37°C and the zones of inhibition were subsequently measured. The mutants could be clearly split into 2 groups according to the size of the zone of inhibition. 50 mutants had a small zone of inhibition (approximately 24 mm) equivalent to that of SL1344, and 25 mutants had a large zone of inhibition (approximately 36 mm) equivalent to MPG203. These results suggested that the majority of the mutants obtained had undergone a single recombination in *ahpF* as they appeared to have wild type Ahp function, as indicated by the small zone of inhibition. This is hardly surprising as the probability of establishing a recombination event in the *ahpF* region must be greater due to the proportionally larger DNA fragment present in the construct (approximately 1.03 kb) compared to that of *ahpC* (0.25 kb).

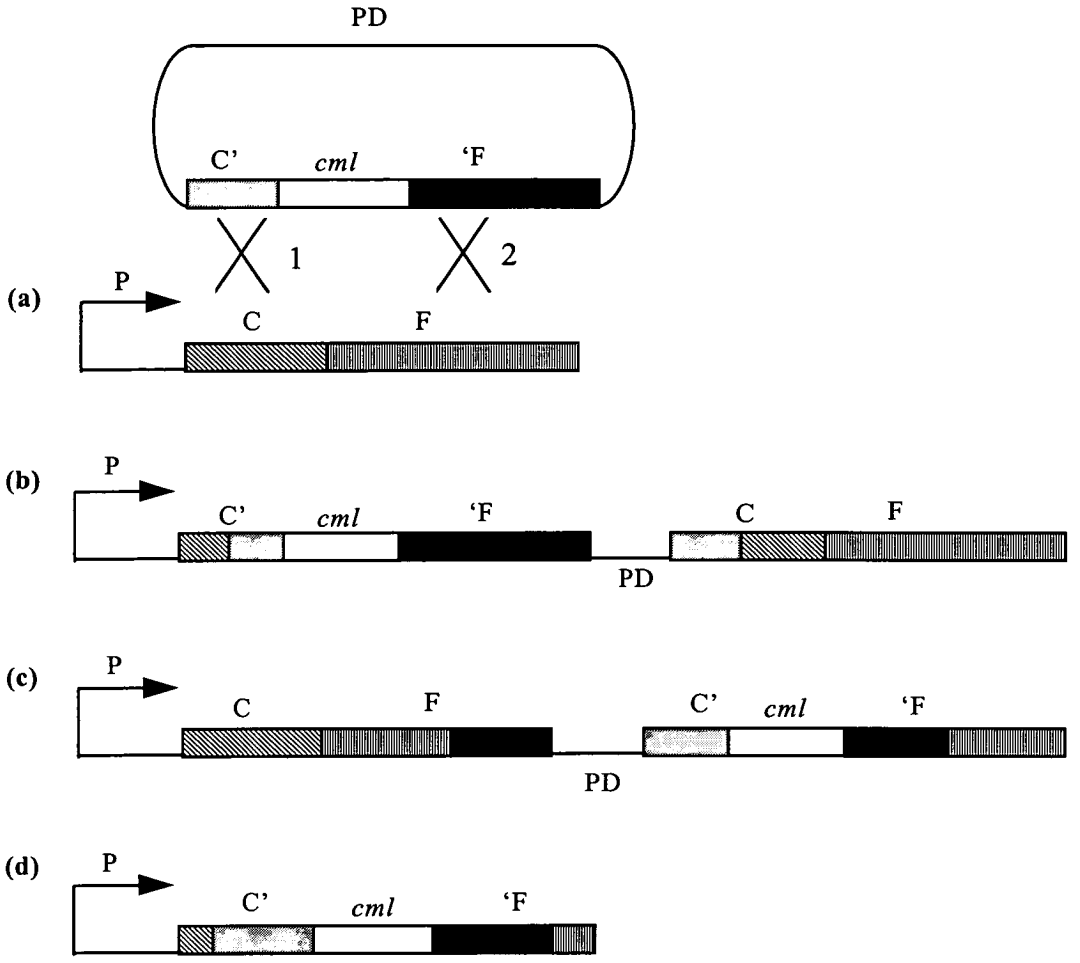


Figure 5.2 A schematic diagram of the anticipated outcome of recombination in the *ahpCF* locus

The promoterless insertionally inactivated copy of the *ahpCF* locus was excised from pPDT6 on a *Pst*I fragment and religated. This recircularised molecule not only contains the *ahpCF* locus but also contains a small 0.7 kb fragment of pBR322 DNA. After transformation of this molecule into *S. typhimurium* SL1344, four possible outcomes were anticipated: no recombination (a); a single cross over event in *ahpC* via X1 (b); a single cross over event in *ahpF* via X2 (c); a double crossover via X1 and X2 (d). In the event of no recombination occurring, the *ahp* locus would remain as wild type (a) and would show Ahp activity. In the event of outcome (b) the chromosome would carry two copies of the *ahp* locus, an inactivated and intact copy respectively. However, the intact copy is distal from the chromosomal promoter and therefore these cells would be devoid of Ahp activity. In contrast, if recombination occurred by single cross over in *ahpF* (c) the cell would also contain two copies of the *ahp* locus but the functional copy would be positioned adjacent to the promoter, therefore these cells would also display Ahp activity (d). Finally, if a double cross event occurred (d) the insertionally inactivated *ahp* locus would be exchanged for the wild type locus, and these cells would be devoid of functional Ahp. Key: P - *ahpCF* promoter; *cml* -chloramphenicol acetyl transferase cassette used to disrupt the *ahp* locus; PD - 0.7 kb fragment of pBR322 DNA; C - functional *ahpC* gene; F - functional *ahpF* gene; C' - non-functional truncated *ahpC* gene; 'F - non-functional truncated *ahpF* gene. The origin of the DNA fragments are shown as follows - diagonal stripes-chromosomal *ahpC*; vertical stripes-chromosomal *ahpF*; dark-grey-*ahpC*'(truncated at the 3' end by the chloramphenicol-resistance cassette); black-'*ahpF* (truncated at the 5' end by the chloramphenicol-resistance cassette). X1 and X2 represent possible positions of recombination.

The large zone of inhibition observed in 25 of the mutants clearly indicated loss of Ahp function. However, it was impossible at this stage to determine whether a single or double cross-over event had occurred in these mutants.

Analysis of recombination events in the *ahp* locus by Southern blotting and PCR

To ascertain whether a single or double recombination event had occurred in the 25 cumene-sensitive transformants, Southern blotting and probe hybridisation were employed (Southern, 1975). After a single recombination event, two copies of *ahpCF* locus, one wild type and the other inactivated, would be detectable by these approaches thus allowing identification and elimination from this study. A suitable restriction enzyme was therefore required to identify the arrangement of the recombination events in the mutants obtained. From the *S. typhimurium* sequence of the *ahp* locus (Tartaglia *et al.*, 1990) it is known that a *Hpa*I restriction enzyme site exists such that the entire *ahpC* gene can be liberated as a 1.38 kb fragment from the wild type locus (Figure 5.3 (a)). In the case of a single recombination event in *ahpC* (Figure 5.3 (b)), the *Hpa*I enzyme cuts to liberate two *ahpC* fragments, one of 1.45 kb containing the *ahpC* gene and the chloramphenicol resistance cassette, and a second fragment of 2.93 kb containing the truncated *ahpF* gene, the fragment of pBR322 DNA and the intact *ahpC* gene. In contrast, in the event of a double recombinant (Figure 5.3 (c)), only the 1.45 kb fragment would be obtained by *Hpa*I digestion. Thus, digestion of DNA from transformant cells, followed by probing with *ahpC*, would identify the nature of the recombination events and allow subsequent elimination of single recombinants from the study.

Genomic DNA was isolated from all 25 of the mutants, which showed the large zone of inhibition, as well as from *S. typhimurium* SL1344 which serves as a control, and

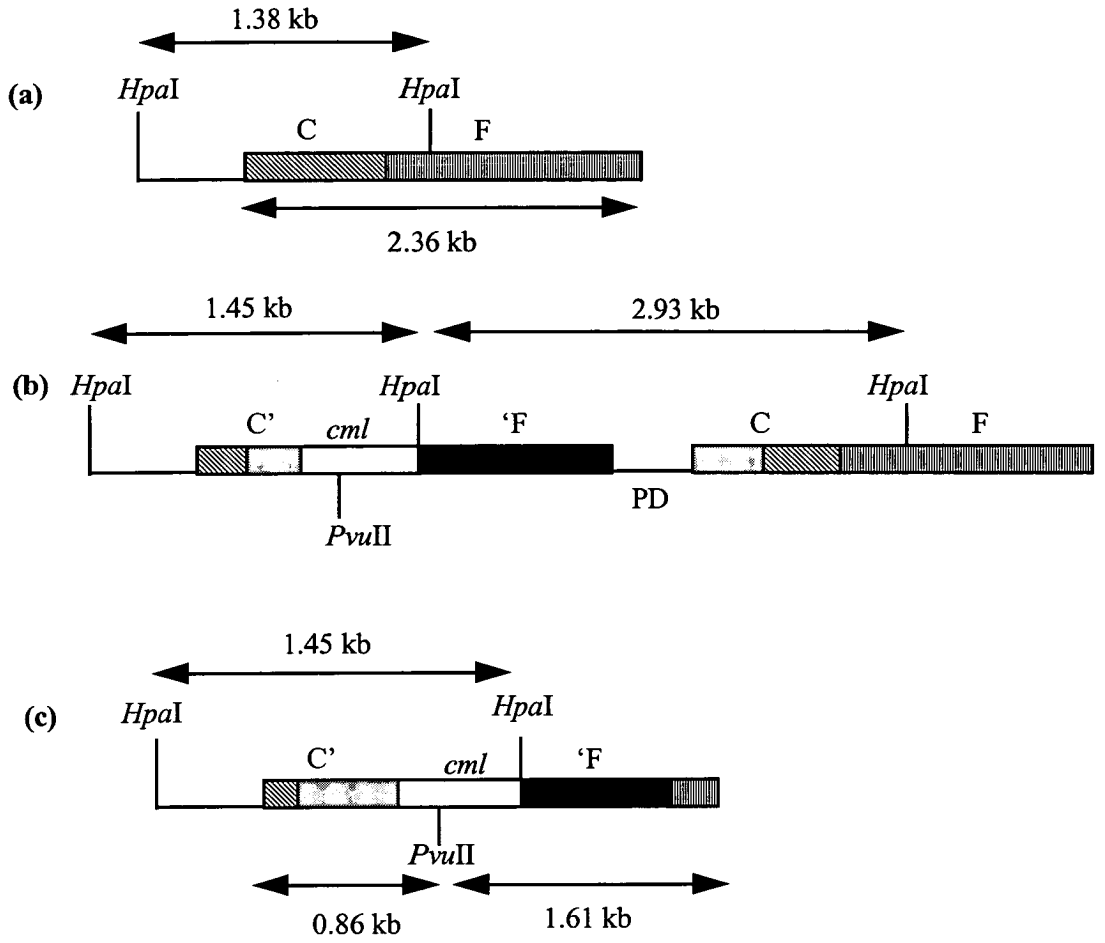


Figure 5.3 Schematic diagram of the anticipated organisation of the *ahpCF* genes and diagnostic restriction sites

In order to determine whether the cumene hydroperoxide-sensitive transformants obtained had undergone a single or double recombination event with the insertionally inactivated *ahpCF* fragment, Southern blot analysis was used. The chromosomal *ahpCF* locus (a) can be cut with *Hpa*I to liberate a 1.38 kb fragment containing the entire *ahpC* gene. For a single recombinant in the *ahpC* gene (b) these sites would result in two fragments, one containing truncated *ahpC* and the *cml* cassette on a 1.45 kb fragment, and a second of 2.93 kb in size, containing truncated *ahpF*, the fragment of pBR322 DNA and the intact *ahpC* gene. In contrast, only the former *Hpa*I fragment (1.45 kb) would be formed in a double recombinant (c). Using *Hpa*I digests and Southern blotting of candidate mutants it was possible to eliminate single recombinant mutants from this study by probing with the *S. typhimurium ahpC* gene (see Figure 5.4a).

Key: *cml* -chloramphenicol acetyl transferase cassette used to disrupt the *ahp* locus; PD - 0.7kb fragment of pBR322 DNA; C - functional *ahpC* gene; F - functional *ahpF* gene; C' - non-functional truncated *ahpC* gene; 'F - non-functional truncated *ahpF* gene. The origin of the DNA fragments are shown as follows:- diagonal stripes-chromosomal *ahpC*; vertical stripes-chromosomal *ahpF*; dark-grey-*ahpC*' (truncated at the 3' end by the chloramphenicol-resistance cassette); black- '*ahpF* (truncated at the 5' end by the chloramphenicol-resistance cassette).

the DNA was treated with the restriction enzyme *Hpa*I. The *Hpa*I digested genomic DNA was electrophoresed on a 1% agarose gel, transferred to a nylon filter and probed with the *ahpC* gene (amplified by PCR from the *S. typhimurium* chromosome using primers N5138 and N5139; see Table 2.1). Of the 25 mutants of interest, only one, *ahp70*, carried the correct mutation, producing a single band after digestion which was slightly larger than that of the parental strain, SL1344 (Figure 5.4a). The remaining mutants produced two bands, indicating that a single recombination event had occurred and were therefore not investigated further. Mutant *ahp44* is shown as an example of this (Figure 5.4a).

As further evidence that the nature of the inactivation of the *ahpCF* locus in the double recombinant resulted from insertion of the chloramphenicol cassette, PCR and *Pvu*II restriction digests were performed. The chloramphenicol cassette introduces a unique *Pvu*II site into the *ahp* locus (Figure 5.3 (c)), therefore if a suitable region is amplified from the wild type and mutant loci, the former should not be cut by *Pvu*II and the latter should form two bands. Using primers G7858 and G7859 (Table 2.1) the *ahp* locus from SL1344 and *ahp70* was obtained by PCR and the fragments were subjected to *Pvu*II digestion. The amplified *ahp* locus (2.47 kb) was slightly larger than the wild type homologue (2.36 kb), prior to *Pvu*II restriction, due to the presence of the chloramphenicol cassette (Figure 5.4b). As predicted, only the inactivated locus formed two bands (0.86 kb and 1.61 kb), confirming that the *ahp* disruption resulted from the insertion of the chloramphenicol cassette (Figure 5.4b). Mutant *ahp70* was subsequently re-designated MPG473.

Testing MPG473 for attenuation using a mouse model

A culture of MPG473 was grown overnight in LB supplemented with chloramphenicol. The cells were then pelleted and washed twice in PBS before being serially diluted to concentrations of approximately 10^1-10^6 organisms per ml. Six groups of six female BALB/c mice (8-10 weeks old) were then each injected i.p. with one of the dilutions of bacteria and the number of bacteria administered in the inoculum was determined by plating equivalent samples on LB agar. The mice were

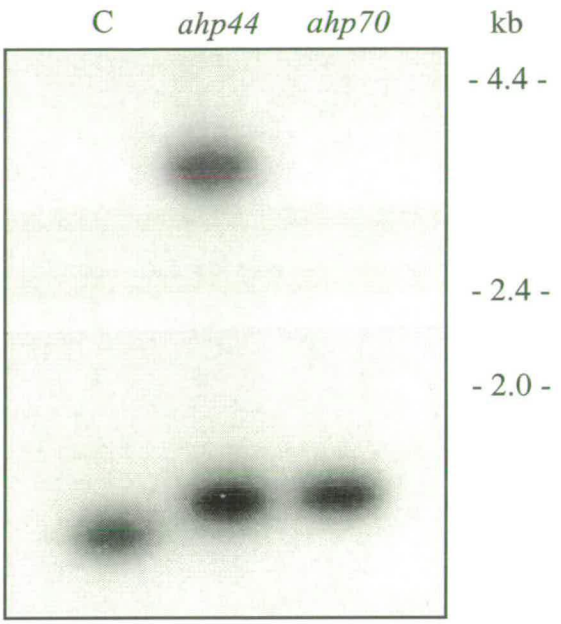


Figure 5.4 (a)

Analysis of recombination events in the *ahpCF* locus by Southern blotting

Genomic DNA was isolated from the virulent *S. typhimurium* strain, SL1344, and the transformants of interest, *ahp44* and *ahp70*. Equal amounts of DNA from these cells were subject to restriction digest with *Hpa*I and electrophoresed on a 1% agarose gel overnight at 25 mV. DNA was then transferred to a nylon membrane and probed with the *ahpC* gene, obtained by PCR from the *S. typhimurium* SL1344 chromosome using primers N5138 and N5139 (see Table 2.1). The positions of *Hin*dIII digested λ DNA molecular size markers (kb) are indicated at the right of the blot.

Tracks are as follows:

C - Control sample containing DNA from the virulent *S. typhimurium* strain, SL1344

ahp44 - DNA from a mutant which has undergone a single recombination event in the *ahpC* gene

ahp70 - DNA from a mutant which has undergone a double recombination event

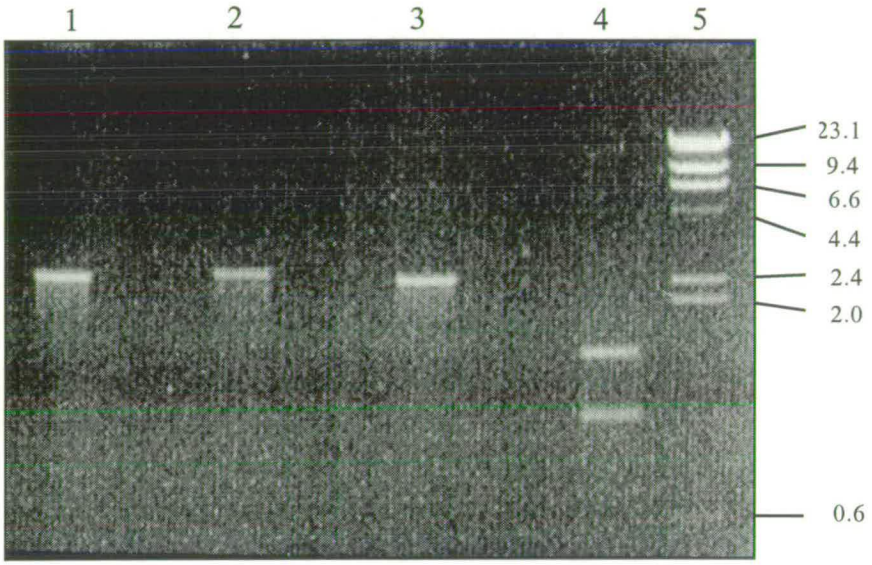


Figure 5.4 (b)

Use of PCR to demonstrate the presence of a *cml* cassette within the *ahpCF* locus

Primers to the 5'- and 3'-end of the *ahp* locus, G7858 and G7859, respectively (see Table 2.1), were used to amplify the *ahp* locus from *S. typhimurium* SL1344 (Lane 1) and *ahp70* (Lane 2). The chloramphenicol cassette introduces a unique *Pvu*II site into the *ahp* locus. After *Pvu*II digestion of the PCR products from the SL1344 and *ahp70* DNA, no change in size was observed for the band from SL1344 (Lane 3). In contrast, the band from *ahp70* formed two fragments of approximately 0.86 kb and 1.61 kb (Lane 4) thus confirming the presence of the chloramphenicol cassette in the *ahp* locus. *Hin*dIII digested phage λ DNA molecular size markers (kb) were included as a guide to size (Lane 5).

then followed for survival over a period of 28 days, and the results of this investigation are shown in Table 5.1 (see page 211). Unfortunately, the level of survival of mice injected with MPG473 was below that required for the calculation of an LD_{50} (Reed and Muench, 1938), therefore it is assumed that this mutant is as virulent as SL1344 (LD_{50} approximately 10^1 bacteria). These results suggest that the *ahpCF* locus has little effect on the gross virulence of *S. typhimurium*, as indicated by the LD_{50} procedure. This suggests that any attenuation present in MPG203 is derived from some (uncharacterised) property of the Mu*dlux* element.

5.2.2 The role of the *oxyR* gene in the virulence of *S. typhimurium*

Cloning of the *S. typhimurium oxyR* gene

Although it appeared that the *ahpCF* locus of *S. typhimurium* plays little or no discernible role in virulence, it seemed likely that the other genes of the OxyR regulon would be involved. Francis and Gallagher (1993) had shown *ahp* was induced during macrophage interaction and therefore, because it constitutes part of the OxyR regulon, and because it is not known to be regulated by any other transcriptional regulators, it would be expected that the other genes of the regulon would also be expressed in a similar manner. This is supported by the demonstration that the OxyR-regulated gene *dps*, is also induced within the macrophage (Valdiva *et al.*, 1996). Moreover, Fields *et al.* (1986) identified a number of oxidative stress mutants which were unable to survive in the macrophage and which were attenuated in mice. It was therefore decided to determine the role of the *oxyR* gene *per se* in virulence by disrupting the gene and testing the resulting mutant for attenuation. This approach would provide an insight into the overall importance of the OxyR regulon in virulence.

The sequence of the *oxyR* gene is known for *E. coli* but not for *S. typhimurium* (Christman *et al.*, 1989). In chapter 3 of this thesis, a description is provided of how the *S. typhimurium oxyR* gene was obtained from *S. typhimurium* strain TA4100 by PCR and cloned into a controllable expression vector using primers based on the

E. coli oxyR sequence, OXYR1 and OXYR3 (Table 2.1). These primers incorporated flanking *Eco*RI sites and were designed such that the resulting *oxyR* gene would be promoterless. The strategy for disruption of the *oxyR* gene was essentially the same as that used for *ahpCF*, in which the gene was cloned and then disrupted by insertion of an antibiotic-resistance cassette. Using this approach, the disrupted gene could then be excised from the plasmid, on a fragment devoid of an origin of replication, recircularised and transformed into SL1344. Those cells that had replaced the wild type *oxyR* gene with the disrupted copy could then be screened for by examining the antibiotic resistance phenotype.

Using OXYR1 and OXYR3 primers, the *oxyR* gene (approximately 1.1 kb) was amplified from the *S. typhimurium* SL1344 genome and cloned into pGEM-T (Promega), a vector specifically designed to directly clone PCR products, to form the plasmid pPDT7 (Figure 5.5). In order to disrupt the *oxyR* gene it was necessary to identify a unique restriction site for inserting an antibiotic-resistance cassette. Partial sequencing was therefore carried out on the cloned DNA in pPDT7 using a primer to the T7 RNA polymerase promoter sequence (P_{T7}) that flanks one side of the multiple cloning site of the plasmid (Figure 5.5). Using this primer, 467 bp of sequence was obtained (Figure 5.6). The original primers to *oxyR* were devised to the *E. coli* sequence, therefore the *S. typhimurium* gene is assumed to be flanked either end with approximately 20 bp of the *E. coli oxyR* sequence. By comparison, the sequence obtained was found to represent the 3'-end of the *S. typhimurium oxyR* gene and corresponded to bases 771-1238 of the published *E. coli* gene sequence (Accession number, J04553; Christman *et al.*, 1989). Excluding the 20 bp of flanking *E. coli* sequence, the *S. typhimurium* DNA showed 82.3% sequence identity to the *E. coli oxyR* gene over this region.

Using the sequence, one possible unique restriction site (not already present in the plasmid) was identified, *Sma*I, which was present in the 3'-end of *oxyR* (Figure 5.6). Since only partial sequencing of the gene had been performed, restriction analysis of pPDT7 was required to determine whether the *Sma*I site was entirely unique.

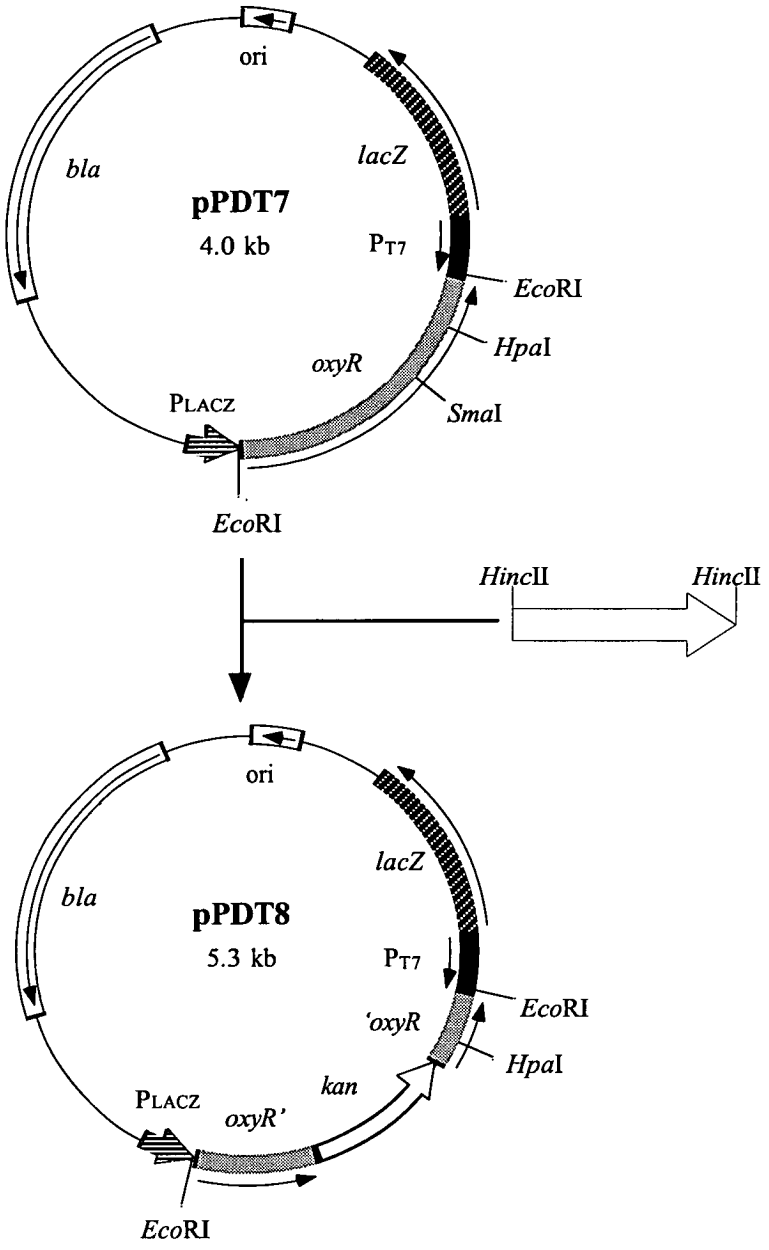


Figure 5.5

A summary of the cloning and insertional inactivation of the *S. typhimurium oxyR* gene

The *oxyR* gene was obtained by PCR from the genome of *S. typhimurium* SL1344 using primers corresponding to the *E. coli oxyR* sequence, OXYR1 and OXYR3 (see table 2.1). The 1.07 kb PCR product was then cloned directly into pGEM-T (Promega), a vector designed to clone PCR generated DNA, to form pPDT7 (see methods section). A unique *Sma*I site was identified in the gene and *oxyR* was subsequently inactivated by the insertion of a 1.3 kb *Hinc*II fragment, containing the kanamycin cassette from pUC4-K (Promega), to generate vector pPDT8. Key: ori - origin of replication; *bla* - β-lactamase gene; *lacZ* - gene encoding β-galactosidase; P_{LACZ} - the promoter of the *lac* operon of *E. coli*; P_{T7} - Phage T7 RNA polymerase promoter; *kan* - kanamycin-resistance cassette from pUC4-K; *oxyR* - gene encoding the *S. typhimurium* OxyR regulatory protein.

Figure 5.6 Partial sequence of the *S. typhimurium oxyR* gene

The partial nucleic acid sequence of the *S. typhimurium oxyR* gene was obtained by sequencing of the DNA inserted in pPDT7, using the T7 primer. The DNA is flanked at one end with *E. coli oxyR* sequence (lower case), from use of the primer OXYR3 (see Table 2.1). Excluding this region, the 467 bp represent the 3'-end of the oxyR gene and the sequence shows 82.3% identity to the published *E. coli* sequence (Christman *et al.*, 1989; Accession number J04553). The amino acids encoded in the open reading frame are specified by the standard one letter abbreviation and are shown beneath the nucleotide sequence. The termination codon is represented by an asterisk. Also shown are three underlined restriction sites; *Eco*RI -used to excise the disrupted copy of *oxyR* from pPDT8; *Sma*I - used to insert the *Hinc*II fragment of pUC4-K containing the kanamycin cassette; *Hpa*I - used as a diagnostic cutting site in Southern blot analysis of cells obtained after recombination with the disrupted OxyR gene.

Digesting pPDT7 with *Sma*I produced a single band after agarose gel electrophoresis, indicating that this site was indeed unique to the plasmid-borne *S. typhimurium oxyR* sequence. Therefore, the *Sma*I site was chosen for use in the disruption. The kanamycin-resistance cassette of pUC4-K (Promega), which contains its own promoter, was therefore excised as a 1.3 kb *Hinc*II fragment and cloned into the *Sma*I site present in *oxyR*, to form pPDT8 (Figure 5.5). Transformants were selected on LB kanamycin plates and the plasmid DNA of resulting colonies was verified by restriction analysis. In order to enhance the efficiency of transformation and the probability of disrupting the chromosomal *oxyR* gene, pPDT8 was then transferred to *S. typhimurium* SL1344, to ensure correct methylation of the DNA.

Transfer of the insertionally inactivated *oxyR* gene onto the *S. typhimurium* chromosome

The disrupted *oxyR* gene was excised from pPDT8 as a 2.37 kb *Eco*RI fragment, recircularised by treatment with DNA ligase, and electroporated into SL1344. Approximately 2, 000 transformants were obtained from this procedure. In previous work it had been noticed that *S. typhimurium* strains deficient in *oxyR* showed a small colony phenotype. Therefore, patching, to eliminate colonies carrying the original plasmid, was biased to selecting colonies that showed this phenotype. 400 colonies were replica patched onto LB plates containing either kanamycin (the antibiotic marker in the disrupted *oxyR* gene) or ampicillin (the antibiotic marker in the plasmid) and incubated overnight at 37°C. 386 of the colonies exhibited a Kan^R Amp^S phenotype indicating that recombination onto the chromosome had been extremely efficient. As in the disruption of *ahpCF*, there were three possibilities for recombination, a double cross-over event, or a single upstream or downstream recombination event. The type of mutant produced was determined, as previously described for *ahp*, using disc inhibition tests. OxyR regulates a number of genes including *ahpCF*. Thus, it was decided to use cumene hydroperoxide for the inhibition test as it produces a very clear zone of inhibition and SL1344 and TA4108 (an *oxyR* deleted strain of *S. typhimurium* LT2) were employed as controls. From 50 colonies that were screened for sensitivity to cumene hydroperoxide, 48 showed a

zone of inhibition equivalent to SL1344 (approximately 24 mm) and only 2 colonies showed a large zone of inhibition equivalent to TA4108 (approximately 31 mm).

Analysis of recombination events in the *oxyR* gene by Southern blotting

The genomic DNA from the two cumene hypersensitive *S. typhimurium* strains was examined by Southern blotting. The unique *Hpa*I restriction site at the 3'-end of the *oxyR* gene (Figure 5.6) provided a means, similar to that of the diagnostic restriction analysis of the *ahp* mutants, to distinguish those cells that contained two copies of the *oxyR* gene (from a single recombination event) or a single copy (as in the wild type or as a result of a double recombination event). For the virulent parental strain (SL1344) a single band would be obtained, the size of which is unknown because the position of the nearest upstream *Hpa*I site is uncharacterised. A double recombinant would also produce a single band but, would be 1.3 kb larger than that of the wild type due to the kanamycin-resistance cassette. If a single recombination event had occurred, then two bands would be seen, one the same size as in the double recombinant and the other of 1.1 kb (because two copies of the *oxyR* gene are present producing two *Hpa*I sites).

*Hpa*I was used to cut the genomic DNA of the mutants, along with DNA from SL1344. DNA digests were then electrophoresed on an 0.8% agarose gel, transferred to a nylon filter and subsequently probed with the *S. typhimurium oxyR* gene, amplified from the chromosome as previously described. When Southern blot analysis was performed, both the isolates that were cumene sensitive, *oxyR12* and *oxyR47*, produced a single band (approximately 6.0 kb) which was larger than the band for SL1344 (approximately 4.7 kb) indicating that the *oxyR* locus was disrupted (Figure 5.7 Panel A). For proof that the *oxyR* gene was disrupted by virtue of the kanamycin cassette, a parallel sample of the same *Hpa*I digested DNA was blotted and probed with the kanamycin cassette, obtained from pUC4-K as previously described. No signal was obtained from the DNA sample from SL1344 (Figure 5.7 Panel B). In contrast, the DNA from the two mutants showed a single band of the same size and alignment as the band produced after probing with *oxyR* (Figure 5.7

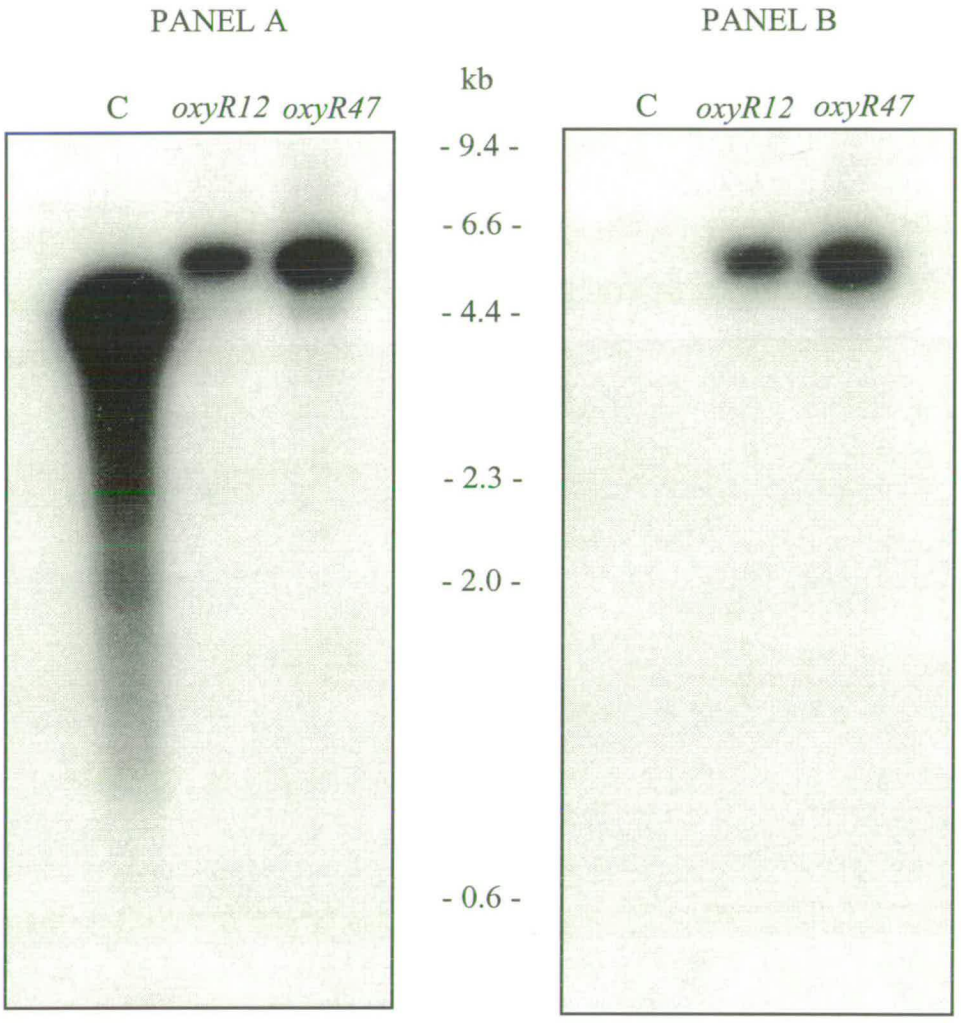


Figure 5.7

Southern blot analysis of mutants from insertional inactivation of the *oxyR* gene by recombination

Genomic DNA was prepared from the parental strain, *S. typhimurium* SL1344, and from two cumene hypersusceptible mutants, *oxyR12* and *oxyR47*. Equal amounts of DNA were treated with the restriction enzyme *Hpa*I and the resulting digests were electrophoresed on a 0.8 % agarose gel overnight at 25 mV. The DNA was then transferred to a nylon membrane and probed with the *oxyR* gene from *S. typhimurium* (Figure 5.7 Panel A) or the kanamycin gene from pUC4-K (Promega) (Figure 5.7 Panel B). For the purpose of probing, the *oxyR* gene was obtained by PCR from the *S. typhimurium* SL1344 chromosome using primers OXYR1 and OXYR3 (see Table 2.1) and the kanamycin gene was prepared from the *Hinc*II digestion of pUC4-K. The positions of *Hin*dIII digested phage λ DNA molecular size markers (kb) are indicated in the middle of the page as a guide to size.

Lanes are as follows:

C - DNA from *S. typhimurium* SL1344

oxyR12 and *oxyR47* - DNA from mutants displaying a cumene hypersensitive phenotype

Panel B). One of these mutants was then used for further study and designated MPG484.

Testing MPG484 for attenuation using a mouse model

MPG484 was cultured overnight in LB supplemented with kanamycin. The cells were then prepared for injection into mice by serial dilution in PBS to approximately 10^1-10^6 cells per ml. Six groups of six female BALB/c mice (8-10 weeks) were then injected i.p. with 100 μl of the appropriate dilution of bacteria and the number of bacteria administered during injection was determined by plating equivalent samples on selective media. The mice were then observed for survival over 28 days, and the results of this investigation are displayed in Table 5.1. From the data it can be seen that the *oxyR* mutant is as virulent as *S. typhimurium* strain SL1344, with an LD_{50} of 1 ($\log_{10}$ cell number). Thus, these results indicate that *oxyR* does not play a substantial role in the virulence of *S. typhimurium*.

Table 5.1

The effect of disrupting the *ahp* or *oxyR* loci upon the virulence of *S. typhimurium*

Number of bacterial cells injected (log_{10})	Number of Mice Surviving at 28 days post-injection			
	SL1344	MPG203	MPG473	MPG484
0-1	3/6	6/6	2/6	2/6
1-2	1/6	6/6	0/6	1/6
2-3	0/6	5/6	0/6	1/6
3-4	0/6	5/6	0/6	0/6
4-5	0/6	3/6	0/6	0/6
5-6	0/6	0/6	0/6	0/6
LD_{50} (log_{10} bacterial cell number)	1.0	4.8	ND*	1.0

Each group was comprised of 6 female BALB/c mice (8-10 weeks of age). The strains to be tested were grown overnight in LB, with selection if required. Bacterial cells were then pelleted and washed three times in sterile PBS before being diluted to between 10^1-10^6 bacteria per ml. Mice in each group were injected intraperitoneally with 100 µl of one of the dilution series. The survival of the mice was then monitored for a period of 28 days post-injection and the LD_{50} calculated by the method of Reed and Muench (1938). Key: SL1344 - mouse-virulent strain of *S. typhimurium*; MPG203 - *ahp*::Mu*dlux*; MPG473 - *ahpCF*::*cml*; MPG484 - *oxyR*::*kan*; ND*- Survival of mice was below that necessary to calculate the LD_{50}.

5.3 DISCUSSION

The investigations performed in this chapter indicate that *S. typhimurium* does not require the activities of AhpCF or OxyR for full virulence in mice. The ability of *S. enterica* serovars to infect and cause invasive disease in human and animal hosts has been attributed to intracellular survival in phagocytic cells, particularly those of the mononuclear phagocytic line (Collins, 1974; Hormaeche, 1979; Buchmeier and Heffron, 1989). Macrophages play an integral role in the prevention of bacterial infection through their phagocytic capabilities and anti-microbial mechanisms. Macrophages are capable of producing a respiratory burst to generate reactive oxygen species such as superoxide and hydrogen peroxide which are capable of damaging the bacterial cell at all fundamental levels (Hassett and Cohen, 1989). Macrophages also induce the production of large quantities of nitric oxide (NO), which plays a major role in the antibacterial activity of these cells (Vidal *et al.*, 1993; Pacelli *et al.*, 1995).

S. typhimurium and *E. coli* have been shown to respond to oxidative stress by the induction of multigenic responses that can prevent and repair damage incurred by reactive oxygen radicals (Christman *et al.*, 1985; Greenberg and Demple, 1989; Walkup and Kogoma, 1989). A number of these genes are under the control of the specific multigenic regulators, OxyR and SoxRS, which are activated under peroxide and superoxide stress respectively. These two different regulators have also recently been demonstrated to respond to the production of nitric oxide (Nunoshiba *et al.*, 1993; Hausladen *et al.*, 1996).

Loss of the specific regulator for the OxyR regulon results in the hypersusceptibility of *S. typhimurium* to many types of peroxides (Christman *et al.*, 1985). OxyR regulates a number of important antioxidant defence genes such as *katG* (HPI catalase), *ahpCF* (alkyl hydroperoxide reductase), and *dps* (DNA binding protein), and loss of any one of these genes also conveys sensitivity to peroxides (Christman *et al*, 1985; Francis, 1993; Ivanova *et al.*, 1994, Altuvia *et al.*, 1994). It would be expected therefore, that OxyR or the genes of the regulon it controls, which protect

against oxidative stress, would be essential for the intracellular survival of *S. enterica* in the macrophage. This is supported by the identification of transposon mutants which were unable to survive within the macrophage and were found to be sensitive to a range of peroxides (Fields *et al.*, 1986). In addition, Francis and Gallagher (1993) and Valdiva *et al.* (1996) have demonstrated that the OxyR regulon genes, *ahpC* and *dps*, respectively, are induced by *S. typhimurium* during interaction with macrophage cell lines, demonstrating that *S. enterica* are likely to encounter such intracellular oxidative stress. This would suggest that the OxyR regulon, or some of the genes that it regulates, may have important roles for the virulence of *S. enterica* upon host infection.

The insertion of Mu*dlux* into the *ahp* locus effectively produced a strain which was deficient in Ahp activity, as MPG203 was sensitive to cumene hydroperoxide (Francis, 1993). When MPG203 was injected into mice by the intraperitoneal route, the calculated LD_{50} was 4.8 (log_{10} cell number), compared to SL1344 which had an LD_{50} of 1 (log_{10} cell number) indicating that MPG203 was attenuated. This difference in LD_{50} of approximately 4 logs was attributed to the loss of Ahp and indicated that the ability to prevent lipid peroxidation, induced by oxidative stress, was an important requirement for survival in the host and therefore for virulence. However, an *S. typhimurium* strain in which the *ahpCF* locus was inactivated by insertion of a chloramphenicol-resistance cassette (MPG473), was found to be as virulent as SL1344 (Table 5.1). This indicated that the attenuation of MPG203 was unlikely to have occurred as a result of loss of Ahp function, but was mediated in some manner by the Mu*dlux* element.

The Mu*dlux* element not only carries the ability to produce luciferase, the enzyme responsible for the bioluminescent reaction, but also carries the *luxC, luxD* and *luxE* genes which produce the aldehyde substrate for the luciferase reaction (reviewed in Meighen, 1991). The synthesis of the aldehyde substrate and the bioluminescence reaction requires FMNH and NAD(P)H. It could be that induction of the *ahp* operon, as has been shown upon macrophage interaction (Francis and Gallagher, 1993), may result in some form of metabolic burden from the channelling of cellular pools of metabolites towards the synthesis of the *lux* proteins or from overproduction of

intracellular aldehyde or alternatively, from depletion of NAD(P)H and FMNH in the luciferase reaction. In the latter instance, the depletion of NAD(P)H is of special importance in oxidative stress as the ability to maintain a reduced cellular environment has been linked to the level of this compound and the control of the superoxide stress response (Liochev and Fridovich, 1992). This suggests that loss of such compounds increases the susceptibility of cells to oxidative stress. Alternatively, the production of the aldehyde substrate may reach sufficiently high levels to be toxic to the bacterial cell. Whether the anomalous osmolarity-dependent expression of the Mu*dlux* element, described in chapter 4, results in the attenuation of MPG203 is uncertain, but cannot be ruled out as a contributory factor.

Since the role of AhpCF appeared to be unessential for the full virulence of *S. typhimurium* in mice, the role of the transcriptional regulator OxyR in virulence was addressed. The ability of the OxyR regulon to produce resistance against oxidative stress has been demonstrated in lab culture, as *oxyR* mutants are susceptible to the addition of various peroxides (Christman *et al.*, 1985). This suggested that although *ahpCF* may not be essential, some of the other functions regulated as part of the OxyR regulon may have an important role in virulence. However, examination of an *S. typhimurium* strain with an insertionally inactivated *oxyR* gene (MPG484) revealed no significant attenuation compared to the virulent strain (Table 5.1). This suggests that the ability of OxyR alone to induce resistance to oxidative stress is unlikely to provide a significant level of protection to the respiratory burst of phagocytic cells. This is supported by the finding that *S. typhimurium* cells deficient in OxyR were no more sensitive to killing by tissue cultured human neutrophils than wild type cells (Papp-Szabo *et al.*, 1994). In addition, *Mycobacterium tuberculosis*, an intracellular pathogen of macrophages, is a natural mutant with a disrupted *oxyR* locus, suggesting that this bacterium also does not require the activities of the OxyR-induced oxidative stress defence system for survival within phagocytic cells (Deretic *et al.*, 1995; Dhandayuthapani *et al.*, 1996). Nevertheless, in contrast to the findings in this chapter, a previous report demonstrated that an *oxyR* mutant of the *S. typhimurium* strain LT2 was significantly attenuated in mice compared with the $oxyR^+$ parental strain (unpublished data cited in Fields *et al.*, 1986). However,

subsequently the parental strain, LT2, has been shown to be only weakly virulent (Lee *et al.*, 1995). This suggests that *oxyR* may play some role in virulence, but not a vital one.

These studies cannot rule out the possibility that OxyR-regulated genes other than *ahpCF* have a role to play in the virulence of *S. typhimurium* as at least four of these genes, *katG*, *dps*, *gorA* and *stiA*, have also been shown to be controlled independently of this regulator (Ivanova *et al.*, 1994; Altuvia *et al.*, 1994; Becker-Hapak and Eisenstark, 1995; Seymour *et al.*, 1996). In these cases, regulation occurs via the stationary phase sigma factor RpoS and these genes help provide resistance to oxidative stress in stationary phase cells. Whether any further regulatory factors are involved in the OxyR-independent induction of the OxyR regulon genes remains to be elucidated. Interestingly, *rpoS* null mutants of *S. typhimurium* are attenuated, and display sensitivity to oxidative stress (Fang *et al.*, 1992; Coynault *et al.*, 1996). In addition, the *rpoS* gene has been shown to be induced to a high level within 2 hours of *S. typhimurium* entering the macrophage (Chen *et al.*, 1996a), suggesting that if survival in macrophages is important to the virulence of *S. enterica*, then resistance to the anti-microbial environment is likely to be mediated by this stationary phase sigma factor to a significant degree. This observation might also explain why the loss of *ahp* or *oxyR* have little effect upon the virulence of *S. typhimurium* since the expression of *ahp* is RpoS-independent and *ahp* is not expressed in the stationary phase of growth. Moreover, it appears that OxyR normally only exerts its regulatory effects in actively growing (exponential phase) cells (Ivanova *et al.*, 1994; Altuvia *et al.*, 1994; Chapter 3 of this thesis).

Whilst it is clear from the present study that the *ahpCF* and *oxyR* genes are not required for full virulence, only a small amount of information exists about the role or possible importance of the other genes in the OxyR regulon, for virulence. Catalase (*katG*) could be considered one of the most important enzymes in protection against hydrogen peroxide as it can directly eliminate this substance. In fact, in early studies, it was demonstrated that one of the major effects of hydrogen peroxide upon the bacterial cell was the elimination of the chemiosmotic potential and the ability to transport compounds such as sugars or amino acids across the cell membrane, and

this phenomenon was particularly severe in cells deleted for *oxyR* (Farr *et al.*, 1988). However, when exogenous catalase was supplied, under the test conditions, the inhibition of cellular uptake of substances was rapidly alleviated, leading the investigators to postulate that catalase was the most important function in the OxyR regulon. Despite this, the lack of a role for *katG* in virulence was recently demonstrated by Buchmeier and colleagues (1995). Moreover, these studies also suggested that the alternative, growth phase regulated catalase (*katE*) also had no role in virulence. This is important as it suggests that irrespective of OxyR or RpoS regulation neither catalases play a significant role in virulence. This would suggest that there must be more important antioxidant defence systems than the ability to simply breakdown hydrogen peroxide. Surprisingly, a *katGkatE* double mutant was found to be extremely susceptible to hydrogen peroxide when added in culture yet displayed no increased sensitivity to killing by macrophages when compared with a wild type strain over both short (2 hours) or long (24 hours) periods of exposure (Buchmeier *et al.*, 1995). This suggests that the ability to survive the oxidative burst of phagocytes does not require catalase. Alternatively, it could be envisaged that even with an antioxidant defence system, the level of oxidative stress might be far in excess of any defence that might be induced. These results would indicate that at least four genes, *ahpCF*, *oxyR*, *katG* and *katE*, involved in responses to oxidative stress, have no visible role in the virulence of *S. enterica.*

Other studies in different bacteria have shown similarities and differences to these results. A correlation between the catalase activity and virulence has been noted for a number of pathogenic bacteria. For example, when fifteen *Staphylococcus aureus* isolates were examined, the most virulent bacteria displayed the highest levels of catalase and the least virulent isolates could be increased in pathogenicity by the addition of exogenous catalase (Mandell, 1975). However, a number of studies have shown that the susceptibility of bacteria to peroxide challenge in lab cultures have not correlated with virulence. An examination of the peroxide susceptibility of six virulent clinical isolates of *Neisseria gonnorhaea* demonstrated survival ranging from 80-0.001% after exposure to 30 mM hydrogen peroxide for 30 minutes even though all six isolates contained the same level of catalase activity (Alcorn *et al.*,

1994). In addition, at least two other organisms appear not to require catalase for full virulence *Shigella* (Franzon *et al.*, 1990) and *Haemophilus influenza* (Bishai *et al.*, 1994). Interestingly, these catalase deficient mutants, like *S. typhimurium*, exhibited susceptibility to peroxide in lab culture but were not affected in virulence, further demonstrating that catalase activity is not a good indicator of virulence.

It is known that *S. typhimurium* and *E. coli* can respond to different forms of oxidative stress (Christman *et al.*, 1985; Greenberg and Demple, 1989; Walkup and Kogoma, 1989) and it may be that OxyR regulates less important protective responses. The SoxRS regulon regulates genes which are activated in response to superoxide (Farr and Kogoma, 1991), which is the first product of the respiratory burst (Adams and Hamilton, 1984). However, the majority of studies investigating this regulon have been performed in *E. coli* therefore the role of SoxRS in *S. enterica* is unclear. Interestingly, an *E. coli soxS::lacZ* fusion was induced within the macrophage environment suggesting that the SoxRS regulon is likely to be induced in the host (Nunoshiba *et al.*, 1993). In addition, an *E. coli* mutant deleted for the *soxRS* locus displayed increased killing by macrophages indicating that the SoxRS regulon may play a role in virulence, however this difference was not seen until approximately 4 hours after *E. coli* had been engulfed by these phagocytic cells (Nunoshiba *et al.*, 1993).

SoxRS regulates a number of functions including a superoxide dismutase (*sodA*), which breaks down superoxide (Demple, 1991). Investigations into the role of *sodA* in *S. typhimurium* have shown that *sodA* mutants exhibit enhanced susceptibility to macrophages and this increased susceptibility can be alleviated by the expression of *sodA* from a plasmid (Tsolis *et al.*, 1995). Nevertheless, *sodA* mutants are only slightly attenuated in BALB/c mice, approximately threefold, indicating that the role of *sodA* in virulence is likely to be negligible. In addition, the removal of the *E. coli sodB* gene, the normal cellular SOD, made no difference to the killing of these cells by human polymorphonuclear leukocytes (Papp-Szabo *et al.*, 1993), further suggesting that SOD activities are relatively unimportant. However, it could be suggested that without testing a SOD double mutant, the exact role in virulence cannot be determined as one SOD activity may compensate for the other.

Interestingly, the SOD activity of *Shigella* has been shown to be important in the ability of this organism to display characteristics of full virulence in intestinal loop models (Franzon *et al.*, 1990). This suggests that like catalases, SOD function in virulence may be dependent upon the particular organism. Whether the other genes of the SoxRS regulon are important for the virulence of *S. enterica* is unknown.

One role of defence against oxidative stress appears to be protection and repair of DNA. DNA is susceptible to single and double stranded breaks and to alteration or loss of bases under both peroxide and superoxide stress (reviewed in Farr and Kogoma, 1991). Interestingly, one of the main observations made about *S. typhimurium oxyR* mutants grown aerobically, is that they show a 10-55-fold higher frequency of spontaneous mutation, especially from T°A→A°T transversions, a base substitution event commonly found after treating wild type cells with chemical oxidants (Storz *et al.*, 1987). This mutation frequency can be alleviated by multicopy expression of *katG* or *ahpCF* genes, and slightly by *sodA*. Similarly, Greenberg and Demple (1988) demonstrated that mutants which overproduced either *katG*, *katE* or *ahpCF* reduced the level of spontaneous mutagenesis in an *oxyR* mutant. Moreover, one of the OxyR regulated gene, *dps*, has been demonstrated to form complexes with DNA (Almiron *et al.*, 1992), although whether this protects DNA directly from the action of hydrogen peroxide is unknown. These results might suggest that the OxyR regulon has an important role to play in preventing damage to, and maintaining the fidelity of, the bacterial the genome, even though it does not provide a key role in virulence.

A number of other enzymes have been shown to be induced by hydrogen peroxide stress independently from the OxyR regulon and include *nfo* (endonuclease IV), *xthA* (exonuclease III), *polA* (DNA polymerase I) and *recA*, *recB* and *recC* (recombination and DNA repair enzymes) (Farr and Kogoma, 1991). Interestingly, *recA* or *recBC* mutants, are very sensitive to hydrogen peroxide even though they possess normal levels of catalase activity. These mutants also survive poorly in the macrophage environment and are attenuated in mice (Buchmeier *et al.*, 1993). Moreover, the sensitivity of the *recA* mutant was directly correlated with the ability of the macrophage to produce a respiratory burst as both the wild type strain and *recA*

mutant survived equally well in a macrophage cell line devoid of the ability to generate superoxide (Buchmeier *et al.*,1993). Bacterial killing by hydrogen peroxide has been shown to be bi-modal, with mode one being characterised by death at low cell density and with low concentrations of the oxidant (1-3 mM) via damage to the DNA (Imlay and Linn, 1986). At higher concentrations (>10 mM), mode two killing occurs where death is actually independent of the peroxide concentration but dependent upon the time of exposure. In this case, death is attributed to a range of factors including damage to DNA, RNA and a number of undefined cellular targets (Imlay and Linn, 1986). Interestingly, Buchmeier *et al.* (1993, 1995) have suggested that, because of the sensitivity of *recA* or *recBC* mutants to peroxide *in vitro* and *in vivo*, the *in vivo* situation whereby *S. typhimurium* is killed by hydrogen peroxide in macrophages can be likened to that of mode one. In fact, Buchmeier *et al.* (1993) have indicated that the DNA repair process in virulence is more important to survival than the ability to directly inactivate hydrogen peroxide.

It could be imagined that damaged proteins are relatively simple to repair as such proteins can be degraded and resynthesised whereas damage to the DNA is a much greater threat, as irreversible or lethal mutations may arise (Storz *et al.*, 1987; Greenberg and Demple, 1988). Interestingly, the OxyR regulon genes appear to be maximally induced by hydrogen peroxide at 0.1-2 mM (Altuvia *et al.*, 1994; Francis, 1993) which is in the range expected to cause mode one killing by DNA damage, and to occur upon interaction with macrophages. Thus, although the OxyR regulon appears not to have a critical role in virulence it could be acting to prevent damage to DNA during the infection cycle, thereby helping to maintain the fidelity of the genome of those bacteria that survive the respiratory burst. The reason why the *oxyR, ahpCF* and *katG* loci may have little effect upon virulence may then simply reflect on the fact that, unlike *recA*, these genes have a supportive but not an essential role in DNA repair.

This discussion has focused on the possible roles of oxidative defence in the virulence of *S. enterica*. However some research has suggested that *S. enterica* serovars, including *S. typhimurium* and *S. typhi,* probably do not require such defence systems for survival in phagocytic cells because they somehow avoid triggering the

oxidative burst upon entry (Miller *et al.*, 1972a; Kossack *et al*, 1981). A number of other studies have also indicated that *S. enterica* serovars may not actually enter macrophages by classical phagocytosis. Gahring and co-workers (1990) identified a transposon mutant of *S. typhimurium* strain which was defective for entry into both macrophages or epithelial cells. This strain was subsequently shown to enter macrophages less effectively than the wild type organism even when opsonised. This suggested that *S. enterica* may possess the ability to actively invade macrophages and this theory has been supported by the identification of a specialised invasion-protein-export system in a number of *S. enterica* serovars, including *S. typhimurium* and *S. typhi* (reviewed in Galan, 1996). This machinery is wholly required for entry into epithelial cells, and the same apparatus was recently shown to be required for the killing of macrophages by *S. enterica* (Chen *et al.*, 1996b, Monack *et al.*, 1996). It is likely therefore, that this machinery helps in the entry process into both epithelial and phagocytic cells. If this is the case, then the classical phagocytic pathway would be circumvented with the possibility that the oxidative burst would not be triggered or would be avoided. Even if it were, then there is also evidence to suggest that *S. typhimurium* escapes the normal endocytic route and enters a unique intracellular location (Garcia-del Portillo and Finlay, 1995). It may therefore be possible that entry by such routes would reduce the requirement for antioxidant defence systems and might explain why *oxyR*, *ahpCF* and *katG* appear to have little effect on *S. typhimurium* virulence.

Against this perspective it must be remembered however, that studies on macrophage infection by *S. typhimurium* show that the number of viable bacteria rapidly diminishes by several orders of magnitude following infection (Buchmeier and Heffron, 1989; Abshire and Neidhart, 1993b; Papp-Szabo *et al.*, 1993; Buchmeier and Libby, 1997). Furthermore, phagocytic cells may be activated by interaction with cytokines, or soluble factors such as f-met polypeptides and LPS (Karlsson *et al.*, 1995), therefore out of a population of macrophages it might be expected, especially during infection, that some phagocytes will be in an active state. It should also be noted that studies including those by Fields *et al.* (1986) and Francis and Gallagher

(1993), suggest that *S. enterica* cells are likely to be exposed to some form of oxidative stress upon macrophage interaction.

One pertinent question to ask is whether the use of an LD_{50} test is a suitable test to determine the importance of genes in virulence. This test is usually performed on mutant strains of *S. typhimurium* in innately susceptible mice. However, a virulent strain of *S. typhimurium* usually kills at a low dose (1-10 organisms) and rapidly (within approximately 7 days) when injected intraperitoneally. In this scenario, only those genes that play a critical role in helping *S. typhimurium* survive in the host can be determined and genes with lesser, but not necessarily unimportant, roles will not be identified. This problem might be circumvented by the use of a less virulent *S. typhimurium* strain, for example LT2. The report that an LT2 mutant carrying an *oxyR* mutation was attenuated in virulence supports this view (Fields *et al.*, 1986).

Other researchers have employed more sensitive methods for determining if bacterial genes are important for infection by, for example, examining the kinetics of infection (Dorman *et al.*, 1989; Collins *et al.*, 1991; Kaniga *et al.*, 1996). This can be performed by monitoring the number of organisms present in the mesenteric lymph nodes, Peyer's patches, liver and spleen over a suitable time period in comparison with the fully virulent organism. This type of test has also been used to demonstrate that mutations with no apparent role in virulence, as determined by an LD_{50} test, can actually affect the ability of the bacterium to colonise the host (Kaniga *et al.*, 1996; Tsolis *et al.*, 1996). For example the *stpP* gene of *S. typhimurium* is believed to modulate the protein tyrosine kinase activities of eucaryotic cells, thus perturbing events like cytoskeletal rearrangements or phagocytosis, but *sptP* mutants showed no difference from the virulent strain in LD_{50} tests. In contrast, examination of the colonisation of the spleens of infected animals showed that an *sptP* mutant was less able colonise this site, suggesting it did affect virulence (Kaniga *et al.*, 1996). With hindsight, these types of investigations may well have shown differences for mutants of the *ahp* or *oxyR* loci compared to the virulent strain, and future studies may well clarify this.

The identification of factors that are important for *S. enterica* virulence has helped in our understanding of how *S. enterica* causes disease and aided the development of vaccines. Recent vaccine design has been orientated, in part, to the development of live attenuated bacterial strains, which have been shown to induce longer lasting and more effective protection than dead bacterial cells. Such attenuated mutants have also been shown to be useful as carriers of a diverse range of heterologous antigens, providing protection against the pathogenic organisms from which the antigen is normally derived. However, since vaccines devised from attenuated mutants have still resulted in some symptoms of disease post-vaccination (Ivanoff *et al.*, 1994), recent research has investigated the possible effects of combining lesions to further attenuate vaccine strains to a level where the vaccine is protective but does not produce reactogenicity or even overt disease in vaccinees (Ivanoff *et al.*, 1994).

From the studies outlined in this chapter and other investigations (Buchmeier *et al.*, 1993) it has been shown that some of the genes involved in the peroxide stress response, including the *ahp* locus, *katG* and *oxyR* genes, have little fundamental role in virulence. However, this does not preclude the use of other genes in the OxyR regulon as targets for vaccine development. Moreover, since the *ahp* and *oxyR* loci are non-essential, they may have a role in already attenuated *S. enterica* vaccine strains by providing a site for the insertion of genes encoding heterologous antigens. Such antigens could then be expressed from the promoters of, for example, the *ahp* promoter, during the course of infection. Finally, although the Mu*dlux* element insertion in MPG203 did attenuate this strain in mouse models, this type of mutation would be unsuitable for vaccine design because the Mu phage may be lost, possibly resulting in reversion to wild type levels of virulence.

CHAPTER 6

The purification of the *S. typhimurium* AhpC and GroEL proteins

6.1 INTRODUCTION

Vaccines consisting of live attenuated *S. enterica* strains are known to produce greater immunological protection than those comprised of killed bacterial cells (Collins, 1974; Mastroeni *et al.*, 1996). This phenomenon has been attributed to the induction of an intensive cell-mediated immune response by the live but not by the dead bacteria (Collins, 1974). However, the mechanism by which live cells can induce a stronger CMI than dead cells is poorly understood.

When bacteria are subjected to environmental stress they respond by increasing the level of a variety of proteins which help them to deal with the potential detrimental effects of exposure to the stress condition (Mahan *et al.*, 1996). When *S. typhimurium* enters into the macrophage, the bacterium responds by inducing around 30-40 proteins and repressing around 100 (Buchmeier and Heffron, 1990; Abshire and Neidhart, 1993a). These studies suggest that *S. typhimurium* regulates a large number of proteins in order to deal with the hostile environment of the macrophage.

The elevated synthesis of stress proteins in host phagocytes would increase their availability for antigen recognition by the host immune system. It has also been suggested that such stress proteins may be dominant antigens, compared with the normal complement of cellular proteins present in non-stressed bacteria, for recognition by both the cell-mediated and humoral response arms of the immune system (Kagaya *et al.*, 1992). Therefore, live bacteria may induce greater protection than dead bacteria because they respond to the host environment by producing antigens that stimulate the immune system.

The *ahpCF* genes encode an alkyl hydroperoxide reductase enzyme which prevents oxidative damage to the bacterial cell membrane (Christman *et al.*, 1985; Jacobson *et al.*, 1989). Previous studies have shown that the *ahpCF* locus of *S. typhimurium* is induced when bacteria come into contact with, and are engulfed by, macrophages, as a result of the hydrogen peroxide produced during the respiratory burst (Francis and Gallagher, 1993; Francis, 1993). This suggests that the Ahp enzyme becomes more

abundant during the process of binding and internalisation of the bacteria into the macrophage and may therefore be a target for immunological recognition. To address this phenomenon it was decided to overexpress the *S. typhimurium* AhpC protein and to examine the immunological responses of the murine host to this polypeptide.

Previous studies have utilised an *oxyR1* mutant of *S. typhimurium*, which overexpresses the genes of the OxyR regulon, to enhance isolation of AhpC (Jacobson *et al.*, 1989). However, the purification method was complex and many steps resulted in a poor yield. In order to circumvent this problem, a His-tag procedure was employed which allows a rapid single-step purification of the protein of interest. This procedure works by creating a fusion polypeptide at the genetic level, in which a 23 amino acid leader sequence (approximately 2-3 kDa in size) that includes a stretch of ten adjacent histidine amino acids is attached to the N-terminus of the protein of interest. The purification system takes advantage of the high affinity interaction of the charged polyhistidine sequence with nickel ions (Ni^{2+}) which can be chelated on a resin, thereby forming a chromatography system. Contaminating proteins can be washed from the resin before a high concentration of imizadole (which competes with the His residues for binding to the Ni^{2+}) is used to elute the highly purified fusion polypeptide. The His-tag has also been reported to be non-immunogenic (Qiagen, 1992), a relevant issue if the resulting polypeptide was to be used in immunological studies.

Previous studies have shown that the *S. typhimurium* AhpC protein is antigenic in rabbits (Storz *et al.*, 1989). Using tissue cultured macrophages it was also shown that the *S. typhimurium ahp* locus was induced upon interaction of the bacteria with these phagocytic cells (Francis and Gallagher, 1993; Francis, 1993). However, it remained to be proven that Ahp was expressed *in vivo* and, that it was recognised by the host immune system during infection. As a control, the GroEL protein was examined in parallel, as previous studies had shown that mice develop both cell-mediated and humoral immune responses to this polypeptide during the course of infection with *S. typhimurium* (Brown and Hormaeche, 1989; Gupta *et al.*, 1996). In addition, GroEL molecules from other bacteria have also been shown to be immunodominant

antigens (Kaufmann, 1990). However, there is conflicting evidence as to whether GroEL is induced within *S. typhimurium* upon interaction with macrophages (Buchmeier and Heffron, 1990; Abshire and Neidhart, 1993a). Nevertheless, the GroEL protein is an abundant protein of the bacterial cell. For example, in *E. coli*, GroEL constitutes approximately 1% of the total cell protein in unstressed cells but this can be elevated to approximately 10% under stress conditions (Hemmingsen *et al.*, 1988). Therefore, the GroEL protein was considered a likely candidate for presentation to the immune system during the course of *S. typhimurium* infection.

GroEL is induced as part of the cellular response to heat shock as well as in response to a number of other stresses (reviewed in Gross, 1996), and in *E. coli*, is encoded in an operon, *groESL*, which also encodes the GroES protein. Together, GroEL and GroES act as a molecular chaperone, refolding misfolded proteins generated under stress conditions. In addition, a useful property of GroEL is that it assumes a multimeric configuration consisting of 14 subunits, giving it the approximate size equivalent to a ribosome (Hendrix, 1979; Gross, 1996) and this has been useful for the selective purification of this protein from *E. coli* (Hendrix, 1979).

6.2 RESULTS

6.2.1 Cloning and overexpression of the *S. typhimurium ahpC* gene

Cloning of the *ahpC* gene from *S. typhimurium*

As stated above an AhpC chimera was constructed, which contained a histidine-tag at the N-terminus, to aid purification of the *S. typhimurium* AhpC protein. In order to create this fusion polypeptide, the *ahpC* gene from *S. typhimurium* SL1344 was cloned into pET-19b (Novagen). To facilitate this process, primers were devised to the 5'- and 3'-end of the *ahp* gene, to include nucleotides 156-179 (N5138) and 761-748 (N5139), respectively (as published in Tartaglia *et al.*, 1990). These primers also incorporated *Nde*I or *Bam*HI sites at their 5' ends respectively, for directional

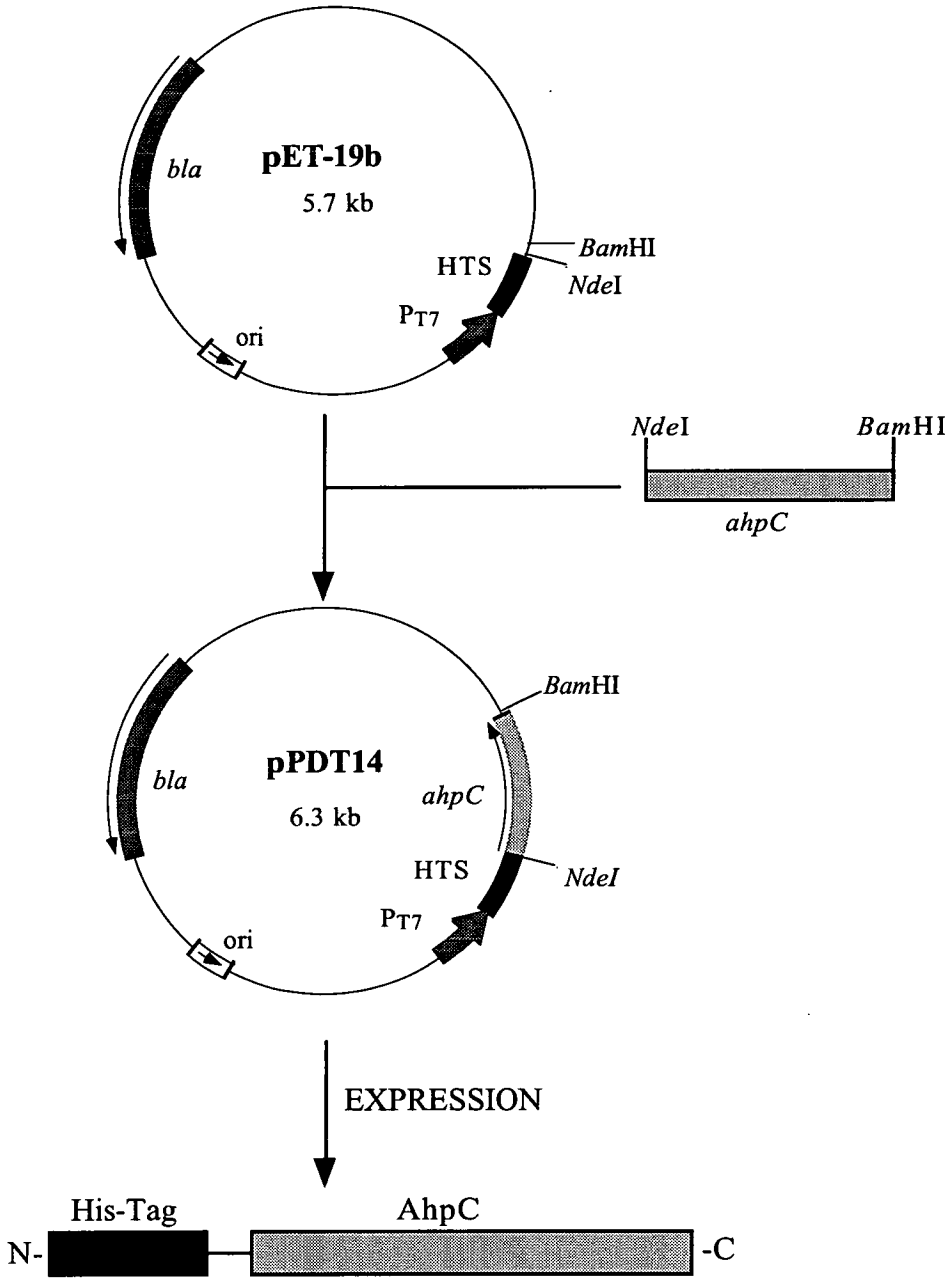


Figure 6.7 Cloning of the *S. typhimurium ahpC* gene into pET-19b to generate a His-tag fusion

The *ahpC* gene was amplified from the *S. typhimurium* SL1344 chromosome by PCR using primers to the 5' (N5138) and the 3' (N5139) ends of the gene which incorporated *Nde*I and *Bam*HI restriction sites, respectively (see Table 2.1). The product was cloned into the *Nde*I and *Bam*HI sites of pET-19b (Novagen) to form pPDT14. The *ahpC* gene was cloned such that the 5' end formed an in-frame fusion to DNA encoding a histidine-tag (His-tag). When the *ahpC* gene is expressed from pPDT14 the resulting AhpC polypeptide is preceded by 23 amino acids, including 10 adjacent His residues, at the N-terminus of the protein. This His-tag can be used to rapidly purify the polypeptide by nickel ion affinity chromatography. Key: *ahpC*, gene encoding alkyl hydroperoxide reductase subunit C; ori, plasmid origin of replication; HTS, DNA sequence encoding the histidine-tag; *bla*, gene encoding β-lactamase resistance; PT7, bacteriophage T7 RNA polymerase promoter.

cloning into pET-19b. The *Nde*I site was devised such that when the *ahpC* gene was cloned into the vector, the region encoding the His-tag would form an in-frame fusion to the start of the gene. Using these primers, the 0.6 kb *ahpC* gene was amplified from the genomic DNA of *S. typhimurium* SL1344 by PCR. The product was then restricted with *Nde*I and *Bam*HI and cloned into the corresponding sites of pET-19b to form pPDT14 (see Figure 6.1).

Overexpression of the *ahpC* gene from *S. typhimurium*

Expression of His-tagged fusion polypeptides from pET-19b requires the T7 RNA polymerase from bacteriophage T7. The plasmid pPDT14 was constructed in *E. coli* strain DH5α, which does not have the ability to produce the T7 RNA polymerase therefore, pPDT14 was moved into the *E. coli* strain BL21(DE3) by transformation. This strain carries the T7 RNA polymerase gene on the chromosome, under the control of the *lac* promoter (lactose operon) and expression of the polymerase is initiated when IPTG is added to BL21(DE3). The polymerasé is very specific and does not initiate significant transcription from *E. coli* DNA sequences. Induction therefore results in specific and high level expression of the gene cloned downstream of the T7 promoter in the vector.

Three strains of BL21(DE3) were used to examine the overexpression of *ahpC*: BL21(DE3); BL21(DE3) containing pET-19b (designated MPG480); BL21(DE3) containing pPDT14 (designated MPG481). BL21(DE3) and MPG480 were used as controls to distinguish host-induced proteins from those which were selectively produced from pPDT14 in MPG481. Briefly, each strain was incubated overnight at 37°C in 5 ml LB medium, containing the appropriate antibiotics. Cells were then pelleted and resuspended in 1 ml of LB and 0.5 ml of this was added to 24.5 ml of Spitzizen minimal medium (Spitzizen, 1958), containing the appropriate antibiotics, and incubated at 37°C until the optical density (OD_{600}) had reached 0.6. Cultures were then split into four aliquots and two of the samples were induced by the addition of IPTG (0.5 mM final concentration). After 30 minutes of incubation, one of the untreated samples and one of the induced samples was treated with rifamycin

(200 μg ml^{-1} final concentration). Rifamycin selectively blocks transcription of host genes by inhibition of the *E. coli* RNA polymerase but not expression from the plasmid which is under the control of T7 RNA polymerase. Protein synthesis *de novo* was then detected by pulse labelling with 5 μCi [S^{35}]-methionine for 5 minutes. Cells from each culture were then spun down and resuspended in LSB and the samples were subjected to SDS-PAGE analysis on a 12.5% (v/v) polyacrylamide gel. After drying on paper, the gel was subjected to autoradiography.

Figure 6.2 shows the radiolabelled products from the three strains under investigation. A comparison of the lanes showed that two bands were particularly obvious. One of these, of approximately 30 kDa, was common to both MPG480 and MPG481 (indicated by the arrow labelled B) and the lower one, of approximately 25 kDa, was only present in MPG481 (indicated by an arrow labelled A). This latter band was considered to be the His-tagged AhpC polypeptide. The wild type AhpC protein has been reported to have a molecular mass of 22 kDa (Jacobson *et al.*, 1989), however the presence of the 23 amino acid His-tag increases the size of the fusion polypeptide by approximately 2-3 kDa. The upper band (B) is thought to be β-lactamase which is responsible for the ampicillin resistance of cultures carrying pET-19b or its derivatives. This is supported by the identification of band B in both MPG480 and MPG481. Quite why the expression of β-lactamase appears to be induced in the IPTG and rifamycin treated culture of MPG481 (lane 2) but not for that of MPG480 (lane 2) is unknown, although the possibility of sample loss during the experimental procedure cannot be excluded. Nevertheless, the labelling experiments clearly showed that AhpC was being produced from pPDT14 under the inducing conditions.

Large scale overexpression and purification of the His-tagged AhpC protein

MPG481 was cultured overnight at 37°C with shaking in 50 ml LB medium containing chloramphenicol (5 μg ml^{-1}) and ampicillin (100 μg ml^{-1}). The bacteria were then pelleted, resuspended in 10 ml LB medium and this sample was then added to 500 ml Spitzizen minimal media containing the appropriate antibiotics and

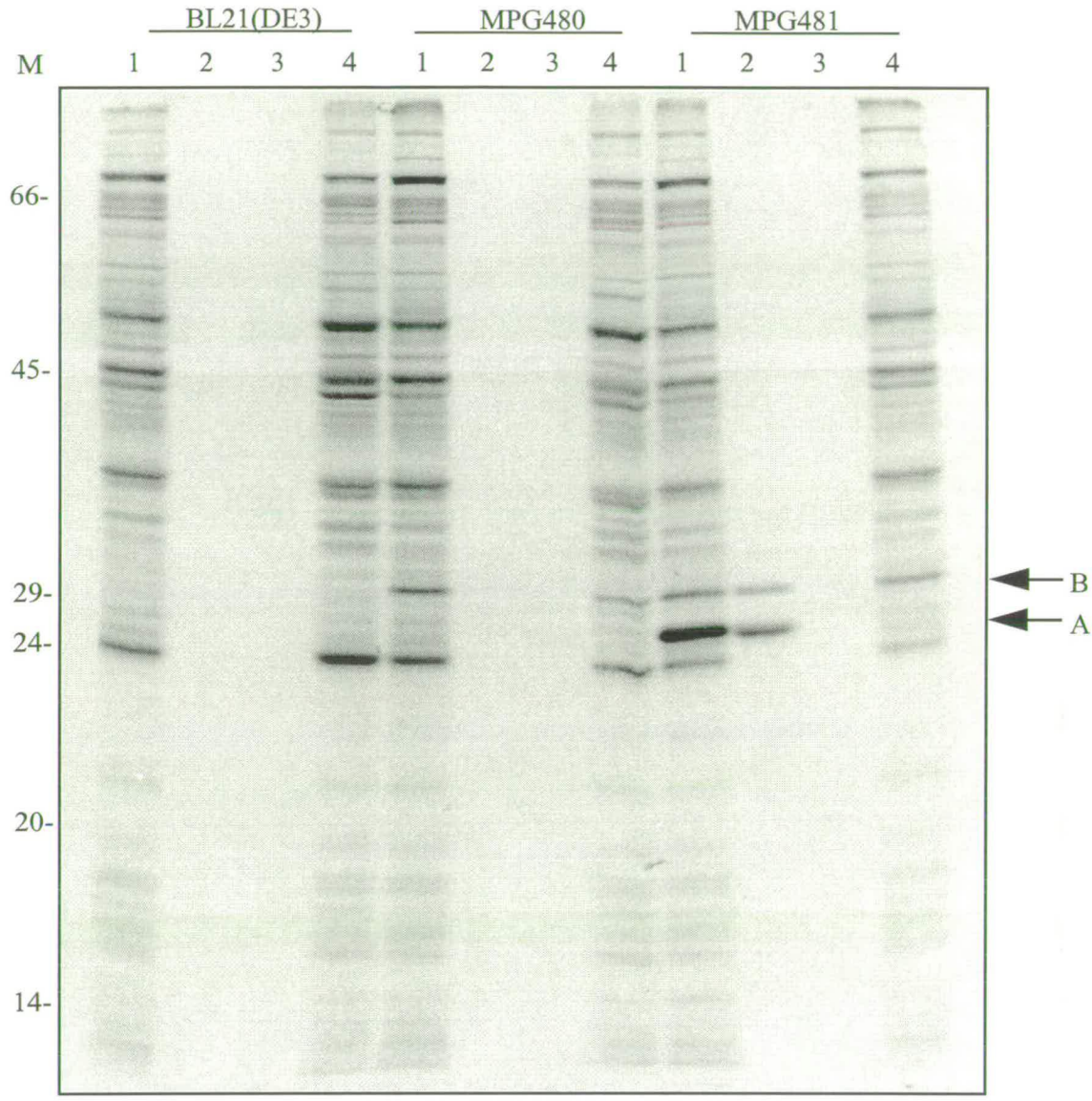


Figure 6.2 Examination of the expression of *ahpC* using radiolabelling

BL21(DE3), MPG480 (pET-19b) and MPG481 (pPDT14) were grown overnight in 5 ml LB at 37°C with shaking in the presence of the appropriate antibiotic. The cells were then harvested, resuspended in 1 ml of fresh LB media and 0.5 ml of this was diluted into 24.5 ml of Spitzizen minimal medium, containing the appropriate antibiotic. Each culture was then grown to an OD_{600} of 0.6 before being split into 4 aliquots. IPTG was added to a final concentration of 0.5 mM to two of the aliquots and the other two aliquots were left untreated. After 30 minutes of incubation, rifamycin (200 μg ml^{-1}) was added to one of the untreated samples and to one of the IPTG induced samples, and incubation was continued for a further 45 minutes. Aliquots were then pulsed for 5 minutes with 5 μCi of [S^{35}]-methionine and the cells were then pelleted, resuspended in LSB and electrophoresed on a 12.5% (v/v) SDS polyacrylamide gel. Protein profiles are shown after autoradiography. As a guide to the size of the polypeptides, molecular mass markers of 66, 45, 29, 24, 20 and 14 kDa were included on the gel, and detected by Coomassie Brilliant Blue staining. The relative position of these markers on the autoradiogram are indicated in lane M. Labelled arrowheads represent the positions of the His-tagged AhpC (A) or β-lactamase (B). Key to lanes: lane 1, I+ R-; lane 2, I+ R+; lane 3, I- R+; lane 4, I- R-. I and R represent IPTG and rifamycin respectively; + and - represent that the cultures were treated or left untreated, respectively.

grown until the OD_{600} of the culture reached 0.6. Expression of the protein was then achieved by adding IPTG to a final concentration of 0.5 mM and incubating the culture for 3 hours. Cells were then harvested and resuspended in 20 ml of Buffer A (see Materials and Methods), sonicated and the soluble fraction obtained by spinning out cell debris by centrifugation at 4, 000 *g* for 10 minutes.

10 ml of supernatant was then subjected to affinity chromatography on a 1 ml column, containing Ni^{2+} ions chelated to the column matrix (agarose), pre-equilibrated with Buffer A. Contaminating proteins with even a slight affinity for the column were removed by extensive washing of the loaded column with 20 ml of Buffer B (see Materials and Methods) containing 20 mM imizadole. The AhpC fusion protein was then rapidly and specifically eluted in 6 ml Buffer B containing 80 mM imizadole and examined by SDS-PAGE (12.5% [v/v] polyacrylamide gel). Lane 1 (Figure 6.3) shows the whole cell extract before passage through the column and lane 2 shows the AhpC protein after specific elution. Two bands were particularly obvious (lane 2), of approximately 25 kDa (the same size as the radiolabelled product; Figure 6.2) and of approximately 21-22 kDa. Previous reports have suggested that some non-His-tagged proteins, such as Manganese-superoxide dismutase, may bind specifically to the column, by virtue of their metal binding properties or because of a high His amino acid content (Hengen, 1995). However the lower band is too small to reflect this impurity (the molecular size of the *E. coli* Mn-SOD is 38 kDa). During radiolabelling experiments, a lower band was sometimes observed and therefore, the lower band is believed to be a degradation product of the His-tagged fusion polypeptide. The fact that it appears in the eluate of the high imizadole wash would indicate that the degraded fragment must still contain the His-tag component.

To increase the potential purity of the protein, the eluate was diluted four-fold (to reduce the concentration of the imizadole) and was then re-passaged through the Ni^{2+}-chelate column. However, this second purification step (Figure 6.3, lane 3) did not appear to greatly enhance the purity already obtained by the single elution. Estimation of the protein concentration, as determined by the Bradford assay

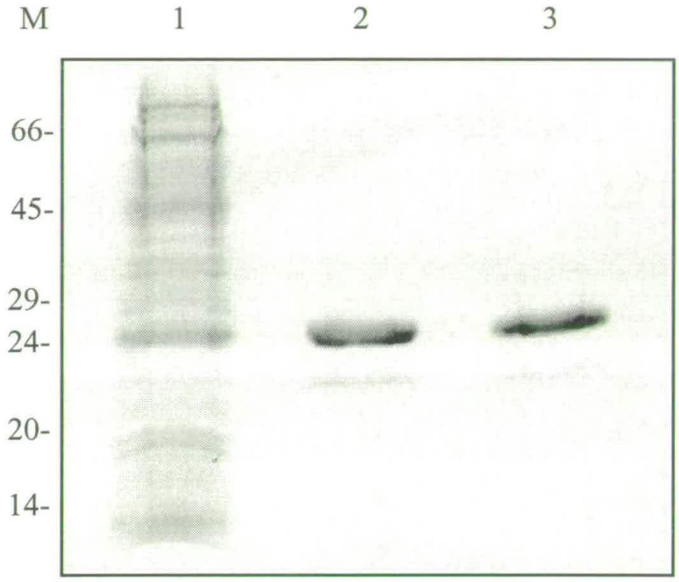


Figure 6.3 Purification of a histidine-tagged AhpC polypeptide using Ni^{2+}-chelate resin affinity chromatography

A culture of MPG481 was grown overnight at 37°C in 50 ml LB medium containing chloramphenicol (5 μg ml^{-1}) and ampicillin (100 μg ml^{-1}). The bacteria were then pelleted, resuspended in 10 ml LB and added to 500 ml of Spitzizen minimal medium, containing the appropriate antibiotic, and incubated at 37°C with shaking until the OD_{600} had reached 0.6. IPTG was then added to a final concentration of 0.5 mM and incubation continued for three hours. After this time, cells were harvested, resuspended in 20 ml of Buffer A (see Materials and Methods), sonicated and the soluble fraction of the cells isolated by centrifugation at 4, 000 *g* for 10 minutes. The extract (10 ml) was then subject to metal ion affinity chromatography using a column containing 1 ml of a Ni^{2+}-chelate resin. Contaminating proteins were removed using 20 ml of Buffer B (see Materials and Methods) containing 20 mM imizadole (low stringency wash) and the histidine-tagged AhpC protein then eluted in 6 ml of Buffer B containing 80 mM imizadole (high stringency wash). This sample was then diluted 1:4 and the column chromatography repeated. The position of molecular mass markers of 66, 45, 29, 24, 20 and 14 kDa are shown as a guide to size (lane M). Key: lane 1, soluble extract of the cell after sonication and centrifugation of the induced culture; lane 2, first elution of AhpC; lane 3, AhpC after repeating the affinity chromatography procedure.

(Bradford, 1976) using a BIORAD standard assay procedure, suggested that approximately 1 mg of protein could be purified from 1 litre of culture following two-rounds of His-tag purification.

The procedure was repeated until approximately 10 mg of AhpC protein was obtained. The protein was then dialysed against several changes of phosphate-buffered saline (PBS) and concentrated using Millipore CX-10 immersible filter units, with a molecular weight cut-off of 10 kDa. During concentration the AhpC protein was observed to precipitate and so further concentration was minimised to prevent loss of the protein. Overall, the dialysis and concentration procedures resulted in a significant loss of protein such that the final yield was approximately 5 mg. The purified protein was stored frozen at -70°C in PBS as a 2.5 mg ml^{-1} solution until further use.

6.2.2 Cloning and overexpression of the *S. typhimurium groEL* gene

Unlike *ahpC*, the sequence of the *S. typhimurium groEL* gene was unknown, preventing direct use of vectors for overexpression. Nevertheless, previous studies have shown that GroEL from *E. coli* can be induced to a high level from its natural promoter, after a period of heat shock (Hendrix, 1979; Hemmingsen *et al.*, 1988), and use of a multicopy plasmid enhanced this expression (McClennan *et al.*, 1993). Thus, an attempt was made to clone the *S. typhimurium groESL* operon on a multicopy plasmid and express it from its natural promoter in the hope that high level production would occur.

An *E. coli* mutant (K090; a gift from N. McClennan, Edinburgh University, Scotland) which carries the *groESL* operon under the control of the arabinose promoter (P_{BAD}) was used to facilitate cloning of the *groESL* operon. GroESL is essential for growth under normal conditions as well as stress conditions (Fayet *et al.*, 1989) and therefore, K090 can only survive if media is supplied with arabinose to stimulate the expression of GroESL from the P_{BAD} promoter. The ability to control the production of GroESL was therefore exploited as a means for isolating the

S. typhimurium groESL operon by complementation of the mutant in the absence of arabinose.

Identification of a *groESL* homologue in *S. typhimurium*

The *groESL* operon of *E. coli* has been extensively characterised (Hendrix, 1979; Hemmingsen *et al.*, 1988; Gross, 1996). The relatively high degree of similarity between *S. typhimurium* and *E. coli* and the highly conserved nature of GroEL suggested that the genetics and properties of the *S. typhimurium groEL* gene should be very similar. However, studies have indicated that some bacteria may carry more than one homologue of either *groEL* or *groES* (Fischer *et al.*, 1993; Rusangawa and Gupta, 1993; Duchene *et al.*, 1994; Thole *et al.*, 1995), therefore, a preliminary investigation was performed to identify whether *S. typhimurium* carries one or multiple homologues of *groESL*, as this would have a bearing on the cloning strategy. Previous studies had shown that the *groESL* operon of *E. coli* DH5α existed on an 8.1 kb *Eco*RI genomic DNA fragment (McClennan *et al.*, 1994). Genomic DNA was therefore prepared from *E. coli* (DH5α) and from a pathogenic strain of *S. typhimurium* (SL1344) and digested with *Eco*RI. The resultant digest was then subjected to electrophoresis overnight in a 0.8% (w/v) agarose gel and the DNA was subsequently transferred to nylon membrane and Southern blotted. A 2.1 kb *Hin*dIII-*Eco*RI fragment from pGT3270 (McClennan *et al.*, 1994) which carries the *E. coli groESL* operon was radiolabelled, and used to probe the filter. The resulting blot (Figure 6.4) shows that only one band is specifically identified by this probe in both the *E. coli* and *S. typhimurium* samples, of sizes 8.1 kb and 6.5 kb, respectively. This suggests that *S. typhimurium* contains one homologue of the *groESL* operon. In addition, the size of fragments and the absence of any other bands on the gel suggested that the complete *S. typhimurium groESL* operon was present on the 6.5 kb *Eco*RI derived fragment.

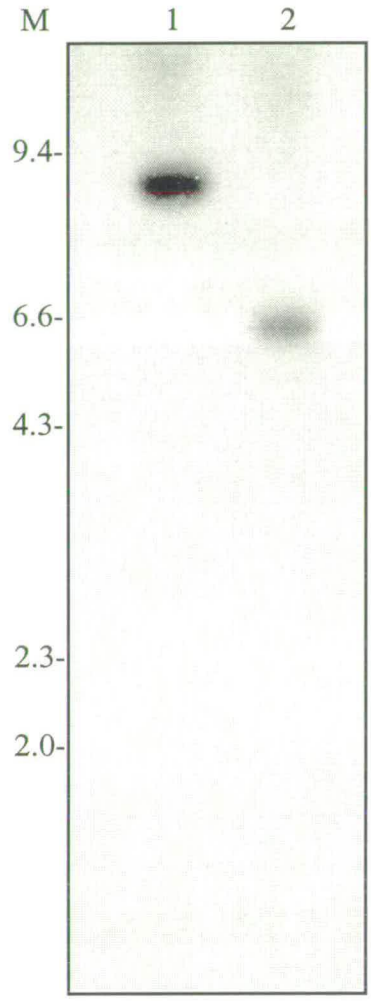


Figure 6.4 Detection of a *groESL* homologue in *S. typhimurium*

Genomic DNA was isolated from *E. coli* DH5α and *S. typhimurium* SL1344 and was subjected to *Eco*RI digestion. The digests were then electrophoresed on a 0.8% (w/v) agarose gel and the DNA was transferred to a nylon membrane for Southern blotting. A 2.1 kb *Eco*RI-*Hin*dIII fragment from pGT3270 carrying the *groESL* operon from *E. coli* (McClennan *et al.*, 1994) was labelled with [^{32}P]-dATP and was used as a probe to detect the presence of *groESL* homologues. Single bands of approximately 8.5 kb and 6.5 kb were observed in the samples from *E. coli* (lane 1) and *S. typhimurium* (lane 2), respectively. The locations of λ *Hin*dIII molecular size markers are indicated (lane M).

Cloning of the *S. typhimurium groESL* operon

The *groESL* operon lies at approximately 94 minutes on the *E. coli* chromosome (Berlyn *et al.*, 1996) and might be expected to lie at a similar position in *S. typhimurium* because of the high degree of genomic similarity between the two organisms. To explore this possibility a specialised Mu*d*-P22 hybrid phage was employed. A collection of hybrid Mu*d*-P22 phage have been constructed, such that the phage have been integrated into defined locations on the chromosome and so, when phage are induced to enter the lytic cycle, large portions of the bacterial chromosome, close to the region of insertion, are packaged in a unidirectional manner into the phage (Benson and Goldman, 1992). Thus, these Mu*d*-P22 phage can be used to enrich for defined regions of approximately 3 genetic minutes of chromosomal DNA. In general, these phage have been used to map the positions of genes on the *S. typhimurium* chromosome. However, since Mu*d*-P22 lysates are also enriched for defined regions of the bacterial chromosome they can also be used to facilitate cloning of genes within these regions.

Strain TT15276, which contains a Mu*d*-P22 lysogen at 93 minutes on the *S. typhimurium* chromosome (Benson and Goldman, 1992), was used to enrich for DNA containing the *groESL* locus. The culture was grown overnight at 37°C with shaking in LB containing chloramphenicol (20 $\mu g\ ml^{-1}$). 5 ml of an overnight culture was then added to 25 ml of fresh LB containing chloramphenicol (20 $\mu g\ ml^{-1}$) and Mitomycin C (2 $\mu g\ ml^{-1}$) and grown overnight with shaking. Mitomycin C induces the lysogenised phage to enter into the lytic cycle and to undergo packaging of DNA (in this case, the bacterial chromosome). The resulting phage particles were liberated from the cells by the addition of chloroform, and were pelleted by centrifugation at 8,000 *g* for 10 minutes. The pellets were then subject to phenol/chloroform extraction, which removes the viral protein coat from the DNA present in the phage. The isolated DNA was subsequently digested with a range of restriction enzymes, including *Eco*RI, *Hin*dIII, *Pvu*II, *Xba*I, *Bgl*II, *Bam*HI and *Sac*I, electrophoresed on a 0.8% (w/v) agarose gel and blotted onto a nylon filter. The filter was probed with the *E. coli groESL* fragment as described previously. The lysogen in TT15276 is inserted

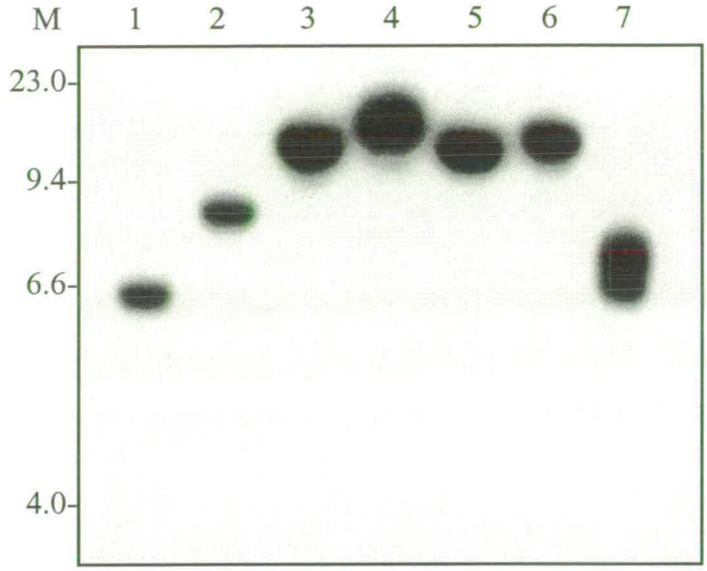


Figure 6.5 Analysis of Mu*d*-P22 DNA isolated from the *S. typhimurium* strain TT15276 for the presence of a *groESL* locus

A 5 ml culture of TT15276, containing a Mu*d*-P22 lysogen at 93 minutes on the *S. typhimurium* chromosome, was grown overnight at 37°C in LB medium containing chloramphenicol (5 μg ml^{-1}). The culture was then diluted into 25 ml fresh LB, containing the same antibiotic and mitomycin C (2 μg ml^{-1}), and incubated overnight at 37°C with shaking. The cells were lysed with chloroform and the phage were isolated by centrifugation at 8, 000 *g* for 10 minutes. Phage proteins were extracted from the DNA with phenol/chloroform and the DNA subject to restriction digestion with *Eco*RI (lane 1), *Hin*dIII (lane 2), *Pvu*II (lane 3), *Xba*I (lane 4), *Bgl*II (lane 5), *Bam*HI (lane 6) or *Sac*I (lane 7). The DNA was then electrophoresed on a 0.8% (w/v) agarose gel and Southern blotted. The *groESL* locus was detected using a 2.1 kb *Eco*RI-*Hin*dIII *groELS* probe (see legend to Figure 6.4) derived from *E. coli* (McClennan et al., 1994). *Hin*dIII digested phage lambda DNA was added as a guide to molecular size (kb) (lane M).

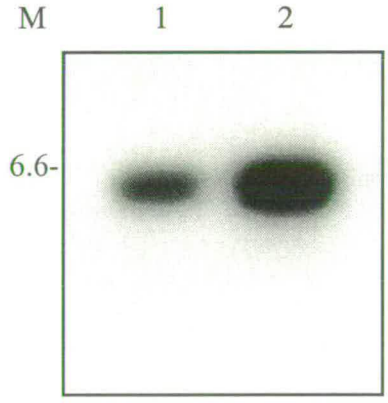


Figure 6.6 Confirmation by Southern blotting that a complementing plasmid contained the *S. typhimurium groESL* locus

The plasmid which complemented an *E. coli groESL* deficient strain, and Mu*d*-P22 DNA, derived from TT15276, were subject to *Eco*RI digestion and the resultant DNA was electrophoresed on a 0.8% (w/v) agarose gel. The DNA was then transferred to a nylon membrane and Southern blotted. The *groESL* locus was detected using a 2.1 kb *Eco*RI-*Hin*dIII *groESL* probe (see legend to Figure 6.4) derived from *E. coli* (McClennan et al., 1994). Identical bands of approximately 6.5 kb can be seen in the lane containing the Mu*d*-P22 DNA (lane 1) or plasmid (lane 2), thus confirming that the plasmid contains DNA which is homologous to the *groESL* operon. The position of the 6.6 kb λ *Hin*dIII molecular size marker is shown (lane M).

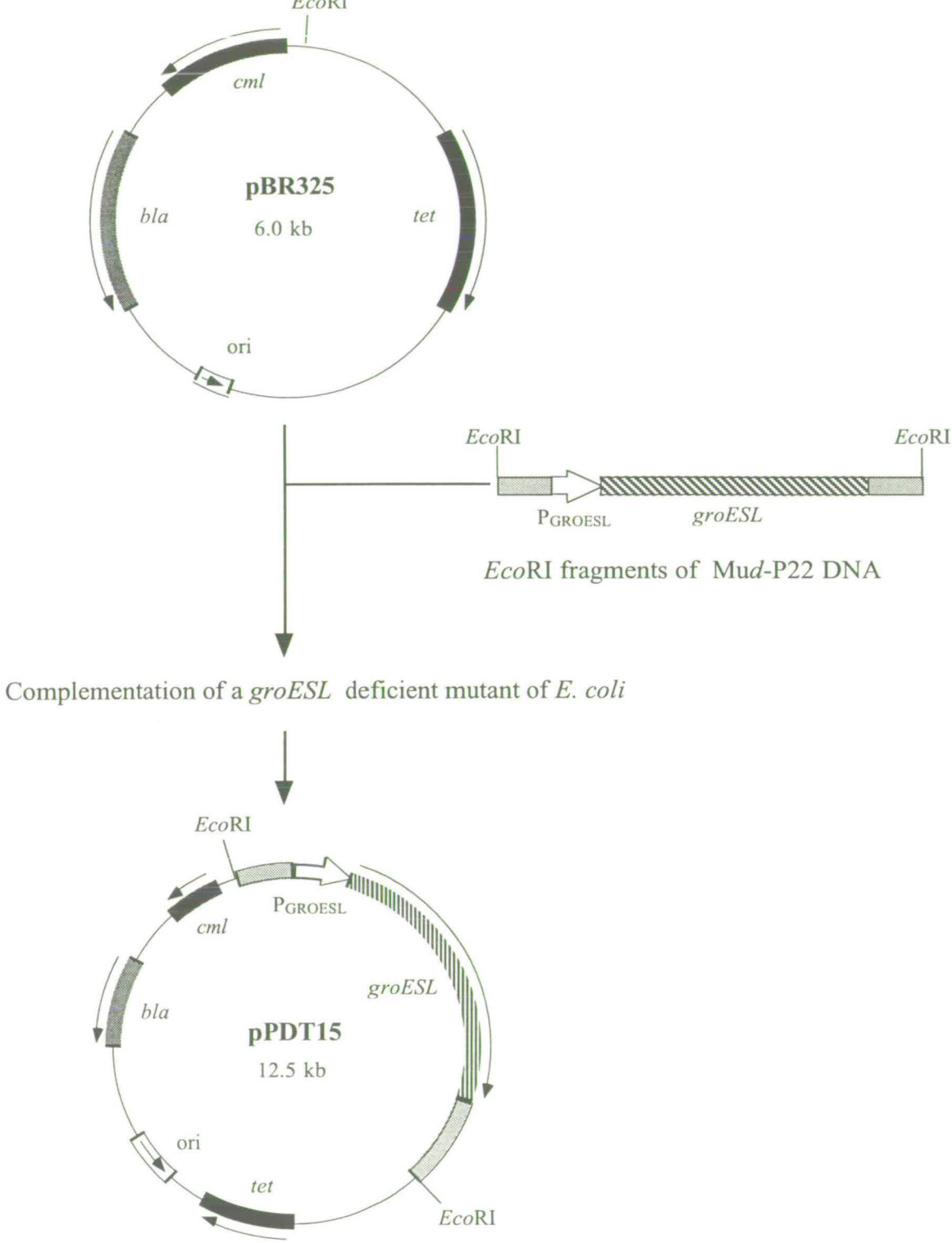


Figure 6.7 Summary of the cloning of the *S. typhimurium groESL* locus

The *groESL* locus had been identified on a 6.5 kb fragment in an *Eco*RI digested sample of Mu*d*-P22 DNA from *S. typhimurium* strain TT15276 (Figure 6.5). *Eco*RI fragments of Mu*d*-P22 DNA of approximately 6-8 kb were excised from a 0.8% (w/v) agarose gel, purified and ligated into *Eco*RI-restricted pBR325. The ligation mixture, containing a heterogenous mixture of inserts, was then transformed into K090, an *E. coli* strain which shows arabinose-dependent expression of the *groESL* locus. Transformants containing complementing plasmids were then selected for in the absence of arabinose. One plasmid, pPDT15, was obtained and proven to contain the *groELS* locus by Southern blotting (Figure 6.6). Key: *bla*, gene encoding ampicillin resistance; *cml*, gene encoding chloramphenicol resistance; *tet*, gene encoding tetracycline resistance; ori, origin of replication; *groESL*, the *S. typhimurium groESL* locus; P_{GROESL}, heat shock-inducible promoter of the *groESL* locus.

at 93 minutes and packages DNA clockwise upon entry into a lytic cycle. Thus, the presence of *groESL* in the DNA (Figure 6.5) suggests that the *S. typhimurium groESL* operon is at approximately 93-96 minutes. Analysis of the restriction pattern indicated that *Eco*RI liberated the smallest fragment (approximately 6.5 kb) and so DNA from this region was used to clone the *groESL* locus.

An area encompassing *Eco*RI fragments of Mu*d*-P22 DNA of between 6-8 kb was excised from the gel. The DNA was purified using GENECLEANII (BIO 101 Inc.) and then ligated into the *Eco*RI site of pBR325. The resultant ligation mix was then electroporated into K090 and transformants were selected on LB plates containing 50 $\mu g\ ml^{-1}$ ampicillin but no arabinose. Only those cells that could express a functional *groEL*S independently of the addition of arabinose, would grow. Only one colony was obtained and the plasmid was isolated from this transformant and subject to *Eco*RI digestion. After agarose gel electrophoresis, two bands were observed, one corresponding to the size of pBR325 (6 kb), and the other to an approximately 6.5 kb fragment (data not shown). As further proof that the 6.5 kb fragment contained the *S. typhimurium groESL* locus, *Eco*RI digested samples of the complementing plasmid and Mu*d*-P22 DNA were examined by Southern blotting, as described previously. From Figure 6.6 it can be seen that DNA from plasmid and the Mu*d*-P22 lysate produced identical bands following autoradiography, thus confirming that the *groESL* operon from *S. typhimurium* had been isolated. The complementing plasmid was subsequently called pPDT15 and was used for the overexpression of GroEL (see Figure 6.7 for a summary diagram of the cloning of the *S. typhimurium groESL* locus).

Expression and purification of GroEL from *S.typhimurium*

The *E. coli* GroEL protein has been isolated previously by anionic exchange chromatography and by centrifugation of the cell extract through a sucrose velocity gradient (Hendrix, 1979). GroEL has an approximate molecular mass of 60 kDa (Hemmingsen *et al.*, 1988) but has been shown to associate into a multimeric particle consisting of 14 subunits with a molecular mass of 840 kDa (Hendrix *et al.*, 1979).

This complex can be separated from the majority of other cellular proteins with the aid of the centrifugation procedure. The value of these approaches for purifying the GroEL protein from *S. typhimurium* was explored. To prevent any chance of the *S. typhimurium* sample being contaminated with *E. coli*-derived GroEL, pPDT15 was transferred into the *S. typhimurium* strain CH23, to form MPG482. This strain is also *recA* deficient, thus preventing any detrimental effects of recombination between the plasmid encoded *groEL* and that on the chromosome of MPG482.

A 5 ml culture of MPG482 was grown overnight at 30°C in LB containing ampicillin (100 $\mu g\ ml^{-1}$). This lower temperature was required because growth at 37°C was found to be detrimental to MPG482, probably as a result of the accumulation of high levels of GroEL. The culture was then diluted 1:100 into 500 ml of LB containing ampicillin (100 $\mu g\ ml^{-1}$), and was grown with shaking at 30°C until the OD_{600} had reached approximately 0.6. The sample was then subjected to a temperature shift to 42°C, which induces a strong heat shock response and results in the high level expression of *groESL* from the plasmid. After 10 minutes the cells were harvested, resuspended in 20 ml of Buffer Z (see Materials and Methods) and sonicated. The majority of the insoluble material was then removed by centrifugation at 4, 000 *g* for 10 minutes to obtain the soluble fraction containing the GroEL.

The supernatant (20 ml) was then loaded onto an 80 x 15mm (approximately 8 ml) DEAE-cellulose (DE52; Whatman) ion exchange column pre-equilibrated with Buffer Z. The column was then washed with 100 ml of Buffer Z to remove any unbound proteins. Bound proteins were then eluted from the column by using 10 ml volumes of Buffer Z containing stepwise increases in NaCl concentration (150 mM, 500 mM and 1000 mM NaCl) and the eluted fractions subsequently collected in 3-4 ml volumes. The initial soluble fraction and fractions obtained from the ion exchange procedure were then examined by SDS-PAGE analysis using a 10% (v/v) polyacrylamide gel (Figure 6.8). The first lane shows the soluble fraction obtained after sonication and centrifugation of the heat-shocked culture. GroEL can be clearly seen and is indicated in the figure. GroES is believed to be one of the two bands which ran to the approximate position of the 14 kDa molecular mass marker (this

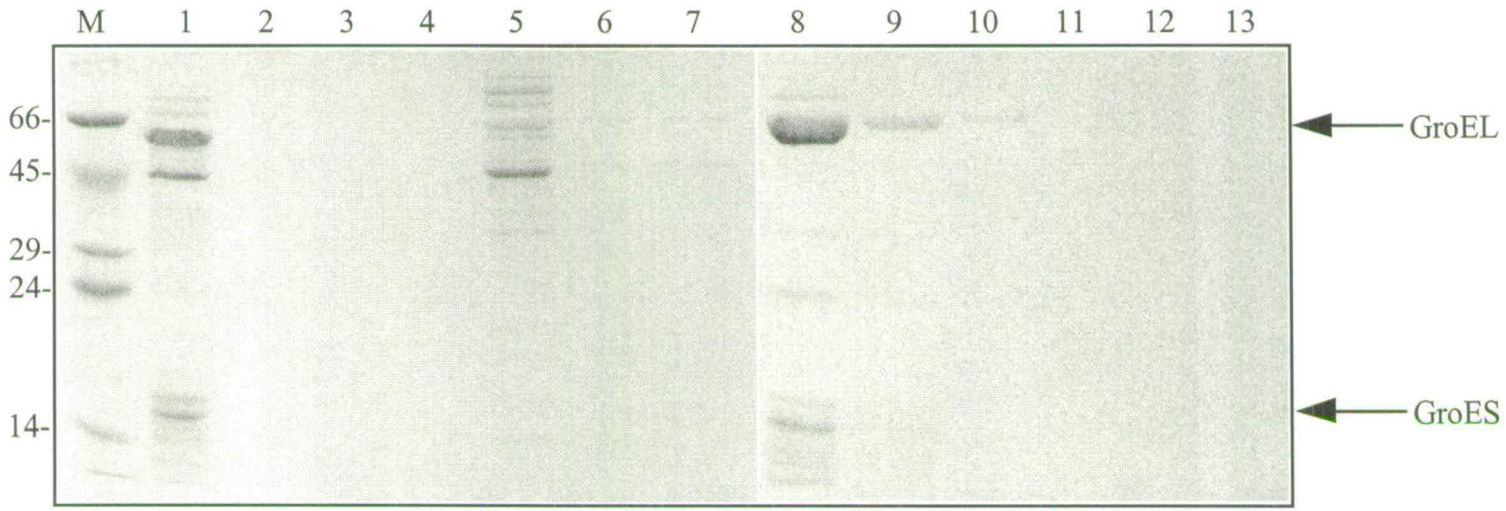


Figure 6.8 Use of anionic exchange column chromatography to purify GroEL

MPG482 (pPDT15) was grown overnight at 30°C in LB containing ampicillin (100 μg ml^{-1}). The culture was diluted 1:100 into the same medium and was incubated at 30°C until the OD_{600} had reached approximately 0.6. The culture was then subjected to a heat shock at 42°C for 10 minutes and the cells were harvested, resuspended in 20ml of Buffer Z (see Materials and Methods) containing 50 mM NaCl and sonicated. The soluble fraction containing GroEL was then isolated by centrifugation at 4, 000 *g* for 10 minutes. 10 ml of supernatant was then subjected to anionic exchange chromatography on a DE52 (Whatman) column (80 x 15mm). The column was extensively washed with 100 ml Buffer Z (50 mM NaCl) to remove any unbound proteins. Bound proteins were then eluted using 10 ml volumes of Buffer Z containing stepwise increases in the concentration of NaCl (150 mM, 500 mM and 1000 mM NaCl). Fractions of samples obtained during this procedure were then subject to SDS-PAGE analysis on a 10% (v/v) polyacrylamide gel. Molecular weight markers of 66, 45, 29, 24, and 14 kDa were included as a guide to size (lane M). Key: lane 1, soluble cell extract after sonication and centrifugation; lanes 2-4, fractions obtained after washing the column with Buffer Z (50 mM NaCl); lanes 5-7, lanes 8-10 and lanes 11-13 represent the eluted fractions obtained after using Buffer Z containing 150, 500 and 1000 mM NaCl respectively. The position of the GroEL and GroES proteins is marked by an arrow.

relative position is also marked). The level of GroEL present suggested that a high level of induction had occurred. Lanes 2-4 show the column wash fractions in Buffer Z (containing 50 mM NaCl) and indicates very few proteins were being washed off the column. However, when the salt concentration of Buffer Z was increased to 150 mM, a number of proteins were seen to elute (lanes 5-7). Importantly a band of 45 kDa, which forms a predominant part of the initial soluble fraction, was eluted and helped to increase the purity of the GroEL sample. Unfortunately, a small amount of GroEL could also be seen in the eluates at this salt concentration. Therefore, the concentration of NaCl was subsequently adjusted to 500 mM to elute as much of the GroEL into as few fractions as possible. The resulting eluate (lanes 8-10) showed that the column had resulted in substantial purification of GroEL, however a number of other bands, including GroES were present. A final elution of proteins in 1 M NaCl resulted in little if any further elution of proteins (lanes 11-13).

The value of subjecting the soluble fraction to sucrose density gradient centrifugation was also explored as a means of purification. 4 ml aliquots of the soluble fraction, obtained after centrifugation of the sonicated cells, were carefully laid onto pre-formed gradients (30 ml) of between 30-10% (w/v) sucrose and centrifuged overnight at an average relative centrifugal force of 86, 000 *g*. 1.5 ml fractions of the gradient were then carefully removed and samples subjected to SDS-PAGE analysis on a 10% (v/v) polyacrylamide gel to ascertain the composition of the proteins present. The composition of proteins from a typical gradient is shown in Figure 6.9. GroEL is seen at the densest region of the gradient (30% [w/v] sucrose) but appears to be relatively dispersed in these lower fractions (lanes 1-7), encompassing approximately 8-10 ml of the 34 ml gradient. Lanes 6-10 show that some contaminating proteins start to appear and are likely to be components of the ribosomes or RNA polymerase as described previously (Hendrix, 1979). The majority of the cellular proteins, including GroES, appear to be confined to the upper reaches of the gradient (lanes 11-18). The overall purity obtained by such a procedure appeared to be >95% (by visual inspection of gels). However, after pooling and concentrating appropriate fractions containing GroEL, a number of contaminants could still be seen. It was decided therefore, that a combination of both anionic

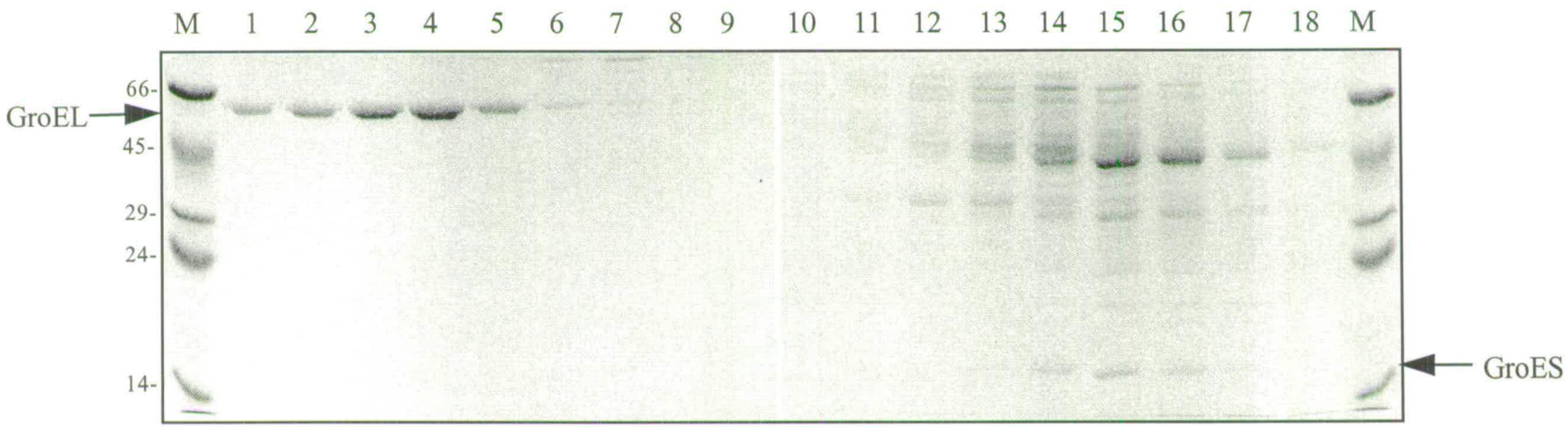


Figure 6.9 The use of sucrose gradient velocity centrifugation to purify GroEL

A soluble fraction containing GroEL was obtained from MPG482 as described in the legend for Figure 6.8. 4 ml of this supernatant was then laid carefully onto a 30-10% (w/v) sucrose gradient (30 ml) and centrifuged for 18 hours at an average centrifugal force of 86, 000 *g*. Fractions of approximately 1.5 ml were isolated from the gradient and the protein composition determined by SDS-PAGE analysis on a 10% (v/v) polyacrylamide gel. Molecular mass markers (kDa) were included as a guide to size and are indicated (lane M). Fractions are numbered from the most dense (30% [w/v] sucrose) to the least dense (10% [w/v] sucrose) region of the gradient. Lane numbers correspond to the fraction numbers. The position of the GroEL and GroES proteins is marked by an arrow.

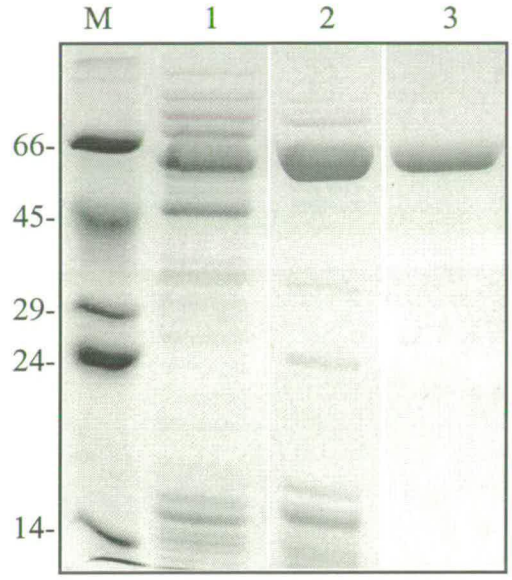


Figure 6.10 Overall purification of the *S. typhimurium* GroEL protein using a combination of ion exchange chromatography and sucrose velocity gradients

A soluble fraction containing GroEL was obtained from MPG482 as described in the legend for Figure 6.8. This fraction was subjected to ion exchange column chromatography followed by sucrose velocity gradient centrifugation (see legends to Figure 6.8 and 6.9 respectively). Samples of the protein were examined by SDS-PAGE analysis on a 10% (v/v) polyacrylamide gel during the purification procedure in order to determine the level of purity. Molecular mass markers of 66, 45, 29, 24 and 14 kDa were included as a guide to size (lane M). Key: lane 1, soluble cell extract after sonication and centrifugation of the induced MPG482 culture; lane 2; GroEL sample after being subjected to ion exchange column chromatography; lane 3; GroEL sample after subsequent sucrose velocity gradient centrifugation.

exchange and velocity gradient centrifugation would be used for maximising the purity of the *S. typhimurium* GroEL. A typical sequential profile of the overall purification of GroEL is shown in Figure 6.10. The combined protocol was repeated until approximately 5 mg of protein had been isolated, as determined by the Bradford assay (Bradford, 1976) using a BIORAD standard assay procedure. The resultant GroEL samples were dialysed against several changes of PBS and were then concentrated using immersible Millipore (CX-10) filter units with a molecular cut off of 10 kDa. GroEL was found to precipitate during the concentration procedure, as similarly found for AhpC, therefore the concentration step was minimised to prevent loss of protein. The resulting protein was stored frozen at approximately 2 mg ml^{-1} at -70°C until further use.

Approximately 2 mg of GroEL was obtained from approximately 1 litre of starting culture, however this was believed to be a rather low overall yield, considering the abundance of the protein in the cell after induction. Nevertheless, the purification strategy, although costly in terms of loss of protein, resulted in a high level of purity.

6.3 DISCUSSION

The *S. typhimurium* AhpC and GroEL proteins were successfully cloned, overproduced and purified and the proteins were estimated to be greater than 95% pure after visual inspection of protein gels. This was an important factor, as the proteins were to be injected into mice for immunological studies and a high level of impurity may affect the characteristics of the immune responses which would be generated.

The use of a His-tag procedure for purification of AhpC proved simple and effective, resulting in a high level of purification from only a small number of steps (Figure 6.3). However, the small additional level of purity gained by a second passage down the Ni^{2+}-chelate column probably resulted in some loss of the protein. In addition, the dialysis and concentration steps appeared to result in a large loss in the overall protein recovery, although the reasons for this are unclear. It is noteworthy that in

previous investigations it was found that the native AhpC polypeptide tended to aggregate during the purification procedure (Jacobson *et al.*, 1989). This might suggest that the His-tag at the N-terminus of the fusion polypeptide was not responsible for the precipitation of the protein observed in the present study. Nevertheless, the ready nature with which the protein precipitated from solution during concentration may have resulted in much of the loss observed.

Very little information exists about the *S. typhimurium* homologue of GroEL. However, taking advantage of an *E. coli* strain that could only produce GroESL in the presence of arabinose, the *groESL* operon from *S. typhimurium* was cloned. A number of bacteria contain several copies of the *groEL* gene, including *Mycobacterium tuberculosis* (Thole *et al.*, 1995), *Bradyrhizobium japonicum* (Fischer *et al.*, 1993), *Streptomyces coelicolor* (Duchene *et al.*, 1994) and *Rhizobium leguminosarum* (Rusanganwa and Gupta, 1993). In *E. coli*, the association of GroEL with GroES is essential for chaperone activity and the two proteins are encoded within an operon. This arrangement also appears in the above bacteria but some of these bacteria, including *M. tuberculosis* and *R. leguminosarum*, also appear to have *groEL* homologues encoded in a locus in which the *groES* gene is absent (Thole *et al.*, 1995; Rusanganwa and Gupta, 1993) This work is the first to report that *S. typhimurium* contains only one homologue of *groEL* and which exists in an operon with *groES*. This was shown by Southern blotting (Figure 6.4) and also by virtue of the complementing plasmid pPDT15, since the *E. coli* strain K090 can only grow in the presence of both genes and neither is significantly produced in the absence of arabinose. Moreover, Southern blot analysis of DNA from a Mu*d*-P22 lysate mapped the locus to approximately 93-96 minutes on the *S. typhimurium* chromosome.

Using the natural heat shock inducible promoter of the *groESL* operon, a high level of induction was obtained after a shift to 42°C. Visual estimates of the amount of protein in the cell extract suggested that as much as 20-40% of the soluble protein was GroEL. The multimeric assembly of GroEL aided rapid and high level purification using sucrose gradient centrifugation (Figure 6.9). However, the anion exchange column was found to reduce the overall level of impurities, if performed

prior to the sucrose gradient spin (Figures 6.8 and 6.10). Interestingly, the GroES protein appeared to elute in the same fractions as GroEL after ion exchange but did not appear to be associated with GroEL after sucrose gradient centrifugation. This supports evidence that GroES only weakly associates with GroEL, as a high centrifugal force is capable of separating these two proteins (Braig *et al.*, 1994; Fenton *et al.*, 1994). The overall purity of the GroEL was high, but considering the level of induction of the protein the yield of 2 mg per litre was considered poor.

GroEL, like AhpC, also precipitated upon concentration. The fact that these proteins precipitated from the solution would suggest that denaturation had occurred. The reason for this precipitation is not known but for the purposes of the immunological studies both proteins required to be in phosphate-buffered saline (PBS). The bacterial cytoplasm has an ionic salt concentration of approximately 0.15 M -0.2 M (at neutral pH) and protein solubility under these conditions is a result of ionic and polar interactions with the surrounding solvent (Scopes, 1994). The concentration of NaCl in PBS is approximately 0.14 M therefore, the insolubility of AhpC and GroEL is unlikely to be a result of the composition of the PBS. Cellular proteins also exist in a reduced environment, and this has important implications for proteins containing cysteine groups. Cysteine amino acids are capable of forming disulphide bridges between one another, following oxidation of the sulphydryl groups they contain. One problem associated with protein purification is that these groups are very sensitive to oxidation once the protein has been removed from the intracellular environment (Scopes, 1994). This may result in the inappropriate formation of such bonds not only within individual proteins but also to adjacent proteins leading to denaturation and the formation of aggregates. During the purification of both AhpC and GroEL, the reducing agent dithiothreitol (DTT) was present in the buffers. However, during dialysis, DTT was excluded and this may have promoted inappropriate intra- and inter-disulphide bond formation, especially when the proteins were subsequently concentrated, leading to denaturation and precipitation. In support of this, Braig and colleagues (1994) reported that they were able to concentrate the GroEL protein to approximately 50 mg ml^{-1} in a buffer containing DTT.

CHAPTER 7

The use of a murine model to examine the immunological properties of AhpC and GroEL

7.1 INTRODUCTION

Initial attempts to prevent *S. enterica*-related diseases, particularly typhoid fever, by vaccination, involved whole cell preparations (reviewed in Ivanoff *et al.*, 1994). However, these were shown to have a poor ability to induce protective immunity to subsequent challenge, although this could be overcome, to a degree, by the use of multiple doses (Collins, 1974; Ivanoff *et al.*, 1994; Plotkin and Bouveretlecam, 1995). This produces problems especially in developing countries where such vaccines are most needed. The cost of transport and staff, to disperse the vaccines over large areas with poor infrastructure would make a multiple dose regimen difficult to implement (Bloom, 1989). Ideally, therefore, there is a need for a vaccine that gives long term protection and that can be administered preferably as a single dose (Bloom, 1989; Ivanoff *et al.*, 1994). Moreover, such a vaccine would find a use in eliminating *S. enterica* infection of animals, which act as reservoirs for this pathogen and which are believed to be a major factor in the spread of *S. enterica* infection to humans via the food chain (Maurice, 1994).

More recent developments have involved the use of live attenuated organisms and these have been shown to induce a greater degree of immunity in individuals than killed bacterial cells (Chatfield *et al.*, 1992a; Mastroeni *et al.*, 1996). However, even the live attenuated vaccines have not provided a complete answer, as a fine balance is required between attenuation and the ability of the organism to persist long enough to induce substantial protective immune responses (Chatfield *et al.*, 1992a; Dougan, 1994; Ivanoff *et al.*, 1994). In addition, field trials have demonstrated that there may be problems associated with the use of live attenuated vaccines. For example, the live attenuated vaccine strain CVD908, an *aroCaroD* mutant of *S. typhi*, has been shown to produce symptoms of typhoid fever in human vaccinees (Miller *et al.*, 1993; Ivanoff *et al.*, 1994). Moreover, although a single dose of this vaccine was protective, multiple doses were still needed for sustained levels of protective immunity (Ivanoff *et al.*, 1994; Plotkin and Bouveretlecam, 1995).

In addition, to the ability to persist in the host, there is a second reason why live bacterial vaccines are believed to be superior to killed-cell vaccines. Live bacteria are capable of responding to the host environment and therefore produce a spectrum of antigens which more accurately equate with the natural infection (Kagaya *et al.*, 1992). However, very few studies have examined the protein antigens from *S. enterica* that are important in generating immunity or the value of such antigens for the development of prophylactic therapies to *S. enterica* infection. Indeed, the potential for developing a subunit vaccine has largely been ignored, perhaps because most of the important recent advances in vaccine development have come from identifying genetic lesions that attenuate *S. enterica*.

Subunit vaccines comprising of a few key molecules, could solve many of the problems associated with whole cell vaccine preparations (Horwitz *et al.*, 1995). Many bacterial molecules are likely to be irrelevant or non-essential to developing immune responses and possibly may even be immunosuppressive. For example, a number of mycobacterial wall components, including lipopolysaccharide and lipoarabinomannan, have been shown to suppress the effective development of protection against this organism (Horwitz *et al.*, 1995). Thus, a vaccine comprised of a few defined components is likely to be safer and less reactogenic and may induce stronger protective immunity than a whole cell vaccine. Also, because such vaccines would be chemically defined, they are more likely to be produced reproducibly, to be easier to assay and to be less expensive to manufacture, transport and store.

In the present study, the murine model of salmonellosis was employed to examine the immune responses of *S. typhimurium*-infected mice to the proteins, AhpC and GroEL. Both of these proteins have been shown to be expressed during interaction with macrophages (Buchmeier and Heffron, 1990; Francis and Gallagher, 1993; Abshire and Neidhart, 1993a), suggesting that AhpC and GroEL are likely candidates for antigen recognition by the host immune system. The importance of these proteins in the generation of immunological responses during infection by *S. typhimurium* was assessed therefore, to further our understanding of the immunogenicity of defined protein antigens and to examine their potential in vaccine design.

Attenuated strains of *S. typhimurium* produce a systemic bacterial infection in innately susceptible (*ity*^{S}) BALB/c mice resembling that of typhoid fever in man (Collins, 1974; Hormaeche, 1979). Moreover, such strains persist long enough to induce immunological protection against a subsequent challenge from a lethal dose of wild type organisms. This property has been associated with the development of both cell-mediated and humoral responses (Collins, 1974; Mastroeni *et al.*, 1993).

Cell-mediated immunity (CMI) can be measured by *in vivo* manifestations such as delayed-type hypersensitivity (DTH) (Abbas *et al.*, 1991). Classically, a DTH reaction occurs when an individual is challenged with an antigen that has previously been recognised by the immune system. For experimental purposes, the challenge is often performed by injecting the antigen into the footpad of a laboratory animal. This produces swelling at the site of injection 24-72 hours later, hence the name delayed-type hypersensitivity. This swelling is a result of the recognition of the antigen by memory T cells, which initiate the process of inflammation and recruitment and activation of macrophages (Abbas *et al.*, 1991).

The humoral response also plays an important role in the immune response to *S. enterica* infection (Mastroeni *et al.*, 1993). The predominant antibody response to *S. enterica* is directed at the surface of the bacteria (*i.e.* the LPS or flagella) but recently a number of reports have shown responses are also directed to intracellular protein antigens (Brown and Hormaeche, 1989). In the present study, the ability of T cells to recognise AhpC and GroEL was addressed by examining the DTH responses to these proteins, after injection into the footpads of mice which were previously infected with *S. typhimurium*. In addition, the antibody response to AhpC or GroEL was determined by sampling the serum of animals at various time intervals post-infection and assessing whether a response had occurred by immunoblotting.

7.2 RESULTS

7.2.1 Examination of the cell-mediated responses of mice infected with *S. typhimurium*

The aim of this section was to determine whether the AhpC and GroEL proteins are recognised by T cells during the course of *S. typhimurium* infection. This was addressed by examining whether mice previously exposed to an attenuated strain of *S. typhimurium* generated a DTH reaction (an *in vivo* manifestation of a cell-mediated immune response) after subcutaneous injection of these proteins. Previous studies have suggested that when BALB/c mice are infected with an attenuated *aroA* strain of *S. typhimurium*, the bacteria persist for approximately 2 months before being fully cleared from the mouse (Mastroeni *et al.*, 1993). The cell-mediated responses were therefore examined at two time points, the first when the mice should still contain viable *S. typhimurium* organisms and the second when they should be pathogen-free. This would indicate whether any immune response to AhpC or GroEL was a transient feature or whether these proteins could be identified as part of the immunological memory to *S. typhimurium* infection.

Construction of an attenuated *aroA* mutant of a pathogenic strain of *S. typhimurium*

Hosieth and Stocker (1981) constructed and characterised an *aroA* mutant of a pathogenic strain of *S. typhimurium*. Injection of this mutant into susceptible mice resulted in protection against a subsequent challenge by a lethal dose of pathogenic organisms and this protection was shown to require both antibody and cell-mediated immune responses (Mastroeni *et al.*, 1993). Therefore, this mutation was used in the present study since a large body of information exists about its attenuating properties. An *aroA* transposon insertion, *aroA554*::Tn*10*, was moved by transduction with phage P22 *HT int4* (Roth, 1970) from SL1346 (Hosieth and Stocker, 1981) into a pathogenic strain of *S. typhimurium* (SL1344) to generate MPG479, for use in the present study.

Cell-mediated responses to AhpC and GroEL 33 days post-infection of mice with *S. typhimurium*

Four groups of six female BALB/c mice, aged 8-10 weeks (15-20 g), were injected intraperitoneally (i.p.) with either 1.78 x 10^5 c.f.u. of MPG479 in 100 µl of phosphate buffered saline (PBS) (Groups 1 and 2) or with 100 µl PBS alone (Groups 3 and 4). MPG479 was prepared on the day by taking an overnight culture, grown in the presence of 10 µg ml^{-1} tetracycline, and washing and diluting it in PBS. The number of organisms injected was determined by sample dilution and plating onto solid LB media containing tetracycline. The dose of organisms was chosen because Hosieth and Stocker (1981) had previously shown that a similar bacterial inoculum had resulted in no visible signs of illness in the mice, yet conferred substantial protection to challenge by a virulent strain of *S. typhimurium* (SL1344), thus indicating that a suitable immune response had been generated.

After infection, mice were placed in cages, no more than three to a cage, and the animals were supplied with a commercial pellet diet and water *ad libitum*. Thirty three days after infection, DTH reactions to the purified AhpC or GroEL proteins (see chapter 6 for purification details) were stimulated by subcutaneously (s.c.) injecting, the left hind footpad (LHFP) of each mouse with 50 µl of PBS containing either 40 µg of heat aggregated AhpC (Groups 1 and 3) or GroEL (Groups 2 and 4). As a control to show that any increase in footpad size was a specific response to the injection of the proteins, the right hind footpad (RHFP) of each mouse in all the groups was injected with 50 µl of PBS only. Uninfected mice, treated similarly were used as an additional control. These experimental details are summarised in Table 7.1.

After 24 and 48 hours, the thickness of the RHFPs and LHFPs were measured in thousandths of an inch, taking measurements of each footpad at three different positions, using dial-type callipers. The thickness of the LHFP (protein injected) was then divided by the thickness of the RHFP (saline injected) to give a footpad

Table 7.1 A summary of the experimental protocol for examining the DTH response to AhpC and GroEL in mice 33 days post-infection with *S. typhimurium*.

Mouse Group	Treatment (Day 0)	DTH challenge (Day 33) (LHFP/RHFP)
1	*S. typhimurium* infected[(a)]	AhpC/PBS[(c)]
2	*S. typhimurium* infected	GroEL/PBS[(d)]
3	Saline control[(b)]	AhpC/PBS
4	Saline control	GroEL/PBS

Each group of mice consisted of six female BALB/c mice, 8-10 weeks of age (15-20 g). Mice were injected i.p. with (a) 1.78 x 10^5 c.f.u. of the *aroA S. typhimurium* mutant, MPG479, in 100 µl of PBS or (b) 100 µl of PBS only and were challenged 33 days later by injecting the LHFP s.c. with 50 µl of PBS containing 40 µg of heat aggregated (c) AhpC or (d) GroEL, and the RHFP was injected with 50 µl PBS only. LHFP, left hind footpad; RHFP, right hind footpad; s.c., subcutaneously.

thickness ratio.The ability of AhpC or GroEL to elicit a DTH response could then be determined by comparing the mean increase in footpad ratio per group between infected and uninfected control mice (Group 1 versus 3 and Group 2 versus 4, for AhpC or GroEL, respectively). The results of this experiment are outlined in Figure 7.1. The statistical significance of the results was calculated by the Student's *t*-test method.

It should be noted that a control group, infected initially with bacteria and challenged on day 33 by the injection of 50 μl of PBS into the LHFP only, showed a small increase in footpad thickness when compared with the uninjected RHFP. This was significant after 48 hours ($p<0.05$) but not at 24 hours (data not shown). This suggests that subcutaneous injection of PBS elicits a small amount of swelling in the absence of antigen. However, the increase was very small when compared to that of mice injected with either antigen.

It was found that injection of AhpC or GroEL into infected mice (Group 1 or 2, respectively) resulted in a significantly greater increase in footpad size than in uninfected mice after 24 hours ($p<0.01$ for each). For example, injection of the LHFP with AhpC resulted in a 32% increase in the footpad thickness compared to the PBS injected RHFP (Group 1) after 24 hours, in infected mice, whereas only a 15% increase was observed in uninfected mice (Group 3). Similarly, injection of the LHFP with GroEL resulted in a 30% increase in footpad thickness compared to the PBS injected RHFP (Group 2) after 24 hours, in infected mice, whereas only a 9% increase was recorded in uninfected animals (Group 4). These relative differences in thickness were maintained after 48 hours ($p<0.001$ for both proteins), even though the overall thickness in both groups declined from that seen at 24 hours. This suggests that AhpC and GroEL are capable of eliciting a DTH reaction and indicates that these proteins, or epitopes thereof, are specifically recognised by a sub-population of the T cell repertoire during the course of *S. typhimurium* infection of mice.

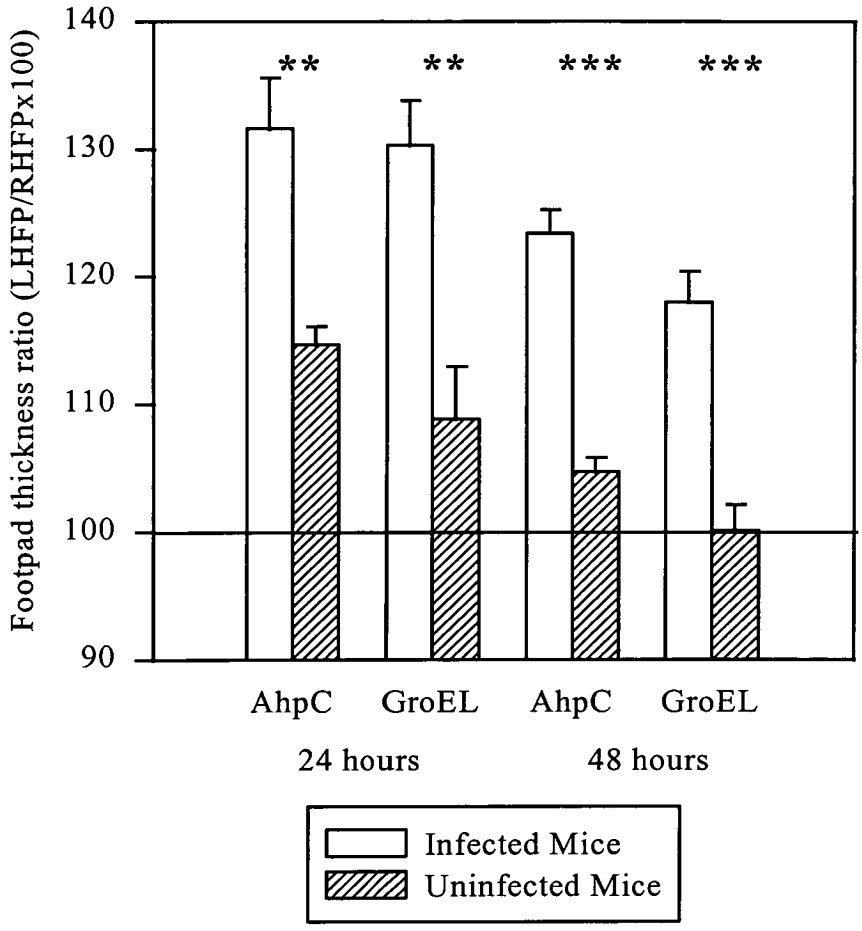


Figure 7.1 Cell-mediated responses of mice challenged with AhpC and GroEL (Day 33)

Four groups of six female BALB/c mice, 8-10 weeks of age (15-20 g), were injected i.p. with 1.78 x 10^5 c.f.u. of an *aroA S. typhimurium* mutant, MPG479, in 100 µl of PBS, or with 100 µl of PBS only. 33 days later mice were challenged by injecting the LHFP with 40 µg of heat aggregated AhpC or GroEL in 50 µl of PBS and the RHFP with 50 µl PBS only (See Table 7.1). After 24 and 48 hours the thickness of the LHFP and RHFP were measured and the increase in the mean left footpad thickness was expressed as a ratio (LHFP/RHFP x 100) ± SEM. Key: LHFP, left hind footpad; RHFP, right hind footpad. Uninfected mice and mice infected with *S. typhimurium* are represented by the shaded and empty bars respectively. The increase in footpad thickness in infected mice after injection of AhpC or GroEL was compared with those in uninfected mice using the Student's *t*-test and the level of statistical significance of this comparison is represented by asterisks: ** $p<0.01$; *** $p<0.001$.

Interestingly, the increase in footpad thickness in uninfected mice injected with either AhpC or GroEL appeared greater than might be expected for animals that were theoretically naive to these antigens. The significance of this enlargement is best examined by comparing the size of the LHFP (injected with antigen) relative to the RHFP (injected with saline) *within* the group because this takes into account any possible effect of injection with saline. An examination of Groups 3 (AhpC injected) and 4 (GroEL injected) suggested that AhpC but not GroEL resulted in a significant increase in footpad thickness at 24 hours in uninfected mice ($p<0.001$ and $p=0.16$, respectively). However, the lack of significance in the increase of footpad thickness in Group 4 may have reflected on the fact that one mouse had an unusually thick RHFP. When the measurements for this mouse were removed and the significance recalculated, the increase in footpad thickness due to injection of GroEL was shown to be significant ($p<0.05$) compared to the saline control. Nevertheless, the effects of AhpC and GroEL were only short lived in these mice and the difference in footpad thickness at 48 hr was not significant. In summary, it is clear that both AhpC and GroEL induce a transient increase in footpad thickness in uninfected animals, greater than that of the saline injected footpads, but this is not nearly as great or as prolonged as the enlargement observed in infected mice. In addition, the induction of a cell-mediated response in these uninfected mice indicates that there may have been previous immunological recognition of AhpC or GroEL (or even homologous antigens).

Cell-mediated responses to AhpC and GroEL 104 days post-infection of mice with *S. typhimurium*

A similar protocol to that described for the antigen challenge at day 33 was used to examine the cell-mediated responses to AhpC and GroEL at 104 days post-infection, a time when the mice should have cleared the bacterial load. Four groups of six BALB/c mice, 8-10 weeks old (15-20 g), were either injected i.p. with 1.78×10^5 c.f.u. of MPG479 in 100 µl of PBS (Groups 5 and 6) or, 100 µl of PBS alone (Groups 7 and 8). Mice were then maintained, as described previously. 104 days after infection, the LHFP of each mouse was injected s.c. with 40 µg of AhpC (Groups 5

and 7) or GroEL (Groups 6 and 8) in 50 µl of PBS and the RHFP of each mouse, in all groups, was injected with 50 µl of PBS alone. These experimental details are summarised in Table 7.2.

After 24 and 48 hours the thickness of the LHFP and RHFP, and the footpad thickness ratio, were determined as previously stated for the antigen challenge at day 33. The results of this experiment are shown in Figure 7.2. As in Figure 7.1 the footpad thickness of infected mice injected with either AhpC or GroEL was significantly greater than that of uninfected animals ($p<0.05$ and $p<0.001$, respectively). For example, after 24 hours the AhpC injected LHFPs were 20% thicker than the PBS injected RHFPs in infected mice (Group 5) but only 13% thicker in uninfected mice (Group 7). Similarly, the GroEL injected LHFPs were 24% thicker than the PBS injected RHFPs in infected mice (Group 6) but only 8% thicker in uninfected mice (Group 8). As with the antigen challenge experiment at day 33, the DTH responses were still apparent 48 hours post-challenge ($p<0.001$ and $p<0.01$, respectively). This again supports a role for these proteins in the initial stimulation of T cells during infection, and in the generation of a cell-mediated response and immunological memory. Nevertheless, the overall increase in thickness after injection with the proteins was less than observed at 33 days post-infection. The mice challenged at 104 days were killed 10 days later and shown to contain no viable organisms in the liver or spleens (data not shown). This may suggest that the reduction in response is linked to the decline in antigenic stimulation following elimination of the bacteria.

In agreement with the results obtained at day 33, injection of either AhpC or GroEL into the left footpad of uninfected animals was shown to result in a small but significant increase in footpad thickness ($p<0.01$ and $p<0.01$, respectively), over that in the saline injected footpad. In addition, this was again shown to be transient as by 48 hours no significant difference could be seen between the footpads injected with antigen or saline. These results again suggest that the mice may have had some prior immunological exposure to these antigens.

Table 7.2 A summary of the experimental protocol for examining the DTH response to AhpC and GroEL in mice 104 days post-infection with *S. typhimurium*.

Mouse Group	Treatment (Day 0)	DTH challenge (Day 104) (LHFP/RHFP)
5	*S. typhimurium* infected[(a)]	AhpC/PBS[(c)]
6	*S. typhimurium* infected	GroEL/PBS[(d)]
7	Saline control[(b)]	AhpC/PBS
8	Saline control	GroEL/PBS

Each group of mice consisted of six female BALB/c mice, 8-10 weeks of age (15-20 g). Mice were injected i.p. with (a) 1.78 x 10^5 c.f.u. of the *aroA S. typhimurium* mutant, MPG479, in 100 µl of PBS or (b) 100 µl of PBS only, and challenged 104 days later by injecting s.c. into the LHFP with 40 µg of heat aggregated (c) AhpC or (d) GroEL in 50 µl of PBS and the RHFP was injected with 50 µl PBS only. LHFP, left hind footpad; RHFP, right hind footpad; s.c., subcutaneously.

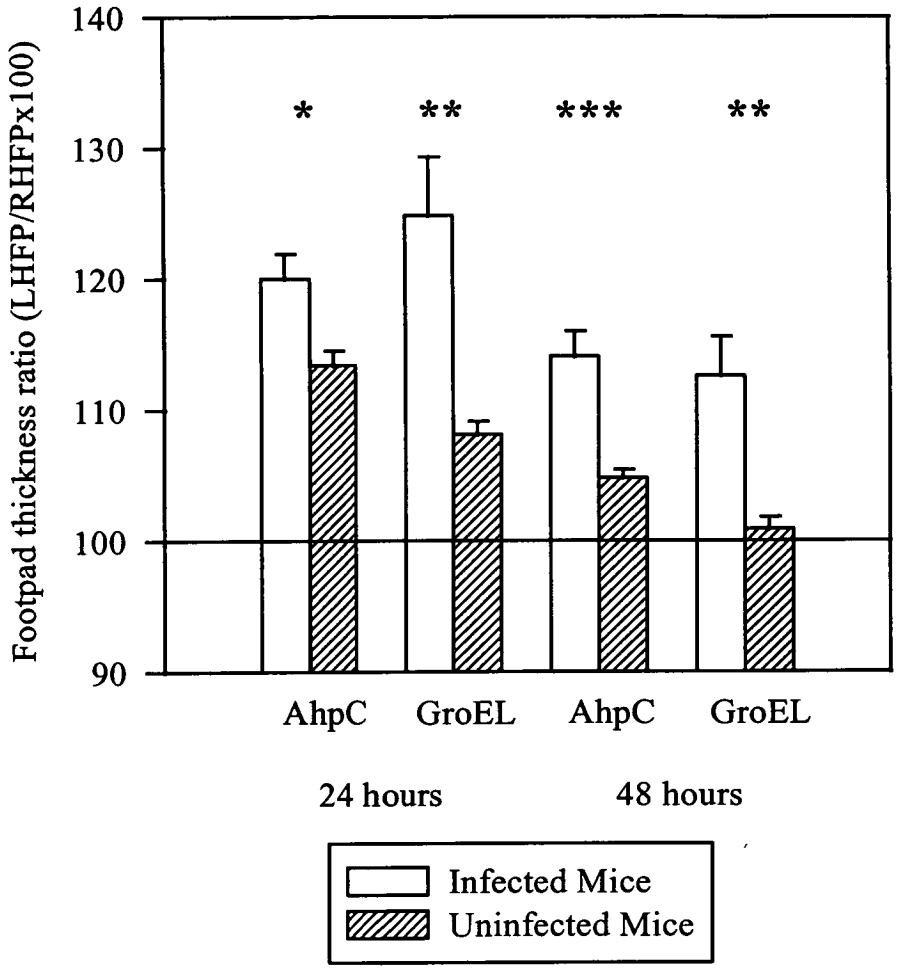


Figure 7.2 Cell-mediated responses of mice challenged with AhpC and GroEL (Day 104)

Four groups of six female BALB/c mice, 8-10 weeks of age (15-20 g), were injected i.p. with 1.78 x 10^5 c.f.u. of an *aroA S. typhimurium* mutant, MPG479, in 100 µl of PBS, or with 100 µl of PBS only. 104 days later mice were challenged by injecting the LHFP with 40 µg of heat aggregated AhpC or GroEL in 50 µl of PBS and the RHFP with 50 µl PBS only (see Table 7.2). After 24 and 48 hours the thickness of the LHFP and RHFP were measured and the increase in mean left foodpad thickness was expressed as a ratio (LHFP/RHFP x 100) ± SEM. Key: LHFP, left hind footpad; RHFP, right hind footpad. Uninfected mice and mice infected with *S. typhimurium* are represented by the shaded and empty bars respectively. The increase in footpad thickness in infected mice after injection of AhpC or GroEL was compared with those in uninfected mice using the Student's *t*-test and the level of statistical significance of this comparison is represented by asterisks: * $p<0.05$; ** $p<0.01$; *** $p<0.001$.

7.2.2 Antibody responses to AhpC and GroEL in mice infected with *S. typhimurium*

Previous investigations into the antibody responses of mice infected with *S. typhimurium* have shown that both surface and intracellular bacterial protein antigens are recognised (Brown and Hormaeche, 1989). In the present study, serum from infected animals was examined for the presence of antibodies to AhpC or GroEL. The mice which were subjected to the DTH experiments also permitted a study into the ability of these proteins to directly stimulate B cell responses. Mice were challenged on two occasions (see section 7.2.1): (i) 33 days after infection ('early' challenge; Table 7.1, Groups 1-4), when *S. typhimurium* was still present (Mastroeini *et al.*, 1993); (ii) 104 days after infection ('late' challenge; Table 7.2, Groups 5-8), when *S. typhimurium* had been cleared from the tissues (Mastroeni *et al.*, 1993). When antigen is injected subcutaneously into the mouse footpad, some of the protein moves to the draining lymph nodes either directly or via antigen presenting cells such as macrophages or Langerhans cells. Here, the protein stimulates memory T cells which recirculate to the footpad where they elicit a DTH reaction in response to the to presence of the protein. The stimulation of T cells in the draining lymph node would also be expected to promote the proliferation of B cells specific to the antigen, and result in antigen-specific antibody. To address this feature, the ability of these proteins to stimulate B cell responses in the draining lymph nodes was determined by comparing serum in infected and uninfected mice after early or late challenge for antibodies to AhpC or GroEL.

Examination of the resident antibody levels to AhpC or GroEL in uninfected mice

Prior to starting the experiments outlined in this chapter, all the mice were bled and the sera from each group was pooled. The presence of antibody specific to AhpC or GroEL was then investigated by Western blot analysis. This was an important step as it would determine whether any of the animals had had previous exposure to these or related antigens prior to the planned infection with *S. typhimurium*. Figure 7.3

displays examples of the results obtained from screening these pre-infection, pooled sera for the presence of AhpC- or GroEL-specific antibodies.

An SDS-polyacrylamide gel (12.5% [v/v]) (Figure 7.3 Panel A) was loaded with approximately 1 μg of purified his-tagged AhpC (lane A) or GroEL (lane G) and was subjected to electrophoresis. Since previous studies had indicated that a number of *S. typhimurium* proteins are identified by infected mice (Brown and Hormaeche, 1989), a whole cell extract of *S. typhimurium* (lane WC), grown to exponential phase, was included as a control to show whether there was a general background level of antibody specific to these proteins in the uninfected mice. Pre-stained molecular mass markers of 120, 75 and 35 kDa were included on the gel as a guide to the size (lane M). These stained markers transfer to the Western blot filter allowing the sizes of any bands identified by antibodies to be determined. This gel template was used throughout the Western blot analysis.

Equivalent gels were blotted onto nitro-cellulose and the filters were incubated in the presence of 1:200 dilutions of the different pooled sera. The presence of specifically bound antibody was then determined by incubation with rabbit anti-mouse alkaline phosphate-conjugated antibody. The sera, which was pooled from each of the mice within a group, showed a negative response with AhpC and either a negative response or, at most, a weak positive response with GroEL (see Figure 7.3, Panels H and I for visible GroEL-specific bands). The result for GroEL supports the evidence from the DTH studies which suggested that the uninfected mice may have been exposed to this or a related antigen prior to infection.

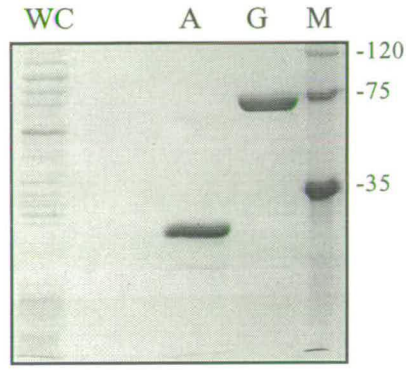
A number of other proteins were detected using the sera from some groups of uninfected mice. Indeed, bands at a different relative position to those of AhpC and GroEL were clearly discernible in the *S. typhimurium* whole cell extracts. The most commonly seen bands appeared to consist of a doublet of approximately 35-40 kDa and a lower band of approximately 15 kDa. The identity of these bands is uncertain, however, in previous studies bands at similar positions were postulated to be porins

Figure 7.3

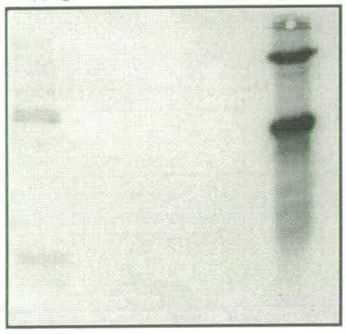
Determination of the presence of AhpC- or GroEL-specific antibody in serum of uninfected mice (pre-infection bleeds)

Western blot analysis was performed on the pooled sera from groups of mice prior to infection by an *aroA*::Tn*10* strain of *S. typhimurium*, MPG479. Panel A represents a standard gel profile used in all the Western blot analysis. The 12.5% polyacrylamide gel was loaded with a whole cell extract of *S. typhimurium* SL1344 (lane WC) and approximately 1 μg of AhpC (lane A) or GroEL (lane G). Pre-stained molecular mass markers of approximately 120, 75 and 35 kDa were included as a guide to size (lane M). Standardly, identical gels to that described above were electrophoresed and blotted onto nitro-cellulose. Each blot was then exposed to a 1:200 dilution of the pooled serum from a group of mice. Specifically bound antibody was then detected using a polyclonal rabbit anti-mouse antibody, conjugated to alkaline phosphatase. Panels B-I represent the blots from using the pooled sera of Groups 1-8 in this procedure. The arrangement of the lanes is identical to that described for the Coomassie Brilliant Blue-stained gel.

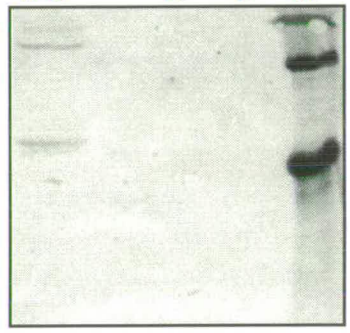
PANEL A



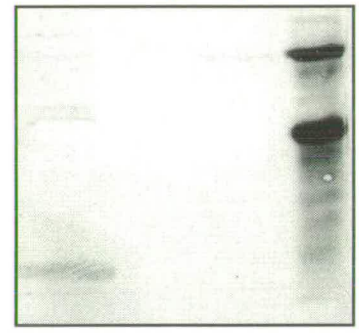
PANEL B



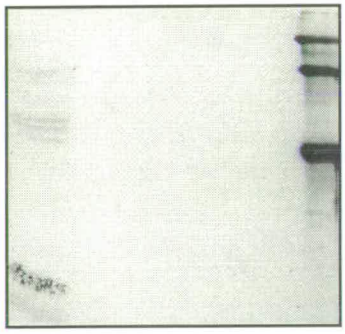
PANEL C



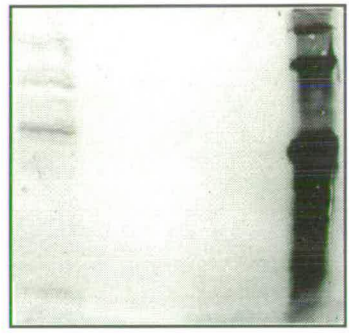
PANEL D



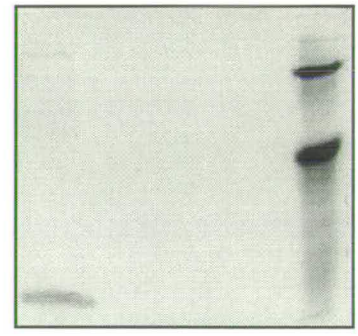
PANEL E



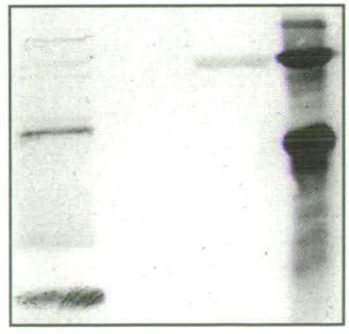
PANEL F



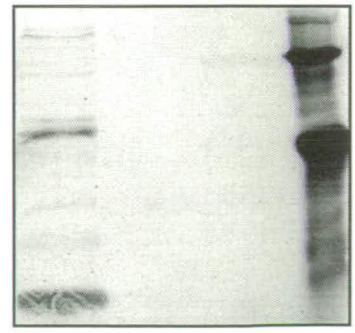
PANEL G



PANEL H



PANEL I



(~35 kDa) or lipoprotein (~15 kDa) (Brown and Hormaeche, 1989).

The pre-infection bleeds suggested that there were likely to be differences in the level of resident antibody to AhpC and GroEL, depending on the particular group studied. However, since pooled sera were analysed in these experiments, it is impossible to tell whether the antibody responses are true for all the mice in a particular group or if the results reflect cage- or even mouse-specific phenomena. As such, these blots should be seen as a reflection of the typical spectrum of background levels of antibody to AhpC and GroEL in uninfected mice.

The antibody responses to AhpC or GroEL in mice infected with *S. typhimurium*

The development of antibody responses to either AhpC or GroEL during the course of an infection was determined by examining the serum from mice at 14 and 28 days post-infection. A group which had displayed no AhpC- or GroEL-specific antibodies prior to infection was chosen for this study (Group 1). However, as stated above, this does not rule out the possibility that any of the mice in this group had previously been exposed to either antigens.

Gels were run and blotted as described previously. The antibody responses of infected mice is shown in Figure 7.4. For the purpose of comparison, the pre-infection bleed (Day 0) was included. This showed that although some *S. typhimurium* polypeptides were detectable using the serum from uninfected mice, AhpC and GroEL were not amongst them (Figure 7.4 Panel A). By Day 14 a band that corresponded to AhpC (Figure 7.4 Panel B) was clearly detectable, but not to GroEL. This suggests that the kinetics of the response to these proteins may be different. As might be expected, the detection of other *S. typhimurium* polypeptides also increased as the immune response developed to the bacterial infection (Figure 7.4 Panel B). After 28 days, from the point of infection, however, responses to both AhpC and GroEL (Figure 7.4 Panel C) were detectable, GroEL more so than AhpC. The response to AhpC appeared weak (given the level of protein loaded in the track), and not much greater than that seen on Day 14. In addition, the lane containing the

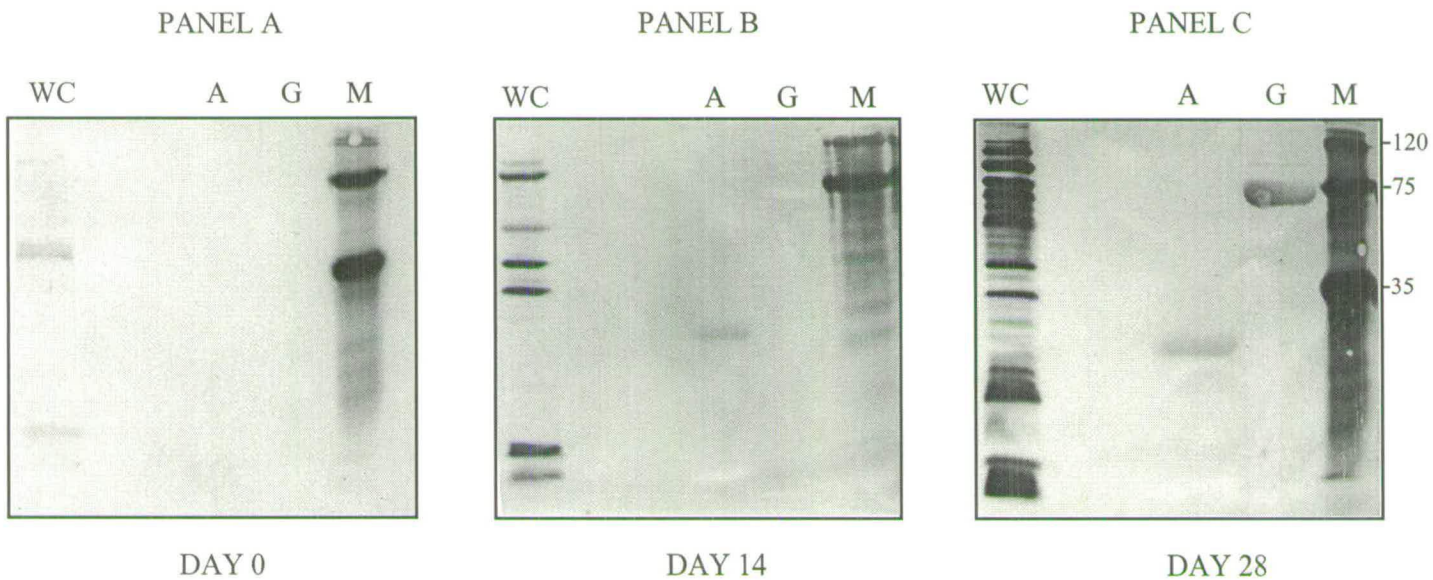


Figure 7.4 The development of an antibody response to AhpC or GroEL in mice after infection with *S. typhimurium*

Western blot analysis was performed on the sera from mice in Group 1, prior to infection (day 0, Panel A) and 14 days (Panel B) and 28 days (Panel C) after infection with an *aroA*::Tn*10* strain of *S. typhimurium,* MPG479. All features of the blots and the identification of specifically bound antibody are described in the legend for Figure 7.3. Blots were treated with a 1:200 dilution of the pooled serum from Group 1. Key to lanes: WC, whole cell extract of *S. typhimurium* SL1344; A, AhpC, G, GroEL; M, pre-stained molecular mass markers of 120, 75 and 35 kDa.

whole cell extract was extremely heavily stained, suggesting that many *S. typhimurium* polypeptides can elicit some form of humoral response in infected mice (Figure 7.4 Panel C).

7.2.3 The antibody responses of mice subcutaneously injected with AhpC or GroEL

Although infected mice can tell us whether immune responses are generated to AhpC or GroEL during infection, it does not indicate how antigenic the native proteins are. In order to explore this issue, the mice challenged in the DTH experiments were sampled for serum preceding and following challenge with AhpC or GroEL and immunoblotting was used to compare serum samples for the presence of specific antibody to these proteins in uninfected and infected mice. This would indicate whether it was possible to stimulate antibody responses in the draining lymph nodes of mice subcutaneously injected with these antigens.

The antibody responses of uninfected mice subcutaneously injected with AhpC or GroEL

Mice previously injected with saline were injected 33 days later with either AhpC or GroEL (Groups 3 and 4). Serum samples from these mice were obtained 10 days post challenge (day 43) and Western blot analysis was performed. As can be seen from Figure 7.5, subcutaneous injection of these antigens resulted in a negative response to AhpC (Figure 7.5 Panel A) and a strong positive response to GroEL (Figure 7.5 Panel B). The post-challenge blot for AhpC effectively resembles a pre-infection bleed, suggesting that within the 10 days post-challenge little or no antibody response has been generated to this antigen. This is in contrast to the observation that AhpC was capable of eliciting a small but significant T cell response after subcutaneous injection of antigen at the same site (the footpad) in uninfected animals. On the other hand, the intensity of the GroEL-specific band suggests that a substantial response had been generated to this antigen. In fact, as a result of this subcutaneous injection, the antibody is capable of detecting GroEL within the whole

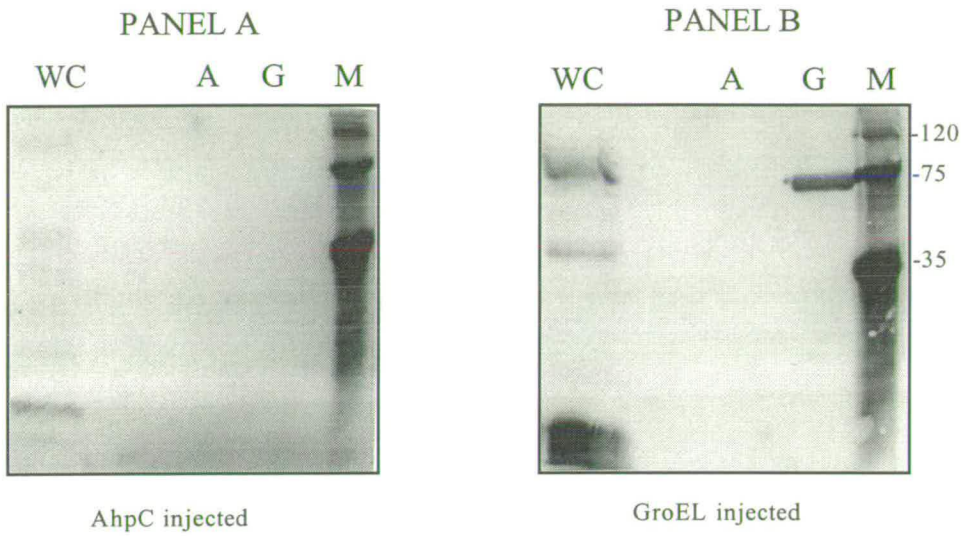


Figure 7.5 Antibody responses to subcutaneously injected AhpC and GroEL in uninfected mice

Western blot analysis was performed on the sera from uninfected mice 10 days post-challenge with AhpC or GroEL. All features of the blots and the identification of specifically bound antibody are as described in the legend for Figure 7.3. Panel A and Panel B show the bands obtained after using a 1:200 dilution of the pooled serum from uninfected mice challenged with AhpC (Group 3) or GroEL (Group 4), respectively. Key to lanes: WC, whole cell extract of *S. typhimurium* SL1344; A, AhpC; G, GroEL; M, prestained molecular mass markers of 120, 75 and 35 kDa.

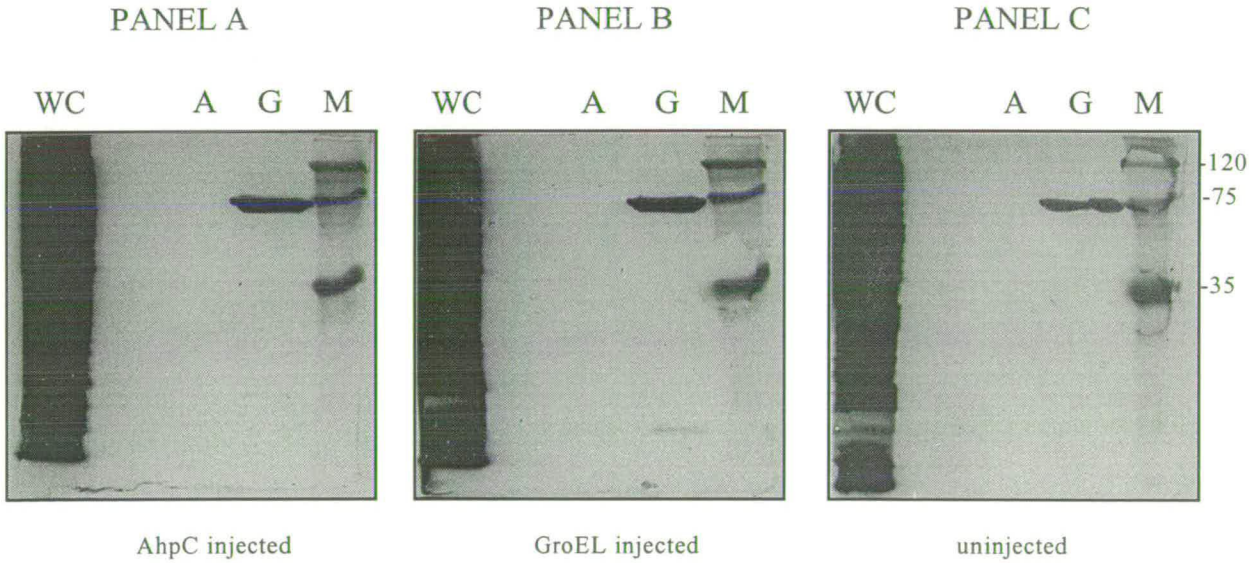


Figure 7.6 Antibody responses to subcutaneously injected AhpC and GroEL in mice challenged 33 days after infection and bled 10 days later

Western blot analysis was performed on the sera from mice infected with MPG479 10 days after challenge with AhpC or GroEL or from infected mice not used in the challenge experiment on day 33. All features of the blots and the identification of specifically bound antibody are as described in the legend for Figure 7.3. Panels A, B and C show the bands obtained with a 1:200 dilution of the pooled serum from infected mice challenged with AhpC (Group 1) or GroEL (Group 2) or infected mice not used in the challenge experiment on day 33 (Group 5), respectively. Key to lanes: WC, whole cell extract of *S. typhimurium* SL1344; A, AhpC; G, GroEL; M, pre-stained molecular mass markers of 120, 75 and 35 kDa.

cell extract, which is at a much lower concentration on the gel than the pure sample (Figure 7.5 Panel B). (It is also noteworthy that no GroEL-specific antisera was detected in pre-immune sera from Group 4 (Figure 7.3, Panel E) prior to GroEL injection).

The antibody responses of infected mice subcutaneously injected with AhpC or GroEL

The results shown in Figure 7.5, obtained from serum in uninfected animals, were compared with the serum samples from infected mice which were challenged at day 33 with AhpC (Group 1) or GroEL (Group 2) and bled 10 days later. An additional control group, infected with MPG479 but not challenged with protein on day 33 (Group 5), was also included. Figure 7.6 shows the antibody responses to AhpC and GroEL in these animals. A negligible response to AhpC was observed, irrespective of whether the animals were challenged with AhpC, GroEL or left unchallenged (Figure 7.6 Panels A, B, C). Importantly, it should be noted that infected mice had been demonstrated to develop some form of antibody response to AhpC at day 28 during the course of infection (Figure 7.4 Panels A, B, C). Nevertheless, irrespective of whether the mice were infected (Figure 7.6) or pathogen free (Figure 7.5), the subcutaneous injection of AhpC appeared to result in poor stimulation of B cell responses.

The situation for GroEL is less clear, the subcutaneous injection of GroEL did not appear to enhance the antibody response, as the GroEL-specific bands from mice left unchallenged (Figure 7.6 Panel C), or challenged with AhpC (Figure 7.6 Panel A) or GroEL (Figure 7.6 Panel B) appeared of approximately equal intensity. Moreover, a comparison of the intensity of the GroEL-specific bands after subcutaneous injection of GroEL into uninfected (Figure 7.5 Panel B) and infected mice (Figure 7.6 Panel B) did not suggest that there was any enhancement of the response to the antigen in the infected animals. However, this may simply reflect that the Western blotting detection procedure was saturated and was not capable of displaying further changes in the level of GroEL-specific antibody. Equally, the relevance of small variations in

the results is difficult to assess by comparing blots which were developed independently.

Interestingly, the examination of the sera from mice which were infected but not challenged (Figure 7.6 Panel C) suggested that the general antibody response to *S. typhimurium* had increased further since day 28, as had the antibody response to GroEL (Figure 7.4 Panel C). In contrast, there was no detection of an AhpC-specific band suggesting that the response to this protein had declined since day 28.

7.2.4 Summary of the antibody responses to AhpC or GroEL in mice

The antibody responses of uninfected and infected mice to AhpC and GroEL are summarised in Table 7.3. Also included in this table are the preliminary results from analysing whether antibody to AhpC (Group 5) and GroEL (Group 6) are present in mice challenged on day 104 post-infection and bled 10 days later. Unfortunately no pre-challenge bleeds were available for these mice. However, after using the sera from these mice, a relatively intense GroEL-specific band was seen on both blots (Figure 7.7 Panels A and B) suggesting that the GroEL-specific antibody present had been generated during the bacterial infection. No AhpC-specific antibody was detected in the sera from the mice challenged with AhpC.

7.3 DISCUSSION

The AhpC and GroEL proteins were shown to be recognised by both the cell-mediated and humoral arms of the immune system in mice previously infected with *S. typhimurium*. When infected mice were challenged subcutaneously with AhpC or GroEL, a significant increase in footpad swelling was observed compared to that of uninfected mice. This was seen for both early (day 33) and late challenges (day 104), indicating that both proteins are recognised by subpopulations of the T cell repertoire. The fact that both proteins also elicited a significant response at day 104 emphasises the likelihood that part of the immunological memory to *S. typhimurium* infection is derived from these proteins. In support of this, the

Table 7.3 Summary of the antibody responses to AhpC and GroEL in uninfected and infected mice

	Detection of antibody to *S. typhimurium* proteins	
Serum sample	**AhpC**	**GroEL**
Pre-infection (day 0)	–	+[(a)]
Post-infection (day 14) [(b)]	+	–
Post-infection (day 28) [(b)]	+	++
Challenged (day33)* uninfected	–	++
Challenged (day 33)* post-infection	–	+++
Post-infection (day 43)	–	+++
Challenged (day 104)* post-infection	–	+++

Results represent a qualitative assessment of the antibody responses in different mice, as interpreted from the intensities of bands on Western blots. Key: *, mice were challenged with either AhpC or GroEL on the day indicated in brackets and serum was obtained 10 days later; (a) GroEL-specific antibody detected in some of the pre-infection blots; (b) sera was examined from a mouse group (Group 1) which had displayed no AhpC- or GroEL-specific antibody prior to infection; –, negative response, no visible band detected; +, weak positive response, faint band observed; ++, positive response, clear band observed; +++, strong positive response, intense band observed.

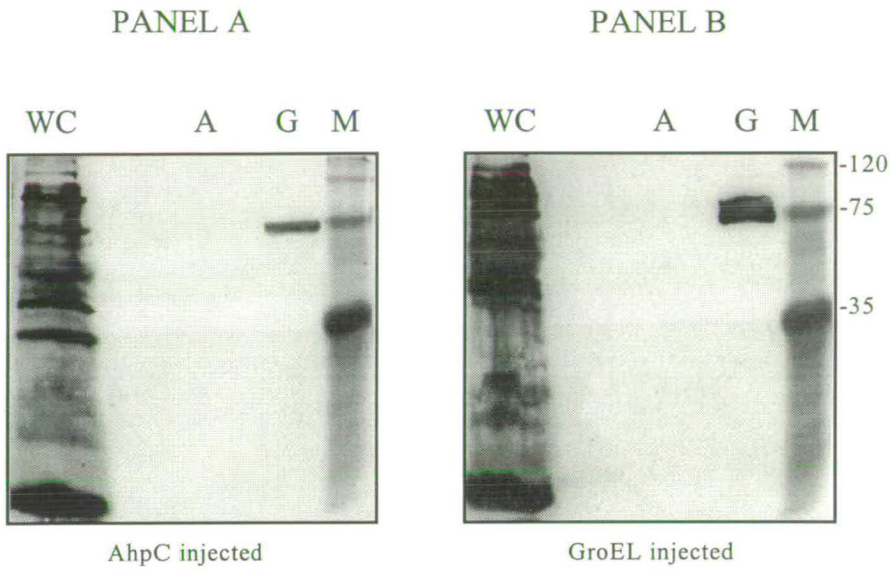


Figure 7.7 Antibody responses to subcutaneously injected AhpC and GroEL in mice challenged 104 days after infection and bled 10 days later

Western blot analysis was performed on the sera from mice infected with MPG479 10 days after challenge with AhpC or GroEL on day 104. All features of the blots and the identification of specifically bound antibody are as described in the legend for Figure 7.3. Panel A and Panel B show the bands obtained from using a 1:200 dilution of the pooled serum from infected mice challenged with AhpC (Group 5) or GroEL (Group 6), respectively. Key to lanes: WC, whole cell extract of *S. typhimurium* SL1344; A, AhpC; G, GroEL; M, pre-stained molecular mass markers of 120, 75 and 35 kDa.

bacteria are believed to be cleared approximately 8 weeks after infection (Mastroeni *et al.*, 1993), and examination of tissues from mice killed in this study at 114 days post-infection proved that no viable *S. typhimurium* organisms were present in the liver and spleens at this time. Therefore, any response observed in the mice at day 104 post-infection, at a time when the immune response to the initial infection has dampened down, is likely to be derived from the stimulation of memory T-cells.

However, the B cell response to AhpC and GroEL differed. The absence of antibody in normal mice injected subcutaneously with AhpC indicated that AhpC was not particularly immunogenic (Figure 7.5). Although the epitopes which B and T cells can recognise are different (Abbas *et al.*, 1991), this result would appear slightly unusual given that the injection of AhpC resulted in a significant T cell-dependent response in both the early and late challenge experiments of the uninfected animals. In these mice, even when taking into consideration the possibility of some form of previous exposure, the number of specific B cells is likely to be low and possibly accounts for the lack of response at the time of examination. It is also likely that the site of administration of the antigen was suboptimal for eliciting such a response, especially since the majority of the injected protein would be expected to end up at the local draining lymph nodes rather than in the spleen. It should be noted however, that these results do not exclude the possibility that an antibody response did form, albeit outwith the test period.

In contrast, infected mice clearly developed antibody responses to AhpC during the course of infection (Figure 7.4), suggesting that a pool of specific B cells were present. Challenge of these infected mice with AhpC would therefore be expected to produce a greater antibody response. However, analysis of serum samples obtained 10 days after challenge failed to show, within the limitations of the technique, that there had been an increase in AhpC-specific antibodies (Figure 7.6 and Figure 7.7 for challenge at 33 and 104 days post-infection, respectively). The reasons for the discrepancies in the T and B cell responses to AhpC are unknown. As mentioned above, it seems likely that the site of administration of the antigen was the major factor for the lack of an antibody response. However, other explanations are also

possible. Perhaps, the 'pure' sample of AhpC contained minor yet immunogenic impurities from the protein purification procedure. Alternatively, the swelling in the mouse footpad may have been a consequence of general irritation from the heat aggregated protein. Either of the scenarios would enhance the level of measured footpad size and would falsely reflect a greater T cell response. It may also be possible that the antibody response to AhpC, which was observed in the early stages of infection (Figure 7.4) but not in the later stages (Figure 7.6), was due to some ability of the infecting *S. typhimurium* to modulate the immune response as the infection ensued. The bacteria themselves, perhaps via the LPS, possibly acted as a B cell adjuvant to boost the ability of B cells to form a response to AhpC. LPS is a B cell mitogen and might well have influenced the response to AhpC when the bacteria were present. Such a scenario would support the fact that AhpC is a poor B cell immunogen *per se* and might explain why uninfected animals failed to elicit a response to subcutaneously injected antigen. This could be tested experimentally by immunising mice with graded amounts of LPS in the presence of a fixed dose of AhpC and examining the sera from these animals for AhpC-specific antibody.

In contrast to the results with AhpC, the antibody response to GroEL, although it developed less rapidly, was of a greater magnitude (as determined by visual inspection of Western blots) and duration, with substantial antibody present even after 114 days (Figures 7.4, 7.6 and 7.7). This suggests that GroEL is highly immunogenic. However, this may reflect heavily on the relative abundance of this protein in the bacterial cell. For example, the normal concentration of GroEL in unstressed *E. coli* cells is approximately 1%, but this rises to become almost 10% under stress conditions (Hemmingsen *et al.*, 1988), while AhpC appears to be a minor component of the cell, even under optimal inducing conditions (See chapter 3 of this thesis). Nevertheless, for the same dose of protein, administered subcutaneously into uninfected mice, only GroEL was shown to elicit an antibody response (Figure 7.5).

The DTH challenges in uninfected animals suggested that the mice had encountered AhpC and GroEL previously. This pre-exposure, at least for GroEL, was further

indicated by the presence of specific antibodies in the pre-infection bleeds from some of the groups of mice. Primary antibody responses are characterised by the predominance of IgM. However, multiple exposures to an antigen result in a secondary response which is characterised by an antibody isotype switch to produce IgG and an increase in the titre of specific antibodies (Weir and Stewart, 1993). Thus, any pre-exposure of the mice to AhpC or GroEL would influence the type and level of antibody present.

A polyclonal rabbit anti-mouse antiserum that detected all the mouse antibody isotypes was used in the Western blot analysis therefore, the type of antibody present in serum samples could not be determined. In addition, this procedure was relatively insensitive to the antibody titre. In order to conclude whether the antibody responses generated were primary or secondary, the isotype and titre would have to be determined, ideally using a germ-free mouse, which could not have been exposed to the *S. typhimurium* antigens, as a control. This could be best performed by using an enzyme linked immunosorbant assay (ELISA), using limiting dilutions of the serum samples and specific anti-isotype antibody. This sort of procedure would also have helped determine whether the GroEL challenge in infected mice resulted in an increase in specific antibody, as no visible increase could be determined by examination of the blots (Figure 7.6 and Figure 7.7), possibly because the blot was saturated with antibody.

The nature of any previous immunological recognition of AhpC and GroEL is uncertain. Since these mice were not germ-free they will have large numbers of microorganisms colonising their external and internal surfaces, including the skin and the mucosal lining of the intestinal tract. It is likely that some of these organisms contain homologues of AhpC or GroEL, as both proteins appear to be conserved in a number of bacterial species. For example, the *S. typhimurium* AhpC protein shows 92% identity to that of *E. coli* and approximately 50% identity to that of *Staphylococcus aureus* (Smillie, 1994; Armstrong-Buisseret, 1995). Although the amino acid sequence of the *S typhimurium* GroEL protein has not been determined,

the equivalent molecule from *S. typhi* has been shown to have 98.5% identity to its *E. coli* counterpart (Lindler and Hayes, 1994).

Normal commensal organisms that colonise the mouse would therefore seem a likely source of cross-reacting antigens which may be encountered by the immune system. In addition, immunological memory has been postulated to involve frequent cross-reactive re-stimulation, possibly by exposure to non-pathogenic organisms (Beverley, 1990). Since *E. coli* forms a predominant part of the normal gut flora, it is quite possible that these *E. coli* proteins provided the mice with immunological memory to AhpC and GroEL prior to infection with *S. typhimurium*, although it must be remembered that no AhpC-specific antibody response was detected in uninfected mice.

An alternative explanation for pre-existing immune responses to the *S. typhimurium* antigens may be the immunological recognition of cross-reacting proteins in the mouse, particularly GroEL. GroEL is highly conserved in both eukaryotes and prokaryotes and has been implicated as a causative agent in a number of autoimmune diseases (reviewed in Kaufmann, 1990 and described later). The *S. typhi* GroEL is approximately 51% identical to that of the mouse mitochondrial GroEL homologue and it could be that some of the humoral response to the *S. typhimurium* GroEL has been generated from immune recognition of the mouse homologue.

Immune responses to *S. enterica* infection are generally controlled by $CD4^+$ helper T cells because the bacterial antigens are processed in an endocytic compartment of antigen presenting cells and presented in the context of the class II Major Histocompatibility Complex (MHCII) (Abbas *et al.*, 1991). However, helper T cells (T_H) have been split according to the cytokines they produce and the immune responses they generate (reviewed in Abbas *et al.*, 1996). T_H1 cells typically produce cytokines such as IFN-γ and IL-2, and generate cell-mediated responses with some antibody production. On the other hand, T_H2 cells typically produce cytokines such as IL-4, IL-5 and IL-10 and the immune response is characterised by a predominance of antibody. However, it has been suggested that these two subsets are not distinct

cell types but reflect the development, dictated by the type of infection, from a common type of T cell (Kelso, 1995). In this way, the infection is believed to select the correct phenotype of T cell to regulate the relevant response. For example, it makes sense that infection by a pathogen capable of surviving inside host cells (*e.g. S. enterica*) would result in a T_H1 response, where activation of macrophages and other cell-mediated responses would help in the killing of intracellular bacteria. In fact, the development of the inappropriate response has been associated with the exacerbation of some disease states, such as seen with the intracellular pathogen *Mycobacterium tuberculosis*, where a T_H2 response leads to prolonged infection and poor resolution of the disease (Kaufmann, 1993; Abbas *et al.*, 1996). The studies performed in this chapter add to the conclusions that the immune responses generated to *S. typhimurium* infection are regulated by T_H1 cells.

Recent research into the development of vaccines to *S. enterica* infection has focused on live attenuated organisms (reviewed in Chatfield *et al.*, 1992a). Attenuated *S. typhimurium* cells produce much greater immunological protection in mice than killed cells, by virtue of the development of a strong cell-mediated immune response (Collins, 1974; Mastroeni *et al.*, 1993). However, the ability of such vaccines to produce better protection has been attributed to the production and recognition of bacterial proteins, induced in response to the host environment (Kagaya *et al.*, 1992). The currently used vaccine against *Mycobacterium tuberculosis* and *Mycobacterium leprae* infection is the live Bacille Calmette Guerin (BCG) vaccine. Like the *S. enterica* vaccines, the ability of the live BCG cells to induce a better protective response than killed cells has been attributed to the selective production of bacterial antigens in the host environment (Andersen and Heron, 1993; Andersen, 1994). These proteins appear to elicit strong cell-mediated responses and are thought to be an important factor in the elimination of *Mycobacteria* from the host. Very little information exists about the ability of individual *S. typhimurium* proteins to induce protective immune responses and, about whether such polypeptides have a potential use in prophylactic therapies to disease. Theoretically, subunit vaccines could be developed to include a small number of polypeptides that would induce protection to

the whole virulent bacterium (Horwitz *et al.*, 1995). The delivery mechanism would no doubt be of primary importance in influencing the outcome of such a strategy.

The immune responses to AhpC and GroEL, described here, raise the question of the relationship between mediators of bacterial virulence and the development of protective immunity. In a study with the intracellular pathogen *Legionella pneumophila*, it was found that vaccination with the Major Secretory Protein (MSP) resulted in strong protective immunity to a subsequent challenge with a virulent strain (Blander *et al.*, 1990). However, MSP itself is not directly involved in virulence since both wild type and *msp*$^-$ strains of *L. pneumophila* display the same level of virulence. Thus, protection does not necessarily depend on immune recognition of virulence determinants, rather, any molecule that has the potential to initiate an immune response in the presence of a pathogen can be seen as a candidate for a subunit vaccine or, as a component of one (Blander and Horwitz, 1991). In chapter 5 of this thesis, it was shown that *ahpC* was not essential for the full virulence of *S. typhimurium*. Nevertheless, in the present chapter, immune recognition of AhpC has been shown. Immune responses to proteins such as AhpC and GroEL or other *S. typhimurium* antigens could therefore be a key step on the road to developing a polypeptide based vaccine and to host protection.

Unfortunately, as yet, only a few *S. enterica*-derived components have been identified that can induce partial or full protective immunity against a virulent bacterial challenge. When *S. typhimurium* outer membrane proteins (OMPs) were subcutaneously injected into BALB/c mice (50 μg OMPs; immunised twice at a 15 day interval), 100% of the mice survived a lethal challenge of 50 times the normal LD_{50} level of the virulent *S. typhimurium* strain C5, one month after the last immunisation. Moreover, the protective immunity derived from the injection of the OMPs was maintained, with up to 70% of the immunised mice surviving the challenge after 6 months (Udhayakumar and Muthukkaruppan, 1987). In a separate study, the intraperitoneal injection of BALB/c mice with a stress induced catalase, KatG (50 μg; immunised twice at a 14 day interval), was shown to provide the mice with 50% protection, as measured by survival, against a challenge of 20 times the

normal LD_{50} level of the virulent *S. typhimurium* strain SH5170, three weeks after immunisation (Kagaya *et al.*, 1992). Also, in the latter study, examination of the liver and spleens of these animals showed that the immunised animals contained 100-1, 000 fold less bacteria than an unimmunised control (Kagaya *et al.*, 1992). Perhaps the most important property of these proteins is that, when suitably delivered, they are capable of inducing a T_H1 type of response, as judged by the development of DTH (Galdiero *et al.*, 1990; Kagaya *et al.*, 1992; Gupta *et al.*, 1996) or, by the detection of T_H1 response-associated cytokines in the tissue culture supernatants of monocytes (TNF-α and IL-1) after exposing these cells to the antigen (Galdiero *et al.*, 1993).

A number of studies have shown that other *S. typhimurium* proteins are also recognised in a T_H1-regulated immune response. For example, DTH reactions have been elicited in mouse footpads following injection with proteins associated with pili and flagella (Gupta *et al.*, 1996), and from the studies in this chapter, AhpC and GroEL. These proteins may therefore be important in the development of protective immune responses and have potential as components of a subunit vaccine. However, the detection of a T_H1 phenotype does not necessarily correlate with protection. For example, the KatE protein of *S. typhimurium* was shown to induce substantial DTH when used for immunisation but was not found to be protective (Kagaya *et al.*, 1992). Thus, although many of these results are encouraging, it is obvious that the protective nature conveyed by individual proteins requires further investigation.

Further clues to developing subunit vaccines to prevent *S. enterica*-related infection may come from examining the preliminary work that has been performed on other bacterial species. A number of groups have investigated the potential of using mycobacterial proteins in the development of a vaccine (Andersen and Heron, 1993; Andersen, 1994; Gelber *et al.*, 1994, Horwitz *et al.*, 1995; Silva *et al.*, 1996; Tascon *et al.*, 1996). In *M. tuberculosis*, it has been shown that proteins which are actively secreted by replicating bacteria, and which are also believed to be released when the bacteria resides within the macrophages, generate protective immunity (Andersen, 1994). Indeed, vaccination of mice with *M. tuberculosis* culture filtrates was found to

prime T cells against a broad spectrum of these proteins (Andersen and Heron, 1993; Horwitz *et al.*, 1995, Andersen, 1994). In addition, this culture filtrate still produced protection six months after immunisation, as measured by bacterial counts from the liver and spleen, and the level of protection was equivalent to that afforded by the BCG vaccine (Andersen and Heron, 1993).

The most efficacious fractions of the *M. tuberculosis* filtrate contained proteins of between 5-12 kDa and 25-35 kDa (Andersen, 1994). Similarly, a soluble extract of *M. leprae* cell wall proteins, which included low molecular weight peptides of between 1-3 kDa, produced protection in mice for up to 12 months post-vaccination (Gelber *et al.*, 1994). This may suggest that a limited selection of secreted mycobacterial proteins is sufficient for the induction of protective immunity, and might be largely responsible for the protective immunity generated after injection of the BCG vaccine (Horwitz *et al.*, 1994). Whether any of these proteins have homologies to *S. typhimurium* proteins is unclear. Nevertheless an encouraging result from these studies is that the protective mycobacterial antigens, like those in the *S. typhimurium* studies, induced DTH reactions and a selection of T_H1 type cytokines such as IFN-γ, IL-2, IL-6, GM-CSF (Andersen and Heron, 1993; Horwitz *et al.*, 1995; Andersen, 1994).

The protection afforded by a number of *Legionella pnemophila* proteins to challenge by this organism has also been shown to involve the development of strong cell-mediated immunity. *L. pneumophila* is an intracellular pathogen that produces a fatal form of pneumonia. Guinea pigs injected with either *L. pneumophila* cell membrane preparations, a heat shock protein homologue of GroEL (MSMP) or a Major Secretory Protein (MSP) showed 80-85% survival 3 weeks post-vaccination after virulent bacterial challenge (Blander *et al.*, 1990; Blander and Horwitz, 1991, 1993). In addition, these preparations were more effective than dead cells (Blander and Horwitz, 1993). Thus, such studies involving other bacteria further the evidence that polypeptides can be effective in inducing protective immunity. Moreover, at least for *M. tuberculosis*, polypeptide preparations can be as effective as the live attenuated organism for vaccination (Andersen, 1994). This would suggest that the

use of polypeptides in a vaccine to prevent *S. enterica* infection warrants further investigation.

There are a number of important factors to consider in the use of polypeptides in vaccine development. One is heterogeneity in the genetic loci that govern immune responses in outbred populations. For example, the ability of mice to respond to infection by *S. typhimurium* has been linked to genetic factors that modulate the immune response (Hormaeche *et al.*, 1985; Fayolle *et al.*, 1994; Lo-Man *et al.*, 1996), such as the *H-2* phenotype (MHC Class II). This in turn may affect the ability of the immune system to identify selected protein antigens. When different laboratory strains of innately susceptible B10 mice were injected with *S. typhimurium* expressing the *E. coli* MalE protein, immune responses to MalE differed. When the *H-2* loci of these mice were mapped for the number of epitopes of MalE that could be recognised, it was found that the recognition of a small or high number of epitopes corresponded to whether mice were low or high responders, respectively (Lo-Man *et al.*, 1996).

The use of a multivalent vaccine may guard against poor responses to individual antigens. However, vaccine dosage may compensate for the heterogeneity of immune response in an outbred population. For example, the *E. coli malE* gene was cloned into two plasmids which permitted different levels of MalE protein expression within *S. typhimurium*. When B10 mice, which had shown a poor immunological response to the MalE protein, were infected with *S. typhimurium* strains bearing these vectors, the greatest immunological response to this protein was observed in those mice infected with bacteria expressing the highest level of MalE (Fayolle *et al.*, 1994). There may also be problems associated with the vaccination dose. For example, adjusting the amount of a *M. tuberculosis* culture filtrate injected into mice from 10 μg to 100 μg per mouse, reduced the level of certain cytokines such as IFNγ. As a result, a shift from a cell-mediated type response (T_H1) to a predominant antibody response (T_H2) was observed and this correlated with a drop in the immunological protection obtained (Andersen, 1994). Thus, any subunit vaccine would require

critical examination in order to optimise the induction of the appropriate immune response.

It should also be noted that successful immunisation with bacterial proteins often depends on the use of adjuvants (Kagaya *et al.*, 1992; Andersen, 1994; Gelber *et al.*, 1994). In the experimental details described in this chapter, only heat aggregated proteins were injected, suggesting that a requirement for adjuvant may not be necessary. However, it is noteworthy that the use of alum as an adjuvant with AhpC enabled high levels of specific antibody to be produced and this antibody was used in the immunodetection studies outlined in chapter 3 and 4. However, the use of an adjuvant is sometimes undesirable, especially in humans, because of the potential side-effects such as toxicity or local ulceration which may occur at the site of injection. Therefore, any effective vaccine for humans would have to evaluate the safety of such adjuvants prior to use (Andersen *et al.*, 1994).

Some researchers have attempted the use of novel adjuvants which are more likely to be acceptable for use in the human host. These include the use of recombinant cytokines. Recently, it was shown that IL-12 acted effectively as an adjuvant when administered with *Yersinia enterocolitica* HSP60, a homologue of GroEL (Noll and Autenrieth, 1996). IL-12 is normally produced by macrophages and B cells, upon stimulation by microorganisms or their products, and is believed to influence the formation of a T_H1 type of response (Abbas *et al.*, 1996; Lamont and Adorini, 1996). The use of such cytokines raises the interesting possibility of specifically directing the development of an immune response of the correct protective phenotype.

A likely candidate for consideration in any potential subunit vaccine against *S. enterica*-related illness is GroEL. The present study suggested that GroEL is highly immunogenic, producing both long-lived T and B cell responses. In addition, GroEL homologues in a number of bacterial species including *M. tuberculosis* (Silva *et al.*, 1996), *M. leprae* (Gelber *et al.*, 1994), *Y. enterocolitica* (Noll and Autenrieth, 1996), *L. pneumophila* (Blander and Horwitz, 1993) and *H. pylori* (Ferrero *et al.*, 1995) have been shown to be highly immunogenic, and induce some degree of

protection to lethal challenge doses of the respective bacteria. Nevertheless, the use of GroEL in vaccine design is controversial (Kaufmann, 1990). HSPs have gained particular interest because of their highly conserved nature (see Figure 7.8) and because of evidence which suggests that immune recognition of these conserved proteins may lead to autoimmune disease (Kaufmann, 1990; Jones *et al.*, 1993). For example, the presence of antibodies to HSP60 (GroEL family) or HSP70 (DnaK family) has been correlated with the generation of rheumatoid arthritis and systemic lupus erythematosus, respectively (reviewed in Kaufmann, 1990).

GroEL belongs to the heat shock family of proteins (HSPs) and molecular homologues appear to be very widespread if not ubiquitous in living organisms (Kaufmann, 1990). HSPs generally act as chaperones helping to repair misfolded proteins, or control their degradation (reviewed in Gross, 1996). However, although these proteins were primarily identified as having a role in heat shock, they have a much wider role in a variety of stresses. In addition, the function of GroEL has been shown to be essential for the growth of *E. coli*, suggesting that this protein is integral to many general cellular processes (Fayet *et al.*, 1989). Host HSPs are generally thought to be hidden intracellularly but under stress conditions, such as during microbial infection, it is thought that they may become displayed on the surface of the cell (Wandwurttenberger *et al.*, 1991). However, the basis by which most autoimmune diseases are generated is poorly understood at present.

The majority of the studies which have investigated the potential for a cross-reactive response between a bacterial HSP and the homologous mammalian host protein have focused on the mycobacterial GroEL (Kaufmann, 1990). Alignments of the *M. tuberculosis* and human GroEL homologues (Jindal *et al.*, 1989) suggest there is approximately 48% identity between these two proteins (Table 7.4; see Figure 7.8 for an alignment) and at least four decapetides of the *M. tuberculosis* protein are identical. In addition, when the protein sequence of GroEL was divided into stretches of 25 amino acids, and these fragments were put into a data base of protein sequence, 86 human polypeptides were identified which exhibited high sequence similarity to many of these fragments. Furthermore, 19 of these polypetides have been implicated

Figure 7.8 An alignment of the protein sequences of HSP60 from different organisms

The amino acid sequences of the HSP60 proteins from different organisms were aligned. The sequences were obtained from the following accession numbers: mouse, X53584 cds 25-1665; rat, X53585.em_ro cds 1-1641; human, M34664.em_hum1 cds 25-1746; *E. coli*, P06139.swissprot; *S. typhi*, P48217.swissprot; *Y. eneterocolitica*, D14078.em_ba; *M. tuberculosis*, P06806.swissprot. The amino acids which form a leader sequence to target the eucaryotic HSP60 derivatives to the mitochondrion, and which are subsequently cleaved upon import, have been excluded from the alignment. The alignment of the eucaryotic HSP60 homologues shown therefore represents only the mature proteins and the nucleotides of the available sequence that encode this region are indicated next to the accession number where appropriate.

in autoimmune diseases such as insulin-dependent diabetes and rheumatoid arthritis (Jones *et al.*, 1993), although a causative relationship has yet to be shown.

One of the greatest concerns about using bacterial GroEL for vaccination, especially mycobacterial GroEL, is that in certain infections, a substantial portion of the immune response would seem to be directed to this single protein (Kaufmann *et al.*, 1987). Indeed, in one study, between 20-40% of all the $CD4^+$ cells isolated from *Mycobacterium*-infected mice were found to respond to GroEL. In addition, γδ T cells are also particularly responsive to this antigen and have been shown to lyse *Mycobacterium*-infected cells. With such a large proportion of cells specifically recognising epitopes of GroEL, it has been suggested that the chances that immune responses can develop to the hosts own GroEL are high (Kaufmann, 1990). In fact, mice primed with synthetic peptides derived from the *M. tuberculosis* GroEL protein were found to generate T cells that could respond to both the mouse and bacterial GroEL proteins. Moreover, cytolytic T cells primed with peptides based on mycobacterial GroEL were capable of lysing uninfected, but stressed, macrophages (Kaufmann, 1990).

It should be noted however, that a recent study has indicated that it is the non-homologous regions of the mycobacterial GroEL that are predominantly recognised during the course of infection (Mustafa *et al.*, 1996). The mycobacterial HSP70 protein also displays a high degree of conservation in bacterial and mammalian cells and has been implicated in the generation of autoimmune diseases. Interestingly, an investigation of mycobacterial HSP70 also indicated that it was the non-homologous regions of this protein which provoked the greatest immunological response during mycobacterial infection of mice (Adams *et al.*, 1997). Thus, this might indicate that although the GroEL protein is extremely immunogenic, only a small proportion of the immune response focuses on the cross-reactive epitopes of HSP60 and may suggest that the likelihood of inducing autoimmune disease is much lower than has been suggested from other studies.

Table 7.4 A comparison of GroEL homologues from different organisms

Origin of homologues	% Similarity	% Identity
Mouse and Human	99	97
Mouse and *E. coli*	71	51
Mouse and *S. typhi*	71	51
Mouse and *M. tuberculosis*	67	48

A table showing the level of similarity and identity of some of the GroEL homologues described in the text. % Similarity and % Identity were calculated using a Bestfit analysis of the reported sequence of the proteins. % Similarity reflects the percentage of amino acid residues between the GroEL proteins of the organisms which are identical or which are chemically related. % Identity represents the percentage of amino acid residues between the GroEL proteins of the organisms which are absolutely identical. The accession numbers for the HSP60 sequences in the data base are indicated in the legend for Figure 7.8.

Concerns about the safety of GroEL would also seem relevant to the use of any *S. enterica* GroEL homologue as the *S. typhi* GroEL shares 75% identity with the *M. tuberculosis* homologue, and also shares 51% identity with the human derivative (Table 7.4; see Figure 7.8 for an alignment). However, some of the problems of homology to human proteins may well be overcome by selectively expressing non-homologous epitopes of these proteins in a candidate vaccine.

In conclusion, more research is required to identify candidate polypeptides from *S. enterica* that can induce protective immunity. The studies performed in this chapter have provided an important step towards this goal and have also enhanced our understanding of immune responses to antigens from *S. enterica*. More information on the level of antigen required, the value of adjuvants and the importance of immunisation route are also required if an effective vaccination strategy is to be developed. In addition, the consequences of incorporating certain proteins into subunit vaccines need to be fully evaluated before such vaccines might be used in human trials.

CHAPTER 8

Concluding discussion

8.1 CONCLUDING DISCUSSION

The ability of bacteria to sense changes in their environment allows such cells to optimise their survival (Mekalanos, 1992; Mahan *et al.*, 1996). Of relevance to this thesis is the ability of *S. typhimurium* to deal with hydrogen peroxide stress. *S. typhimurium* and *E. coli* have both been shown to induce around 30 genes involved in protecting the cells from the detrimental effects of hydrogen peroxide (Christman *et al.*, 1985; Greenberg and Demple, 1989). A subset of these genes (the OxyR regulon), which are regulated by the OxyR regulatory protein, have been shown to be essential for the development of resistance to peroxide stress (Christman *et al.*, 1985). The classical view of the transcriptional activation of the genes of the OxyR regulon, is that activation occurs through the activity of OxyR and the σ^{70}-containing RNA polymerase ($E\sigma^{70}$), after treatment of bacterial cells with hydrogen peroxide (Christman *et al.*, 1985; Tao *et al.*, 1993). Each of the genes of the OxyR regulon contains a conserved but degenerate consensus sequence stretching approximately 45 bp upstream of the σ^{70} -35 hexamer (Toledano *et al.*, 1994). The binding of OxyR to this target sequence is believed to stabilise the interaction of RNA polymerase and is essential for transcriptional activation (Tao *et al.*, 1993). Interestingly, it was recently shown that the OxyR regulon genes, *dps*, *katG* and *gorA*, can show expression independently of OxyR and σ^{70} in the stationary phase of growth, by the alternative sigma factor RpoS (Altuvia *et al.*, 1994; Ivanova *et al.*, 1994; Becker-Hapak and Eisenstark, 1995). Thus, not only do *dps*, *katG* and *gorA* form part of the inducible resistance to peroxide stress in growing cells, but they contribute to the resistance of stationary phase cells.

The *ahpCF* locus of *S. typhimurium* encodes a heterodimeric protein which protects the cell membrane from lipid peroxidation during exposure to hydrogen peroxide (Christman *et al.*, 1985; Tartaglia *et al.*, 1990). Interestingly, the present study showed that *ahp* is not induced upon entry into stationary phase and that expression is independent of RpoS (see Chapter 3). As such, *ahp* is the first example of an OxyR-regulated gene which is not RpoS regulated. Although these studies were performed with *S. typhimurium* cells, a comparison of the *E. coli* and *S. typhimurium*

ahp promoter regions would suggest that if it is the -10 and -35 consensus sequences which determine regulation by RpoS, then the regulation of *ahp* in these two genetically-related organisms is likely to be similar (Figure 3.9). Moreover, these findings are supported by examining the *ahp* locus in *B. subtilis* which shows σ^B-independent expression. σ^B regulates many equivalent aspects of stationary phase to σ^S (Boylan *et al.*, 1993). These results raise the question of why *ahp* should be required in the exponential phase but is not required in the stationary phase and further work would be needed to determine if this feature simply reflects redundancy of *ahp*, due to the large number of stress-resistance genes expressed in stationary phase cells, or possibly that there is an, as yet unidentified, enzyme present which mediates protection to the membrane. In support of this latter possibility, stationary phase cultures of an *ahp* mutant of *B. subtilis* were shown to be no more sensitive to wild type cells after challenges with cumene, yet a σ^B mutant was extremely sensitive, suggesting that σ^B regulates a cumene resistance function (Antelmann *et al.*, 1996).

The ability of hydrogen peroxide to induce the genes of the OxyR regulon only appears possible in the exponential phase. This study also attempted to determine whether this reflects on a lack of OxyR protein or an inability of OxyR to achieve an active configuration. Interestingly, when cells were provided with high levels of either wild type OxyR or an OxyR molecule capable of constitutive expression of the OxyR regulon, no expression of *ahp* could be obtained (Figure 3.7), suggesting that the requirements for OxyR-dependent induction in the exponential phase are absent in the stationary phase. It is noteworthy that, although *gorA*, *katG* and *dps* are induced upon entry into the stationary phase, their promoter sequences do not bear the stereotypical characteristics of RpoS-dependent genes (Figure 3.9) therefore, determining the molecular mechanism(s) by which *gorA*, *katG* and *dps*, but not *ahp*, can show RpoS-dependent expression, may yield further clues as to the nature of the regulatory determinants required for stationary phase-inducibility and gene regulation in general.

In the present study, a number of experiments were dependent upon the use of the Mu*dlux* transcriptional reporter system to monitor expression of the *ahp* locus. The advantages of such a system is that gene expression can be followed in real-time without the need to disrupt cells or perform time-consuming biochemical tests (Carmi *et al.*, 1987; Francis and Gallagher, 1993). However, in several instances, the *lux* reporter system has been shown to cause anomalous expression of the tagged gene (Owen-Hughes *et al.*, 1992; Forsberg *et al.*, 1994). In this study, the hydrogen peroxide-inducible *ahp* locus was found to be osmotically regulated (Figure 4.2). However, a Western blot procedure conclusively demonstrated that the expression of the wild type AhpC protein was not influenced by the osmotic environment (Figure 4.9) and suggested that the Mu*dlux* element imposed some form of anomalous regulation upon the *ahp* locus. This result was all the more intriguing because, initially, the osmotic regulation of the *S. typhimurium ahp* locus fitted well with data from *S. aureus* and *B. subtilis*, in which the expression of the *ahp* locus had been shown to be osmotically-sensitive (Armstrong-Buisseret *et al.*, 1995; Antelmann *et al.*, 1996).

Whilst anomalies in gene expression using a *lux* reporter system have been reported previously (Forsberg *et al.*, 1994), the findings in this thesis represents the first example where a non-osmotically induced gene has developed osmotic regulation as a result of the use of such a reporter system. The unusual influence of *lux* reporter genes has been attributed to the *luxA* gene and specifically to the interaction of the DNA-binding protein H-NS, to a region of DNA curvature in the first 200 bp of the coding sequence (Owen-Hughes *et al.*, 1992; Forsberg *et al.*, 1994). However, in the present study, the anomalous expression of *ahp* was shown to be independent of H-NS (Figure 4.5), although due to the nature of the *hns* mutation, the complete absence of H-NS could not be fully excluded. In the Mu*dlux* fusion, the distance (approximately 4 kb) of the *ahp* promoter from *luxA*, would make it unlikely that changes to the promoter would occur directly by, for example, transmission of supercoiling-dependent changes through the DNA helix. It would seem more realistic then, that other known DNA-binding proteins with related activities (*e.g.* StpA; Zhang *et al.*, 1996) or as yet unidentified DNA-binding proteins, are involved in the

anomalous expression of *ahp*. The regulatory circuit involved in converting a non-osmotically-inducible gene into an osmotically-sensitive gene warrants further investigation. The Mu*dlux* element obviously introduces a factor which is capable of altering the behaviour of the *ahp* promoter, identifying the nature of this factor may be an important step in furthering our understanding of gene regulation.

Unravelling the complexities of gene regulation also has important implications in understanding how bacterial pathogens such as *S. enterica* are capable of successfully infecting a host. The host-pathogen interaction is a very dynamic one in which *S. enterica* must promote its survival and multiplication, yet, at the same time, deal with the considerable array of anti-microbial factors which are present in the host to prevent such a scenario (Finlay and Falkow, 1989a). Macrophages are believed to be the major factor in preventing infection by *S. enterica* (Collins *et al.*, 1974; Fields *et al.*, 1986; Buchmeier and Heffron, 1989). These phagocytic cells are equipped with an arsenal of anti-microbial effector mechanisms including a respiratory burst, which generates toxic oxygen radicals. As such, the ability to survive the anti-microbial environment of the macrophage might be considered of prime importance in the virulence of *S. enterica.* A major aspect of this study, was to attempt to examine the role of oxidative stress resistance genes in virulence and to assess whether such loci could be beneficial to vaccine development.

From the perspective of the bacterium, loss of either the *ahp* or *oxyR* loci did not significantly alter the virulence of *S. typhimurium* in a murine model (Table 5.1). The reason for these results are unclear. It is of interest that RpoS, the major regulator of stationary phase, has been shown to be induced within the macrophage (Chen *et al.*, 1996a) and *rpoS* mutants are attenuated in the murine model (Fang *et al.*, 1992 Coynault *et al.*, 1996) yet, *ahp* is not regulated by RpoS and OxyR appears only to exert its effects in growing bacterial cells. This perhaps explains the lack of a role for these loci in virulence. However, contradictory to the role of RpoS, it is clear from other studies that *ahp* is induced upon interaction of the bacterium with macrophages (Francis and Gallagher, 1993). Also, in the present study, the *in vivo* expression of *ahp* was indicated by the development of immune responses to this protein over the

course of *S. typhimurium* infection (Figures 7.1, 7.2 and 7.4). Whether these features reflect on the mechanism(s) by which *S. enterica* cells enter macrophages or, on the intracellular niches in which they are found, is uncertain.

It is still unclear as to whether *S. enterica* resides in a phagosome and subsequently prevents phagolysosomal fusion (Buchmeier and Heffron, 1991) or whether these bacteria may enter into cells via a phagocyte-independent process involving a specialised invasion apparatus, such as that used for entry into epithelial cells (Alpuche-Aranda *et al.*, 1994; Chen *et al.*, 1996b; Monack *et al.*, 1996). Indeed, it remains possible that both modes of entry occur. It is also of interest that Libby and Buchmeier (1997) recently demonstrated that the dynamics of a population of *S. typhimurium* within the macrophage demonstrated both rapid growth and death throughout a 20 hour period. The fact that there appears to be both growth and death would suggest that different proportions of the population are in both active and limited growth states at the same time point and would imply that the observations that both *rpoS* and *ahp* are induced within the macrophage need not necessarily be contradictory. Determining the intracellular niche in which *S. enterica* survives in would be an important step towards understanding bacterial pathogenesis. The identification of the genes responsible for providing access to such a compartment, and which promote the survival of the bacterial cells, may provide a more focused search for virulence factors.

Although, the disruption of the *ahp* and *oxyR* genes did not attenuate the full virulence of *S. typhimurium*, this does not necessarily exclude the further investigation of these loci for potential in vaccine development. In fact, these loci would seem ideal sites for inserting genes expressing heterologous antigens (from other pathogens) and could be used in *S. enterica* strains already attenuated by well-characterised lesions, such as *aroA*. The advantage of the *ahpC* and *oxyR* loci is that their disruption would be unlikely to alter the level of attenuation of the strain they were introduced into, a factor which is important as over-attenuation may lead to the poor generation of protective immunity. Secondly, at least for *ahp*, this locus has been shown to demonstrate low basal expression in the absence of the inducing

stimulus and perhaps more importantly, is known to be expressed upon interaction with macrophages (Francis and Gallagher, 1993). The advantage of this is that the insertion of a gene encoding a heterologous antigen in this locus, is likely to be stable, since the antigen would not be highly expressed in the absence of the oxidative stimulus and would not therefore generate some form of negative selection (such as that of a metabolic burden). Indeed, the selective induction of the *ahp* locus within the macrophage would provide an ideal location for the expression of heterologous antigens because these cells are of prime importance in antigen presentation to T cells.

Much of the recent work into developing an effective vaccine to *S. enterica* has focused on the use of attenuated bacterial strains (Chatfield *et al.*, 1992a; Ivanoff *et al.*, 1994). Live attenuated bacteria provide much greater protective immunity than killed cells, but this ability has been attributed to the fact that the live bacteria express immunogenic proteins in response to the host environment (Kagaya *et al.*, 1992). It seems surprising then, that very little work has examined the potential of using purified immunogenic proteins to stimulate protective immunity. A subunit vaccine would eliminate many of the problems associated with vaccines involving killed or live attenuated bacteria. However, very little information exists about the proteins from *S. enterica* which are recognised by the immune system and their potential for use in a subunit vaccine. Another major aspect of the present study therefore, was to increase our understanding of the immunological response to individual polypeptides thereby contributing to this relatively new angle of research into vaccine development with regards to *S. enterica*.

The successful elimination of *S. enterica* from the host involves the generation of a T_H1 response (Kagaya *et al.*, 1992; Mastroeni *et al.*, 1993). Such a response, is characterised by the presence of cytokines such as IFN-γ, TNF-α and IL-12 and the main effector of this response is the macrophage (Abbas *et al.*, 1996; Lamont and Adorini, 1996). Thus, any form of polypeptide-based vaccine would have to stimulate a cell-mediated response to be effective. Nevertheless, the exciting possibility of an effective polypeptide vaccine has been indicated from preliminary

studies on *S. typhimurium* and other bacteria. For example, the injection of *S. typhimurium*-derived outer membrane porin proteins and a hydrogen peroxide-inducible catalase (KatG) has been shown to induce varying degrees of protective immunity in mice (Udhayakumar and Muthukkaruppan, 1989; Kagaya *et al.*, 1992). In addition, the injection of porins into mice was shown to cause the induction of cytokines associated with cell-mediated immunity, such as IFN-γ and TNF-α (Galdiero *et al.*, 1993), and importantly, suggests that, in the absence of bacterial cells, proteins alone may be able to trigger the cytokines required for a T_H1 response (Galdiero *et al.*, 1993).

In addition to the work performed on *S. typhimurium*-derived proteins, other studies using different bacteria such as *L. pneumophila* (Blander *et al.*, 1990; Blander and Horwitz, 1991, 1993), *M. tuberculosis* (Andersen and Heron, 1993; Andersen, 1994), *M. leprae* (Gelber *et al.*, 194), *Y. enterocolitica* (Noll and Autenrieth, 1996) and *H. pylori* (Ferrero *et al.*, 1995) have indicated that protective immunity can be achieved by the injection of purified components from these bacteria. Especially, encouraging is the preliminary work that has been performed in *M. tuberculosis* which showed that proteins from this bacterium could stimulate DTH reactions and elicit the release of cytokines associated with a T_H1 response (Andersen and Heron, 1993; Horwitz *et al.*, 1995). Moreover, it was shown that the protection afforded by the injection of purified protein components was equivalent to that induced by the live attenuated mycobacterial vaccine strain, BCG (Andersen, 1994).

Importantly, in the present study, the *S. typhimurium*-derived AhpC and GroEL proteins were shown to induce significant DTH responses 33 and 104 days post-infection, suggesting that these polypeptides are recognised in the context of a T_H1 response (Figures 7.1 and 7.2). This may well indicate that these proteins have potential in the development of a subunit vaccine. However, it is important to note that the ability to form a DTH reaction to an injected protein does not necessarily correlate with the induction of protective immunity. For example, KatE, the normal cellular catalase was shown to induce a DTH reaction in mice which were previously

infected with *S. typhimurium* yet, immunisation of mice with KatE failed to protect mice against a subsequent challenge with virulent organisms (Kagaya *et al.*, 1992).

The development of polypeptide-based vaccines presents several interesting challenges. Immune responses to bacteria are likely to result from the recognition of a large number of polypeptides and therefore individual polypeptides, although immunogenic, would be less likely to stimulate such a large repertoire of immune cells, particularly T cells. In previous studies, it was shown that *S. typhimurium* induces approximately 30-40 proteins in response to the macrophage environment and reduces around 100 (Buchmeier and Heffron, 1990; Abshire and Neidhart, 1993a). This aspect raises the fascinating question of how many proteins need to be recognised in order for a suitable level of immune response to be generated to a bacterium, considering the large number of potential immunological targets. Infection of mice with the mycobacterial vaccine strain BCG, induces a selective T_H1 response to proteins of between 6-12 kDa and 25-35 kDa, suggesting that only a limited selection of the total complement of cellular proteins may be responsible for the protective immunity generated by BCG (Andersen and Heron, 1993; Andersen, 1994). Similarly, an examination of antigenic fractions from *S. typhi* demonstrated that the proteins which stimulated CMI most effectively were those of between 29-32, 41-45 and 63-71 kDa (Perez *et al.*, 1996). Thus, it would appear that with the use of a few defined polypeptides, it may be possible to induce effective immunity, equivalent to that of whole bacterial cells or at least to a level that is protective.

The number of polypeptides used in any vaccine is itself an important issue, due to the heterogeneity of the Major Histocompatibility Complex in an outbred population (Lo-Man *et al.*, 1996). The ability to identify and respond to peptides is dependent upon MHC molecules and therefore, the injection of a single protein may stimulate a substantial immune response in one individual but not in another. As such, subunit vaccines are likely to be more effective for wide-scale administration, only if they consist of a number of defined polypeptides

Bacterial cells such as those of *S. typhimurium* are capable of persisting within the host and therefore there is a greater probability for bacterially-derived antigens to be displayed over a long period. Moreover, factors associated with the bacterium are likely to stimulate the immune system. For example, tissue damage is likely to cause the release of cytokines to attract macrophages and other cells to the site of infection. Moreover, bacterial components such as LPS are known to up-regulate immune responses by stimulating B cells to undergo isotype switching, such that they produce complement fixing antibody, or, by activating macrophages (Baldridge and Ward, 1997). Thus, bacteria have natural adjuvant properties. The major disadvantages of proteins *per se* is that they are unlikely to persist for very long nor are they likely to optimally up-regulate the immune system, to release the cytokines required for the development of a T_H1 response (Baldridge and Ward, 1997). Indeed, it is noteworthy that much of the work into the ability of polypeptides to generate protective immunity has shown that protection is most effective in the presence of an adjuvant (Kagaya *et al.*, 1992; Andersen, 1994; Gelber *et al.*, 1994; Noll and Autenrieth, 1996). Thus, a critical assessment of the potential of available adjuvants may be an important step in creating a protein-based vaccine.

Adjuvants have a number of attractive properties ranging from the ability to increase the antigen depot and persistence of the antigen to the ability to modulate the nature of the immune response (reviewed in Cox and Coulter, 1997). This latter ability of adjuvants has particular appeal because, in the absence of bacterial cells which induce production of T_H1 response-associated cytokines, the adjuvant may be able to compensate. In fact, T_H1 response-associated cytokines such as IL-12 have actually been used successfully as adjuvants to skew the immune response to the desired phenotype (Noll and Autenrieth, 1996). It is possible therefore, that a combination of different adjuvants may be able to optimise the magnitude of the immune response and the T_H1/T_H2 balance, even in the absence of bacterial cells.

Bearing in mind the relevant issues concerning polypeptide-based vaccines, there are a number of interesting experiments which could be performed using the purified AhpC and GroEL proteins. Of major importance, would be to determine whether the

recognition by T_H1 cells correlates with an ability of these proteins to induce protective immunity. Such an assessment could also explore the efficacy of combining these proteins with each other or even, with proteins already shown to induce protective immunity (*e.g.* porins or KatG), as a multivalent approach is more likely to generate an efficient immune response. Also, given that adjuvants are known to stimulate the immune system, some form of assessment of different adjuvants such as cytokines (especially T_H1-associated cytokines) or immune stimulating complexes, individually or in combination, would be beneficial to our understanding of the potential of adjuvants in formulating vaccine preparations.

Of particular appeal to the development of a polypeptide subunit vaccine against infection by *S. enterica*, would be the inclusion of GroEL. Preliminary experiments have demonstrated that GroEL from a variety of bacteria, including *M. tuberculosis* (Silva *et al.*, 1996), *M. leprae* (Gelber *et al.*, 1994), *Y. enterocolitica* (Noll and Autenrieth, 1996) and *H. pylori* (Ferrero *et al.*, 1995), is immunogenic and capable of affording some degree of protective immunity against the respective bacteria. GroEL is an abundant protein of the cell (up to 10% of the total cell protein under stress conditions) and such abundance obviously aids isolation and purification (Hendrix, 1979; Hemmingsen *et al.*, 1988). In this study, the amount of GroEL was further enhanced by cloning the *groE* locus onto a multicopy plasmid, and the amount of this protein in a soluble cell fraction was estimated (visually) to be approximately 40%. Another advantage of GroEL is that because it forms a large multimeric structure (840 kDa), it can be separated from the majority of the other components of the cell by a relatively simple and rapid procedure involving density gradient centrifugation (Hendrix, 1979). Such abundance and ease of purification would be advantageous in large-scale vaccine preparations.

Infection by *S. enterica* contributes to the massive incidence of diarrhoeal disease recorded in the world each year (Pang *et al.*, 1996). Unfortunately, although antibiotics have proved useful in combating *S. enterica*-related illness, the emergence of antibiotic resistance makes antibiotic therapy impractical. A prime objective therefore, is to devise suitable prophylactic therapies to reduce the incidence of

S. enterica infection. A polypeptide vaccine is likely to have wide-scale use in both humans and animals and be safer, less reactogenic and may induce stronger protective immunity than whole cell vaccines. In addition, because such vaccines would consist of defined polypeptides, they are more likely to be prepared reproducibly, easier to assay, and less expensive to manufacture and store. Thus, the development of a polypeptide-based vaccine may be a key step in the eradication of *S. enterica*-related illness.

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