Monitoring stress responses in *Salmonella typhimurium* using a Mu*dlux* transcriptional reporter system

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Dedication

To my daughter Leanna whose love and devotion kept me going.

Declaration

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

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ABBREVIATIONS

Ap^R	ampicillin resistant
ATP	adenosine triphosphate
dATP	deoxyadenosine triphosphate
ddATP	dideoxyadenosine triphosphate
bp	base pairs
°C	degrees Celsius
cm	centimetre
CFU	colony forming units
Cm^R	chloramphenicol resistant
dCTP	deoxycytidine triphosphate
ddCTP	dideoxycytidine triphosphate
Ci	Curies
Da	Daltons
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetate
g	grams
x g	times unit gravity
dGTP	deoxyguanosine triphosphate
ddGTP	dideoxyguanosine triphosphate
dH_2O	distilled water
H_2O_2	hydrogen peroxide
$HOO^{\cdot}$	hydroperoxyl radical
hr	hours
IPTG	isopropyl-β-D-thiogalactoside
kb	kilobases

Km^R	kanamycin resistant
kv	kilovolts
LB	Luria Bertani
LSB	Laemmli sample buffer
M	molar
mA	milliamps
min	minutes
mm	millimetres
mM	millimolar
mg	milligram
ml	millilitre
MW	molecular weight
µl	microlitre
dNTP	deoxynucleotidetriphosphate
nM	nanometres
O_2	molecular oxygen
O_2^-	superoxide radical
$^1O_2$	singlet oxygen
OD	optical density
$OH^·$	hydroxyl radical
PBS	phosphate buffered saline
PCR	polymerase chain reaction
psi	pounds per square inch
RNA	ribonucleic acid
RNase	ribonuclease
sec	seconds
sdH_2O	sterile distilled water
SDS	sodium dodecyl sulphate
Tc^R	tetracycline resistant

TEMED	N,N,N'-tetramethylethylenediamine
Tris	tris(hydroxymethyl)aminomethane
UV	ultraviolet
V	volts
w/v	weight per volume
w/w	weight per weight
X-Gal	5-bromo-4-chloro-3-indoyl-β-D-galactoside
2D-PAGE	two-dimentional polyacrylamide gel electrophoresis

ABSTRACT

A Mu*dlux* transcriptional reporter system was used to construct a random pool of fusions in a virulent strain (SL1344) of the facultative intracellular pathogen *S. typhimurium*. This pool was then used to identify and isolate fusions which responded to different stress conditions *in vitro* with light production. Two of these fusions, MPG203 and MPG361, which were found to produce light in response to exposure to either hydrogen peroxide or a temperature downshift (cold shock), respectively, were further characterised. MPG203 was additionally shown to produce light following interaction with cells of the mouse macrophage cell line J774.2. Furthermore, the macrophage-induced light response from MPG203 cells was greatly reduced in the presence of catalase, directly implicating hydrogen peroxide as the eliciting agent produced by the macrophage cells, and also, the involvement of the hydrogen peroxide-induced bacterial stress response in the infection process. The gene responsible for directing light production from MPG203 was identified as *ahpC*, which encodes the smaller sub-unit of alkylhydroperoxide reductase. Further characterization of the second of these stress-inducible fusions, MPG361, demonstrated that the Mu*dlux* construct found in this strain lay within a gene that was selectively induced at low temperature (10°C) or by low concentrations (1 μg ml^{-1}) of the antibiotic tetracycline. This gene was subsequently identified as a homologue of the *E. coli* cold shock-inducible gene *cspA*, and was designated as *cspS*. MPG361 was additionally subjected to Tn*10* mutagenesis. This procedure resulted in the isolation of 3 mutants (MPG386, MPG401 and MPG402) whose light production was significantly altered by the presence of the second transposon. The chromosomal map positions of the Tn*10*s located in each of the above strains were determined as laying between 0 - 3 minutes for MPG386 and between 83 - 86 minutes for both MPG401 and MPG402. More extensive

characterisation of the phenotype of the Tn*10* insertion of the (dark) mutant MPG386, showed that this mutation not only abolished cold shock-induced light production in MPG386, but had a similar effect in every cold shock-inducible fusions isolated during this study. The insertion of this latter Tn*10* in *S. typhimurium* (any SL1344 derivative) was also found to give a number of other phenotypic changes, including slower growth, altered colony morphology, and reduced survival at low temperature. Complete phenotypic complementation of the effects of this Tn*10* could be achieved by the introduction of a plasmid carrying a region of the *S. typhimurium* chromosome which mapped to approximately 0 - 1 minute.

CHAPTER 1

Chapter 1: Introduction.

1.1 Overview.

For the majority of organisms, life is a continuous struggle to combat the rigours associated with environmental change. This is particularly pertinent to intracellular pathogens, such as the Gram-negative bacterium *Salmonella typhimurium*. This bacterium, like many parasites, is able to survive in the unfavourable environments found both outside the host (*eg* soil and water) and within host cells, in particular, macrophages. Its ability to survive within these phagocytic cells is partly dependent on its capacity to adapt to, and overcome stresses encountered during invasion. This chapter will discuss the pathogenesis of enteric bacteria, and will compare and contrast how certain bacteria, including *S. typhimurium*, contend with the environmental stresses encountered in both pathogenic and nonpathogenic surroundings.

1.2 Stress as a universal phenomenon.

All cellular organisms, from bacteria and fungi to plants and animals, respond to a wide range of environmental conditions by inducing the synthesis of specific stress proteins. The purpose of these proteins is thought to be twofold: to initiate repairs to cellular damage inflicted by the stress, and to protect the organism from a further and potentially lethal stress challenge, thereby enhancing the organisms survival under the altered environmental conditions (Watson, 1990).

In past years, a great deal of work has focused on the regulation and specific function of stress proteins, however many questions still

remain unanswered. Part of the reason for this lack of information has been due to the sheer complexities associated with studying stress protein induction. This problem has been compounded by the fact that many stress-inducible proteins, such as the heat shock proteins Hsp60 and Hsp70 (Lindquist and Craig, 1988; Craig *et al*, 1993), are synthesized in response to a range of different environmental conditions, and also, are found at a basal level in unstressed cells, under normal physiological conditions (Bukau and Walker, 1989). The regulation of stress proteins has been particularly well studied in the bacteria *E. coli* and *S. typhimurium* (Christman *et al*, 1985; Morgan *et al*, 1986; Jenkins *et al*, 1988; Jenkins *et al*, 1990; Foster, 1992).

The list of stresses that induce selective protein synthesis in organisms is extensive, including: starvation, temperature, pH, U.V, osmotic fluctuations, oxidation, and oxygen limitation within the cell (Watson, 1990). Studying these stresses in bacteria has two advantages. Firstly, bacteria provide a model organism for increasing our understanding of the roles of stress proteins in higher organisms. Secondly, by broadening our knowledge of how bacteria survive stresses, we may increase our potential to combat bacterial infection in higher organisms. This is particularly pertinent with regard to those diseases caused by enteric bacteria such as *S. typhimurium*. This latter class of bacterial pathogens and the disease mechanisms that they adopt within the host organism will be discussed more fully below.

1.3 The pathogenesis of enteric bacteria.

Of all bacterial species capable of causing an invasive disease in mammals, only certain pseudomonads and a few opportunistic pathogens (most of which possess only a limited invasive capacity) can also

compete effectively in natural environments (*eg* soil) in competition with indigenous saprophytes. Most of the remaining pathogenic organisms are, by definition, obligate parasites ultimately dependent upon their host for perpetuation (Brubaker, 1985). Not all pathogenic bacteria have an equal probability of causing infection and disease. While some bacteria regularly cause disease in a proportion of non-immune individuals with intact host defense systems, others do not. For example, *Pseudomonas aeruginosa* can infect compromised patients (*eg* burn victims or individuals with cystic fibrosis) and cause overwhelming disease. However, it is of marginal concern to those with intact host defenses. Probably any bacterium which has the capacity to sustain itself in humans will occasionally cause disease in compromised individuals and act as an opportunistic pathogen. Thus, infection and disease are as dependent on the host as on the bacterium (Finlay and Falkow, 1989a).

A common outcome of a bacterial infection, is sufficient multiplication by the pathogen to secure its transient or long-term colonisation of the host or to bring about its successful transmission to a new host. Death of the host would seem an inadvertent and unfavourable outcome in many cases. It is important to recognise that a bacterium can be exceptionally equipped to cause infection but with minimal disease (Finlay and Falkow, 1989b). However, circumstances occasionally dictate that disease results. The likelihood of developing disease as a consequence of infection is a reflection of the balance between the bacterium's ability to outwit the host (and multiply), and the host's capacity to control or limit bacterial proliferation. Understanding the outcome of the host-parasite relationship is further complicated by the fact that neither the host nor the bacterium is a static entity. Indeed, the global setting of host-parasite interaction represents an evolving dynamic process (Falkow *et al*, 1992).

One fundamental requirement for bacterial pathogenicity is the necessity of the microorganism to enter the host. Each host surface is the target for different microorganisms that use them as the portal for entry to other locations within the host (Hoepelman and Tuomanen, 1992). The bacterium's surroundings may change profoundly during the infection process and pathogens must possess the appropriate genetic machinery to grow in, or at least tolerate, several different environments as a prerequisite for survival and subsequent multiplication within the host (Mekalanos, 1992). Moreover, specialized pathogenic traits may not be expressed until the infecting organism encounters a particular environment within its host (Miller *et al*, 1989b).

The first specific interaction between a pathogenic bacterium and its host entails attachment to a eukaryotic cell surface. Bacteria may express alternative adherence proteins dependent upon the conditions encountered at different host surfaces (Isberg, 1991; MacBeth and Lee, 1993). Specific adherence of a microbe to different host cells is often important, not only during the initial encounter between the pathogen and its host, but throughout the infection cycle. There are several potential outcomes following bacterial attachment. Many pathogens, including the opportunistic bacterium *Pseudomonas aeruginosa*, bind to epithelial cells lining certain mammalian mucosal surfaces, replicate locally, and remain affixed to the host surface for the remainder of the infection (Isberg, 1991). Bacterial pili usually are associated with this form of attachment; these microbial attachment factors are highly adapted surface proteins that interact with specific host target molecules, typically polysaccharide (Clegg and Gerlach, 1987; Finlay and Falkow, 1989b). Alternatively, the microbial cell may possess receptors that bind host-encoded secreted polysaccharides or proteins that, in turn, bind host receptors and indirectly lead to binding of the pathogen to the host cell surface

(Falkow, 1991; Mroczenski-Wildey *et al*, 1989). Such receptors and proteins are primarily found in strain of *E. coli* associated with urinary tract infections (Isberg, 1991). Some bacteria, including species of *Salmonella* (Finlay and Falkow, 1989a), bind to the host cell surface and subsequently become internalized, a property that is often referred to as microbial invasion. (Falkow *et al*, 1992). However, the majority of bacterial invasion mechanisms are extremely complex and do not just involve passing through the cell membrane, but represent entry by mechanisms requiring some kind of participation by both the host and the parasite.

1.3.1 Invasion of host cells.

Entry into host cells involves a number of specialized strategies for survival and multiplication, strategies utilized by a multitude of different pathogens. Besides avoiding the host (defense) immune system, intracellular (cytosolic) localization places the organism in an environment that, in the majority of cases, is potentially rich in nutrients, yet devoid of competing organisms (Moulder, 1985). It appears that many invasive bacteria exploit existing eukaryotic internalization pathways for entry. For example, if an organism adheres tightly to a receptor on a eukaryotic cell and this receptor is then internalized, the bacterium may also gain entry into the host cell. However, simple adherence is not sufficient and the nature of the receptor appears important; bacteria which tightly adhere to animal cells by means of type 1 pili are not internalized (Miller and Falkow, 1988). Possibly because of the large size of the invading organism (compared with normal endocytosed particles), there is usually cytoskeletal rearrangement accompanying bacterial invasion (Finlay *et al*, 1991; Francis *et al*, 1992; Tilney and Tilney, 1993).

Internalization of most pathogenic organisms is inhibited by cytochalasins (Elsinghorst *et al*, 1989), agents which inhibit microfilament function, but microtubules and intermediate filaments do not appear to be involved in bacterial invasion (Finlay and Falkow, 1988). Once inside the host cell, the organism must be able to survive and spread to other cells; intracellular multiplication usually takes place to some degree but is not necessarily a requirement (Finlay and Falkow, 1989b).

Enteroinvasive bacteria enter humans and animals by the oral route (Finlay and Falkow, 1989b). Once ingested, the infecting bacteria may often proceed to invade the host's tissues and cause disease, a process that is not only dependent on the genera of the infecting bacteria but, in the majority of cases, also on the number (LD_{50}) of bacteria associated with the ingested material (the incidence of disease commonly increasing with the bacterial dose) (Finlay and Falkow, 1989b; Jay, 1992). In a typical disease scenario, a high percentage of the infecting bacteria travel through the stomach (surviving the low pH) to the intestines, where they interact with the intestinal mucosa and typically invade the columnar epithelial cells (Wick *et al*, 1991), or other more specialized cells of the intestinal lining, *eg* M cells (discussed in more detail below) (Finlay and Falkow, 1989a; Bliska *et al*, 1993). Again, depending on the genera of bacteria, the infection may be confined to the superficial layer of the intestinal mucosa, as is predominantly the case with enteroinvasive *E. coli* or *Shigella* species, or it may proceed through the surface intestinal epithelial cells into deeper tissue and often enters the mononuclear phagocytes of the reticuloendothelial system, as in the case of certain *Salmonella* and *Yersinia* species. Ironically, these phagocytic cells are normally one of the host's major defences against bacterial infection (Adams and Hamilton, 1984; Forman and Thomas, 1986).

In the case where the infection is confined to the surface epithelial

layer, the bacteria frequently spread throughout the epithelial cells and are capable of causing a great deal of tissue damage (ulceration), loss of fluid uptake or secretion (due to interaction with bacterial toxins) and inflammation, producing the clinical manifestations of dysentery (diarrhoea with blood and mucus). In the case where the bacteria enter the mononuclear phagocytes of the reticuloendothelial system, proliferation usually occurs within these latter cells, especially within macrophages (Finlay and Falkow, 1989b). These cells then act as an immunological shield and allow dissemination of the bacteria throughout the body, in particular to the spleenic cells where, in the case of *Salmonella* infections at least, a reported immunological 'safe-site' exists (Dunlap *et al*, 1991). In some cases the disease may progress to produce life threatening symptoms, such as those associated with typhoid fever, induced by bacteraemia (*ie* high fever, diarrhoea, exhaustion and delirium (Wakelin and Blackwell, 1988)).

Multiplication of bacteria within the reticuloendothelial system is an essential virulence feature of several bacterial pathogens, including species of *Salmonella*, *Yersinia* and *Mycobacterium* (Finlay and Falkow, 1989a; Charnetzky and Shuford, 1985; McDonough *et al*, 1993), *Listeria monocytogenes* (Camilli *et al*, 1993), *Legionella pneumophila* and *Legionella micdadei* (Dowling *et al*, 1992), *Coxiella burnetti* (Maurin *et al*, 1992), *Neisseria gonorrhoeae* (Brubaker, 1985), *Chlamydia trachomatis* (Lundemose *et al*, 1991), *Rickettsia prowazeki* (Falkow *et al*, 1992) and *Bordetella pertussis* (Friedman *et al*, 1992). Ordinarily, professional phagocytes such as neutrophils (polymorphonuclear leukocytes), monocytes (mononuclear phagocytes) and macrophage ingest and kill bacteria by producing a vast range of toxic compounds (Reeves, 1987). However, many bacterial pathogens not only survive within macrophages but actively replicate and destroy these cells (Moulder,

1985; Williams *et al*, 1988; Falkow, 1991; Falkow *et al*, 1992). How these bacteria accomplish this is extremely diverse and complex.

Electron microscopic studies of macrophages infected with different bacteria have revealed that at least three alternative survival strategies exist. For example, *M. tuberculosis* and *L. pneumophila* inhibit phagosome fusion with lysosomes, thereby preventing exposure to toxic lysosomal contents (McDonough *et al*, 1993; Frehel and Rastogi, 1987; Horwitz, 1983). In contrast, *L. monocytogenes* lyses the phagosomal membrane and escapes into the cytoplasm (Tilney and Portnoy, 1989; de Chastellier and Berche, 1994). A third group of bacteria, including *M. lepraemurium*, is found within the macrophage phagolysosomal compartment, where they apparently resist inactivation by lysosomal factors (Lowrie *et al*, 1979). *Salmonella* species are thought to fall into this final category (Finlay and Falkow, 1989a), as are *Yersinia* species (Rosquist *et al*, 1990; Plano *et al*, 1991). However, as will be discussed later, a great deal of ignorance still surround the facts concerning survival of these species within macrophages. The remainder of this section will deal mainly with the bacterial pathogen *S. typhimurium*.

1.3.2 Salmonellosis.

Salmonellae are motile, Gram-negative, rod-shaped bacteria that can infect both humans and animals (Baird-Parker, 1991). In humans, salmonellae give rise to a variety of disease conditions known collectively as human salmonellosis. The organisms most frequently cause a self-limited gastroenteritis (food poisoning), but the spectrum of disease ranges from asymptomatic intestinal colonization to major extraintestinal illness such as meningitis or osteomyelitis (Cohen and Tauxe, 1986). Although approximately 2000 different *Salmonella*

serotypes exist, ten account for more than 70% of reported human isolates each year in the United States. In 1984 the most frequently isolated serotype from infected patients was *Salmonella typhimurium*, accounting for approximately 35% of such isolates (Cohen and Tauxe, 1986). Some serotypes are highly host-specific and rarely cause disease in other species. For example, *S. typhi*, which causes typhoid fever, only infects humans; there are no known animal reservoirs. In contrast, serotypes such as *S. typhimurium*, have a broad host range and cause disease in many species, including livestock, poultry and humans (Jay, 1992).

Salmonella typhimurium is one of the major causes of bacterial gastroenteritis in humans. Its genome consists of approximately 4000 kilobases and has been extensively characterised. Furthermore, it is readily amenable to genetic manipulation. Preliminary studies have shown *S.typhimurium* to be a 'model' organism for understanding how bacteria invade, survive, and replicate within host cells, features of fundamental importance to our understanding of more devastating serotypes such as *S.typhi* (Groisman and Saier, 1990).

Various studies have shown that there are a number of factors which contribute to the virulence of *S. typhimurium*. Many of these, such as intact lipopolysaccharide (LPS) and the ability to invade eukaryotic cells have been shown to be chromosomally encoded (Hackett *et al*, 1986; Fields *et al*, 1989). However, for a number of strains of *S. typhimurium*, invasion and intracellular growth is additionally dependent on a large (approximately 90 kb) virulence plasmid (Taira and Rhen, 1989a). This plasmid has been demonstrated to contribute to a number of pathogenic properties in infected mice, including enhancement of *Salmonella* to multiply within the spleen and suppression of lymphocyte responsiveness to both T-cell mitogens and specific *Salmonella* antigens

(Hoertt *et al*, 1989), alterations to LPS (Kawahara *et al*, 1989), movement through tissue (Gulig, 1990), and enhancement of growth rate during infection (Gulig and Doyle, 1993).

One particular region of this plasmid, spanning 6.2 kb, has been shown to encode five genes that are essential for conferring virulence (Gulig and Doyle, 1993). This same region has been identified in several other salmonellae, including *S. dublin* (Fang *et al*, 1991). The exact virulence function(s) encoded by these genes has not been determined. However, one particular gene, *mkaC* (Taira and Rhen, 1989b), has been shown to encode a 34 kDa protein which possesses significant amino-terminus homology with the LysR family of transcriptional activator proteins (Henikoff *et al*, 1988).

Finally, Ou and colleagues (1987) have tested a number of parental strains and plasmid-cured strains of *S. typhimurium* in mice. They concluded that there are two sets of chromosomal genes involved in virulence: one set determining high-level virulence in cooperation with the plasmid genes, and another set determining virulence independent of the plasmid genes.

i) Infection of intestinal epithelia by *Salmonella* species.

The importance of motility in bacterial invasion is thought to vary between *Salmonella* species. This is most obvious between *S. typhi*, which require intact motility to enter epithelial cells (Finlay and Falkow, 1989a), and *S. typhimurium* which only requires contact to enter (Khoramian-Falsafi *et al*, 1990). *S. typhimurium* has also been shown to demonstrate positive chemotaxis to damaged epithelial cells which release attractants such as glycine (Uhlman and Jones, 1982). Work by Jones and colleagues (1981) has shown that *S. typhimurium* adherence to HeLa

cells consists of two phases of attachment (reversible and irreversible), each varying in its affinity for the epithelial cell surface. Various inhibitors of bacterial RNA and protein synthesis have been reported to prevent adherence and translocation of *S. choleraesuis* and *S. typhimurium* through polarized epithelial cell monolayers (MacBeth and Lee, 1993). Furthermore, the number of salmonellae which adhere to or invade epithelial cells increases dramatically if bacteria are incubated with a monolayer of epithelial cells and then transferred to a second monolayer of the cells (Finlay and Falkow, 1989a).

Only viable, metabolically active salmonellae appear able to adhere to host cell surfaces, in contrast to other invasive bacteria such as *Yersinia* species and enteropathogenic *E. coli.* Moreover, adherence by salmonellae is followed immediately by internalization. This entire process is very efficient and extremely rapid. For example, when 10^7 *S. typhimurium* cells are used to infect an epithelial cell monolayer, 10^5 bacteria are internalized after only 20 minutes (Finlay *et al,* 1991). In addition to this, Pace *et al* (1993) have shown that the interaction of *S. typhimurium* with HeLa cells induces a dramatic increase in the level of intracellular Ca^{2+} within the epithelial cell, and that the addition of calcium antagonists to this interaction results in abolishment of bacterial entry.

In the mouse model, the precise portal of entry of *S. typhimurium* from the terminal ileum of the host has not been defined. However, there is mounting evidence that M cells within Peyer's patches (rather than columnar absorptive epithelial cells) preferentially ingest invasive organisms, in what is thought to be a receptor-mediated phenomenon (Galan *et al*, 1992b; Bliska *et al*, 1993). Gahring and colleagues (1990) have speculated that M cells could additionally contribute to the host-specificity of various *Salmonella* species by providing a specialised

epithelial cell with which to interact.

The major role of the M cells, in conjunction with other specialized cells of the intestinal lining, is to allow the epithelium to function as both gatekeeper to the mucosal immune system and as exporter of secretory antibodies for mucosal defence (Neutra and Kraehenbuhl, 1992). M cells, in particular, cover the mucosal lymphoid follicles (sites of induction of the mucosal immune response) and continually endocytose material from the lumen. The architecture of these cells is striking: the basolateral plasma membrane is deeply invaginated to form a large intraepithelial pocket that usually contains a cluster of T cells, B cells and macrophages (Ermak *et al*, 1990). This invagination brings the basolateral plasma membrane within a few microns of the apical membrane, so that endocytic vesicles need travel only a short distance to deliver samples of luminal material (including bacteria) into the intraepithelial pocket (Neutra and Kraehenbuhl, 1992).

The processes involved in transporting bacteria across the apical borders of columnar epithelial cells and M cells are extremely complex, and are primarily initiated by the bacteria being surrounded by polymerized actin filaments (Finlay *et al*, 1991; Neutra and Kraehenbuhl, 1992). It is thought that the *Salmonella* cells cause rearrangement of the intracellular actin, thereby disrupting the microfilaments supporting the microvilli in epithelial cells (Finlay *et al*, 1991). Drugs which inhibit microfilament polymerisation, such as cytochalasins B and D, prevent invasion, indicating that *Salmonella* internalization into eukaryotic cells requires the active participation of host microfilaments (Elsinghorst *et al*, 1989). Furthermore, anaerobic growth of *S. typhimurium* results in increased uptake by epithelial cells (Schiemann and Shope, 1991) which correlates with enhanced morphological and cytoskeletal changes (Francis *et al*, 1992). Actin microfilament rearrangement has also been reported

as essential for the pathogenicity of *Yersinia* (Rosqvist *et al*, 1991) and *Listeria* (Tilney and Tilney, 1993).

What is known about *Salmonella* invasion and replication within eukaryotic cells is somewhat contradictory and probably reflects real differences between *Salmonella* serotypes and the range of target eukaryotic cells used in the studies (Buchmeier and Heffron, 1989). For example, Elsinghorst and co-worker (1989) have shown that it is possible to confer an invasive phenotype upon a normally non-invasive *E. coli* strain by introducing a cosmid containing a single continuous piece of *S. typhi* DNA. However, this group found that the related chromosomal segment from *S. typhimurium* did not confer this same invasive phenotype when transferred to *E. coli*. Furthermore, attempts to clone directly functional *S. typhimurium* invasion determinants, by screening a cosmid library for recombinants that would allow *E. coli* to invade epithelial cells, was also unsuccessful. Subsequent restriction analysis of the two related chromosomal regions demonstrated that, although both regions produced very similar patterns suggesting that a locus equivalent to the invasion loci (*inv*) of *S. typhi* should be present in *S. typhimurium* (see below), some differences between the DNA sequences must exist. This variation was particularly obvious between the *invA* locus identified in *S. typhi* and its homologous region identified in *S. typhimurium*.

Further work concerned with the identification of the genetic loci responsible for invasion in *S. typhimurium* has been conducted by Finlay and Falkow (1989a). By using transposon mutagenesis, this group found that approximately 0.5% of all insertions affected bacterial invasion. The genetic locus (*inv*), also present in *S. typhi* (as discussed), has been identified by Galan and Curtiss (1989a). This locus has been shown to be specifically involved with the entry of *S. typhimurium* into epithelial cells, but has no effect on bacterial adherence. *Salmonella* with a deletion

at this locus were found to produce elevated LD_{50} level in orally challenged mice, but an LD_{50} level similar to wild-type bacteria when administered intraperitoneally (Galan and Curtiss, 1990).

Recently, two genes of the *inv* locus, *invA* and *invE*, have been identified and characterized with respect to their role in *Salmonella* invasion of epithelial cells (Galan *et al*, 1992a; Ginocchio *et al*, 1992). In contrast to the parental strain, *invA* and *invE* mutants were unable to enter epithelial cells, failed to change the intracellular free calcium levels or the distribution of polymerized actin in cultured epithelial cells, and neither mutant could alter the normal architecture of the microvilli of polarized epithelial cells (Galan *et al*, 1992b; Ginocchio *et al*, 1992). Pace and co-workers (1993) have demonstrated that altering the level of intracellular free calcium is fundamental in the invasion of epithelial cells by *S. typhimurium*. Interestingly, the predicted amino acid sequence of InvE shows significant homology to one of the *Yersinia* outer membrane proteins (YopN) (Ginocchio *et al*, 1992). Yop proteins have also been shown to induce actin microfilament disruption in eukaryotic cells (Rosqvist *et al*, 1991), and are induced, in part, by low Ca^{2+} concentration (Goguen *et at*, 1984).

As with the Inv proteins in *Salmonella* species, the Yop proteins of certain *Yersinia* species are not thought to be involved in the attachment of the bacterium to the epithelial cell surface (Bliska *et al*, 1993). The protein responsible for this function in *Y. pseudotuberculosis* has been shown to be ModV (Rosqvist *et al*, 1990). Similarly, Gahring and co-workers (1990) have found a mutant that is unable to adhere to epithelial cells and has reduced invasive capacity in *S. typhimurium*. The disrupted locus responsible for this mutation, originally isolated by Fields *et al* (1986) during work concerned with *S. typhimurium* survival within macrophages, was shown to be at a locus other than *inv*.

Small *et al* (1987) have suggested that *S. typhimurium* cells do not multiply intracellularly within epithelial cells. However, Finlay and Falkow (1989a) have found this not to be the case, suggesting that the discrepancy between these two sets of results probably arose because Small *et al* (1987) only followed the first few hours of infection. At such early time points, some invasive bacteria such as *Shigella* species multiply rapidly; in contrast, there is a lag period of several hours before *Salmonella* species reach maximal intracellular division rates in epithelial cells. More recently, using transposon mutagenesis, Leung and Finlay (1991) isolated a replication-defective mutant that could not be distinguished from the parental strain apart from its ability to multiply inside epithelial cells. This mutant was found to be highly attenuated in mice.

Salmonellae are internalized individually within epithelial cells into vacuoles which at a later time appear to coalesce. Bacteria within these epithelial membrane-bound inclusions have been found to multiply extensively. After 12-24 hours of intracellular multiplication, most epithelial cells have large vacuoles filled with salmonellae. Such massively infected cells have often been found to lift off the monolayer without disruption of the cytoplasmic membrane (Finlay *et al*, 1988). How these bacteria obtain their nutrients and other components necessary for intracellular growth is not clear. Mutations which affect aromatic amino acid (*aro*) or purine (*pur*) biosynthesis cause these strains to be attenuated and have decreased virulence (Hoiseth and Stocker, 1981; Edwards and Stocker, 1988). Portillo and colleagues (1992) have recently shown, using *lacZ* transcriptional fusions and the highly sensitive substrate fluorescein-di-β-D-galactoside, that the vacuole levels of both Fe^{2+} and Mg^{2+} appear to be low for bacterial growth. However, levels of lysine, oxygen and pH were found to be sufficiently high to stop

induction of the gene-fusions examined.

ii) Entry and survival of *S. typhimurium* in macrophages.

Macrophages are cells that specifically function to defend the body against infection. As such, microbial life within macrophages requires many adaptions to deal with the adverse environmental conditions found therein. Some of the microbial stresses encountered within macrophages will be discussed in more detail later in this section.

Macrophages readily ingest particles, including bacteria, by phagocytosis. In the case of salmonellae, this process is probably eased by the fact that these bacterial species are capable of invading most kinds of animal cells (they do not invade red blood cells). Supporting this hypothesis, work by Gahring and colleagues (1990) has led to the isolation of an *S. typhimurium* Tn*10* mutant that, in addition to not adhering to or entering epithelial cells, shows reduced adherence to and replication within macrophages when compared to the parent strain, even when bacteria are opsonised. This may indicate that salmonellae have two entry pathways into macrophages.

Survival and replication of salmonellae within macrophages is more complex than in non-phagocytic cells, presumably because of the harsher intracellular environment (Adams and Hamilton, 1984; Forman and Thomas, 1986). From a total of 9516 independent Tn*10* insertion mutations in *S. typhimurium*, Fields and colleagues (1986) identified 83 avirulent mutants with reduced survival or replication within macrophages. Twenty-two of the least-virulent mutants entered and multiplied normally within epithelial cells (Gahring *et al*, 1990), indicating that additional bacterial genes are needed for survival within phagocytic cells which are not required for intracellular life in epithelial

cells. Further characterisation of these 83 Tn*10* lesions identified mutants with alterations in serum sensitivity, LPS, motility, responses to oxidative stress, and auxotrophy (Fields *et al*, 1986).

Further work by Fields and colleagues (1989), continuing with the analysis of the same 83 Tn*10* lesions in *S. typhimurium*, found that three mutants with increased serum sensitivity, and some of the highest corresponding LD_{50} values, contained insertions in the regulatory locus *phoP*. These findings were confirmed by Miller and colleagues (1989a), again using transposon mutagenesis. This group found that the *phoP* locus, first identified by Kier and co-workers in 1979, is composed of two regulatory proteins, PhoP and PhoQ. Under conditions such as low pH or starvation (Groisman *et al*, 1989; Foster and Hall, 1990) these proteins activate the expression of a set of genes known as the *pag* genes (Miller *et al*, 1989a), and repress another set known as the *prg* genes (Miller, 1991). Mutations in both *phoP* and *phoQ* were found to convey extreme sensitivity to antimicrobial peptides (defensins) isolated from phagocytes (Fields *et al*, 1989; Miller *et al*, 1990a), and had reduced survival within macrophages (Miller, 1991), as did *phoP* constitutive mutants (Miller and Mekalanos, 1990). However, *phoP* mutants could still enter and replicate normally in epithelial cells (Gahring *et al*, 1990).

From the above findings it might be inferred that some of the 40 or so gene products regulated under *phoP* control in *S. typhimurium* (Miller and Mekalanos, 1990) are involved in sustaining life within phagocytic cells, but not necessarily within epithelial cells. However, recent work by Behlau and Miller (1993) has shown that the PhoP/Q regulon of *S. typhimurium* is also involved with the invasion of host epithelial cells. Again using transposon mutagenesis (Tn*phoA*), the latter workers identified five unlinked PhoP-repressed (*prg*) loci. Further characterization of these loci, showed that one of the locus in particular,

prgH, promoted *S. typhimurium* invasion of epithelial cells and also contributed to the virulence of this bacteria in mice. *prgH* was located at 59 minutes on the *S. typhimurium* chromosome (Behlau and Miller, 1993), a region where other genes essential to invasion of epithelial cells are clustered (Lee *et al*, 1992). Behlau and Miller (1993) further showed that both *prgH* and *phoP* constitutive mutants were defective in induction of endocytosis by epithelial cells and that the invasion defect of *prgH* mutants, but not that of the *phoP* constitutive mutant bacteria, was complemented by plasmids containing *prgH* DNA. Taken together, these data (Fields *et al*, 1986; Fields *et al*, 1989; Miller *et al*, 1989a; Miller *et al*, 1990a; Gahring *et al*, 1990; Miller and Mekalanos, 1990; Behlau and Miller, 1993) indicate that two virulence properties of *S. typhimurium*, induction of endocytosis by epithelial cells and survival within macrophages, are oppositely modulated by the PhoP/Q virulence regulon, further emphasising the complexities of this pathogenic process.

Additional work involving the PhoP/Q regulon has been conducted by Alpuche Aranda and colleagues (1992). This group found that several hours after phagocytosis by macrophages, PhoP-activated gene transcription in *S. typhimurium* increased more than 50-fold. This same induction was not observed in epithelial cells, and could be abolished in macrophages by the addition of weak bases that raise the pH of acidic compartments. Measurements of pH indicated that *S. typhimurium* delayed and attenuated acidification of its intracellular compartment. Phagosomes containing live bacteria required 4-5 hours to reach pH $<$ 5.0, which corresponds to the time taken for maximal PhoP-dependent gene expression. In contrast phagosomes containing heat-killed bacteria reached pH $<$ 4.5 in less than 1 hour. From the above results, Alpuche Aranda and colleagues have speculated that phagosome acidification is an intracellular inducer of PhoP-regulated gene expression and suggest

that *Salmonella* survival is partly dependent on its ability to attenuate phagosome acidification.

Although there is a great deal of evidence to indicate that the PhoP/Q regulon of *S. typhimurium* plays an important role in the resistance of this bacteria to the battery of cationic peptides encountered within certain phagocytic cells during infection, none of the five PhoP-regulated genes identified so far have been attributed with encoding this particular protective function (Fields *et al*, 1989; Groisman *et al*, 1989; Miller *et al*, 1990a). However, recent work by Groisman and colleagues (1992) has identified a second set of genes from *S. typhimurium*, which are not only required for resistance to a range of antimicrobial peptides (including melittin and protamine) analogous to those found in the lysosomal granules of certain phagocytic cells, but are also essential for the full virulence of this bacterium in mice. As with a number of related studies conducted previously (*eg* Fields *et al*, 1986; Miller *et al*, 1989a), these mutants were isolated by transposon mutagenesis, in this case by isolating a library of 20,000 Mu*dJ* transposon insertion mutants of *S. typhimurium* and screened it for strains hypersensitive to protamine. This procedure resulted in the isolation of twelve mutants with reduced virulence in mice and heightened susceptibility to one or more peptides from a group of six tested (bovine protamine, rabbit defensin NP-1, frog magainin 2, pig cecropin P1, and the insect venom-derived peptides mastoparan and melittin). These were subsequently designated as *sap* (sensitive to antimicrobial peptide) mutants.

Further characterisation of the twelve *sap* mutants discussed above, showed that a number of these strains had a transposon insertion that mapped near *pyrF* at 33 minutes on the *S. typhimurium* chromosome (Parra-Lopez *et al*, 1993), indicating that this particular region of DNA

might be of some importance to the survival of this bacterium during it's exposure to this latter class of antimicrobial peptide. Sequence analysis of a 5.7 kb fragment which mapped to this same region of the *Salmonella* chromosome, showed five open reading frames: *sapA*, *sapB*, *sapC*, *sapD* and *sapF*, organized in an operon structure and transcribed in a 5.3 kb mRNA. Interestingly, SapD and SapF exhibited a high degree of similarity with several members of the 'ATP binding cassette' family of transporters, including Opp (Hiles *et al*, 1987) and SpoOK (Perego *et al*, 1991), which participate in the uptake of oligopeptides in *S. typhimurium* and *B. subtilus*, respectively. Furthermore, SapA shows identity with other periplasmic solute binding proteins involved in peptide transport (Abouhamad *et al*, 1991). This has lead Parra-Lopez and colleagues (1993) to speculate that the SapABCDF system constitutes a novel transporter for enteric bacteria, the first one identified harbouring a periplasmic component with a role in virulence.

In contrast to the subtle defence mechanisms discussed above for the survival of *S. typhimurium* within the harsh environment of the macrophage cell, Buchmeier and Heffron (1991) have proposed that this intracellular pathogen persists by adopting a more dramatic survival strategy. *S. typhimurium* has previously been reported to persist within the macrophage phagolysosomal compartment, where it apparently resists inactivation by lysosomal factors (Finlay and Falkow, 1989a). However, Buchmeier and Heffron (1991) contend that examination of *S. typhimurium*-infected macrophages by electron microscopy in comparison to infections with avirulent *E. coli* strains and heat-killed *S. typhimurium*, shows that virulent *S. typhimurium* cells actively inhibit phagosome-lysosome fusion and are able to proliferate within unfused phagocytic vesicles. This group also state that the inhibition of macrophage phagosome-lysosome fusions by *S.typhimurium* was not

blocked by opsonization with fresh normal mouse serum, and was not due to lipopolysaccharide.

Careful examination of the data provided in the above report (Buchmeier and Heffron, 1991), however, might equally provide an alternative explanation to that proposed by the latter workers. For example, since the number of unfused phagosomes containing bacteria was only twice as many in macrophages infected with live *S. typhimurium* cells, compared to macrophages infected with *E. coli* or heat killed *S. typhimurium* cells (each set of data recorded 4 hours post-infection), it is equally possible that this increased number of unfused phagosomes containing bacteria was due to one to two division of live *S. typhimurium* in these unfused, but still normally acidified compartments. This is substantiated by the fact that Buchmeier and Heffron (1991) were able to show that unfused phagosomes infected by live *S. typhimurium* divided in accordance with bacterial cell division.

Although the mechanisms that allow *S. typhimurium* to survive within the harsh intracellular environment of macrophage cells are not fully understood, this bacterium has been reported to achieve this ability by a strategy other than lysing the phagosomal membrane and escaping into the cytoplasm (Finlay and Falkow, 1989a), as is reported to be the case for *L. monocytogenese* (Tilney and Portnoy, 1989; de Chastellier and Berche, 1994). However, work by Libby and colleagues (1994) has shown that *Salmonella*, *Shigella*, and enteroinvasive *E. coli* all possess a cytolysin, and that this protein (termed salmolysin in *Salmonella* strains) is required for *S. typhimurium* to survive in macrophages and for virulence of this same bacterium in mice. Furthermore, by fusing the gene encoding salmolysin (*slyA*) to *lacZ*, the latter workers have demonstrated that the expression of this gene was tightly regulated and was independent of both *phoP* and *katF* regulation. These findings have

led Libby and colleagues (1994) to speculate that *slyA* is probably only expressed *in vivo* (*ie* during *S. typhimurium* infection of macrophage cells) and that, due to its structure, salmolysin might function as a pore forming protein which permeate the membranes of certain target host cells (*eg* macrophages) to disrupt their correct functioning and so allow the survival of the infecting bacteria.

As can be seen from the majority of the above reports (Fields *et al*, 1986; Fields *et al*, 1989; Miller *et al*, 1989a; Miller *et al*, 1990a; Gahring *et al*, 1990; Miller and Mekalanos, 1990; Groisman *et al*, 1992; Parra-Lopez, 1993) the advent of genetic techniques such as transposon mutagenesis has greatly increased our knowledge as to how *Salmonella* and other intracellular bacteria survive within phagocytic cells. Furthermore, a large number of the survival strategies adopted by these bacteria are now well understood. This understanding has in turn been put to good use through the development of several novel vaccines. Many of these vaccines consist of live attenuated auxotrophic bacteria. For example, in 1981 Hoiseth and Stocker reported that an aromatic-dependent mutant (*aroA*) of *S. typhimurium*, created by transposon mutagenesis, was attenuated in mice and effective as a live vaccine. Several other groups have also developed *aroA* based vaccine strains of a number of different intracellular pathogens *eg* *B. pertussis* (Roberts *et al*, 1990), *Y. enterocolitica* (Bowe *et al*, 1989), and as part of a double mutant in *S. typhi* (Edwards and Stocker, 1988; Fairweather, *et al*, 1990; Tacket *et al*, 1992).

The reason why *aroA* mutants are so effective as vaccine strains, whereas other auxotrophic mutants are not, is that pathogenic bacteria such as *S. typhimurium* cannot assimilate exogenous foliate and must synthesis it from *p*-amino-benzoic acid. However, the latter compound is almost absent in vertebrate tissue. Thus under circumstances where

p-amino-benzoic acid is not present, such as during infection of the host, bacterial pathogens such as *S. typhimurium* synthesis this compound from chorismate, the final product of the aromatic biosynthetic (*aro*) pathway. Hence, disrupting the aromatic biosynthetic pathway inhibits the synthesis of chorismate and leads to the absence of *p*-amino-benzoic acid, foliate, and bacterial growth (Bacon *et al*, 1950; Hoiseth and Stocker, 1981).

The information gained through the analysis of transposon-generated mutants in macrophages (Fields *et al*, 1986; Fields *et al*, 1989; Miller *et al*, 1989a; Miller *et al*, 1990a) has led to the development of several vaccine strains that are not auxotrophic mutants. For example, several groups have created mutations within the genes of the PhoP/Q regulon and tested these as potential vaccine strains (Galan and Curtiss, 1989b; Miller *et al*, 1990b). One of the advantages of using attenuated prototrophic vaccine strains, is that some auxotrophies are too crippling to the bacterium and so reduce the immunogenicity of the vaccine (Sigwart *et al*, 1989). Furthermore, several groups have found that *aroA* mutants are not fully attenuated in the host (Hormaeche *et al*, 1990; Miller *et al*, 1990b) and that an additional disruption within a gene of the PhoP/Q regulon further attenuates *aroA* mutants in *S. typhimurium* by at least 100-fold (Miller *et al*, 1989a).

Despite the large volume of information that has been gleaned in recent years, concerning the various strategies adopted by certain pathogenic bacteria for surviving within phagocytic cell, there still remains a large degree of ignorance as to the intricacies involved in the majority of the underlying survival mechanisms. Nevertheless, a great deal of work has been directed towards both oxygen-dependent and oxygen-independent killing mechanisms within phagocytic cells and their effects on certain intracellular pathogens. A fuller account of the bactericidal environment created within certain phagocytic cells in

response to bacterial interaction is discussed below.

1.3.3 The environmental stresses found within macrophages.

The major professional phagocytes involved in destruction of invading bacteria are neutrophils (also refered to as granulocytes when grouped with eosinophil and basophil cells), monocytes, and macrophages. Neutrophils and monocytes derive from common progenator cells in marrow. Upon maturation and release, the former circulate for a few hours and then migrate into tissues where they function for 4 to 5 days. In contrast, monocytes circulate for 1 to 3 days before maturing into macrophages that can survive for months either as mobile forms capable of migration into soft tissues or as sessile forms comprising an efficient antibacterial component of the reticuloendothelial system (Brubaker, 1985; Lydyard and Grossi, 1989). In addition to differing in size and morphology from neutrophils, macrophages have the additional key function of presenting antigen to T cells (Reeves, 1987).

Both macrophages and neutrophils generally respond to infective stimuli with the following sequence of activities: chemotaxis, target recognition, ingestion, killing and degradation. When activated, macrophages engulf bacteria in membrane-bound vacuoles or phagosomes which then fuse with azurophilic lysosomes to form phagolysosomes. The invading microorganisms are so subjected to an arsenal of oxygen-dependent and oxygen-independent killing mechanisms (Badwey and Karnovsky, 1980; Groisman and Saier, 1990).

i) Oxygen-dependent killing mechanisms of phagocytic cells.

When the oxygen-dependent killing mechanisms were first

recognised in phagocytes, the initial product of the respiratory burst was identified as hydrogen peroxide (H_2O_2), because it was found to oxidize formate. However, it was subsequently shown that the generation of H_2O_2 was actually due to dismutation of superoxide (O_2^-), the one-electron reduction product of oxygen (Fridovich, 1978). During the process of bacterial phagocytosis, O_2^- is produced in the region of the plasma membrane in direct contact with the bacterium. Furthermore, the discovery that free radicals were also produced in large amounts within the phagocytic vacuole implicated the reduced oxygen species themselves in microbial killing (Segal and Abo, 1993).

Production of activated oxygen species is a regulated process which appears closely linked to phagocytosis and the release of bactericidal proteins *via* oxygen-independent mechanisms. However, these processes can be separated by selective interference with cellular stimulus-response mechanisms, cytoskeletal movement, or by granule depletion (Forman and Thomas, 1986). The first regulatory step in the physiological stimulation of reactive oxygen species by the phagocyte is through receptor specificity. Interestingly, Newman and Tucci (1990) have shown that there is enhanced phagocytosis and killing when bacteria are opsonized. However, this is due to increased CR (complement) and FcR (antibody) receptor activity and not enhancement of the respiratory burst.

Binding of bacterial antigen (*eg* fMetLeuPhe) to the phagocyte receptor leads to an immediate depolarization of the plasma membrane potential, followed by a transient increase in free Ca^{2+} (Rossi, 1986) (The importance of depolarisation in respiratory burst stimulation was first suggested by failure of neutrophils from chronic granulomatous disease (CGD) patients to both depolarise and produce O_2^- (Segal, 1989)). This increase in cytosolic Ca^{2+}, and its subsequent binding to calmodulin,

results in stimulation of the phagocyte respiratory burst and to the generation of reactive oxygen species (Forman and Thomas, 1986) (Interestingly, infection of neutrophils with *L. pneumonophila* has been shown to inhibit the rise in intracellular Ca^{2+} (Dowling *et al*, 1992). This could help to account for this pathogens increased survival within phagocytic cells). The respiratory burst in phagocytic cells has also been reported to be induced by protein kinase C agonists, such as phorbol myristate acetate (PMA). This induction, however, has been reported to be by a Ca^{2+} independent mechanism (Segal and Abo, 1993).

The respiratory burst is facilitated by an NADPH oxidase, which derives its energy from NADPH generated *via* the hexose monophosphate shunt (Rossi, 1986). The transfer of energy from this electron donor (NADPH) to molecular oxygen is achieved by the catalytic actions of at least two oxidases (Forman and Thomas, 1986), which in turn leads to the formation of superoxide. In this scenario, NADPH acts an alternative electron donor to ATP, and explains why inhibitors of the electron transport chain (*eg* azide and cyanide) do not affect this mechanism (Sbarra and Karnovsky, 1959).

The respiratory burst induced in phagocytic cells leads to the generation of several reactive oxygen species, including singlet oxygen ($^1O_2$), hydroxyl radicals ($OH^{\cdot}$), hydrogen peroxide (H_2O_2), and superoxide anions (O_2^{-}). Each of these oxygen species has been implicated as an important factors in the killing of intracellular pathogens (Hassett and Cohen, 1989). $^1O_2$ and $OH^{\cdot}$ have been implicated as causative agents in lipid peroxidation and are also known to cause oxidation and damage to nucleic acids (Badwey and Karnovsky, 1980). Though highly bactericidal, the activity of H_2O_2 can be markedly increased by its combination with certain halides (Cl^-, I^- and Br^-) mediated by the granule-associated protein myloperoxidase (MPO) (Brubaker, 1985). The actual microbicidal agents

in this process are toxic compounds such as $HOCl^-$, $HOCl_3$ and chloramines (Segal and Abo, 1993). It has been estimated that at least 28% of the oxygen consumed in the respiratory burst of neutrophils is converted to active chlorinating agents (Foote *et al*, 1983). These compounds have been shown to cleave peptide bonds and oxidatively decarboxylate amino acids (Badwey and Karnovsky, 1980), oxidize plasma membrane sulfhydryl groups (Schraufstatter *et al*, 1989), and damage the succinate oxidase system (Rosen *et al*, 1987). Lastly, O_2^- has not been shown to have microbicidal activity within phagocytes and the importance of this radical probably relates to its ability to interact with H_2O_2 to generate $^1O_2$ and OH.

Recently, a great deal of attention has been focused on the production of nitric oxide (NO) by macrophages (Lorsbach and Russell, 1992), especially in reference to its antimicrobial activity on certain bacterial pathogens, including *M. leprae* (Adams *et al*, 1991). NO is a noxious, free radical gas produced in a range of cells by the activities of two major classes of nitric oxide synthases. In neurons and endothelial cells the enzyme is expressed constitutively, and is activated by calmodulin and calcium. By contrast, the macrophage enzyme is only produced after simultaneous activation of the macrophages by bacteria and various cytokines (Lorsbach and Russell, 1992), but its activity is independent of calmodulin and calcium, and it is always fully active (Prince and Gunson, 1993).

A key function of NO in endothelial cell is to regulate vascular tone, whereas in neurons it acts as a novel messenger. In macrophages, NO has been implicated in mediating bactericidal and tumoricidal activities (Adams *et al*, 1991; Noronha-Dutra *et al*, 1993). Inducible NO synthase also occurs in neutrophils. The oxygen free radicals formed by activated neutrophils and macrophages can combine with NO to form

substances substantially more toxic than NO itself. For example, NO combined with O_2^- yields peroxynitrite that decomposes to the free radicals OH^- and NO_2 (Lowenstein and Snyder, 1992). NO also combines with H_2O_2 to produce $^1O_2$ (Noronha-Dutra *et al*, 1993). NO is derived from arginine, and as a consequence, the addition of certain arginine derivatives, or removal of arginine from the incubation medium, result in the reduction of the bactericidal action of macrophages. This implies that NO can be a crucial mediator of the macrophages bactericidal functions (Lowenstein and Snyder, 1992).

As will be discussed in more detail later in this Chapter, *S. typhimurium*, as with most enteric bacteria, have enzyme activities, such as superoxide dismutase and catalase, that may protect the cell from oxidative damage in host phagocytes (Kagaya *et al*, 1992). In addition, other enzymes such as exonuclease 3 and recA protein appear to be important in repairing DNA lesions resulting from oxidative damage (Christmas *et al*, 1985). Buchmeier and Heffron (1990) have reported the synthesis of over 30 *Salmonella* proteins to be selectively induced during infection of macrophages, including the heat shock proteins GroEL and DnaK, both major immunodominant antigens for many infectious organisms (Buchmeier and Heffron, 1990; Watson, 1990; Garbe, 1992), and one of which, DnaK, is also induced by O_2^- and H_2O_2 (Farr and Kogoma, 1991).

Kwaik and colleagues (1993) have reported similar finding to those stated above in the intracellular pathogen *L. pneumophila.* This group found that infection of macrophages by this pathogen selectively induced the synthesis of at least 35 *Legionella* proteins. Thirteen of these macrophage-induced proteins were shown also to be induced by one or more of several stress conditions *in vitro*, including heat shock, osmotic shock, and hydrogen peroxide. Two of these proteins were the heat

shock GroEL- and GroES-like proteins. Furthermore, Kagaya *et al* (1992) have shown that mice inoculated with a sublethal dose of *S. typhimurium* develop an intensive protective immunity associated with both a H_2O_2-induced catalase (catalase II related to HPI/HPII) and a constitutively produced catalase (catalase I related to HPIII). However, only purified catalase II was found to give protective immunity in vaccine trials. Hence, it appears that certain oxidative/stress induced proteins, as well as being important for survival within macrophages are, ironically, the key to cellular immunity.

Recently, Kantengwa and Polla (1993) have shown that phagocytosis of microorganisms represents a stress not only for the phagocytosed agent but also for the host cell. Phagocytosis of *S. aureus* induced host cell HSP70, superoxide dismutase, and *de novo* synthesis of heme oxygenase in human macrophages. This group concluded that the coordinate upregulation of two scavenging enzymes and of HSP70 suggests that all three are part of the cellular protection mechanisms against phagocytosis-related oxidative injury to host cells.

ii) Oxygen-independent killing mechanisms of phagocytic cells.

As already discussed above, the discovery that free radicals were produced in large amounts within the phagocytic vacuole, implicated the reduced oxygen species themselves as the most likely candidates in microbial killing. However, cytoplasts (enucleated, granule-free bodies) also phagocytose bacteria and produce a normal respiratory burst, but kill the bacteria much less efficiently (Roos *et al*, 1983). Similarly, the absence of myeloperoxidase in phagocytes such as ovine neutrophils, does not overtly effect microbial killing (Segal and Abo, 1993). Therefore, unless nitric oxide is the essential component for mediating

killing within phagocytic cells, other factors beside reactive oxygen species must also be important.

Oxygen-independent mechanisms of microbial killing have not been as extensively studied as oxygen-dependent mechanisms, and are thought to be extremely complex. Past studies have tended to focus on areas such as acidification of the phagosome and the degradation of its contents by lysozyme and acid hydrolases (Reeves, 1987). However, Elsbach (1990) has reported that there is a much wider range of cytotoxic (antibiotic) proteins and peptides within mammalian phagocytes and from phagocytic organisms all along the evolutionary scale. For example, the list of antimicrobial polypeptides found within the cytoplasmic granules of human neutrophils is quite extensive and includes, defensins, lysozyme, azurocidin, lactoferrin, cathepsin G, elastase, bactericidal-permeability increasing protein (BPI), and proteinase 3 (Elsbach, 1990; Lehrer and Ganz, 1990). In contrast, the list of antimicrobial polypeptides found within human macrophages is much shorter and includes lysozyme and certain cationic peptides that have been found to be distinct from defensins (Watanabe *et al*, 1991).

The majority of the antimicrobial agents produced by the oxygen-independent mechanisms of professional phagocytes are extremely diverse, varying in size, structure and effect. This diversity makes it almost impossible to find common molecular determinants to account for their cytotoxic action (Elsbach, 1990). In addition to the structural and functional differences reported between these various antimicrobial agents, there are also considerable differences reported among their distribution within different phagocytic cell types (Hiemstra *et al*, 1993). This diversity has even been demonstrated to exist between species, where identical phagocytic cell types from two different species have been reported to contain different agents (Eisenhauer and Lehrer,

1992). For example, human neutrophils (but not macrophages (Watanabe *et al*, 1991)) have been reported to contain at least four types of defensins (Ganz *et al*, 1990). Similarly, defensins have been isolated from the neutrophils of rabbits, but they are also present in rabbit macrophages (Ganz *et al*, 1985). Mouse neutrophils, however, do not contain defensins, nor have any been reported in mouse macrophages (Eisenhauer and Lehrer, 1992).

Recently, Hiemstra and colleagues (1993) have analysed the antimicrobial peptides produced by cultures of activated mouse macrophages and identified three murine microbicidal proteins (MUMPs). These proteins are very similar (structurally) to certain histone proteins (*eg* H1) and were found to kill a range of bacteria, including *S. typhimurium*, *E. coli*, *S. aureus*, *L. monocytogenes*, and *M. fortuitum*. Similarly, histone proteins have also been found to be bactericidal (Hirsch, 1958). As with defensins, both MUMPs and histone proteins have cationic carboxyl termini (Hiemstra *et al*, 1993). However, in many other respects MUMPs are very different from defensins, as are similar proteins found in human macrophages (Watanabe *et al*, 1991).

The pH regulation of phagocytic vesicles is believed to be essential to the bactericidal capacity of phagocytes during infection (Foster, 1992; Falkow *et al*, 1992). However, due to the complexities of studying this phenomenon, it has been very poorly documented, and those few reports extremely contradictory.

Cellular acidification has been shown to occur during the respiratory burst of macrophages (Seguin *et al*, 1990), probably in relation to the function of the endocytic vesicles (Seguin *et al*, 1991). The precise mechanisms for this vacuole acidification are not known, but result in a rapid increase of H^+ ions (Garbe, 1992). This in turn leads to a decrease in pH within the phagolysosome, which is reported to drop to

less than pH 4 (Foster, 1992). Many bacterial pathogens, which have evolved to grow within a vacuole, utilize mechanisms to prevent acidification of the vacuole and/or the fusion of the vacuole with lysosomes (Falkow *et al*, 1992). Alternatively, *C. burnetti*, an obligate intracellular pathogen, has evolved to the point that it actually requires an acidified phagolysosome for growth (Hackstadt and Williams, 1981). Furthermore, there is mounting evidence that *L. monocytogenes* requires an acidified phagosome to induce vacuolar lysis by listeriolysin O (de Chastellier and Berche, 1994).

Alternatively, Segal and Abo (1993) state that the pH within the phagocytic vacuole is in fact neutral, and that the mechanisms behind activation of the bactericidal capability of the vacuole is remarkably simple and is based on alkalinisation. In brief, the pumping of millimolar concentrations of electrons, unaccompanied by protons, into the vacuole results in the consumption of hydrogen ions within the lumen, thus increasing the pH. The granule contents are normally maintained in an inactive state at a pH of about 5.0 (Ohkuma and Poole, 1978), and the neutral proteinases are activated when exposed to the relatively alkaline environment within the vacuole. This would explain why the optimal pH for certain phagocytic proteinases, such as defensins, is neutral (Elsbach, 1990; Lehrer *et al*, 1990).

As discussed above, several groups of workers have reported the isolation of transposon generated mutants of *S. typhimurium* with heightened susceptibility to a number of cationic peptides extracted from, or similar to those found in, a range of phagocytic cells (Fields *et al*, 1986; Fields *et al*, 1989; Miller *et al*, 1989a; Miller *et al*, 1990a; Gahring *et al*, 1990; Miller and Mekalanos, 1990; Groisman *et al*, 1992; Parra-Lopez, 1993). At least two genetic systems have been identified (*ie* the PhoP/Q regulon (Fields *et al*, 1989) and the SapABCDF system

(Parra-Lopez *et al*, 1993)) that are known to be involved with the bacterium's resistance to these peptides and its increased virulence in mice (Galan and Curtiss, 1989b; Miller *et al*, 1990b; Groisman *et al*, 1992), however, neither is well understood.

The best characterized of the two known genetic systems involved in the survival of *S. typhimurium* upon its exposure to certain antimicrobial peptides, is the PhoP/Q regulon. This regulon has been shown to be induced by a number of stimuli, including phosphate starvation, low pH, and macrophage interaction (Groisman *et al*, 1989; Foster and Hall, 1990; Miller *et al*, 1989a). Furthermore, Alpuche Aranda and colleagues (1992) have demonstrated that, in addition to encoding proteins responsible for the anti-defensin (or anti-defensin-like) capabilities seen within phagocytic cells, certain genes of this regulon are also involved in directing alterations in the phagocytic pH. In brief, these workers measured the pH within the endocytic compartments of macrophages (recorded by measuring the fluorescence excitation spectrum of internalised fluorescein isothiocyanate-conjugated dextran) to demonstrate that *S. typhimurium* delays and attenuates acidification of macrophage phagosomes. Additionally, it was found that the duration of this response correlates well with the induction time of PhoP-dependent gene expression (Miller *et al*, 1989a; Alpuche Aranda *et al*; 1992). These results imply that the PhoP/Q regulon might be responsible for sensing and adjusting pH within the phagosome.

The complex interplay that occurs within phagocytic cells between the antimicrobial proteins of the oxygen-independent mechanism and the reactive oxygen species of the oxygen-dependent mechanism is not well understood. Ganz and co-workers (1990) have reported the occurrence of individuals where microbicidal deficiencies exist in one or both of the above killing mechanisms. In patients suffering from Chronic

Granulomatous Disease, the phagocyte's respiratory burst is absent. This condition results in a selective and partial decrement in the microbicidal activity of phagocytes and is often associated with recurrent and severe bacterial and fungal infections. Alternatively, patients with either Chediak-Higashi syndrome or Specific Granule Deficiency, in which the microbicidal capacity of the phagocytic cells is again reduced, have been shown to have phagocytes with a normal respiratory burst but antimicrobial granule components that are defective or missing. This suggests that both the respiratory burst and the constituents of granules are required for optimal phagocytic killing of microbes.

iii) Additional factors affecting killing in macrophages

The importance of iron in the intracellular multiplication of bacterial pathogens has been demonstrated in a number of genera, including *Salmonella, Yersinia, Listeria, Legionella, Neisseria,* and *Bordetella* (Griffiths, 1987; Carniel *et al*, 1987; Adams *et al*, 1990; Byrd and Horwitz, 1993; Gebran *et al*, 1994; Holbein, 1981; Gorringe *et al*,1990). The two major sources of iron in phagocytes, are iron-saturated lactoferrin (LF) (an iron-binding glycoprotein which is endocytosed bound to LF receptors) and ferritin (an intracellular iron storage protein). Ferritin can be degraded to haemosiderin, and the former is present as insoluble granules within secondary lysosomes (Crichton, 1984).

During infection, neutrophils are stimulated to release LF which, in its iron-free state, captures iron from transferrin (TF) (an iron-binding glycoprotein with lower affinity for iron than LF, found mainly in blood). The LF-iron complex is then picked up by fixed or circulating macrophages and the latter are removed rapidly from the circulation by the reticuloendothelial system (Otto *et al*, 1992). In contrast to this

iron-clearing function of macrophages, Byrd and Horwitz (1993) have reported that gamma-interferon-activated human monocytes inhibit intracellular multiplication of *L. pneumophila* by limiting the intracellular iron. This limiting of iron is achieved by downregulation of the LF receptors on the cell surface, with a consequent decrease of the intracellular concentration of ferritin.

Many intracellular bacteria are capable of removing iron from TF or LF by the synthesis of low molecular weight, high-affinity iron chelators, termed siderophores (*eg* enterobactin and aerobactin) (Griffiths, 1987). Iron-siderophore complexes are taken up by such bacterium *via* specialized iron-repressible outer membrane proteins (IROMPs) (Tigyi *et al*, 1992; Otto *et al*, 1992). In *E. coli*, one type of IROMP has been reported to be encoded by the *fep* gene, which is part of the enterobactin gene cluster (*ent, fep, fes*) and is responsible for the biosynthesis, transport, and hydrolysis of enterobactin. This iron transport system is regulated by the product of the *fur* gene (a universal regulator of all known iron transport systems in *E. coli*), which has been strongly associated with enhanced virulence in *E. coli* and *Corynebacterium diphtheriae* and plays a role in controlling the expression of certain toxins (DiRita and Mekalanos, 1989).

It is interesting to note that, besides haemoglobin and myoglobin, the proteins with the highest iron content in the body are enzymes that are involved in the elimination of reactive oxygen species from host cells. These enzymes, including catalases, peroxidases, and some oxygenases (Crichton, 1984; Otto *et al*, 1992), help to inhibit oxidative damage to nucleic acids, proteins, and lipids (as similarly discussed with oxidative damage in bacterial systems) (Imlay and Linn, 1988; Demple, 1991; Ahern, 1991). This helps to explain the dichotomy found with listeriae infections of macrophages, where iron has been shown to be essential in

both supporting macrophage listericidal mechanisms, and also in allowing the intracellular growth of listeriae (Adams *et al*, 1990).

In recent years a great deal of research has focused on the complex interplay between macrophages and helper T-cells (T_H cells). The activation of macrophage effector functions is regulated by an intricate lymphokine network with positive and negative signals. Production of lymphokines is a major function of T_H cells (Kaufmann and Flesch, 1992). These cells recognise antigenic peptides in association with major histocompatibility complex (MHC) class II molecules, and respond by secreting gamma interferon (IFN-τ) and a range of interleukins (IL) (Kaufmann and Flesch, 1992). IFN-τ has been shown to protect mice against *L. monocytogenes* (Buchmeier and Schreiber, 1985), and cause inhibition of growth in *M. bovis* and *M. tuberculosis* (Kindler *et al*, 1989).

The antibacterial activity of IFN-τ has been shown to be enhanced significantly by the macrophage product, tumour necrosis factor-α (TNF-α) (Kindler *et al*, 1989). Nauciel and Espinasse-Maes (1992) have shown that mice infected with a sublethal dose of *S. typhimurium*, die when either IFN-τ- or TNF-α- monoclonal antibodies are subsequently injected. These findings have been substantiated by Edwards and colleagues (1992), who have shown that IFN-τ enhances macrophage killing of *S. typhimurium* by increasing the production of reactive oxygen intermediates. Similarly, NO production by macrophages requires either simultaneous or sequential exposure to IFN-τ and lipopolysaccharide (Lorsbach and Russell, 1992).

IL-4, derived from T cells, has also been shown to increase the degree to which macrophages are activated during infection, enhancing both antigen presentation and antigen expression by murine peritoneal macrophages. This interleukin has also been reported to induce stasis of

M. tuberculosis in macrophages (Kaufmann and Flesch, 1992). The pleiotropic mediator, IL-6, is not only produced by T-cells but also by fibroblasts, epithelial cells and monocytes/macrophages. Like IL-4, IL-6 also activates tuberculostatic functions in infected macrophages. Since TNF-α and IL-6 are produced by macrophages themselves, they might control macrophage antibacterial function at the autocrine level (Kaufmann and Flesch, 1992).

In addition to the range of lyphokines and cytokines that enhance the antimicrobial functioning of macrophages, there are also a number that reduce macrophage activity, including transforming growth factor-β (TGF-β). This cytokine is again produced by macrophages themselves with the effect that, macrophages exposed to TGF-β fail to produce reactive oxygen- and reactive nitrogen intermediates and are not able to kill intracellular parasites after activation (Nelson *et al*, 1991; Lorsbach and Russell, 1992).

Finally, the genetic status of the host has been shown to be important in innate resistance to several intracellular bacteria in mice. For instance, by selectively breeding hybrid mice which show a 37.4% mortality to natural *S. typhimurium* mouse typhoid infection under standard conditions, mice can be bred which display mortality rates of approximately 15 and 85% following infection (Wakelin and Blackwell, 1988). More specific breeding experiments have been conducted on particular strains of mice, selected by the characteristic killing capacities of their macrophages. *L. pneumophila* has been shown to proliferate in peritoneal macrophage cultures derived from A/J mice but not in macrophages cultures derived from many other strains, including C57BL/6 mice. By selectively crossing these mice and their progeny, it was shown that resistance and susceptibility are controlled by a single gene or closely linked genes which are autosomal, and that the resistance

allele is dominant. This susceptibility-resistance gene was mapped to the proximal part of mouse chromosome 15 (Yoshida *et al*, 1991).

Probably the best characterized locus implicated in innate resistance in mice is the *ity* gene, located on mouse chromosome 1. This gene has been shown to regulate the extent to which a range of intracellular bacteria, including *S. typhimurium*, *M. bovis*, and *M. lepraemurium*, replicates within the reticuloendothelial cell system during the first days of infection (Skamene *et al*, 1982). In animals that are homozygous for the ity^s susceptibility allele, *S. typhimurium* undergo rapid net multiplication, and mice die of typhoid fever-like disease by day 10 of infection. Animals that are heterozygous or homozygous for the resistance allele, ity^r, control net *Salmonella* replication and survive the first phase of salmonellosis (Lissner *et al*, 1983). Indirect studies have implicated the resident macrophages as the effector cells for regulation of early salmonellae growth *in vivo* (Swanson and O'Brien, 1983). This was verified using macrophages from inbred ity^r and ity^s mice and from *ity* congeneic mice. Phagocytosis of *S. typhimurium* by ity^r and by ity^s macrophages was the same. However, bacteria grew to a greater extent in ity^s macrophages than in ity^r cells, and ity^r macrophages killed intracellular salmonellae more efficiently than did ity^s macrophages (Lissner *et al*, 1983).

1.3.4 Conclusions

Intracellular bacteria are, by their very nature, extremely well adapted to life within host eukaryotic cells. In *S. typhimurium*, the ability to survive within these hostile intracellular environments appears to be encoded by a number of genetic loci that may be induced by intracellular stresses, such as low pH and oxidative stress (Hassett and Cohen, 1989;

Miller, 1991; Farr and Kogoma, 1991). These stress-induced loci can be subdivided into sets of genes that are co-ordinately controlled by distinct regulatory proteins (each set being termed a regulon). One of the most characterized regulons connected with *S. typhimurium* pathogenesis in mice is the PhoP/Q regulon. This set of genes has been implicated in *S. typhimurium* invasion of murine epithelial cells (Behlau and Miller, 1993), in survival within murine macrophages (Fields *et al*, 1986; Miller *et al*, 1989a) and in pathogenisity in mice (Galan and Curtiss, 1989; Miller *et al*, 1990b; Behlau and Miller, 1993). Furthermore, this regulon has been demonstrated to be responsible for controlling resistance to defensins (Fields *et al*, 1989; Miller *et al*, 1990a) and has been implicated in regulating the intracellular pH of macrophages (Alpuche Aranda *et al*, 1992).

The task of identifying and characterising bacterial genetic loci essential for pathogenicity within the host is extremely arduous. For example, identification of the *phoP* locus in *S. typhimurium* involved the screening of approximately 10,000 independent colonies carrying Tn*10* insertions, for reduced survival in murine macrophages (Fields *et al*, 1986). Furthermore, since the *phoP* locus is responsible for regulating the synthesis of over 40 different proteins (Miller and Mekalanos, 1990), it is not surprising (from a statistical point of view) that the disruption of this locus should have such a dramatic effect on *S. typhimurium*. Identification of a locus that only affected the synthesis of a single protein might be more difficult under the same circumstances. In fact, extensive studies of the genes regulated by the PhoP/Q regulon have demonstrated this, with only one virulence locus, *pagC*, encoding a single outer membrane protein, clearly being identified to date (Pulkkinen and Miller, 1991). (A second virulence locus, *prgH*, which is involved with the invasion of epithelial cells, has also been identified recently (Behlau

and Miller, 1993). However, this locus has not been fully characterized as yet). Additionally, it has been shown that *pagC* mutants are not as attenuated as *phoP* mutants (Miller, 1991), further emphasizing this point.

From the above information, it is likely that similar infection studies to those reported by Fields and colleagues (1986) and Miller and co-workers (1989a), will result in the discovery of more regulons with genes involved in virulence, rather than specific virulence genes. An alternative approach, is not to identify bacterial loci that are essential for survival within macrophages, but to determine which bacterial proteins are specifically synthesized during macrophage infections. This can be accomplished by several different methods, some of which are described in more detail towards the end of this Chapter.

As discussed above, one of the major stresses to be encountered by enteric bacteria during their infection of host cells, is oxidative stress. This stress, however, is also frequently encountered in non-pathogenic environments and is a key feature in the survival of the majority of organisms. The effects of oxidative stress and some of the defence mechanisms adopted by bacteria are discussed in more detail below.

1.4 Oxidative stress in bacterial cells.

1.4.1 Active oxygen species.

Organisms which employ oxygen as the terminal electron acceptor in oxidative phosphorylation, must deal with the toxic side-effects of molecular oxygen (O_2), which are mediated through the avid participation of this gas in radical-generating reactions (Fridovich, 1978). Furthermore, organisms which are adept at surviving as intracellular pathogens within phagocytic cells, must also be able to cope with the toxic oxygen species

that occur as a consequence of the host's defenses (Adams and Hamilton, 1984; Moulder, 1985; Finlay and Falkow, 1989b). In both cases, these reactive oxygen species can readily diffuse across cell membranes and are capable of causing extensive cellular damage (Imlay and Linn, 1988; Demple, 1991; Ahern, 1991).

In the ground state, O_2 has two unpaired electrons in separate outer orbitals, both rotating in the same direction. In this state, molecular oxygen is at its lowest energy level, and is relatively unreactive. However, each of its outer orbitals can accommodate an additional electron, with each addition increasing the reactivity of this molecule. This increased reactivity can be most commonly seen in aqueous environments at neutral pH, with the formation of reactive oxygen species such as superoxide radical (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radicals (OH^-) (Badwey and Karnovsky, 1980). The reactivity of molecular oxygen can also be increased when an electron undergoes a spin flip to give singlet oxygen ($^1O_2$), and under more acidic conditions, when O_2 can be protonated by hydrogen to form hydroperoxyl radicals (HOO^-) (Brunori and Rotilio, 1984).

As already stated, reactive oxygen species are predominantly produced within the cell as an inescapable by-product of normal aerobic metabolism, in particular, by oxidative phosphorylation. The production of these reactive oxygen compounds within cells has been shown to result from the natural activity of a variety of enzymes, including NADH dehydrogenase, succinate dehydrogenase, and D-lactate dehydrogenase (Imlay and Fridovich, 1991). Nonenzymatic production of reactive oxygen species (*eg* O_2^-), can also be induced in cells by redox-cycling agents such as menadione and plumbagin which causes autooxidation of several cellular components (Farr *et al*, 1985; Pryor, 1986). High energy photons (usually associated with the UV spectrum of natural light) have

also been shown to induce the formation of reactive oxygen species (*eg* H_2O_2 and OH^-) within the cell (Eisenstark, 1989).

1.4.2 The cellular damage caused by oxidative stress.

Oxidative stress can be functionally defined as the damage caused by an excess of oxidizing agents in the cell. Active oxygen species have been reported to cause damage to DNA, RNA, proteins, and lipids (Imlay and Linn, 1988; Demple, 1991; Ahern, 1991). Toxicity due to reactive oxygen species, results when the degree of oxidative stress exceeds the capacity of the cell's defense systems to combat cellular damage. In mammals, oxidative stress has been strongly implicated in a number of diseases such as rheumatoid arthritis, inflammatory bowel disorders, and atherosclerosis (Halliwell and Gutteridge, 1990). It is also emerging as one of the main causative agents of mutagenesis, tumorigenesis, and ageing (Adelman *et al*, 1989; Floyd, 1990).

Virtually all aerobic organisms have evolved complex defence and repair mechanisms to dissipate the damaging effects of reactive oxygen derivatives. These mechanism have been extensively studied in a range of organisms, including a number of bacterial species (Farr and Kogoma, 1991; Hartford and Dowds, 1992; Lee *et al*, 1993). This has been made easier in recent years by the powerful genetic techniques available for manipulating bacteria, and tremendous progress has been made toward understanding the genetic and physiological responses of cells to oxidative stress. The majority of (if not all) aerobic bacteria appear to encode multigenic responses to oxidative stress (Bol and Yasbin, 1990; Lee *et al*, 1993). These have been most extensively studied in *E. coli* and *S. typhimurium* (Christman *et al*, 1985; Walkup and Kogoma, 1989).

1.4.3 Oxidative stress responses in *E. coli* and *S. typhimurium.*

As previously discussed, active oxygen species naturally arise in aerobic cells from a variety of sources (Fridovich, 1978; Moulder, 1985). It is not surprising, therefore, that cells maintain a strong defense against the damaging effects of these molecules. For example, aerobically grown *E. coli* cells are equipped with two superoxide dismutases (SODs), an O_2^- -inducible MnSOD encoded by *sodA* (Touati, 1983) and a constitutively produced FeSOD encoded by *sodB* (Sakamoto and Touati, 1984). SODs catalyse the dismutation of superoxide to hydrogen peroxide, which is in turn converted into molecular oxygen and water by the action of a variety of peroxidases, including two catalases: the H_2O_2-inducible HPI catalase, encoded by *katG* (Loewen *et al*, 1985b), and the constitutively produced HPII catalase, encoded by *katE* (Loewen, 1984). Alkylhydroperoxide reductase (Ahp), encoded by *ahpC* and *ahpF* (Storz *et al*, 1989) is thought to provide additional defense by reducing organic hydroperoxides.

An extensive study by Walkup and Kogoma (1989) using two-dimensional protein gel analysis, has shown that *E. coli* cells which are exposed to elevated levels of superoxide, respond by inducing approximately 40 proteins. These O_2^--induced proteins include the heat shock proteins GroEL, GroES and DnaK, the two hydrogen peroxide inducible proteins HPI catalase and Ahp, and at least 30 other proteins that are not seen with H_2O_2 induction (Greenberge and Demple, 1989).

At least nine of the 40 superoxide inducible proteins form a co-regulated group (regulon) under the control of an *E. coli* locus named *soxR* (standing for superoxide radical response (Tsaneva and Weiss, 1990; Greenberg *et al*, 1990)). By analyzing proteins encoded within this region, Tsaneva and Weiss (1990) identified two gene products, termed

SoxR and SoxS, which are essential for inducing the genes of the so called, SoxRS regulon (Wu and Weiss, 1991). This regulon is considered to be a fundamental part of the superoxide stress response, regulating proteins such as endonuclease IV, which increases the DNA repair capacity of the cell (Chan and Weiss, 1987). Furthermore, Farr and colleagues (1985) have demonstrated that there is an adaptive response to superoxide exposure, where cells pretreated with a sub-lethal dose of the redox cycling agent plumbagin, display enhanced survival upon subsequent exposure to a normally lethal dose of this latter compound.

Exposure of *E. coli* or *S. typhimurium* cells to hydrogen peroxide also results in a multigenic stress response. The cellular concentrations of approximately 30 proteins become elevated over the basal levels. Of those 30 proteins, the synthesis of 12 (early proteins) is dramatically increased during the first 30 minutes, whereas 18 (late proteins) continue to be synthesized at an elevated rate in the second 30 minutes (Christman *et al*, 1985). As with the superoxide response, in *E. coli* the heat shock proteins GroEL, GroES and DnaK are also elevated (Greenberg and Demple, 1989).

Christman and colleagues (1985) have shown that, of the 30 proteins induced by hydrogen peroxide, at least eight proteins in *E. coli* and at least nine proteins in *S. typhimurium* are positively regulated by the *oxyR* locus thereby forming a regulon. *oxyR* constitutive mutants, isolated by chemical mutagenesis, were shown to have elevated level of the latter proteins, whereas both *E. coli* and *S. typhimurium* deleted for the *oxyR* gene failed to induce these proteins. Furthermore, Christman and colleagues (1985) have demonstrated that, as with superoxide pre-treatment of cells, both *E. coli* and *S. typhimurium* treated with a sublethal dose of H_2O_2 display enhanced resistance to subsequent lethal challenge doses of H_2O_2.

In many respects the superoxide- and the hydrogen peroxide stress responses in *E. coli* are very similar. Both induce a clearly defined set of some 30 to 40 proteins upon stimulation, including some commonly induced proteins (*eg* GroEL, GroES, DnaK). Furthermore, both oxidative stress responses induce a subset of stress proteins that are co-ordinately regulated by a distinctive regulon (*ie* SoxRS or OxyR). Nevertheless, in other respects these two stress responses are quite different. For example, cells pretreated with H_2O_2 and cells preinduced with O_2^- generators do not develop cross-resistance: that is, H_2O_2-treated cells show no enhanced survival when exposed to plumbagin, and *visa versa* (Farr *et al*, 1985). Similarly, H_2O_2-pretreated cells reactivate H_2O_2-damaged phage but not O_2^--damaged phage, whereas O_2^--pretreated cells reactivate O_2^--damaged phage but not H_2O_2-damaged phage (Demple and Halbrook, 1983). Thus, it appears that in each case the induction of specific proteins in response to a particular stress (*eg* O_2^- or H_2O_2) is essential for the organisms survival of that stress. The OxyR regulon is discussed in more detail below.

1.4.4 The OxyR regulons of *E.coli* and *S. typhimurium.*

As discussed above, Christman and colleagues (1985) demonstrated that the expression of a limited number of the *S. typhimurium* proteins induced with H_2O_2 are regulated by the *oxyR* locus. This was shown by the isolation of an *S. typhimurium* mutant, *oxyR1*, in which the expression of 9 of the 12 early proteins was constitutively elevated. The *oxyR1* mutation was mapped to approximately 89.5 minutes on the *S. typhimurium* chromosome, and *oxyR* deletions were then generated in both *S. typhimurium* and *E. coli*. The deletion of this locus in *S. typhimurium* was found to abolish the inducibility of the same nine

proteins overexpressed by the *oxyR1* mutant. From these results it was inferred that the *oxyR* gene product is a positive factor essential for activation of the genes of the OxyR regulon. Further work by Christman and colleagues (1989) and Tao and colleagues (1989), was conducted to clone and sequence the *oxyR* gene of *E. coli*. This work revealed that the *oxyR* gene encoded a 305 amino acid protein of 34.4 kDa with homology to a family of positive regulatory proteins (Christman *et al*, 1989; Tao *et al*, 1989), including LysR of *E. coli* (Henikoff *et al*, 1988) and NodD of *Rhizobium* (Horvath *et al*, 1987). Like the genes encoding these latter proteins, *oxyR* appears to be negatively autoregulated (Tao *et al*, 1991).

Regulation by OxyR operates primarily at the transcriptional level. Indeed, the *katG* transcript is elevated by more than 50-fold in *S. typhimurium oxyR1* constitutive mutants (Morgan *et al*, 1986). There is also a 50-fold increase in catalase activity observed in this mutant (Christman *et al*, 1985), as well as elevated levels of both the *ahp* transcript and AhpC and AhpF proteins (Tartaglia *et al*, 1989). Furthermore, induction of β-galactosidase by H_2O_2 in strains carrying a *katG::lacZ* operon fusion, is completely blocked by introduction of an *oxyR* deletion mutation (Tartaglia *et al*, 1989).

Tao and colleagues (1991) have studied the *in vivo* regulation of *oxyR* expression in merodiploid *E. coli* cells using a plasmid carrying an *oxyR'-'lacZ* gene fusion. This showed that the expression of *oxyR* was not induced by treatment with the low concentrations of H_2O_2 that induced the genes of the OxyR regulon. Furthermore, the basal expression of the *oxyR'-'lacZ* gene fusion was higher in an *oxyR*-deletion strain than in the parental strain ($oxyR^+$), and was repressed by the overexpression of the OxyR protein. These results suggest that the OxyR protein functions as a repressor of the *oxyR* gene, in addition to its function as a transcriptional activator for the other genes of the OxyR

regulon.

The above results would seem to represent something of a quandary. In the first instance, OxyR protein activates transcription of the genes of the OxyR regulon in response to an elevated flux of H_2O_2 (Tartaglia *et al*, 1989), and yet, in the second instance, OxyR protein is not synthesized in response to this same H_2O_2 flux (Tao *et al*, 1991). However, a plausible solution to this oddity has been proposed by Storz and colleagues (1990). This group purified OxyR protein from *E. coli*, and showed that oxidized but not reduced OxyR protein activates transcription of OxyR-induced genes, such as *ahpC/F* and *katG*. Furthermore, these workers found that oxidation of purified OxyR protein occurs in air, and that the addition and removal of dithiothreitol (a reducing agent), permits conversion between the active and inactive forms of the protein. Finally, the conversion of the protein to the active form by removal of the reductant was found to be prevented by the addition of catalase (Storz *et al*, 1990). Hence, it appears that as soon as OxyR protein is removed from the reducing environment within the cell, it is oxidized, perhaps by H_2O_2 that is present under the aerobic conditions. Thus, in conclusion, oxidative stress induced by increased flux of H_2O_2 converts OxyR protein to an oxidized form, which in turn activates transcription of the genes in the OxyR regulon. In this scenario, OxyR protein may serve as both the sensor and the transducer of an oxidative stress signal which is H_2O_2 itself, and high level induction of the *oxyR* gene is not required (Farr and Kogoma, 1991) .

Tartaglia and colleagues (1989) constructed a set of deletions in the *ahpC* promoter to further define the mechanisms by which *oxyR* regulated proteins are produced *in vitro*. Analysis of these deletions by DNA footprinting with purified OxyR protein, revealed the location of sequences that are involved in OxyR binding to the *ahpC* promoter

region. This same approach was used to identify OxyR binding sites in the promoter regions of *katG* and *oxyR* itself. The OxyR-binding sites extended into the -35-σ^{70} binding region, suggesting that the OxyR protein interacts with RNA polymerase to activate transcription. Furthermore, recent work by Tao and colleagues (1993) has shown that mutated RNA polymerase, containing α subunits carrying C-terminal truncations or defined amino acid substitution, were unable to bind to the *katG* promoter. Together, these results suggest that direct protein-protein contact between the OxyR protein and the C-terminal contact site 1 region of the RNA polymerase α subunit plays an essential role in transcriptional activation at OxyR-dependent promoters (Tao *et al*, 1993).

Tartaglia and colleagues (1992) have demonstrated that OxyR protein purified in its oxidized form, binds to non-homologous, functional DNA-binding sites in the promoter regions of *ahpC*, *katG*, *oxyR*, and *orfO* (an overlapping open reading frame transcribed in the opposite direction to the *ahp* operon (Tartaglia *et al*, 1990)), with over 10^6-fold higher affinity than to random DNA sequences. Intriguingly, inspection of the sequences involved in OxyR-binding in these promoter regions, has revealed only a few conserved nucleotides (Tartaglia *et al*, 1989). However, there is an unusually high number of degenerate homologies (positions at which only two of the four possible base pairs are represented):

```
ahpC          TTGTTAGTTAACGCTTATTGATTTGATAATGGAAACGCATTAGCCGAATCAGCAA
katG          ATGTAAGATCTCAACTATCGCATCCGTGGATTAATTCAATTATAACTTCTCTCTA
oxyR          ACGATAGTTCATGGCGATAGGTAGAATAGCAATGAACGATTATCCCTATCAAGCA
orfO          ACGCCAGCTCTTACCTATGTCTGTGATAGGCATCATCATTAATACTCTTTTCGCT

perfect
consensus     --G--AG-T-------AT--------T------------T-A------------

degenerate
consensus     DDD--DD-DDDDD-DDDD-D-D---DDDD---D-D-D-DDDDDD---DDD--D-D
```

Tartaglia and colleagues (1992) have suggested that these degenerate homologies are the reason why OxyR protein specifically recognizes seemingly disimilar sequences. This hypothesis has been partially substantiated by methylation interference assays on two OxyR-binding sites. These assays indicate that recognition of OxyR-binding sites is achieved through the use of a multidegenerate recognition code.

1.4.5 Conclusions.

Oxidative stress is an inescapable feature of life in any aerobic environment. The reactive oxygen species that are generated within a cell as a consequence of normal oxidative metabolism, can cause damage to proteins, lipids and nucleic acids (Imlay and Linn, 1988). Thus, in order for an organism to survive oxidative damage it must be equipped with a range of enzymes and other proteins that can protect the cell(s) from these toxic oxygen species. The regulation of these proteins has been extensively studied in a number of organisms, including *S. typhimurium* and *E. coli* (reviewed by Farr and Kogoma, 1991). These bacteria help to modulate oxidative stress *via* two regulons, OxyR and SoxRS, which have been identified and characterized (Christman *et al*, 1985; Tsaneva and Weiss, 1990).

1.5 Temperature stress in bacterial cells.

1.5.1 The heat shock phenomenon.

All organisms respond to heat (temperature upshift) by inducing the synthesis of a group of proteins called heat shock proteins. This response is one of the most highly conserved stress response systems known, and has been found to exists in every organism in which it has been sought,

from archaebacteria to eubacteria, from plants to animals (Lindquist and Craig, 1988). Most notable are the heat shock proteins encoded by the *hsp70* and *hsp90* gene families, which are induced significantly in response to a temperature upshift, and which appear to be universally produced in all organisms so far examined (Craig *et al*, 1993). In *E. coli*, some heat shock proteins are also induced to different degrees by stresses which result from exposure to stimuli such as ethanol, anoxia, certain heavy metal ions, production of abnormal proteins, viral infection, nutrient starvation, oxidants, DNA-damaging agents, alkaline shift, acid shift and osmotic shock (Neidhardt *et al*, 1984; Taglicht *et al*, 1987; Jenkins *et al*, 1990; Foster, 1992; Bakau, 1993). Furthermore, heat shock proteins are also present in all organisms at normal temperatures and play a vital role in normal cell functions (Lindquist and Craig, 1988; Bukau and Walker, 1989b).

The generally accepted view for the functions of heat shock proteins, is that they protect the cell from the toxic effects induced by heat (Neidhardt *et al*, 1984) and other stresses, such as those mentioned above (Neidhardt *et al*, 1984; Taglicht *et al*, 1987; Jenkins *et al*, 1990). Since these stresses are often encountered suddenly and may be extremely disruptive to the organism's normal cellular functions, it is not surprising that the heat shock response is often thought of as an emergency response, essential for the organism's survival (Ellis and van der Vies, 1991).

The most compelling argument that heat shock proteins have a protective function is the strong correlation between their induction and the induction of thermotolerance. The basic observation in a wide variety of organisms, is that a group of cells or organisms is killed rapidly when shifted directly from their normal growing temperature to a much higher temperature, whereas a matched group, with prior exposure to a sublethal

temperature upshift (to induce heat shock proteins) is killed much more slowly when subsequently heat shocked (Lindquist and Craig, 1988). Moreover, prior exposure to a sublethal temperature upshift induces tolerance to other forms of stress, such as oxidative damage (Watson, 1990), and other forms of stress induce tolerance to heat, such as treatment of cells with ethanol or ultraviolet radiation (Watson, 1990), or starvation (Jenkins *et al*, 1988; Hengge-Aronis, 1993).

Interestingly, a great variety of dimorphic pathogens that cycle between relatively cool temperatures in one phase of their life cycle and the warmer temperatures of their mammalian host in another phase, show a strong induction of heat shock proteins during infection, many of which are immunodominant antigens (Bianco *et al*, 1986; Garbe, 1992). The most likely explanation for this antigenicity, is that heat shock proteins are extremely abundant proteins at high temperatures and are therefore processed by macrophages as major foreign antigens for presentation to lymphocytes. The induction of the heat shock proteins might be further enhanced by the hostile environment found within the phagolysosome of macrophages, which engulf and serve as host cells for some of the organisms (Moulder, 1985; Finlay and Falkow, 1989b).

1.5.2 Heat shock in *E. coli*

E. coli is capable of maintaining growth in rich medium over the temperature range from 8°C to 49°C. Raising the temperature above 40°C or lowering it below 20°C results in progressively slower growth, until growth ceases at the maximum temperature of growth, 49°C, or the minimum, 8°C (Ng *et al*, 1962). The steady-state levels of most cellular proteins do not change greatly within the normal temperature range (20°C to 40°C), but many proteins exhibit significantly increased or decreased

levels near the temperature extremes (Herendeen *et al*, 1979).

The heat shock response in *E. coli* has been well characterised. When cell cultures are transferred from 30°C to 42°C, more than 17 heat shock proteins are transiently expressed (Neidhardt *et al*, 1984). This induction is accomplished primarily as a result of increasing the levels of an alternative subunit of RNA polymerase (σ^{32}), encoded by *rpoH* (*htpR*) (Grossman *et al*, 1984). σ^{32} has been shown to recognize specific heat shock promoters which differ from those recognised by σ^{70} at lower temperatures (*eg* 30°C) (Cowing *et al*, 1985), and so result in the selective synthesis of the heat shock proteins. Of particular importance to the survival of the cell are the heat shock proteins DnaK (Hsp70) and GroEL (Hsp60) which, in conjunction with the heat shock proteins DnaJ and GrpE, have been shown to act as molecular 'chaperons', aiding the proper folding of other proteins and the assembly and disassembly of protein structures which may be damaged as a result of increased temperature (Langer *et al*, 1992).

Recently, Mizushima and co-workers (1993) have demonstrated that heat shocking *E. coli* from 30°C to 42°C leads to a transient relaxation of negatively supercoiled plasmid DNA, and that there is no (or a delayed) recovery of DNA torsional strain in *gyrA* mutant cells following temperature upshift. Furthermore, after heat shock, DnaK and GroEL are synthesized continuously at an elevated level in a *gyrA* mutant cell, whereas in a wildtype cell the increased synthesis of these two proteins is only transient. This implies that change in superhelical density of the DNA may, in part, also account for temperature-induced expression of heat shock proteins.

1.5.3 Cold survival in microorganisms.

More than 80% of the Earth's biosphere is permanently cold, therefore those organisms that are best able to cope with low temperature are its most successful colonizers. Representatives of all cellular microbial groups (*eg* bacteria, yeasts, algae and fungi) are found in both aquatic and terrestrial cold environments, even being found in snow and ice. They play key roles in the ecology of permanently cold places (*eg* polar regions), and are also important in the spoilage of many foods stored at refrigerated temperatures. Therefore, microorganisms capable of growing at low temperature merit concerted investigation because of their overall contribution to global ecology and Man's well-being (Russell, 1990).

Like most higher organisms, the majority of microbial species are mesophiles, usually growing over a temperature span of some 20°C to 40°C. However, as a group, microorganisms can grow at temperatures ranging from sub-zero to boiling point and, although they represent a thermal continuum, it is useful to classify individual species as psychrophiles, mesophiles or thermophiles according to their growth temperature range (low temperature range to high temperature range, respectively) (Gounot, 1991).

Psychrophilic (cold-loving) microorganisms, capable of growing at 0°C, can be divided into two groups: obligate and facultative psychrophiles, depending on whether they grow optimally below or above 20°C (Ingraham and Stokes, 1959). This latter group of microorganisms, the facultative psychrophiles, are also known as the psychrotrophs (Morita, 1975), and include a large number of species with extended growth ranges that may frequently be classified as mesophiles, such as *Listeria monocytogenes* (Walker *et al*, 1990). In contrast with psychrophiles, phychrotrophs are characteristic of cold habitats where the

temperature fluctuates (Russell, 1990).

As discussed, true psychrophilic microorganisms are restricted to temperatures below 20°C and are thermolabile at room temperature (Ingraham and Stokes, 1959). In contrast, psychrotrophic microorganisms are widespread in natural environments and in foods that undergo thermal fluctuations during storage, such as meat and dairy products. The psychrotrophic population in dairy products is composed mainly of Gram-negative rod-shaped bacteria, such as *Pseudomonas, Acinetobacter, Alcaligenes, Chromobacterium* and *Flavobacterium* species (Gounot, 1991). Strains of *Pseudomonas* are the most troublesome psychrotrophs in the dairy industry because of their pronounced ability to produce undesirable flavours, odours and pigments. Furthermore, they produce thermally-resistant enzymes, lipases and proteases, which resist pasteurization and even ultra-high temperature treatment (Poffe and Mertens, 1988).

Special attention must be given to the growth of pathogens or toxin production in food or biological products at refrigerated temperatures. *Listeria monocytogenes* can grow at low temperature in a range of meat and dairy food products, and has been shown to cause higher mortality in mice when grown at low temperature prior to infection (Stephens *et al*, 1991). Other bacteria which are of particular concern to the food industry, include *Yersinia enterocolitica, Clostridium botulinum*, and *Bacillus cereus*, which are renowned for producing toxins in food (Gounot, 1991).

The ability of phychrotrophic bacteria to grow at low temperature depends on changes in the cellular composition of proteins and lipids. These adaptions may be genotypic or phenotypic, and are reliant on three factors: preservation of the structural integrity of macromolecular complexes such as membranes; provision of the necessary energy supply,

metabolites and intracellular environment for metabolism; and regulation of metabolism in response to the changing needs of the organism (Russell, 1990). Although it is useful to consider the adaption of individual cellular systems, for example, those responsible for generating energy, it is the maintenance of functional enzymes and structural molecules which are ultimately required, since these represent the mechanics of the cell. Therefore, qualitative and quantitative changes in proteins and lipids underpin the ability of psychrotrophs to grow at low temperature.

Work by Julseth and Inniss (1990) has demonstrated that a temperature shift from 21°C to 5°C in the psychrotrophic yeast *Trichosporon pullulans* increases the synthesis of at least 26 cold shock proteins. However, the synthesis of only six of these proteins is increased significantly in response to a shift from 15°C to 5°C. Furthermore, if cells are grown at 24°C, that is, above the optimum temperature for growth of this organism, and then shifted to 5°C, only 10 proteins are induced. This reduction in the number of proteins induced by cold shock, subsequent to heat stress, implies that in these eukaryotic cells heat stress has a direct effect on cold shock. Hence, it is possible that heat stress acts to either inhibit production of certain cold shock proteins, or that these same proteins are also induced by heat stress, and hence, their synthesis is masked during pulse labelling of newly synthesized proteins. Interestingly, cold shock-inducible genes that are also heat shock-inducible have been reported in *Saccharomyces cerevisiae* by Kondo and Inouye (1991).

Various groups of workers, including Jones and colleagues (1987), and VanBogelen and Neidhardt (1990), have implicated ribosomes and certain cold shock proteins to be involved in protein synthesis at low temperature (10°C to 15°C) in *E. coli* (discussed in more detail below). These findings correspond well with discoveries made in psychrophiles and psychrotrophs. Krajewska and Szer (1967) showed that a cell-free

protein-synthesizing system prepared from a psychrophilic *Pseudomonas* species had a very low miscoding rate compared with cell-free systems from mesophiles and thermophiles at the same low incubation temperatures. The ability of the system to function at 0°C was a property of the ribosomes *per se* rather than the soluble fraction of cell free extract (*ie* the ribosomes were the critical factor in allowing protein synthesis to proceed at low temperature). This was concluded by monitoring various combinations of *Pseudomonas* and *E. coli* ribosomes and supernatants, which showed that a system of psychrophilic ribosomes and *E. coli* supernatant was active at 0°C, whereas a combination of *E. coli* ribosomes and psychrophilic supernatant, was not. Similarly, Bobier and co-workers (1972) showed that differences in thermal sensitivity of protein synthesis in two psychrophilic *Bacilli* was also a property of the ribosomes rather than association factors.

A somewhat contradictory finding to that of Krajewska and Szer (1967) reported above, has been documented in further work by Szer (1970). In this study, it was found that washed ribosomes of the same psychrophilic *Pseudomonas* as that used by Krajewska and Szer (1967), while retaining activity at 25°C to 37°C, largely lost their capacity to function at 0°C. Addition of the protein washings, however, was found to restore this capacity at 0°C. Thus, it appears that both the ribosomes and specific cellular proteins are necessary for the full functional capacity of psychrophiles at low temperature.

In marked contrast to the lack of information pertaining to psychrophilic proteins, there is abundant data concerning the lipids found in both psychrophiles and psychrotrophs (reviewed by Russell (1990) and Gounot (1991)). Most information is on fatty acid composition, which is relatively easy to measure and, moreover, is relevant to thermal studies because it is the acyl composition of lipids that has some of the most

dramatic effects on membrane properties such as fluidity (Russell, 1990). Chan and colleagues (1971) compared two thermophilic, a mesophilic and a psychrophilic species of *Clostridium*, and found that the proportion of unsaturated fatty acids increased in the following order: thermophiles (10%), mesophiles (37%), psychrophiles (52%). Interestingly, recent work by Grau and deMendoza (1993) has shown that increasing the proportion of unsaturated fatty acids in lipids, lowers their gel-liquid-crystalline phase transition temperature, and leads to increased membrane fluidity in the bacterium at low temperatures.

Changes in the proportions of saturated and unsaturated fatty acids, as a consequence of temperature shift, have also been observed. For example, when the mesophilic bacterium *Bacillus subtilus* is grown at 37°C, it almost exclusively synthesizes saturated fatty acids. However, when cultures are transferred from 37°C to 20°C, the *de novo* synthesis of a C-16 unsaturated fatty acid is recorded (Grau and deMendoza, 1993). This thermal desaturation of lipids is seen in a number of species, including *E. coli*, where desaturation occurs by thermal modulation of the activity of a soluble enzyme that converts palmitoleic acid to cis-vaccenic acid (deMendoza *et al*, 1983). Again, this desaturation at low temperatures has been reported to increase fluidity in the lipid layer of both *E. coli* and *B. subtilus* cell membranes (deMendoza *et al*, 1983; Grau and deMendoza, 1993).

Not surprisingly, there have been many attempts to isolate mutants of one thermal group (*eg* mesophiles) that are able to grow at temperatures characteristic of another thermal group (*eg* psychrophiles). This has been studied predominantly as a way of spotlighting the molecular determinants of psychrophily. However, such mutants are extremely difficult to obtain. It is presumed that so many mutations are required to achieve the latter goal (*ie* a mesophile gaining psychrophily)

that the chance of them occurring together in one cell is extremely small (Russell *et al*, 1990). Kawamoto and co-workers (1989) have attempted to create psychrotrophic mutants of *E. coli*. However, the lowest minimum growth-temperature obtained from a pool of *E. coli* mutants was only 7°C, 1°C lower than the parental strain (Shaw *et al*, 1971). Nevertheless, mutants with extended minimum growth-temperature have been found in other genera of bacteria, including *Pseudomonas* (Azuma *et al*, 1962). By U.V irradiating cultures of *P. aeruginosa*, Azuma and colleagues (1962) showed that it was possible to gain mutants of this bacterium that were capable of slow growth at 0°C, whereas, in contrast, the parental strain was unable to grow at temperatures below 10°C. *P. aeruginosa* is almost unique among the various species of *Pseudomonas* in not itself being a psychrotroph, and it has been suggested by these authors that, since *P. aeruginosa* is closely related to psychrophiles, the number of mutational changes necessary to gain psychrophily might be expected to be small. This hypothesis has been further substantiated by demonstrating that psychrophily can be transduced from *P. fluorescens* to *P. aeruginosa* (Olsen and Metcalf, 1968).

1.5.4 Cold shock in *E. coli*.

In 1934, Sherman and Cameron reported the effects of suddenly chilling cultures of *E. coli* from 37°C to 4°C. They found that the majority of the bacteria from exponentially growing cultures that had been treated in this way were subsequently unable to form colonies on nutrient agar, whereas a stationary phase culture was relatively unaffected by this treatment. These results were confirmed by Hegarty and Weeks (1940), who also described cyclical fluctuations in susceptibility during the exponential phase of growth which were attributed to partial

synchronization of division. This chilling experiment was again repeated by Meynell in 1958, who, in addition to confirming a stationary phase effect, demonstrated that susceptibility to cold shocking, shown by exponentially growing bacteria, could be averted by either gradual cooling or osmotic adjustment. In addition to this, Meynell (1958) also showed that sudden cooling of *E. coli* infected by phage, had the same effect as ultrasonic disruption, leading to the release of intracellular mature phage.

These early experiments clearly demonstrate that there is a difference between the varying phases of bacterial growth in *E. coli* and its ability to survive chilling. However, it was not until 1978 that Lemaux and co-workers, by the use of O'Farrell two-dimentional polyacrylamide gel electrophoresis (2D-PAGE), showed that temperature shift both up and down induces proteins synthesis. These findings were more rigourously tested by Jones and colleagues (1987) who, again using 2D-PAGE, demonstrated that specific proteins were induced by decreasing the temperature of an exponentially growing culture of *E. coli* from 37°C to 10°C. This response, analogous to that already known in *E. coli* for heat shock, was termed 'cold shock'.

Exponential cultures of *E. coli* cells growing in rich medium at 37°C, which are shifted to 10°C, undergo a 4 hour lag period before a resumption of exponential growth occurs, albeit with a new generation time of approximately 24 hours (Ng *et al*, 1962). Jones and colleagues (1987) have demonstrated, by labelling newly synthesized proteins with [^{35}S]methionine, that only 28 proteins are detectably synthesized after 2 hours at 10°C (during the lag period), 27 of which are produced at 37°C. This number increases dramatically shortly before resumption of growth, with the synthesis of an additional 50 polypeptides. Of the 28 proteins produced during the lag phase, each can be categorized into one of three groups: those whose differential rate of synthesis either decrease, stays the

same, or increase. This last group constitute the true cold shock proteins.

During the lag period described above, the synthesis of at least 13 cold shock proteins are transiently increased in *E. coli*. A number of these proteins have been identified (Jones *et al*, 1987; La Teana *et al*, 1991; Jones *et al*, 1992a), and are listed below with their general functions and their location on the *E. coli* chromosome:

- Polynucleotide phosphorylase (responsible for degrading single stranded RNA *in vitro* in the 3' to 5' direction, and may function as an RNase in cells (69 minutes) (Regnier *et al*, 1987)).

- NusA (involved in both termination and antitermination of transcription (69 minutes) (Ishii *et al*, 1984a)).

- Translation initiation factors 2α and 2β (responsible for mediating binding of charged $tRNA^{fmet}$ to the small ribosomal subunit prior to initiation of translation (69 minutes) (Ishii *et al*, 1984b; Sacerdot *et al*, 1984)).

- Dihydrolipoamide acetyltransferase and pyruvate dehydrogenase (two enzymes of the pyruvate dehydrogenase complex which catalyses the decarboxylation of pyruvate to yield acetyl coenzyme A for entrance into the tricarboxylic acid cycle (3 minutes) (Guest, 1974)).

- RecA (involved in recombination and in the induction of the SOS response needed for DNA repair (58 minutes) (Horii *et al*, 1980; Sancar *et al*, 1980)).

- H-NS (DNA binding protein with a high affinity for curved DNA which has also been implicated in condensation of the chromosome and in organization of the nucleoid (27 minutes) (Pon *et al*, 1988; Hulton *et al*, 1990, La Teana *et al*, 1991)).

- GyrA (essential for maintaining the level of DNA supercoiling which is itself fundamental for the regulation of processes such as transcription, recombination, and replication (48 minutes) (Drlica *et al*, 1980))

- CS7.4 (possible cold shock regulatory protein (79 minutes) (Goldstein *et al*, 1990; La Teana *et al*, 1991))

The majority of the proteins listed above which are induced by cold shock in *E. coli*, are relatively well characterized at 37°C, both in terms of their regulation and functions. However, it has not yet been established whether these characteristics differ significantly at lower temperatures such as 10°C. For example, although induction of RecA is itself under SOS control, the expression of this latter protein at 10°C does not appear to result in induction of other SOS proteins, but instead appears similar to the increased synthesis of RecA that occurs as cells enter the stationary phase (Jones *et al*, 1987).

The function of CS7.4 has still not been clearly demonstrated. However, this protein has been found to correlate with the increased synthesis of a number of other cold shock proteins, including NusA, H-NS, GyrA, and the two as yet unknown proteins, G55.0 and G41.2 (La Teana *et al*, 1992; Jones *et al*, 1992b). It has also been speculated that, since CS7.4 is not induced to any significant level at 37°C (Jones *et al*, 1987), this protein may have a more fundamental role in the bacterium's survival at lower temperatures than simply regulating the induction of

other proteins (Goldstein *et al*, 1990). This will be discussed in more detail later in this section.

Recent work by Qoronfleh and co-workers (1992), has identified several loci with low-temperature-inducible promoters, by utilizing a P1::Tn5-*lac* delivery system to generate strains with transcriptional fusions at random locations in the *E. coli* chromosome. This approach identified several cold shock strains, three (WQ3, WQ6 and WQ11) with fusions at novel map locations (81 minutes, 12 minutes and 34 minutes, respectively). Both WQ3 and WQ11 (WQ6 was not tested) were found to have CCAAT boxes in their promoter regions, the relevance of which will be discussed later.

Work by Das and Goldstein (1968) has demonstrated that shifting exponentially growing cultures of *E. coli* from 37°C to 0°C results in incorporation of labelled leucine into protein at a decreasing rate for a period of approximately 4 hours and also, the subsequent accumulation of 70S ribosomal particles. They postulated that while elongation of the polypeptide occurred at predictable rates at 0°C, initiation of translation was blocked, resulting only in the completion of proteins for which translation had been initiated prior to the temperature downshift (*ie* until the ribosomes had run off their respective mRNA). Similar work was conducted by Friedman and colleagues (1969), who observed that ribosomal subunits accumulated in *E. coli* cells incubated at temperatures below 8°C. This group also concluded that low temperature blocks the initiation of protein synthesis.

The dramatic effects on protein synthesis that occur when exponentially growing cultures of *E. coli* are shifted to subminimal growth temperature, has been reported to take place in a range of mesophilic bacteria (Broeze *et al*, 1978). Since this reduction appears to be substantially dependent on translation being inhibited in the cell at low

temperature (Das and Goldstein, 1968; Friedman *et al*, 1969; Szer, 1970), and because some of the identified cold shock proteins are involved in transcription or translation (NusA and IF2), or are on operons containing other transcriptional and translational genes (Polynucleotide phosphorylase) (Jones *et al*, 1987), the cold shock response has been interpreted as an adaptive response to facilitate expression of genes involved in translation (Jones *et al*, 1992a).

As with the relationship between heat shock and starvation (Neidhardt *et al*, 1984) discussed above, there appears to be a relationship between cold shock and nutrient upshift. Jones and co-workers (1992a) have stated that pattern of protein synthesis in *E. coli* which follows a shift to lower temperatures, is reminiscent of the pattern of protein synthesis following nutrient upshift. In both cases there is a decrease in the level of (p)ppGpp (guanosine 5'-triphosphate-3'-diphosphate and guanosine 5'-diphosphate-3'-diphosphate), regulated by *relA* and *spoT* [(p)ppGpp synthetase I and II]. This decrease in (p)ppGpp (also referred to as an alarmone) leads to a corresponding increase in the rate of RNA synthesis, which in turn leads to a directed increase in protein synthesis after a suitable interval in time (Mackow and Chang, 1983). Furthermore, shifting *E. coli* from 41°C to any lower temperature results in a decrease in the (p)ppGpp level; the greater the drop in temperature, the greater the decrease in (p)ppGpp level (Pao and Dyess, 1981).

It has been suggested by Mackow and Chang (1983) that the level of (p)ppGpp as a function of temperature shifts has a role in regulating RNA synthesis in *E. coli.* This hypothesis was tested by Jones *et al* (1992a) who showed, by over-expressing (p)ppGpp in *E. coli* (using a strain containing a multicopy plasmid carrying a truncated *relA* gene fused to a *p-tac* promoter, and treated with IPTG) during cold shock, that synthesis of most cold shock proteins was significantly reduced in the

presence of excess (p)ppGpp. In contrast, the level of certain heat shock proteins, including DnaK and GroEL, was increased (derepressed). *E. coli relA spoT* double mutants that did not synthesis significant levels of (p)ppGpp, produced substantially higher steady-state levels of CS7.4 at 24°C than wildtype cells at this temperature (Jones *et al*, 1992a). Furthermore, *relA spoT* double mutants showed increased levels of most cold shock proteins over wildtype strains at 10°C. These double mutants also showed pre-adaption to cold shock, since there was no lag in growth when an exponentially growing double mutant was cold shocked from 24°C to 10°C (comparable wildtype cells showed a growth lag of 2 hours) (Jones *et al*, 1992a).

As discussed previously, an alternative or additional mode of modulating temperature-induced proteins is *via* the ribosomes (Krajewska and Szer, 1967; Bobier *et al*, 1972; VanBogelen and Neidhardt, 1990). This hypothesis was proposed on the basis that certain alterations in the translational capacity of the cell exclusively induce heat shock- or cold shock proteins (VanBogelen and Neidhardt, 1990). To test this hypothesis, two groups of antibiotics (termed H and C), known to effect specifically the ribosome, were tested. H group antibiotics, including puromycin, streptomycin, and kanamycin, produce a response that is virtually indistinguishable from heat shock (only the time-course is different). C group antibiotics that include chloramphenicol, tetracycline, erythromycin, spiramycin, and fusidic acid, produce a response consistent with that of cold shock. 10 of the 14 cold shock proteins are synthesized to a level similar to that seen in a temperature shift from 37°C to 10°C (Jones *et al*, 1987; VanBogelen and Neidhardt, 1990).

The simplest explanation as to how antibiotics affect the synthesis of heat shock- and cold shock proteins *via* the ribosome is by altering the frequency of ribosome stalling. This normally occurs due to factors such

as amino acid starvation, carbon starvation, deficiencies in charged tRNAs, and nutrient down-shift (Pao and Dyess, 1981). Under these circumstances the stalled ribosome induces (p)ppGpp. (p)ppGpp in turn might help the cell respond more quickly to enable it to adapt and survive (Jones *et al*, 1992a). This same conclusion has also been hypothesized by Jiang and colleagues (1993) during recent work specifically pertaining to chloramphenicol induction of the major cold shock protein, CS7.4. This is discussed in more detail below.

1.5.5 The major cold shock protein of *E. coli*, CS7.4.

The major cold shock protein of *E. coli* was first identified by Jones and co-workers in 1987. As discussed above, this group found that, when an exponentially growing culture of *E. coli* was shifted from 37°C to 10°C, only one new polypeptide was uniquely synthesized following this temperature shift. From its apparent 2D-PAGE location Jones and co-workers (1987) concluded that this cold shock protein was approximately 10.6 kDa. Hence, this protein was termed F10.6.

The above experiment was repeated by Goldstein and colleagues (1990), who subsequently cloned and sequenced the gene encoding this protein by reverse genetics. This technique involved synthesizing a set of degenerate oligonucleotide DNA probes related to a partial amino-terminal sequence of the protein. A mixture of these degenerate oligonucleotides was then used to probe a southern blot of chromosomal DNA digested with restriction enzymes. A 2.4 kb fragment of chromosomal DNA, corresponding to that which hybridized with the probe, was then cloned and sequenced. This fragment was found to encode a transcript (*cspA*) of 1.205 kb, with an open reading frame of 210 nucleotides and a promoter region that is recognised by RNA

polymerase containing the σ^{70} subunit. The 70 amino acid protein encoded by *cspA* was accurately sized as 7.402 kDa, and re-termed CS7.4. Finally, *cspA* was mapped to approximately 79 minutes of the *E. coli* chromosome (Goldstein *et al*, 1990).

CS7.4 is the most highly expressed cold shock protein in *E. coli*, accounting for as much as 13% of total protein synthesis upon temperature shift from 37°C to 15°C (Goldstein *et al*, 1990). This protein has also been found to be hydrophilic, with more than 20% of the protein consisting of amino acids with charged side chains (Goldstein *et al*, 1990).

The kinetics of CS7.4 induction have been studied by pulse-labelling with [^{35}S]methionine (Goldstein *et al*, 1990; Jiang *et al*, 1993). These experiments showed that CS7.4 is synthesized within the first 30 minutes of shifting a culture from 37°C to 10°C or 15°C. The time of peak induction and the maximal rate of CS7.4 synthesis which occurs, depends on both the initial growth temperature and also on the extent of the temperature downshift. As already stated, after a shift from 37°C to 15°C, CS7.4 represents approximately 13% of the total newly synthesized protein and peak induction occurs 30-60 minutes after the shift. Following a shift from 37°C to 10°C, CS7.4 represents approximately 8.5% of the total newly synthesized protein, reaching a peak 60-90 minutes after the shift. Returning cold shocked cultures to 37°C does not overtly affect the functional stability of CS7.4 (Goldstein *et al*, 1990), indicating that its absence at 37°C is not due to protein instability.

In 1991, La Teana and colleagues demonstrated, by gel retardation, that CS7.4 binds to the promoter region of the *E. coli hns* gene. Furthermore, this group showed that the addition of purified CS7.4 to a coupled transcription-translation system prepared from control cell extract

(*ie* wildtype *E. coli* cells grown at 37°C) and programmed with a plasmid carrying the *hns* promoter fused to a promoterless *cat* gene, stimulated expression of chloramphenicol acetyltransferase at 37°C (as did cold shock treatment of cells carrying this *hns-cat* plasmid), indicating *hns* to be positively regulated by CS7.4. These latter workers also noted that the primary structure of CS7.4 was strikingly homologous to a conserved region of a class of eukaryotic transcriptional factors, comprising the Y box binding proteins (Wistow, 1990). One of these proteins, YB-1, has been shown to specifically recognise the Y box, a short region of DNA (designated as CTGATTGGCCAAAG) which lies upstream of HLA class II genes. (These latter genes encode the major histocompatibility complex class II in man) Furthermore, the presence of an inverted CCAAT (ATTGG) sequence in the Y box (Didier *et al*, 1988) is an absolute requirement for recognition by YB-1. Interestingly, a CCAAT sequence has been found in the promoter region of *hns*, at the presumed leading edge of the RNA polymerase binding site (La Teana *et al*, 1991).

As stated earlier, Jones and co-workers (1992b) have utilized a high-copy-number plasmid expressing the *cspA* gene to show that, in addition to *hns*, four further proteins are induced following CS7.4 overexpression. These proteins were identified as NusA, GyrA, and two unknown proteins with 2D-PAGE locations of G55.0 and G41.2. Furthermore, analysis of the *gyrA* sequence, showed that the promoter region of this gene contained three ATTGG sequences. Previous work by Tafuri and Wolffe (1992) has shown that competition with ATTGG sequences eliminates CS7.4 protein from binding to an ATTGG-containing oligonucleotide, further establishing the specific nature of this interaction. Direct and inverted CCAAT sequences have also been found in the promoters of several other cold shock genes, including *nusA* (Granston *et al*, 1990), *recA* (Horii *et al*, 1980), *pnp*

(Regnier *et al*, 1987) and *cspA* itself (Goldstein *et al*, 1990).

Tanabe and co-workers (1992) have shown that CS7.4 is regulated at the level of transcription in *E. coli*. This was determined through primer extension experiments, which identified *cspA* mRNA from whole cell RNA prepared using bacterial cultures grown at 37°C and 15°C. No *cspA* mRNA was detected in RNA preparation from cells grown at 37°C, but *cspA* mRNA was readily detectable in RNA prepared from cultures grown at 15°C (its functional half life was estimated as approximately 15 minutes at the latter temperature (Jiang *et al*, 1993)). Furthermore, this group demonstrated that the maximum level of *cspA* mRNA was not seen until 35-65 minutes after temperature shift from 37°C to 15°C. This pattern of *cspA* mRNA induction corresponds well with the previously reported pattern of CS7.4 production (Goldstein *et al*, 1990). Most importantly, synthesis of CS7.4 is abruptly halted in cultures shifted back from 15°C to 37°C, with no new protein synthesis detectable even in the first 5 minutes of the increased temperature shift. These findings indicate that the absence of CS7.4 at 37°C is probably due to the rapid degradation of *cspA* mRNA at this temperature or that translation of *cspA* mRNA is inhibited at 37°C (Tanabe *et al*, 1992).

Recently, Jiang and colleagues (1993) have shown that the stability of *cspA* mRNA can be substantially increased by the addition of chloramphenicol to *E. coli* cell cultures at 15°C. Furthermore, as previously reported by VanBogelen and Neidhardt (1990), this former group found that this antibiotic was additionally capable of inducing transcription of *cspA* mRNA. Moreover, this induction was found to be most dramatic when cells were treated by this antibiotic at 15°C, for under these conditions the synthesis of CS7.4 was found to be constitutive (a control culture was shown to produce this protein only transiently). This work also confirmed that induction of CS7.4 by both

cold shock and chloramphenicol largely reflected alterations at the level of transcription or RNA stability rather than at the level of translation.

From the transcription initiation site of *cspA*, -35 and -10 regions of the promoter were identified which showed similarity to the consensus sequences found in other σ^{70} regulated promoters. Since *cspA* expression appeared to be regulated at the level of transcription or RNA stability, at 37°C the *cspA* promoter must either be nonfunctional without a transcriptional factor induced at low temperature (or repressed by a repressor), or perhaps the instability of the *cspA* transcript is sufficient to cause the loss of CS7.4 production at 37°C. To distinguish between these possibilities, Tanabe *et al* (1992) performed *in vivo* footprinting, and found a region upstream of the transcription initiation site to be protected from chemical modification at 14°C but not at 37°C, indicating that there was a cold shock factor(s) binding to this region. Characterization of the cold shock factor by gel retardation in conjunction with proteinase K or heat treatment, demonstrated the factor to be a protein. Furthermore, disruption of protein synthesis by certain antibiotics confirmed that synthesis of this factor occurs *de novo* during cold shock treatment (Tanabe *et al*, 1992; Jiang *et al*, 1993).

A major cold shock protein with close identity to CS7.4, has been identified in two Gram positive bacteria, *Bacillus subtilis* and *Streptomyces clavuligerus*. Using reverse genetics, Willimsky and co-workers (1992) cloned, sequenced, and characterized the *B. subtilis* cold shock gene, *cspB*. This gene encodes a 67 amino acid, 7.365 kDa polypeptide, with 61% identity to CS7.4 of *E. coli*. *cspB* mRNA is present at low copy at 37°C, but increases 20-fold when cells are shifted from 37°C to 10°C for 2 hours. No growth lag was observed to occur in this species following temperature downshift from 37°C to 10°C and disruption of *cspB* does not have any obvious affects at normal growth

temperatures. However, analysis of thermotolerance reveals that this mutant strain is more sensitive to freezing than the parental strain, an affect that is surprisingly nullified by preadaption of both strains at 10°C for 2 hours.

The protein analogue of CS7.4 in *S. clavuligerus* was identified by Av-Gay and colleagues (1992). This group noticed that, during a study of the thioredoxin system in this bacterium, the protein thioredoxin co-purified with another low molecular weight protein with an apparent molecular mass of approximately 8-10 kDa (by PAGE). The later protein was subsequently designated as SC7.0, and used to carry-out reverse genetics. This procedure identified SC7.0 as a 7.008 kDa polypeptide of 66 amino acids. Moreover, SC7.0 is strikingly similarity to CS7.4 of *E. coli*, with 56% sequence identity and more than 80% sequence similarity.

1.5.6 Conclusions.

Cold shock is a stress that, unlike heat shock, has not been extensively studied. Preliminary studies in *E. coli* (Jones *et al*, 1987; Goldstein *et al*, 1990; VanBogelen and Neidhardt, 1990; La Teana *et al*, 1991; Tanabe *et al*, 1992; Jones *et al*, 1992a; Jones *et al*, 1992b) have indicated that cold shock induces a stress response that in some respects is characteristic of other multigenic stress responses, such as those seen with heat shock and oxidative stress (Watson, 1990). For example, when an exponentially growing culture of *E. coli* is shifted from 37°C to 10°C, a set of cold shock proteins is induced. This is somewhat analogous to the set of heat shock proteins induced by shifting an identical culture of *E. coli* from 30°C to 42°C, or the set of oxidative stress proteins induced by treatment of these cells with an oxidizing agent, such as hydrogen peroxide.

The major cold shock protein of *E. coli*, CS7.4, has been implicated as a DNA binding protein capable of inducing the synthesis of a number of other cold shock proteins (La Teana *et al*, 1991; Jones *et al*, 1992b). However, the importance of this protein in the bacterium's survival at low temperatures (10°C) has not been determined. Studies of an analogous cold shock protein in *B. subtilis* (Willimsky *et al*, 1992) have indicated that this protein is not essential for survival at room temperature but may play some protective role during freezing. Nevertheless, a great deal more work will be necessary in order to understand the role played by this major cold shock protein both in *E. coli* and other organisms.

1.6 Methods for reporting bacterial stress.

1.6.1 Conventional methods for identifying stress-induced loci or virulence loci in bacteria

In the majority of cases the ability for a bacterium to survive a particular environmental stress, usually depends on a complex series of functions requiring a number of co-ordinately expressed bacterial proteins. Therefore, in order to gain a greater understanding of how a bacterium survives a particular stress, it is essential to identify and characterize these proteins and their corresponding genetic loci. One approach to define a protein of interest is to screen banks of mutants (usually constructed with a transposon) for reduced survival in response to a particular stress. An appropriate mutants can then be isolated and the mutated gene cloned by recombinant DNA techniques (possibly using the transposon as a tag), and the parental gene subsequently isolated and studied (Finlay, 1992).

There are several advantages to using transposon mutagenesis over other types of mutagenesis, such as U.V or chemical mutagenesis, to characterize stress responses. Firstly, transposons allow defined genetic lesions to be constructed that eliminate expression of the gene into which they are inserted. Secondly, they can be constructed to contain an identifiable marker (*eg* antibiotic) which may allow them to be manipulated more easily, and thirdly, transposons can be used to create transcriptional fusions with the use of an easily detected reporter genes. Furthermore, the stability of the transposon mutation can be increased by removing the transposase genes from the construct and creating a mini-transposon, where transposition only occurs when the transposase gene are supplied *in trans*. In contrast, U.V or chemical mutagens act by forming point mutations that are revertible and extremely difficult to manipulate.

Tn5-directed *lacZ* transriptional fusions are by far the most utilized transposon systems used in applied studies of enteric Gram-negative bacteria. By inserting this transposon (in the correct orientation) downstream of the putative regulatory sequence of a specific gene encoding a stress or virulence protein, the expression of that gene can be monitored under various environmental conditions (*eg* cold shock or interaction with a host cell) by assaying for β-galactosidase activity. Other enzymes extensively used for this purpose include chloramphenicol acetyltransferase, alkaline phosphatase, phosphoribosyltransferase, galactokinase, thymidine kinase, and catechol oxygenase (Thompson *et al*, 1990 and Carmi *et al*, 1987).

1.6.2 Methods for monitoring phagocytosis.

Existing methods for determining the precise series of biochemical events that occur during the activation of professional phagocytes (including both the oxygen-dependent and oxygen-independent microbicidal mechanisms), are based predominantly on biophysics and biochemistry. Biophysical methods for analyzing phagocytic events are usually quite intricate, involving techniques such as nuclear magnetic resonance (NMR) of ^{31}P(Seguin *et al*, 1990) and ^{13}C (Seguin *et al*, 1991). These studies are often extremely revealing and can give very precise data concerning the intracellular environment of phagocytes during events such as the respiratory burst (*eg* glucose oxidation, activity of the hexose monophosphate shunt, or the formation of hydrogen peroxide, superoxide, or aldehyde (Forman and Thomas, 1986)). However, the majority of studies involving biophysical methods are mainly (though not exclusively) concerned with the functioning of the phagocyte in its own right, regardless of the activating agonist.

In contrast to biophysical methods, biochemical methods for studying phagocytic events are often more concerned with comparing the effects that a specific pathogen and non-pathogen may have on the functioning of the phagocyte during infection (*eg* an *S. typhimurium* infection of macrophages compared to a similar *E. coli* infection). These methods, usually involving techniques such as chemiluminescence (Takahashi *et al*, 1991) or fluorescence (Whitin *et al*, 1981), are also extremely revealing and can be used to monitor phagocytic events such as the respiratory burst, much in the same way as biophysical methods.

An alternative method for monitoring the activation of professional phagocytes by a specific pathogen, is not to examine the phagocytic response directly, as discussed above, but to observe the response of the

phagocytosed pathogen with regard to its changing intracellular environment. This method can be facilitated by using a reporter system (*eg* a Tn5-directed *lacZ* fusion) that will allow the responses of the pathogen to be monitored in relation to a particular phagocytic stress. For example, if a strain of *S. typhimurium* is constructed with a *lacZ* gene fused to the putative regulatory sequence of its *katG* gene (normally encoding HPI catalase), this strain can be used to report oxidative stress (such as that induced by hydrogen peroxide) by its production of β-glactosidase (Miller, 1972). Similarly, if this *lacZ* fusion strain is used to infect phagocytes, it can be used to report the oxidative stress resulting from the respiratory burst of the phagocyte. Hence, this method allows both the response of the phagocyte and that of the pathogen to be monitored.

One of the limitations of using a reporter such as β-galactosidase in the above experiment is that, in order to assay for this enzyme, both the phagocytes and the bacterial cells must be disrupted. This means that it is not possible to continuously assess the whole infectious process in one set of phagocyte-bacterial cells and that, in order to gain a fuller assessment, a series of identical sets of cells must be set-up together and then disrupted and sampled at different times. Furthermore, β-galactosidase is quite a stable enzyme and will accumulate within the bacterial cells in accordance with the expression of the gene to which *lacZ* is fused. This means that it is extremely difficult to record accurately the induction profile of a transiently expressed gene.

In order to resolve the aforementioned problems, it would be an advantage to develop a test system capable of non-destructive, real-time analysis (Carmi *et al*, 1987) of bacterial gene expression from within phagocytes. A light producing reporter system would fulfil such demands, and indeed, the elements of such a system have already been employed

to demonstrate that light can be detected from within mammalian cells (Thompson *et al*, 1990; Gossen and Bujard, 1992). Furthermore, due to the technology available to measure and record light, it is potentially possible to distinguish between two relatively close peak wavelength emissions of light (Eckstein *et al*, 1990), introducing the possibility of using two light producing reporters in the same test, each with different peak wavelengths.

Over the past two decades a great deal of work has been directed toward the identification and isolation of genes responsible for bioluminescence. This has resulted in the cloning of bioluminescence genes from both bacteria (*lux*) and insects (*luc*). The majority of the *lux* studies have been conducted on the marine bacteria, *Vibrio fischeri* (Engebrecht *et al*, 1983; Engebrecht and Silverman, 1984) and *Vibrio harveyi* (Baldwin *et al*, 1984). *Lux* genes from each of these species have subsequently been inserted into transposon derivatives of bacteriophage *Mu* (Engebrecht *et al*, 1984) and Tn*5* (Boivin *et al*, 1988; Lorenzo *et al*, 1990) respectively, and these constructs have been transfered into a large number of diverse non-luminous bacteria, including *S. typhimurium*.

1.6.3 Bacterial bioluminescence.

Bioluminescent organisms are widely distributed in nature and include species of bacteria, dinoflagellates, fungi, fish, insects, shrimp, and squid. Luminous bacteria are the most abundant and widespread of the light-emitting organisms and are found in marine, freshwater, and terrestrial environments. These bacteria are all Gram-negative motile rods and can function as facultative anaerobes (Baumann *et al*, 1983). The majority of luminous bacteria have been classified into the three genera *Vibrio, Photobacterium*, and *Xenorhabdus*, with most of the species being

marine in nature (Meighen, 1988).

The light-emitting reaction in luminous bacteria has been extensively studied by Hastings and colleagues (1985). A reduced flavin, $FMNH_2$, bound to the enzyme luciferase, reacts with O_2 to form a 4a-peroxyflavin. This complex interacts with a long-chain fatty aldehyde to form a highly stable intermediate, which decays slowly, resulting in the emission of light along with the oxidation of the substrate. This reaction (Ziegler and Baldwin, 1981) can be summarised as follows:

$$FMNH_2 + RCHO + O_2 \quad ---> \quad FMN + H_2O + RCOOH + hv_{490nm}$$

The reaction is highly specific for $FMNH_2$, with the natural aldehyde believed to be tetradecanal (Szittner and Meighen, 1990). Luciferase is a heterodimeric enzyme composed of one α and one β subunit. (Meighen, 1988).

In all known cases, the *luxA* and *luxB* genes code for the luciferase subunits and the *luxCDE* genes code for a fatty acid reductase complex. In addition, other *lux* genes (*luxF,G,H,I* and *R*) as well as unlinked *lux* genes have been identified in specific luminescent strains (Swartzman *et al*, 1990).

1.6.4 Applications of *lux* genes within non-host bacteria.

i) Growth, distribution, and viability.

Work by Stewart and colleagues (1989), has demonstrated *lux* to be an good reporter system for assessing bacterial food spoilage. Infection of *Salmonella* and *Escherichia* species by viral vectors containing recombinant *luxAB* DNA (Ulitzur and Kuhn, 1987), has

shown that within 1 h after infection as few as 10 to 100 cells could be detected (Stewart *et al,* 1989). Further work by Stewart and colleagues (1991), has focused on the development of an antibiotic susceptibility test, using *Listeria monocytogenes* as a model organism. This test is relatively simple, relying on the fact that agents that disrupt or kill the bacterium will also interfere with its metabolism, thus eliminating light emission.

The expression of the *lux* genes in different bacterial species also provides a simple and sensitive system for monitoring the growth and dissemination of the bacteria in the environment. In plants, the bacterial luminescent phenotype has been used to observe the movement of *Xanthomonas* species which cause black rot in cauliflower and to observe the infection of potato slices by *Erwinia* species (Shaw and Kado, 1986). Furthermore, O'Kane and co-workers (1988) have shown that it is possible to detect and localize single *Rhizobium*-infected plant cells by using bacterial bioluminescence. They clearly demonstrated that the availability of reduced FMN and molecular oxygen, within bacteroides, was non-limiting for the bioluminescence reaction. Thus, they confirmed that bioluminescence is a precise marker for *in vivo* bacterial gene expression, even in complex environments where bacteria reside within eukaryotic cells.

ii) Reporters of gene expression.

By using the *lux* genes as reporters of gene expression, the strength and regulation of transcription from various promoters can be readily monitored. Light emission has been used to detect and measure the strength of promoters in a number of instances, such as during germination and sporulation of *Bacillus* species (Stewart *et al*, 1989),

mycelium development of *Streptomyces* species (Schauer *et al*,1989), lateral motion in *V. parahaemolyticus* (Engebrecht *et al*, 1985), osmotic regulation in *E. coli* (Park *et al*, 1989), nitrogen-fixation in *Bradhyrhizobium* species (Legocki *et al*, 1986), and opine catabolism in *Agrobacterium* species (Hirooka *et al*, 1987).

The *lux* genes can also be used as reporters in assays that are not directly involved in measurement of the control of gene expression at the promoter level. For example, Nussbaum and Cohen (1988) used two *luxA* genes inactivated by mutations at different sites, in conjunction with *luxB*, to measure recombination in *E. coli* by emission of light. Similarly, *E. coli*, containing *luxA* and *luxB* with a nonsense mutation has been used to study tRNA-mediated nonsense suppression. Light emission was found to be at least 80 times more sensitive than expression of β-galactosidase as a sensor of nonsense suppression (Schultz and Yarus, 1990).

1.6.5 Instrumentation to monitor bioluminescence

The application of *lux* genes in any field of biology requires the availability of instrumentation for the visualisation and quantification of photon emission. In some cases, simply determining whether or not an organism is producing light under a certain set of circumstances can be achieved by exposing that organism to X-ray film. However, most experimental procedures require more accuracy in their analysis, and hence, more sophisticated equipment. The most sensitive detector of photon emission available in most laboratories is the scintillation counter, which can be configured for the detection of less than 10 bioluminescent bacteria per ml of sample. Alternatively, a dedicated luminometer can be used. However, these instruments may be less sensitive by at least an

order of magnitude (Stewart and Williams, 1992).

Recently a number of photon imaging devices have been developed. These devices have the ability to visualize light in two or even three dimensions, and can detect single bioluminescent *E. coli* cells (Masuko *et al*, 1991). Using these devices, spatial information on gene expression within micro-colonies, colony differentiation during development, or during bacterial-plant interactions can be gained in real-time and in a non-disruptive manner (Stewart and Williams, 1992).

1.6.6 Conclusions.

Transcriptional reporter systems enable an immense volume of information to be gleaned from any non-essential gene into which they are fused. Probably the most commonly used transcriptional reporter in bacterial systems is β-galactosidase (encoded by *lacZ*). However, assaying for this enzyme often necessitates termination of the experiment. This makes it extremely difficult to monitor the transient responses that frequently occur in circumstances such as adapting to a new environment and/or to stress. Furthermore, monitoring bacterial *lacZ* fusions in response to infection of macrophages is even more difficult. Transcriptional reporters which utilize *lux* allow the majority of these difficulties to be resolved by providing a non-disruptive, real-time analysis of expression of the gene into which the *lux* reporter is fused.

1.7 Project aims.

In order to combat an infectious disease it is first essential to understand the mechanisms involved in the particular host-parasite interaction. This is particularly pertinent when the interaction involves an

intracellular parasite such as *S. typhimurium* which has evolved specifically with the ability to survive within the hostile environments of the very host cells designed for the destruction of such pathogens *eg* macrophages. How this bacterium survives within such a harsh intracellular environment is largely unknown. However, their ability to rapidly adapt to cellular stress is thought to be rudimentary in this process, therefore, a greater insight into the genetic basis of stress adaptation in this genus of bacteria might prove very rewarding in the fight against salmonellosis and related enteric diseases.

The initial aim of this project was to use a Mu*dlux* transcriptional light reporter system to identify gene fusions in *S. typhimurium* that were induced by stresses analogous to those found within activated macrophages. Theoretically, these fusions could then be further characterised, with particular emphasis being placed on determining their mechanisms of regulation. This aspect was made of greatest importance because most global stress responses are often coordinately regulated by common mechanisms and pathways, and hence, identifying these regulatory loci is important in gaining a greater understanding of stress responses and survival.

Using the above system would also allow certain stress-induced fusions to be monitored within eukaryotic host cells, such as epithelial and macrophage cells, without disrupting any part of the infective process, except that function disrupted by the fusion. In this way certain stress and virulence factors that were initiated by pathogen-host cell interaction could be monitored *via* light production. This information might then be used to gain a greater insight into bacterial survival during pathogenesis and might ultimately lead to better control of food-borne and intracellular bacterial pathogens.

As discussed throughout this chapter, oxidative stress is probably

one of the most common stresses encountered by bacteria, especially by intracellular parasites such as *Salmonella.* Therefore, this stress was chosen as a good candidate for selecting Mu*dlux* fusions in *S. typhimurium* to observe and analyse both with and without macrophage interaction. The reason for studying cold shock, a seemingly unrelated stress phenomenon, will be made plain during the course of the proceeding chapters.

CHAPTER 2

Chapter 2: Materials and Methods

2.1 Materials

Enzymes, Isotopes and Chemicals

All chemicals used were of analytical grade and, unless otherwise stated, were purchased from the UK divisions of Sigma Chemical Company, British Drug House (BDH), Gibco-BRL, Fisons, or Pharmacia LKB Biotechnology. Stabilised aqueous solutions of α^{32}-P-dCTP (3000 Ci mM^{-1}; 10 $\mu Ci\ \mu l^{-1}$) and α^{35}-S-dATP (400 Ci mM^{-1}; 10 $\mu Ci\ \mu l^{-1}$) were purchased from Amersham International plc., UK. Standard agarose was supplied from Northumbria Biologicals Ltd. UK. Isopropyl-β-D -thiogalactoside (IPTG) and 5'-Bromo-4-chloro-3-indoyl-β-D-galactoside (X-Gal) were purchased from Boehringer Mannheim UK as were all enzymes used during this study, unless otherwise stated. Zeta-Probe nylon filters were purchased from Bio-Rad Laboratories Inc. UK. X-ray film (Curix RPi and CRONEX Pelicula) was purchased from AGFA, UK and Du Pont, UK, respectively.

Unless otherwise stated synthetic oligonucleotides were purchased from the OSWEL DNA Service. Oligonucleotides used for sequencing and PCR are listed in table 2.1.

Bacterial strains and Plasmids

Bacterial strains and plasmids and their sources are shown in tables 2.2 and 2.3, respectively. The Kukral Tn*10* mapping strains (Kukral *et al*, 1987) were supplied by K. Sanderson (Salmonella Genetic Stock Centre, Canada).

Table 2.1. Oligonucleotides used in this study.

Shown is a list of oligonucleotides that were used during the course of this study. The numbers that are shown in brackets, with the exception of that marked by an asterisk (*), represent the position at which each of the oligonucleotides lay with respect to the start of the published gene sequence from which they are derived (*cspA* (A211, A212 and A926) (Goldstein *et al*, 1990); *ahpC* (823W) (Tartaglia *et al*, 1990); pBluescript SK and KS (T7 and T7 reverse) (Short *et al*, 1988)). The number that is shown in brackets and is marked by an asterisk (*), represent the position at which the Mu oligonucleotide lays with respect to the host DNA to which it is fused (404X) (Kahmann and Kamp, 1979).

Primer number	Primer sequence	Comments and usage
A211 (917-900)	5'- ACGGGATCCATTATTTAT - 3'	PCR of W3110 *cspA* (3'-5')
A212 (0-15)	5'- ATTAAGCTTCGATGCAAT - 3'	PCR of W3110 *cspA* (5'-3')
A926 (617-634)	5'- ATGTCCGGTAAAATGACT - 3'	PCR of MPG361 Mu*dlux* fusion (5'-3')
823W (150-167)	5'- ACACGGAGGAAGTATAGA -3'	PCR of MPG203 Mu*dlux* fusion (5'-3')
404X (64-83*)	5'- ATACATCTGTTTCATTTGAA - 3'	PCR & sequencing of MPG203 & MPG361 Mu*dlux* fusions (3'-5')
T7 (736-720)	5'- AATACGACTCACTATAG - 3'	Sequencing cloned PCR products
T7 Reverse (823-808)	5'- AACAGCTATGACCATC - 3'	Sequencing cloned PCR products

Bacteriophage

Bacteriophage P22 HT *int4* (Roth, 1970) and lysogens for the production of P1 Tn*9*clr 100 were gifts from C. Higgins (Oxford University). The Mu*d*-P22 rapid mapping strains (Benson and Goldman, 1992) were supplied by K. Sanderson (Salmonella Genetic Stock Centre, Canada).

Macrophages

The mouse macrophage cell line, J774.2 (Ralph and Nakoinz, 1975), was obtained from the Sir William Dunn School of Pathology (Oxford).

Solutions

Solutions made up in distilled H_2O were normaly autoclaved at 15 psi for 20 minutes prior to use. Labile components were filter sterilized and added to the main solution after the latter were autoclaved.

Tris-HCl

Tris base (tris [hydroxymethyl] aminomethane, Sigma) was dissolved in H_2O, and the pH was adjusted to the required value by addition of concentrated HCl. H_2O was added to give a 1 M stock solution.

Table 2.2. Bacterial strains used in this study.

Shown is a list of bacterial strains that were used during the course of this study. All strains are derivatives of *S. typhimurium* except those marked with an asterisk (*) which are *E. coli*. Abbreviations are as follows: MuP1 = Mu *cts* *h*P1; Mu*dlux* = Mu*d*1 (Km^r *lux* *cts*62); Tn*10* = Tn*10* *Δ16Δ17*.

Strain	Genotype	Source/Reference
HB101* (Mu*dlux*)	*hsd*S20($r_{B^-}m_{B^-}$) *sup*E44 *rec*A13 *ara*14 *pro*A2 *rps*L20 *xyl*-5 *mlt*-5 Mu*dlux*	Engebrecht *et al* (1983)
CH424	*his*-6165 *gal*E496 *ilv*-452 *hsd*L16 *hsd*SA29 *met*E55 *trp*B2 *xyl*-404 *rps*L120 *fla*A66 *omp*D1012	Hiles *et al* (1987)
MPG200	*his*-6165 *gal*E496 *ilv*-452 *hsd*L16 *hsd*SA29 *met*E55 *trp*B2 *xyl*-404 *rps*L120 *fla*A66 *omp*D1012 Mu*dlux*	This work
CH463	*gal*E503 *bio*-561/MuP1	Hiles *et al* (1987)
MPG201	*gal*E503 *bio*-561/MuP1 Mu*dlux*	This work
MPG202	*gal*E503 *bio*203::Tn*10* *his* SL1344 derivative	This work
MPG203	Mu*dlux* SL1344 derivative	This work
MPG278-MPG322	*gal*E503 *bio*203::Tn*10* Mu*dlux* SL1344 derivative (for specific phenotypes see tables 3.1, 3.2 and 3.3)	This work
MPG351	*oxyR1* Mu*dlux* TA4100 derivative	This work
MPG352	*oxyΔ2*[*oxy*Δ(*oxyRargH*)*2*] Mu*dlux* TA4108 derivative	This work
MPG353	*his* Mu*dlux* pDSA23 SL1344 derivative (see table 2.2 for details of plasmid)	This work
MPG354-MPG377	Mu*dlux* SL1344 derivative (for reference to specific phenotypes see tables 7.1, 7.2 and 7.3)	This work

Strain	Genotype	Source/Reference
MPG386	Mu*dlux* Tn*10* SL1344 derivative (Tn*10* D12 phenotype)	This work
MPG401	Mu*dlux* Tn*10* SL1344 derivative (Tn*10* C1 phenotype)	This work
MPG403	Mu*dlux* Tn*10* SL1344 derivative (Tn*10* C4 phenotype)	This work
MPG404	Mu*dlux* Tn*10* SL1344 derivative pKPF12 (see table 2.2 for details of plasmid)	This work
MPG407	Tn*10* SL1344 derivative (Tn*10* D12 phenotype)	This work
MPG408	Tn*10* SL1344 derivative (Tn*10* C1 phenotype)	This work
MPG409	Tn*10* SL1344 derivative (Tn*10* C4 phenotype)	This work
MPG410	Tn*10* pKPF12 SL1344 derivative	This work
MPG411*	*hsd*S20(r_B-m_B-) *sup*E44 *lac-pro*AB *thi* F'*pro*AB *lacZ* M15 NM522 derivative	This work
MPG412*	*ara lac-pro*AB *rspL* FSO, *lacZ* M15 (r_K+, m_K+) JM83 derivative	This work
SL1344	*his*	Hosieth and Stocker (1981)

Strain	Genotype	Source/Reference
NM522*	*hsd*S20(r_B-m_B-) *sup*E44 *lac-pro*AB *thi* F'*pro*AB *lacZ* M15	Gough and Murray (1983)
JM83*	*ara lac-pro*AB *rspL* FSO, *lacZ* M15 (r_k+, m_k+)	Vieira and Messing (1982)
TA4100	*oxyR1* LT2 derivative	Christman *et al* (1985)
TA4108	*oxyΔ2*[*oxyΔ*(*oxyRargH*)*2*] LT2 derivative	Christman *et al* (1985)
TT10423	*pro*AB-471 F'128 *pro*$^+$ *lac*$^+$ zzf-1831::Tn*10Δ16Δ17* LT2 derivative	Roth (1970) Way *et al* (1984)
TT10427	LT2 derivative carrying pNK972 (derivative of pBR322 carrying transposase genes 16 & 17)	Roth (1970) Way *et al* (1984)
PY13579	MC1061 carrying pJS28 (containing gene 9 of P22 encoding the tail protein)	Youderian *et al* (1988)

Table 2.3. Plasmids used in this study.

Shown is a list of plasmids that were used during the course of this study. SK and KS derivatives of pBluescript represent two orientations of the polylinker within the N-terminal coding region of the *lacZ* gene.

Plasmids	Relevant Features	Source/Reference
pBluescript SK (M13+/-)	ColE1-based replicon Ap^R carries *lacZ* α containing a multiple cloning site.	Short *et al* (1988)
pBluescript KS (M13+/-)	ColE1-based replicon Ap^R carries *lacZ* α containing a multiple cloning site.	Short *et al* (1988)
pKPF1	pBluescript SK (M13+/-) derivative containing cloned DNA from the PCR product of the MPG361 fusion junction (*cspS*::Mu)	This work
pKPF2	pBluescript KS (M13+/-) derivative containing cloned DNA from the PCR product of the MPG203 fusion junction (*ahpC*::Mu)	This work
pKPF12	pBR325 derivative containing cloned DNA from *Sau3A* digest of *S. typhimurium* and complementary to Tn*10* D12.	This work
pDSA23	pACYC184 derivative containing subcloned DNA from lambda 166 (Kohara *et al*, 1987) carrying *E. coli ahpC* and *ahpF* genes.	Smillie *et al* (1992)

EDTA

A stock solution of 0.5 M EDTA (ethylenediaminetetramino acid, di-sodium salt, Fisons) was made by dissolving solid EDTA in H_2O, adjusting the pH to 8.0 with NaOH, and adding water to the required volume.

TE

A solution of TE buffer, consisting of 10 mM Tris-HCl (pH 7.5) and 1 mM EDTA, was prepared by diluting an appropriate volume of each of the above stock solutions in H_2O. This solution was routinely used as a solvent for DNA.

Phenol

Phenol (Rathburn) was pre-equilibriated by mixing an equal with 1 M Tris. Cl (pH 8.0), followed by an equal volume of 0.1 M Tris-HCl (pH 8.0). To retard oxidation of the phenol solution 0.1% (v/v) 8-hydroxyquinoline (BDH) and 0.2% (v/v) β-mercaptoethanol (BDH) were added. The solution was stored at 4°C in the dark.

Sodium acetate

Sodium acetate (Sigma) was dissolved in H_2O, the pH was adjusted to 5.0 with HOAc, and H_2O was added to a final acetate buffer concentration of 3 M.

Ethidium bromide

Ethidium bromide (Sigma) was dissolved as a stock solution of 10 mg ml^{-1} in H_2O, and stored at room temperature in the dark.

Loading buffer

6X loading buffer for gel electrophoresis of nucleic acids was prepared as 0.25% (w/v) bromophenol blue (BDH) and 15% (w/v) ficoll (Sigma) in H_2O. The solution was stored at room temperature.

TBE

TBE (Tris-borate/EDTA), containing 54 g of Tris base (Sigma), 27.5 g of boric acid (Oxoid), and 20 ml of 0.5 M EDTA (disodium salt), was routinely made up as a 10X stock solution with H_2O (1 l; pH 7.4), and stored at room temperature.

PBS

Dulbecco's phosphate buffered saline, pH 7.4 (Oxoid), used for washing macrophage cell sheets and diluting in, was prepared by dissolving 1 tablet of PBS (containing 800 mg NaCl, 20 mg KCl, 144 mg Na_2HPO_4, 24 mg KH_2PO_4) per 100 ml H_2O.

Antibiotic solutions

All antibiotics were purchased from Sigma. Ampicillin and kanamycin were made up as 10 mg ml^{-1} stock solutions in H_2O, these were filter sterilized and used at a final concentration of 50 µg ml^{-1} unless otherwise stated. Chloramphenicol and tetracycline were made up as 10 mg ml^{-1} stock solutions in absolute ethanol and used at a final concentration of 25 µg ml^{-1} unless otherwise stated. Antibiotics used for routine tissue culture were all tissue culture grade, and were prepared by reconstituting a sterile vial containing antibiotic with 20 ml of sdH_2O. These latter antibiotics were used at a final concentration of 200 U ml^{-1} penicillin, 100 µg ml^{-1} streptomycin and 100 µg ml^{-1} gentamicin. All of the above stock solutions were stored at -20°C.

IPTG

IPTG was dissolved in H_2O to give a 2% (w/v) stock solution. This solution was filter sterilized and used by spreading 50 µl volumes on LB plates.

X-Gal

X-Gal was dissolved in dimethylformamide to give a 2% (w/v) stock solution. This solution was used by spreading 50 µl volumes on LB plates pre-spread with IPTG.

Recipes for all other solutions are included in the appropriate method sections.

2.2 Growth Media

Tissues culture medium

Tissue culture medium, routinely used to culture both bacterial and macrophage cells, was composed of RPMI 1640 medium containing L-glutamine (Gibco) and 10% (v/v) heat-inactivated (56°C, 30 min) foetal calf serum (Gibco). The pH of 1 litre of this solution was maintained at between 7.2 and 7.8 by the addition of 2 g of sodium hydrogen carbonate dissolved in H_2O (filter sterilized). When used to culture macrophage cells, the antibiotics penicillin (200 U ml^{-1}) and streptomycin (100 µg ml^{-1}), both sterile tissue culture grade (Sigma), were added prior to use.

Bacterial Growth Media

Bacterial media was composed as follows:

Luria broth (LB)

LB, used for routine bacterial cell culture, was composed of 5 g tryptone (Difco), 2.5 g yeast extract (Difco), 5 g NaCl (BDH), made up to 500 ml with H_2O. This was sterilized by autoclaving at 15 psi for 20 minutes, and stored at room temperature.

LB agar

LB agar was prepared by adding 7.5 g of agar to 500 ml of LB prior to autoclaving.

LB top agar

LB top agar was prepared by adding 0.7 g of agar to 100 ml of LB prior to autoclaving.

M9 salts

M9 salts, used for selective growth of bacterial cells, was composed of 6.5 g of Na_2HPO_4 (BDH), 1.5 g of KH_2PO_4 (BDH), 0.25 g of NaCl (BDH), and 0.5 g of NH_4Cl (BDH), made up to a final volume of 500 ml with H_2O and sterilized by autoclaving. When used for pulse-labelling of bacteria, the above solution was supplemented with 0.4% glucose (w/v) and 1 mM concentrations of all the common amino acids except methionine. Both glucose and amino acid solutions were filter steralized before addition to the M9 salts.

Bochner plates

Bochner plates (Bochner *et al*, 1980), used for the selection of tetracycline-sensitive transductants in Mu*d*-P22 mapping experiments, were made as follows. Two solutions, A and B, were made up separately, autoclaved (10 psi for 30 minutes), and then mixed to give a final volume of 1 litre. Solution A contained 15 g of agar, 5 g of tryptone (Difco), 5 g of yeast extract (Difco), and 50 mg of chlortetracycline-HCl (Sigma) in a final volume of 500 ml of water. Solution B contained 10 g of NaCl (Difco), 10 g of $NaH_2PO_4.H_2O$ (BDH) in a final volume of 500 ml of water. After mixing, 5 ml of 20 mM $ZnCl_2$ (aqueous) (BDH) and 12 mg of fusaric acid (Sigma) dissolved in 1 ml of N,N-dimethyl-formamide were added. These plates were used within 48 hours of being poured.

2.3 Methods

2.3.1 Maintenance and storage of macrophage cells

All tissue culture procedures were conducted under a positive pressure laminar flow hood using stringent sterile techniques.

Routine culturing of macrophage cells

A 1 ml aliquot of macrophages of the mouse cell line J774.2, at a density of approximately 10^7 macrophages per ml., was taken from the liquid nitrogen stock and thawed at 37°C. This was added to a 250 ml tissue culture flask containing 50 ml of pre-warmed (37°C) tissue culture medium, to which penicillin (200 U ml^{-1}) and streptomycin (100 μg ml^{-1}) had been added. This flask was then placed in a 5% CO_2 incubator (Forma Scientific, 3157) at 37°C until the macrophage cell sheet was just

confluent (determined by microscopy after approximately 48 - 72 hours). At this stage the overlying tissue culture medium was carefully decanted into a 50 ml conical bottomed centrifuge tube (Falcon), and the macrophage cell sheet was stripped by incubating the cells at room temperature for 10 minutes in 10 ml of a 0.05% disodium EDTA solution made up in phosphate buffered saline (pH 7.4) (Hudson and Hay, 1989). Stripped macrophage cells were poured into a second 50 ml conical bottomed centrifuge tube and balanced against the first tube by exchanging an appropriate volume of spent media. Macrophages were then gently pelleted by centrifugation at 600 x g for 15 minutes in a bench-top MSE Centaur 1 centrifuge. The supernatant was discarded and the macrophages were resuspended in 1.5 ml of fresh tissue culture medium. A cell count was taken using a haemocytometer, and the cells were either reseeded at 10^5 macrophage ml^{-1} in fresh tissue culture medium containing both of the above antibiotics, or the suspension was made up to 5 ml with 1 ml of dimethyl sulfoxide (Koch-Light Laboratories, UK) and 2.5 ml of heat-inactivated (56°C, 30 min) foetal calf serum, to be frozen down in liquid nitrogen in 1 ml volumes.

Coating glass cavity slides with macrophage cells

J774.2 cells, prepared as above, were detached from flasks by gentle agitation in 0.05% disodium EDTA (prepared in PBS), gently pelleted by centrifugation at 600 x g for 15 minutes in a bench-top MSE Centaur 1 centrifuge, and then resuspended in fresh tissue culture medium, containing gentamicin (100 µg ml^{-1}), at a density of approximately 10^7 cells ml^{-1}. Sterile cavity slides, placed in dry petri dishes, were coated with 200 µl of this suspension for four hours at 37°C, in a 5% carbon dioxide incubator. Slides were subsequently washed with

PBS, to remove unattached macrophages and residual antibiotic, and then submerged in fresh tissue culture medium, without gentamicin. Incubation was continued until the following morning, by which time the macrophage cell sheet was confluent.

2.3.2 Manipulations of bacteria and phage

Growth of bacterial cultures

Overnight liquid cultures of *S. typhimurium* and *E. coli* were prepared by inoculating a 5 ml volume of LB (plus a suitable antibiotic where appropriate) with a single bacterial colony. Cultures were grown with shaking at 37°C. Large cultures were prepared by diluting overnight cultures 100-fold in conical flasks with a total capacity 5-10-fold that of the final culture volume and cultures were then grown as above.

Storage of bacterial cultures

For long term storage, 1 ml volumes of fresh overnight cultures of bacteria (grown as above) were mixed with 70 µl of 100% DMSO, and stored in a sterile vial at -80°C. Cultures were recovered by using a sterile stick to transfer cultures to an appropriate agar plate. (NM522 was streaked onto minimal agar to maintain the F' plasmid.) After overnight incubation at 30°C or 37°C (depending on whether the strain was temperature sensitive) a single colony was picked to propagate a fresh bacterial culture. For short term storage (4-6 weeks) bacteria were stored as streaks on agar plates at approximately 6°C.

Preparation of phage P22 lysates

Strains containing a transposon with a selectable marker, were cultured overnight at 30°C, with aeration, in LB medium containing an appropriate antibiotic. A 50 µl volume of each culture was diluted into 5 ml volumes of LB medium and incubated for 90 minutes (O.D. 0.2-0.3), at 30°C with aeration. 5 µl of P22 stock (titre 10^9 ml^{-1}) was added to each culture and incubation was continued for a further 6 hours. A 200 µl volume of $CHCl_3$ was added to each culture, samples were inverted to mix and then left at 4°C for at least 2 hours. Each sample was centrifuged at 3,000 x g for 15 minutes in a bench-top MSE Centaur 1 centrifuge, supernatants were decanted, and a further 200 µl volume of $CHCl_3$ was added and mixed as before. The samples were again left at 4°C for at least 2 hours, before being centrifuged and stored at 4°C with 100 µl chloroform until further use.

Phage P22 titration

An appropriate host strains was cultured overnight at 37°C with aeration. 100 µl volumes of culture were pipetted into sets of 9 sterile eppendorf tubes, and 100 µl volumes of a serially diluted P22 phage lysate (dilution range 10^0 - 10^8 ml^{-1} in LB medium) were added. Tubes were mixed and then incubated at 30°C for 30 minutes. 100 µl volumes of each mixture were added to 5 ml volumes of LB top agar (46°C) and poured on LB plates. Controls for both P22 phage only and cells only were also poured. Plates were incubated overnight at 37°C, and the following morning plaques were counted and the phage titres calculated.

Phage P22 transduction

Strains to be transduced were cultured overnight in LB medium at 30°C with aeration. 100 µl volumes of each culture were pipetted into sets of 5 sterile eppendorf tubes, and 100 µl volumes of a serially diluted P22 phage lysate (dilution range 10^0 - 10^4 ml^{-1} in LB medium) were added. Tubes were mixed and then incubated at 30°C for 30 minutes. 100 µl volumes of each mixture were spread on 5 LB plates containing an appropriate antibiotic, and incubated overnight at 30°C. The following morning transductants were counted and the optimal frequencies recorded. Transductants were colony purified twice on LB plates containing an appropriate antibiotic and then inoculated into 5 ml volumes of LB medium to be cultured overnight and frozen at -80°C.

N.B. In all cases, *galE* strains were incubated with filter sterilised solutions of 0.4% glucose (w/v) and 0.4% galactose (w/v). This ensured the presence of P22 phage receptors on the host cells.

Preparation of phage P1 Tn*9*clr 100 lysates

A 5 ml volume of LB medium was inoculated with a single colony of a P1 Tn*9*clr 100 lysogen and incubated overnight at 30°C. 50 µl of this culture was then used to inoculate a fresh 10 ml volume of LB medium and this was incubated at 30°C until the cells had reached an OD_{600nm} of approximately 0.2. This early-log phase culture was transfered to 42°C and incubation was continued for a further 20 minutes, followed by incubation at 37°C for approximately 1 - 2 hours until the cells had lysed. 200 µl of $CHCl_3$ was added and the suspension was mixed thoroughly to ensure that any remaining bacteria were lysed. The suspension was then

centrifuged at 3,000 x g for 15 minutes in a bench-top MSE Centaur 1 centrifuge, after which the supernatant was decanted, a further 200 µl volume of $CHCl_3$ was added, and the lysate stored at 4°C encased in tin foil to keep the light out.

Phage P1 titration

Titrations of P1 phage were conducted in the same manner as P22 phage titrations, except for the addition of 5 mM $CaCl_2$ and 10 mM $MgSO_4$ in the LB top agar.

Phage P1 transduction

Strains to be transduced were cultured overnight in 5 ml LB medium at 30°C with aeration. Each overnight was centrifuged at 3,000 g for 10 minutes in a bench-top MSE Centaur 1 centrifuge, and the respective pellets resuspended in 2.5 ml of LB medium containing 5 mM $CaCl_2$ and 10 mM $MgSO_4$. 100 µl volumes of each culture were pipetted into sets of 5 sterile Eppendorf tubes, and 100 µl volumes of a serially diluted P1 phage lysate (dilution range 10^0 - 10^4 ml^{-1} in LB medium) were added. Tubes were mixed and then incubated at 30°C for 30 minutes. To each tube was added 100 µl volumes of 1 M sodium citrate, and then each re-incubated for 30 minutes at 30°C to allow expression of antibiotic resistance. Each mixture were spread on 5 LB plates containing an appropriate antibiotic, and incubated overnight at 30°C. The following morning transductants were counted and the optimal frequencies recorded. Transductants were colony purified twice on an LB plates containing an appropriate antibiotic and then inoculated into 5 ml volumes of LB medium to be cultured overnight and frozen at -80°C.

Preparation of Mu*d*-P22 lysates

Mu*d*-P22 lysates used for mapping in strains of *S. typhimurium* were prepared as described by Benson and Goldman (1992), as modified from the procedure of Youderian and colleagues (1988). A 5 ml overnight culture of the desired Mu*d*-P22 lysogen (grown in LB broth plus 40 μg of chloramphenicol per ml) was added to 25 ml of LB broth. Mitomycin C was added to a final concentration of 2 μg/ml, and the mixture was shaken overnight at 37°C. After overnight shaking, 3 ml of $CHCl_3$ was added and the flask was vigorously shaken for 3 min. The LB broth was decanted from the $CHCl_3$, and the cell debris was removed by centrifugation at 8,000 x g in a Beckman J2-21 (JA-20 rotor) for 10 min. The supernatant was then incubated with excess tail protein (0.1 ml, prepared as below) for 2 h at 37°C. Fully assembled phage were pelleted by centrifugation at 35,000 x g for 1 h in a Beckman J2-21 (JA-20 rotor). The pelleted phage particles were then resuspended by shaking with 5 ml of phosphate-buffered saline. Lysates were stored over 0.2 ml of $CHCl_3$, at 4°C.

Preparation of P22 tails

The strain PY13579 (MC1061 carrying pJS28, which has gene 9 of P22 encoding the tail protein) was grown up overnight at 37°C in 6 litres of LB broth plus 50 μg/ml ampicillin. Cells were pelleted by centrifugation at 2,000 x g in a Beckman J-6B (JS-4.2 rotor) for 10 min. Pelleted bacteria were then resuspended by shaking with 20 ml of 0.1 M Tris solution (pH 7.5) containing 10 mM EDTA. To this suspension was added 20 mg of lysozyme, and the solution was incubated at 37°C for 1 h, followed by a further incubation at 65°C for 4 h. The lysed cell debris

was removed from the supernatant by centrifugation at 35,000 x g for 30 min in a Beckman J2-21 (JA-20 rotor). The crude tail protein was stored with 0.4 ml of $CHCl_3$, at 4°C.

Rapid mapping of Tn*10* insertions

Tn*10* insertions were mapped using Mu*d*-P22 lysates (Benson and Goldman, 1992). The recipient carrying the Tn*10* at an unidentified locus was grown to approximately 5 x 10^8 c.f.u ml^{-1} in LB media containing 40 μg ml^{-1} tetracycline. The cells were washed once in phosphate-buffered saline, diluted 10-fold in the latter solution, and then 0.1 ml of the suspension was plated on fresh Bochner plates. The complete set of 54 Mu*d*-P22 transducing lysates were each diluted 10-fold in PBS, and 5 μl volumes were spotted onto the prespread Bochner plates. The plates were then incubated at 42°C for 24 to 48 h. Typically, the Mu*d*-P22 insertion close to the site of the Tn*10* insertion gave a confluent spot of tetracycline sensitive transductants, while the remaining spots appeared similar to the background of cells that did not receive any phage.

Preparation of chromosomal DNA from bacterial cells

The method used is as described by Silhavy and colleagues (1984). A 500 ml flask containing 100 ml of LB was inoculated with a single bacterial colony and incubated overnight at 30°C with aeration. Cells were harvested by centrifugation at 10,000 x g for 10 min at 4°C in a Beckman J2-21 centrifuge (JA-14 rotor) and resuspended in 5 ml of T.E.. The cell suspension was place at -20°C until just frozen. 0.5ml of fresh lysozyme solution (10 mg ml^{-1} in T.E.) was added to the cells in a room temperature waterbath, and occasionally mixed until just thawed. This

solution was then put on ice for approximately 45 min. 1 ml of STEP[1] solution was added, mixed well, and then the sample was heated at 50°C for 1 hour with occasional, gentle mixing. 6 ml of Tris-buffered phenol was added, mixed gently to emulsify, and centrifuged at 3,000 x g for 15 min in a bench-top MSE Centaur 1 centrifuge to separate the layers. The top, aqueous layer containing the nucleic acid was transferred to a clean tube, and a 0.1 volume of 3 M sodium acetate was added and the solution was gently mixed. 2 volumes of ethanol were added and the tube was inverted several times. The glob of precipitated nucleic acid was carefully removed with a sterile glass rod and the DNA was re-dissolved in 5 ml of T.E. containing RNase (200 $\mu g\ ml^{-1}$; Sigma, Type IIA), and left overnight at 4°C. An equal volume of chloroform was added, gently mixed to emulsify, and centrifuged at 3,000 x g for 15 min in a bench-top MSE Centaur 1 centrifuge to separate layers. The top aqueous layer, containing the DNA was transferred to a clean tube and a 0.1 volume of 3 M sodium acetate was added with gentle mixing. 2 volumes of ethanol was added and the tube was inverted several times. DNA, which precipitated as long threads, was again spooled out using a sterile glass rod and re-dissolved in T.E.. The DNA was quantified by spectrophotometry (see below), and its quality checked by agarose gel electrophoresis.

1 STEP: 0.5% SDS
50 mM Tris-HCl, pH 7.5
0.4 M EDTA (disodium salt)

Proteinase K powder (12.9 U mg^{-1} protein) (Sigma) was added to the above solution at a final concentration of 1 mg ml^{-1} immediately before use.

Transformation of competent bacterial cells with plasmid DNA

A frozen 200 µl aliquot of competent cells (prepared as below) was thawed on ice for approximately 10 minutes - until just liquid. 10 µl of plasmid DNA (less than 1 µg) was added, and the sample was mixed and left on ice for 20-40 minutes. Cells were then heat shocked in a 42°C water bath for 90 seconds, and 0.8 ml of pre-warmed L-broth was added before incubation at 37°C for 1 hour. This allowed expression of the antibiotic resistance gene. A sample of the cells (100-200 µl) were spread onto L-agar plates containing the 50 µg ml^{-1} of the appropriate antibiotic (determined by the transforming plasmid), and the plates were incubated overnight at 30-37°C.

N.B When selecting for pBluescipt plasmids, plates were pre-spread with 50 µl volumes each of X-Gal (2% w/v in DMSO) and IPTG (2% w/v in H_2O).

Preparation of competent bacterial cells for transformation with plasmid DNA

A single bacterial colony was picked and used to inoculate 5 ml of LB medium. The cells were grown at 37°C with shaking until the culture had reached an OD_{600nm} = 0.3. This culture was then used to inoculate 100 ml of LB medium and incubation continued at 37°C with shaking until the OD_{600nm} = 0.4. The cells were chilled on ice for 10 minutes before pelleting in a pre-chilled rotor at 3,000 g for 5 minutes in a bench-top MSE Centaur 1 centrifuge. After re-suspension in 25 ml of ice cold 0.1 M $MgCl_2$ the cells were again pelleted at 3,000 x g for 5 minutes. The cells were re-suspended, this time in 25 ml of ice cold 0.1 M $CaCl_2$,

before re-pelleting at 3,000 x g for 5 minutes. Finally, the cell were re-suspended in 5 ml of 0.1 M $CaCl_2$ containing 14% glycerol (v/v), and aliquots of 200 µl were stored at -80°C in Eppendorf tubes.

Small scale plasmid DNA preparation

The method used (TELT "miniprep") was described by He and colleagues (1990, 1991). A single colony was picked and used to inoculate 5 ml of L-broth containing the appropriate antibiotic. The culture was then grown overnight at 30-37°C with shaking. The following morning 1.5 ml was pipetted into an Eppendorf tube and centrifuged for 2 min at 20,000 x g. The pellet was re-dissolved in 400 µl of TELT[1] and incubated at room temperature for 2 minutes with 40 µl of 100 mg ml^{-1} lysozyme solution (made up in TELT[1]). The cell suspension was boiled for 90-120 seconds, and then cooled on ice for 10 minutes. Cell debris was removed by centrifugation for 15 minutes (20,000 x g), and the supernatant was transferred to a clean Eppendorf tube. After adding 300 µl of isopropanol, the solution was cooled on ice for 2 minutes, and the precipitated plasmid DNA was pelleted by centrifugation for 20-30 minutes (20,000 x g) at 4°C. The DNA pellet was washed in 70% ethanol (v/v in H_2O) and then desiccated, before being resuspended in 50 µl of H_2O.

1 Telt: 50 mM Tris-Cl, pH 7.5
62.5 mM EDTA, pH 7.5
2.5 M LiCl
0.4% triton X100

Large scale plasmid DNA preparation

This protocol is a modification of the methods of Birnboim and Doly (1979) and Ish-Horowicz and Burke (1981). A single colony was picked and used to inoculate 1 litre of L-broth containing the appropriate antibiotic. The culture was then grown overnight at 30-37°C with shaking. Cells were harvested by centrifugation at 2,000 x g for 10 min at 4°C in a Beckman J-6B centrifuge, and resuspended in 36 ml of solution I[1]. To this suspension was added 4 ml of lysozyme solution (prepared at 20 mg ml^{-1} in solution I[1]). The sample was mixed thoroughly but gently, and then left at room temperature for 10 min. 80 ml of freshly made solution II[2] was added, and the mixture was left on ice for 5-10 min. 40 ml of cold solution III[3] was added, the solutions mixed well and then incubated on ice for 15-30 min. The mixture was then centrifuged at 10,000 x g for 10 min at 4°C in a Beckman J2-21 centrifuge (JA-14 rotor), and the supernatant was filtered through cheese cloth into a graduated cylinder. A 0.6 of a volume of isopropanol was added, with mixing, and the solution was centrifuged at 10,000 x g for 10 min at 4°C. The pellet was resuspended in 8 ml T.E., and 1 g of CsCl was added per ml of solution and dissolved. For every 10 ml of CsCl-nucleic acid-T.E. mixture, 0.8 ml of EtBr (10 mg ml^{-1}) was added. This solution was then mixed, and centrifuged at 3,000 x g for 10 min at room temperature in a bench-top MSE Centaur 1 centrifuge. The supernatant was subsequently centrifuged at 800,000 x g for 3 hr in a Beckman XL-90 ultracentrifuge (in a NVT-90 vertical rotor), and the plasmid band was removed with a sterile syringe. EtBr was extracted with T.E. saturated butanol, until the DNA solution was clear. 6 ml of T.E and 18 ml of absolute ethanol were added, and the solution was centrifuged at 12,000 x g for 30 min. The resulting DNA pellet was washed in 70% ethanol, prior to desiccation, and then the pellet

was resuspended in 2 ml of T.E.. The DNA was quantified by spectrophotometry (see below), and examined by agarose gel electrophoresis. DNA was routinely stored in TE solution at -20°C.

1 Solution I: 50 mM glucose
25 mM Tris, pH 8.0
10 mM EDTA, pH 8.0

2 Solution II: 0.2 M NaOH
1% SDS

3 Solution III: 3 M KOAc
2 M acetic acid

2.3.2 Nucleic acid manipulations and detection procedures

Extraction of proteins from nucleic acid by phenol and chloroform

Proteins were removed from DNA solutions by extraction with an equal volume of phenol. Traces of phenol were then removed by a further extraction with an equal volume of chloroform. Extractions were carried out by adding a volume of phenol equal to that of the DNA solution in a eppendorf tube. The two solutions were mixed thoroughly by vortexing to form an emulsion, and separated by centrifugation for 2 minutes at 20,000 x g at room temperature. The aqueous phase was then transferred to a fresh tube carefully avoiding protein at the interface of the two phases. Traces of phenol were then removed by adding an equal volume of chloroform and repeating the above procedure of vortexing and

centrifugation. Again the aqueous phase was transferred to a clean tube, and the DNA was recovered by precipitation as described below.

Precipitation of nucleic acids with ethanol

DNA was precipitated from solution by the addition of a 0.1 volume of 3 M sodium acetate buffer (pH 5.0), followed by 2.5 (total) volumes of absolute ethanol. The solution was mixed thoroughly by vortexing, and then placed at -80°C for 15 minutes. Precipitated DNA was pelleted by centrifugation at 20,000 x g for 15 minutes at room temperature. To remove trace amounts of salt remaining from the precipitation, the DNA pellet was washed in 100 µl of 70% (v/v) ethanol. The DNA pellet was then air dried, and dissolved in an appropriate volume of H_2O or TE buffer.

Quantification and quality control of nucleic acids

DNA was assayed by spectrophotometry. This involved taking an absorbence reading at wavelengths 260 nm and 280 nm. The reading at 260 nm allows calculation of the concentration of nucleic acid in the sample. An A_{260} of 1 corresponds to approximately 50 µg ml^{-1} for double stranded DNA. The ratio between the reading at 260 nm and 280 nm provides an estimate of purity of the nucleic acid. Pure preparations of DNA have ratios of 1.8. Any ratios less than this value, indicate protein and/or phenol contamination of the sample.

Cleavage of DNA with restriction enzymes

All DNA restrictions were performed using Boehringer Mannheim restriction endonucleases and buffers. Usually, 1 - 5 μg of DNA was incubated with 10 units of restriction enzyme in the appropriate buffer, in a final volume of 10 μl, at 37°C for 1 - 15 hours. For double digests involving enzymes with different recommended buffers, DNA was cleaved first with the lower salt buffered enzyme followed by cleavage with the second enzyme in higher salt buffer.

Dephosphorylation of DNA

Plasmid DNA which was to be used in cloning was in some cases dephosphorylated to remove the 5'-phosphate and prevent self-ligation of the vector ends. The plasmid (5 - 10 μg) was restricted with the chosen endonuclease in a 20 μl reaction mix, and an aliquot (1 μl) was checked for completion, by agarose gel electrophoresis. The remainder of the reaction mixture was made up to 89 μl with H_2O, and 10 μl of phosphatase buffer and 1 μl (1 U) of calf intestinal phosphatase (Boehringer Mannheim) was added, the mixture was then incubated for 1 hour at 37°C. The reaction was stopped by heat inactivation of the phosphatase at 65°C for a 1 hour. DNA was extracted with phenol/chloroform, ethanol precipitated and redissolved in 20 μl H_2O.

Ligation of plasmid DNA

T4 DNA ligase catalyses the ligation of DNA ends by forming a phosphodiester bond between the 3'-OH group of one end and the 5'-phosphate of another end (Weiss *et al*, 1968). This enzyme was

therefore used to ligate various DNA fragments into plasmid vectors. Ligation of DNA was performed using 1 µl (2 U) of T_4 DNA ligase (Boehringer Mannheim) in the appropriate concentration of ligation buffer. Routinely between 1 - 10 µg of DNA insert was ligated with 0.1 - 1 µg of vector, in a volume of 10 µl. Ligation reactions were incubated overnight at room temperature in a waterbath.

Agarose gel electrophoresis

For the analysis of all DNA preparations, 0.8 - 2.5% (w/v) agarose gels were used. Agarose (NBL) was dissolved in a 1X TBE buffer by brief boiling, and ethidium bromide was added at a final concentration of 0.5 µg ml^{-1}. DNA samples were mixed with a 1/6 volume of loading buffer, and loaded into the wells of the gel. Routinely 0.5 µg of λ *HindIII* molecular weight marker (Boehringer Mannheim) was loaded to assess the size and amount of DNA fragments. Gels were electrophoresed horizontally with an applied voltage of 4 - 8 V cm^{-1} for 1 - 3 hours. DNA bands were visualized with a UV trans-illuminator emitting at 313 nm, and photographs taken with a Mitsubishi Video Copy Processor Model P65B.

Purification of DNA fragments

DNA fragments (approximately 0.5 µg - 5 µg) were purified from agarose gels using the GeneClean II™ kit (Vogelstein and Gillespie, 1979) according to the manufacturer's instructions (Stratatech Scientific). In brief, the required fragment of DNA was cut from the gel, placed in an Eppendorf tube, weighed, and then dissolved in an equal weight of saturated NaI at 37°C (approximately 10 minutes). (If the gel was made

appropriate volume of TBE modifying buffer was additionally added prior to incubation.) Once the gel slice had completely dissolved, 5 - 10 µl of glass milk (containing glass powder) was added to the solution and the tube was inverted several times to mix. The solution was briefly centrifuged at 13, 000g for 10 seconds and the pellet was then washed three times in 1 ml volumes of wash solution (50% ethanol and 50% buffer solution composed of 20 mM Tris-HCl, pH7.2 containing 0.2 M NaCl and 2 mM EDTA), again at 13, 000g for 10 seconds. Finally, the purified DNA was eluted from the glass pellet in 20 µl H_2O, centrifuged as before. The DNA solution was removed and stored at -20°C until further use. Routinely, this procedure gave between 20% and 80% yield of the original quantity of DNA contained within the gel (efficiency was found to be lowest with small DNA fragments).

Random-primed labelling of DNA fragments

This method is based on that described by Feinberg and Vogelstein (1983, 1984). The method uses random hexa-nucleotides which bind to the DNA fragments, and initiate DNA polymerase reactions with the Klenow fragments of DNA polymerase 1. One of the nucleotides incorporated is radiolabelled, and thus the newly synthesized DNA becomes radiolabelled. The DNA fragment to be random-primed, was either, cut from a plasmid with appropriate restriction endonucleases, or synthesized by PCR. The DNA fragment(s) were then run on a 0.8% agarose gel (w/v in 1X TBE buffer), and the appropriate band cut out and GeneCleaned. Recovery and quantification of DNA was checked by visualisation of a proportion of the GeneCleaned product on an agarose gel as before. Approximately 0.1 - 0.5 µg of purified DNA (1 µl) was made up to 5 µl with H_2O in an Eppendorf tube, and denatured by heating

for 10 minutes at 100°C. The sample was subsequently cooled on ice. The following was added (Random Primed DNA Labeling Kit, Boehringer Mannheim):

1 µl of each of dATP, dGTP, dTTP (each 0.5 mM)
2 µl of reaction mix
5 µl of [α^{32}P]dCTP (Amersham 10 µCi µl^{-1})
4 µl of H_2O
1 µl of Klenow enzyme (2 U µl^{-1})

The mixture was incubated for 30 minutes at 37°C, and the reaction was then stopped with 2 µl 0.2 M EDTA (pH 8.0). The probe was stored at -20°C until needed.

Sequencing double-stranded DNA

Dideoxy-DNA sequencing reactions using T7 DNA polymerase were performed with a Sequenase Version 2.0 DNA Sequencing Kit (United States Biochemical Corporation), according to the manufacturer's instructions. This method depends on base-specific termination, by dideoxynucleotides, of enzyme catalysed primer extension reactions (Sanger *et al*, 1977). Four reactions are performed, all containing primer, template, and four deoxyribonucleotides; each contains a chain terminating dideoxyribonucleotide and T7 DNA polymerase. This results in a stochastic mixture of extended fragments, each terminated by the particular dideoxyribonucleotide added to the reaction. When the products of the four reactions are separated by electrophoresis side by side, a 'ladder' of progressively larger fragments is formed. The base pair

sequence of the DNA molecule can be deduced by reading the order of this ladder, which is visualised by radiolabelling.

N.B Unless otherwise stated, all reagent used for sequencing were supplied in the Sequenase Version 2.0 DNA sequencing Kit.

Approximately 2 - 5 μg of purified, RNA-free (incubated for 30 minutes at 37°C in 200 μg ml^{-1} of RNase (Sigma, Type IIA)) plasmid DNA was made up to 100 μl with H_2O. This was denatured by the addition of 25 μl of an alkaline-denaturing solution, containing 1 M NaOH, 20mM EDTA, and incubating the mixture at 37°C for 30 minutes. Denatured DNA was then ethanol precipitated. The denatured DNA pellet was redissolved in 7 μl H_2O, and 2 μl of reaction buffer and 1 μl (approximately 10 ng) of primer were added. This mixture was then incubated at 37°C for 30 minutes to allow the primer to anneal. Whilst the primer was annealing to the denatured DNA template; six Eppendorf tubes were labelled 'L', 'E', 'A', 'C', 'G', and 'T', and into each was pipetted:

'L'	4 μl of H_2O + 1 μl of labelling mix
'E'	5 μl of enzyme dilution buffer + 1 μl of T7 polymerase
'A'	2.5 μl of termination mix 'A'
'C'	2.5 μl of termination mix 'C'
'G'	2.5 μl of termination mix 'G'
'T'	2.5 μl of termination mix 'T'

N.B Each of the termination mixtures were prewarmed at 37°C for at least 2 minutes prior to use.

On completion of the annealing reaction, the annealed DNA was labelled for 3 minutes at room temperature by mixing the following:

10 µl of annealed DNA mix
1 µl of 0.1 M DTT
2 µl of diluted labelling mix ('L')
1 µl of [α ^{35}S]dATP (Amersham, 10 µCi)
2 µl of diluted enzyme ('E')

3.5 µl of labelling reaction was then pipetted into each of the termination tubes ('A', 'C', 'G', 'T'), and incubated at 37°C for 5 minutes. 4 µl of stop solution was added, and the samples were frozen at -20°C if they were not being run immediately.

Electrophoresis was carried out using the Bio-Rad Sequi-Gen nucleic acid sequencing gel electrophoresis system. The gel[1] (40 cm x 30 cm x 0.5 mm) was pre-run at a constant 50 mA (2 kV) for 30 minutes in 1X TBE buffer. Samples were boiled for 3 minutes prior to loading, and 3 µl volumes were loaded into each lane. Gels were then run at 50 mA (2 kV) for between 2 and 7 hours. The gel was transferred to a sheet of Whatman filter paper and covered with SaranWrap, before drying on a vacuum gel dryer at 80°C for approximately 2 - 4 hours. The dried gel was then processed by autoradiography overnight at room temperature.

1 Sequencing gel

urea	52.5 g
10X TBE	12.5 ml
30% acrylamide	25 ml
25% ammonium persulphate	300 µl

TEMED 32 µl

Make up to 125 ml with H_2O

Polymerase chain reaction

The polymerase chain reaction (PCR), as described by Saiki and colleagues (1988), was used for the amplification of DNA fragments. Reactions were carried out in a 100 µl mix containing:

Template DNA	1 µl (500 ng)
Primer 1[1]	1 µl (100 pM)
Primer 2[1]	1 µl (100 pM)
Reaction buffer[2]	10 µl (Boehringer Mannheim)
dNTP mix[3]	10 µl
H_2O	76.5 µl
Taq DNA polymerase	0.5 µl (2 U) (Boehringer Mannheim)

To prevent evaporation each reaction mix was overlaid with 50 µl of mineral oil (PCR Grade, Sigma). Amplification was then performed over 30 cycles using a Hybaid OmniGene Thermal Reactor programmed as follows: denaturation at 94°C for 1 minute, annealing at 55°C for 1 minute and elongation at 72°C for 2 minutes. A further 1 cycle of 72°C for 10 minutes and 22°C for 30 minutes completed the amplification. After the PCR reactions had finished, the DNA product was removed from the overlaying oil, and monitored by electrophoresis.

1 Primers: The oligonucleotides used as primers in PCR reactions are listed in table 2.1

2 Reaction buffer:	20 mM Tris Cl (pH 8.0)
	100 mM KCl
	1 mM DTT
	0.1 mM EDTA

3 dNTP mix: A working solution of dNTP's was made up by mixing 25 µl of 100 mM concentrations of dATP, dCTP, dGTP, and dTTP (Boehringer Mannheim)

Cloning of PCR products

To enable PCR products to be cloned, the "ragged ends" of these DNA fragments were first repaired using Klenow fragments (Tabor and Struhl, 1988). PCR products were gel purified and GeneCleaned. T4 polynucleotide kinase was then used to transfer phosphate groups from ATP to the 5' termini ofPCR DNA fragments by incubating the following mixture in an Eppendorf tube for 45 minutes at 37°C:

PCR Product (0.1 - 1.0 µg DNA)	23 µl
Tris Cl, pH 8.0	3 µl
20mM ATP	1.5 µl
0.1 mM DTT	1.5 µl
0.3 M $MgCl_2$	1 µl
T4 polynucleotide kinase	0.5 µl (5 U)

Klenow polymerase was then used to blunt end the DNA by adding dNTP's to kinase treated DNA. This was achieved by adding 1 µl of Klenow polymerase (Boehringer Mannheim) and 8 µl of dNTP's to the above mixture, followed by incubation for a further 30 minutes at room

temperature. The blunt ended PCR DNA was then extracted with phenol/chloroform, ethanol precipitated, and resuspended in 20 µl of H_2O. Ligation was as previously described.

DNA Dot Blotting

DNA dot blotting was used as a method for mapping DNA fragments to regions of the *S. typhimurium* chromosome by hybridization, using the Mu*d*-P22 lysates of the rapid mapping kit (Benson and Goldman, 1992). 100 µl volumes of each of the Mu*d*-P22 lysates and DNA controls (1 µg made up to 100 µl with H_2O), were denatured by heating each at 100°C for 10 minutes in a total volume of 0.5 ml in 0.4 M NaOH containing 10 mM EDTA . Nylon membrane (BioRad) was immersed in H_2O to wet, and then inserted into a 96 well microfiltration apparatus (BioRad), which was tightened under vacuum to insure no cross-well contamination. To check that the apparatus set-up was correct, 0.5 ml of H_2O was pipetted into each well and sucked through under vacuum. 0.5 ml of each denatured DNA sample was then pipetted into appropriate wells, and the vacuum was once again applied until the wells were empty but not dry. Wells were rinsed with 0.5 ml of 0.4 M NaOH, and then the vacuum was applied until all wells were quite dry. The apparatus was disassembled, and the membrane was removed, washed briefly in 2X SSC[1] and dried at 65°C for 30 minutes in an oven. The DNA was then UV cross-linked to the membrane using a Stratagene UV stratalinker 1800, set at 0.12 Joules. The membrane was immediately probed by hybridization with a random primed DNA fragment.

1 2X SSC: 0.3 M NaCl
30 mM trisodium citrate

Analysis of genomic DNA by Southern hybridization

Localization of particular sequences within genomic DNA may be accomplished by the transfer techniques described by Southern (1975). In essence, genomic DNA is digested with one or more restriction enzymes, and the resulting fragments are separated according to size by electrophoresis through an agarose gel. The DNA is then denatured *in situ* and transferred from the gel to a solid support *eg* nylon membrane. A radiolabelled DNA or RNA fragment is then hybridized to the DNA attached to the membrane, and autoradiography is used to locate the position of the band complementary to the probe.

In the present study, approximately 10 μg of genomic DNA was digested overnight with an appropriate restriction enzyme, and electrophoresed through a 0.8% agarose gel (w/v in 1X TBE). The gel was photographed, and the distance migrated by each of the DNA molecular weight markers from the well was recorded. A small portion of the top left corner was then removed so that the gel could be correctly orientated. The DNA was depurinated by soaking the gel in 0.25 M HCl for 15 minutes, followed by a brief rinse in H_2O to remove excess HCl. The DNA was then denatured by placing the gel in a bath of 0.5 N NaOH solution containing 1 M NaCl, on a moving platform for 30 minutes at room temperature. The gel was then neutralized by bathing it in 0.5 M Tris-HCl (pH 7.4) containing 3 M NaCl for 30 minutes at room temperature on a moving platform. Two sheets of Whatman 3MM paper and one sheet of Hybond-N nylon membrane (Amersham) were cut to the exact size of the gel. A further three sheets of Whatman 3MM paper were cut to the width of the gel, but approximately double its length. A dish large enough to contain the gel was half filled with 20X SSC buffer[1] and the three longer sheets of Whatman 3MM paper were soaked in this then

laid over the back of a gel tray so that an equal length of paper hung over either end. (The gel tray was placed face down in the dish of 20X SSC, ensuring that the overhanging Whatman 3MM paper was in contact with the solution on either side.) The gel was careful laid up-side-down on the back of the gel tray, on the overlaying Whatman 3MM paper, and any air bubbles removed by gently pressing them to the side. The four edges of the gel were covered by 2 cm strips of Saran Wrap to ensure that subsequent capillary action only occurred through the gel. The nylon membrane was wet in H_2O and overlaid on the gel, again removing any air bubbles. This was repeated with the two sheets of Whatman 3MM paper cut to the same size as the gel. A stack of paper towels, approximately 15 cm high, was carefully placed on top of the Whatman paper, and a glass plate and weight secured on top of these. The DNA was transferred overnight at room temperature, and the blot was then carefully dismantled. The nylon membrane was separated from the gel, rinsed in H_2O, dried at 65°C for 30 minutes in an oven, and then the DNA was cross-linked to the membrane by UV light using a Stratagene UV stratalinker 1800, set at 0.12 Joules. The membrane was immediately probed by hybridization with a random primed DNA fragment.

1 20X SSC: 3 M NaCl
0.3 M trisodium citrate

Hybridization protocol for DNA probes

The membrane was placed in a glass hybridization roller, with 25 ml of prehybridization solution[1]. Both roller and solution preheated to 65°C, and the membrane was incubated for 5 minutes at 65°C in a Techne Hybridizer HB-1, hybridization oven. The prehybridization solution was

replaced with the fresh solution (25 ml), and denatured probe (prepared by boiling in a waterbath for 10 minutes) was added. Hybridization was conducted for 12 - 24 hours at 65°C. To ensure the removal of the remaining probe that had not hybridized, the membrane was then washed four times for 30 minutes at 65°C: twice in wash 1[2] and twice in wash 2[3]. After washing, the membrane was placed in SaranWrap and monitored overnight by autoradiography at -80°C.

1 Prehybridization solution:

0.25 M Na_2HPO_4, pH 7.2
7% SDS

2 Wash 1:

20 mM Na_2HPO_4, pH 7.2
5% SDS

3 Wash 2:

20 mM Na_2HPO_4, pH 7.2
1% SDS

2.3.3 Protein detection procedures

SDS-polyacrylamide gel electrophoresis of proteins

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970) allows separation and visualisation of proteins that have been

dissociated into their individual polypeptide subunits. This is achieved by treatment of the protein with the strongly anionic detergent SDS, in combination with a reducing agent and heat. The denatured polypeptides bind SDS and become negatively charged. Because the amount of SDS bound is almost always proportional to the molecular weight of the polypeptide and is independent of its sequence, SDS-polypeptide complexes migrate through polyacrylamide gels in accordance with the size of the polypeptide.

SDS-PAGE was carried out using the Bio-Rad Protean II xi slab cell system. Glass plates were cleaned with H_2O and ethanol, separated by, and sealed by side clamps. This assembly was then placed in the casting stand and tightened onto a sealing strip ready for pouring. The 15% resolving gel[1] was poured, and allowed to polymerize by overlaying with butanol. After polymerization was complete, the butanol was removed, and a 5% stacking gel[2] poured directly onto the surface of the polymerized resolving gel. A comb was then pressed firmly into the stacking gel, and the gel allowed to polymerize. While the stacking gel was polymerizing, the samples (see pulse-labelling of MPG361, below) were prepared by heating them to 100°C for 3 minutes in 1X SDS gel-loading buffer[3] to denature the proteins. When polymerization was complete, the gel was placed in the buffer tank, and Tris-glycine buffer[4] added. Samples were loaded (50 µl) into wells, and the gel was run overnight at a constant current of 10 mA. Once the dye front had reach the bottom of the resolving gel, the apparatus was disassembled, and the gel was fixed and stained with Coomassie blue and then dried under vacuum. Gels containing sample that had been pulse-labelled with ^{35}S-methionine were then autoradiographed overnight at room temperature.

1 15% resolving gel (30 ml):

30% acrylamide mix	15 ml
H_2O	6.9 ml
1.5 M Tris (pH 8.8)	7.5 ml
10% SDS	300 µl
10% ammonium persulphate	300 µl
TEMED	12 µl

2 5% stacking gel (10 ml):

30% acrylamide mix	1.7 ml
H_2O	6.8 ml
1.0 M Tris (pH 6.8)	1.25 ml
10% SDS	100 µl
10% ammonium persulphate	100 µl
TEMED	10 µl

NB 30% acrylamide mix was a solution of acrylamide/bisacrylamide at a ratio of 19:1.

3 1X SDS gel-loading buffer:

50 mM Tris-HCl (pH 6.8)
100 mM dithiothreitol
2% SDS
0.1% bromophenol blue
10% glycerol

4 Tris-glycine buffer:

25 mM Tris
250 mM glycine (pH 8.3)
0.1% SDS

Staining SDS-polyacrylamide gels with Coomassie blue

Polypeptides separated by SDS-PAGE were simultaneously fixed with methanol/glacial acetic acid/H_2O solution (ratio 45:45:10) and stained with Coomassie blue. The SDS-PAGE protein gel was immersed in staining solution[1] and placed on a slowly moving platform for a minimum of 4 hours at room temperature. This solution was replaced by destaining solution[2] and the gel was destained on a slowly moving platform for a further 4-8 hours at room temperature, whilst changing the destaining solution three or four times. The gel was then rinsed briefly in H_20, examined by eye, and the distance migrated by each the molecular weight protein markers was recorded.

1 Staining solution (1 litre):

Coomassie brilliant blue	2.5 g
Methanol	450 ml
H_2O	450 ml
Glacial acetic acid	100 ml

The solution was filtered through a Whatman No. 1 filter to remove any particulate matter.

2 Destaining solution (1 litre): Destaining solution was prepared as staining solution, but minus stain.

2.3.4 Methods for the construction, isolation and characterization of *S. typhimurium* Mu*dlux* fusions

Construction of a Mu*dlux* pool in MPG202.

The bacteriophage Mu derivative, Mu*dlux*(Km), when inserted into a gene in the correct orientation, results in a transcriptional fusion that directs the synthesis of Lux enzymes necessary for light production (Engebrecht *et al,* 1983). *S. typhimurium* is normally resistant to phage Mu. However, previous studies have shown that this restriction may be circumvented by using a *galE* derivative for infection (Hiles *et al*, 1987). It has been shown that phage P1-sensitive (*galE*) derivatives of *S. typhimurium* can be infected with Mu that has been given P1 host-range, and that P1 host-range can be conferred by the helper phage Mu*cts*62hP1 (abbreviated MuP1) which directs the synthesis of P1 tail fibres (Csonka *et al*, 1981).

A dilysogen, containing Mu*dlux* and MuP1, was therefore constructed in a sequential manner as previously described by Hiles *et al* (1987). Mu*dlux* (Km^R) was transduced from the *E. coli* strain, HB101 (Mu*dlux*), to a restriction-deficient P1-sensitive *S. typhimurium* strain (CH424) by P1-mediated transduction. A P22 lysate of the resultant Mu*dlux* lysogen (MPG200) was then used to transduce the *S. typhimurium* strain CH463 (containing MuP1) to kanamycin resistance (Km^R). The resultant dilysogen, MPG201, when temperature induced, gave high titre MuP1 lysates that could efficiently transduce a *galE* derivative of virulent *S. typhimurium* strain, SL1344 (designated

MPG202); during this transduction, Mu*dlux* is able to transpose and insert more or less at random into the recipient chromosome. A random pool of Mu*dlux* insertions was therefore obtained. Approximately 65,000 kanamycin-resistant transductant colonies were pooled and washed before storage at -80°C in DMSO (7% v/v). This poolate was constructed by M. P. Gallagher with the help of P. Meaney.

Isolation of fusions which exhibit stress-induced light production.

Mu*dlux* transcriptional fusions can be used to monitor the strength and regulation of promoters, when correctly inserted downstream of their putative regulatory sequence, by the production of light. Therefore, fusions that are within a particular stress-induced gene may be isolated by monitoring selective light production from cells when that stress is applied.

Cells from the random bank of MPG202 (Mu*dlux* Km^R) fusions were plated on LB kanamycin plates at a density of 10^3 c.f.u. per plate and were incubated overnight at 30°C. Colonies were examined by eye to eliminate fusions which produced light prior to hydrogen peroxide stimulation. Plates were then treated in one of the following manners according to the particular stress screened for:

a) overlaid with Whatman No.3 cellulose discs (9 cm dia.), soaked in 90 mM hydrogen peroxide (Sigma), and left at room temperature for one hour.

b) overlaid with Whatman No.3 cellulose discs (9 cm dia.), soaked in 100 mM sodium hypochlorite (Aldrich), and left at room temperature for one hour.

c) overlaid with Whatman No.3 cellulose discs (9 cm dia.), soaked in LB pH 5.6 (citrate or acetate buffered), and left at room temperature for one hour.

d) overlaid with Whatman No.3 cellulose discs (9 cm dia.), soaked in 100 mM menadione solution (sodium salt, Sigma), and left at room temperature for one hour.

e) placed in a cold room overnight.

Fusions exhibiting light induction in response to the stimulus were isolated, colony purified, and re-tested for light production as above. The Mu*dlux* marker was then transduced into a clean background with phage P22, and re-tested for the correct phenotype prior to subsequent characterisation.

Determining the working concentration of hydrogen peroxide for inducing light in MPG203

Hydrogen peroxide is a toxic oxidizing agent that readily kills bacteria at high concentration. However, at low concentrations bacteria are able to adapt to oxidative stress by inducing the synthesis of protective stress proteins (Demple, 1991; Farr and Kogoma, 1991). This experiment was designed to determine the working concentrations of hydrogen peroxide required for inducing light in different densities of exponentially growing cultures of MPG203.

An overnight culture of MPG203 was diluted by a factor of ten in LB and incubated for one hour at 30°C with aeration to re-establish exponential growth. 100 µl volumes of this culture were pipetted into the top row (all 12 wells) of a white 96-well microtitre plate containing 100 µl volumes (all 96 wells) of LB, and then sequentially diluted down the remaining seven rows (approximately 10^8 to 10^6 c.f.u. ml^{-1} diluted from

rows A-H in 0.3 log steps). The first eleven of these bacterial dilution ranges (columns 1-11) were then overlaid with an equal volume of a H_2O_2 dilution range (40 mM to 40 μM H_2O_2 diluted from columns 1-11 in 0.3 log steps), the end column (12) acting as a control for background light. The plate was left at room temperature for one hour, and subsequent light emission was observed by eye and recorded by exposing the plate to X-ray film for 10 minutes.

Hydrogen peroxide light induction assay

Exponentially growing cultures of MPG203 and its derivatives were tested for their light emission in response to exposure to hydrogen peroxide. Overnight cultures were grown in LB medium plus the appropriate antibiotic and viable counts were determined by plating on LB. Cells were subsequently diluted to the required density (as stated in the results) in LB medium or tissue culture medium, and incubated for one hour at 30°C with aeration to allow for media readjustment and to re-establish exponential growth. Samples were then activated by the addition of an appropriate concentration of hydrogen peroxide (Sigma), and light production was recorded at appropriate intervals using an 'in-house' single tube luminometer or a Beckman liquid scintillation counter (Model LS1701) set on the tritium channel, at room temperature.

Peroxide disk inhibition assay

Peroxide disk inhibition assays (Storz *et al*, 1989) were performed to determine whether the strains MPG203 and MPG353 had altered sensitivity to killing by hydrogen peroxide and cumene hydroperoxide in comparison to the parental strain, SL1344. For each strain, 100 μl

volumes of overnight culture were added to 2 ml of soft agar and overlaid on LB plates. On the centre of each plate was placed a 6 mm disk of Whatman No.3 cellulose, and 10 µl of a solution containing 3% hydrogen peroxide (Sigma) (v/v) dissolved in water or 3% cumene hydroperoxide (v/v) (Sigma) dissolved in dimethyl sulfoxide (Koch-Light Laboratories, UK) were carefully pipetted onto each. The diameter of the zone of killing was measured after plates were incubated overnight at 30°C.

Influence of temperature and gentamicin on light production

The viability of overnight cultures was established by plating. Cultures were diluted in tissue culture medium to approximately 10^5 c.f.u. per ml and incubated for one hour at 30°C, prior to the experiment. Samples were then incubated at 30°C or 37°C for an hour in the presence of 100 µM H_2O_2. Gentamicin was added to relevant samples after 40 minutes and incubation was then continued as before for the remaining samples. After this period, samples were directly counted in a liquid scintillation counter, to assess light emission.

Interaction of MPG203 with J774.2 cells.

In order to examine the interaction of MPG203 with macrophages, J774.2 cells were seeded onto two flasks (250 ml), at a density of $5x10^6$ cells per flask, in tissue culture medium containing penicillin (200 U ml^{-1}) and streptomycin (100 µg ml^{-1}). The cells were then allowed to adhere to the flask by incubating overnight at 37°C in a 5% CO_2 incubator (during this time the cell number approximately doubled). Overnight cultures of MPG203 were diluted to approximately 10^7 cells ml^{-1} in tissue culture medium (containing no antibiotics) and grown at 30°C for one hour, with

aeration. Macrophage cell sheets were washed repeatedly in PBS and subsequently infected with the *S. typhimurium* cells at 37°C in tissue culture medium at a multiplicity of approximately 20:1 (bacteria: cells). Incubation was continued for 40 minutes at 37°C and both cell sheets were gently but extensively washed in PBS, to remove the majority of unbound bacteria. Fresh tissue culture medium, with or without gentamicin, was added and incubation was continued at 37°C for a further 20 minutes. After this period, cell sheets were stripped by vigorous hand agitation and light emission was subsequently recorded by liquid scintillation counting at room temperature. (The effectiveness of macrophage release was assessed microscopically.) As a control, duplicate cultures of MPG203 alone were grown for 40 minutes at 37°C and then gentamicin was added to one of the two cultures, to equate with conditions for macrophage interaction. Incubation was continued for a further 20 minutes at 37°C, prior to liquid scintillation counting at room temperature.

After a three hour interval from the initial point of infection, hydrogen peroxide was added to a concentration of 100 μM to both macrophage and bacterial samples, and light production was again recorded for approximately one hour. Macrophage samples, containing gentamicin, were subsequently washed three times by low speed centrifugation (600 x g for 5 minutes in a bench-top MSE Centaur 1 centrifuge) in tissue culture medium, to remove residual gentamicin. High speed centrifugation (3,000 x g for 10 minutes in a bench-top MSE Centaur 1 centrifuge) was then carried out to lyse macrophages and the pellets were resuspended in fresh tissue culture medium. Hydrogen peroxide was added, as before, and samples were measured by liquid scintillation counting.

Influence of catalase on light production.

In order to assess whether J774.2 cells elicited light induction in MPG203 cells by producing hydrogen peroxide, macrophage cells and cultures of MPG203 were grown overnight and prepared for infection studies, as described above. Two flasks (250 ml) containing J774.2 cell sheets were washed repeatedly in PBS, as before, and each was then infected, at 37°C, with *Salmonella typhimurium* cells in tissue culture medium, at a multiplicity of infection of approximately 20:1 (bacteria:cells). Catalase (Sigma) was added simultaneously to one of the flasks (final activity 100 U per ml of tissue culture medium) and both flasks were incubated for 40 minutes at 37°C. The cell sheets were then washed extensively to remove unbound bacteria and fresh tissue culture medium was added, with and without catalase (100 U per ml). Incubation was continued at 37°C for a further 20 minutes, after which cell sheets were stripped by vigorous hand agitation and light emission was subsequently recorded in a liquid scintillation counter at room temperature in the presence or absence of catalase.

Assay of respiratory burst activity in J774.2 cells.

The respiratory burst of J774.2 macrophage cells was monitored by reaction with luminol (Sigma), as previously described (Gentle and Thompson, 1990). Fresh tissue culture medium was added to overnight sheets of J774.2 cells (prepared as above in 250 ml flasks) which were then stripped by vigorous hand agitation and resuspended at a density of approximately 6 x 10^4 cells per ml. Overnight cultures of SL1344 cells were diluted in tissue culture medium to approximately 10^7 cells per ml and grown at 30°C to approximately 4 x 10^9 bacteria per ml, prior to the

experiment. Samples of bacteria were then diluted, mixed with J774.2 cells at appropriate ratios and further incubated at 37°C. (Bacterial densities were verified by plating). At appropriate intervals, phorbol myristate acetate (PMA) (Sigma; 500 ng ml^{-1} final concentration dissolved in H_2O) and luminol (Sigma; 0.1 mM final concentration dissolved in dimethylformamide) were added. Thereafter, light production was recorded by monitoring chemiluminescence in a liquid scintillation counter, at room temperature. Peak light emission occurred approximately twenty minutes after PMA addition.

Visualisation of bacterial-macrophage interaction.

An overnight culture of MPG203 cells was diluted to approximately 10^7 cells ml^{-1} in tissue culture medium and exponential growth was re-established for one hour at 30°C. 1 ml of this suspension was then used to infect a macrophage-coated cavity slide (washed in PBS) at 37°C for 40 minutes, to enhance interaction. Unbound bacteria were subsequently removed by extensive washing in PBS and fresh tissue culture medium, containing gentamicin, was added. Incubation was continued for a further 20 minutes, at 37°C, in a carbon dioxide incubator. Cavity slides were then examined at intermittent intervals for light emission, with the aid of a photon video camera (Model 2400, Hamamatsu Photonics Ltd.) attached to a Zeiss Axioskop microscope.

Pulse-labelling of MPG361 cells during cold shock treatment.

Pulse-labelling of *S. typhimurium* proteins induced during cold shock was conducted as described by Goldstein and colleagues (1990). Overnight cultures of MPG361 and SL1344 cells were diluted 100-fold

in minimal medium (M9 salts), supplemented with 0.4% glucose (w/v in H_2O) and all of the common amino acids (1 mM) except methionine. Cells were then grown at 30°C to an optical density at 420 nm of approximately 0.3 - 0.5 and each culture was divided into 1 ml aliquots. All but one aliquot from each culture was then placed in a 10°C waterbath. The aliquot remaining at 30°C was immediately pulse-labelled with 10 µCi of ^{35}S methionine (> 1000 Ci/mM, Amersham) for 5 minutes, and then chased with 1 mM methionine (nonradioactive) for a further 5 minute. Cells were pelleted by centrifugation for 2 minutes at 20,000 x g at room temperature and were resuspended in 100 µl of 1X SDS gel-loading buffer (see SDS-PAGE method above). The samples were boiled for 3 minutes and then stored at -80°C.

The above procedure was repeated with 1 ml aliquots of each culture placed at 10°C. However, in the latter case each culture was pulse-labelled with 10 µCi of ^{35}S methionine for 1 hour after 1, 2, 3 and 4 hours at 10°C, before being chased with 1 mM methionine (nonradioactive). Samples were pelleted, denatured in sample buffer, and stored at -80°C as before. Samples were then boiled for 3 minutes and 50 µl volumes were loaded onto a 15% SDS-polyacrylamide resolving gel. A 10 µl volume of broad range molecular weight protein marker was loaded into a parellel well (BioRad). Electrophoresis and Coomassie staining were performed as above.

Construction of a mini-Tn*10* pool in MPG361

The *S. typhimurium* strain MPG361 was randomly mutagenised using Tn*10*Δ*16*Δ*17* (Way *et al*, 1984). This transposon is a tetracycline-resistant, mini-Tn*10* derivative that lacks transposition functions unless the transposase is provided *in trans*. Phage P22 lysates

were prepared from cultures of TT10427 containing pNK972 (which expresses the transposase genes 16 and 17 of Tn*10*) and TT10423 (containing Tn*10*Δ*16*Δ*17*) (Both lysates were prepared by E. Allan and contained approximately 10^9 phage ml^{-1}). Phage from the TT10427 lysate were then used to transduce the plasmid pNK972 into an overnight culture of MPG361 cells (multiplicity of infection was approximately 1), selecting for inheritance of the plasmid on LB ampicillin plates at 30°C. A transductant was colony purified, and a subsequent overnight culture of this strain was infected with phage P22 (carrying Tn*10*Δ*16*Δ*17*) from the TT10423 lysate (again the multiplicity of infection was approximately 1). The latter phage-bacteria mixture was spread on 100 pre-warmed LB tetracycline plates and incubated overnight at 30°C. Approximately 55,000 transductants were obtained and these were subsequently screened.

Microscopic examination of bacteria

The motility of bacterial strains was determine using phase contrast microscopy. More precise morphological characterization of cells was also determined by phase contrast microscopy, but by the examination of fixed bacteria. This was achieved by adding an equal volume of an overnight culture to a 20% formalin solution (v/v) made up in phosphate buffered saline. A 10 µl volume of this mixture was then pipetted onto an agar block, premounted on a glass slide, and examined at a magnification of X100 using a Zeiss Axioskop microscope on phase contrast.

CHAPTER 3

Chapter 3: The isolation in *Salmonella typhimurium* of Mu*dlux* fusions which exhibit stress-induced light production.

3.1 Introduction.

As discussed at length in Chapter 1, *S. typhimurium* is a facultative intracellular parasite of certain host and is capable of persisting within professional phagocytes such as macrophages (Finlay and Falkow, 1989a). Relatively little is known however, about the mechanisms by which this bacterium and many other intracellular parasites (Moulder, 1985; Finlay and Falkow, 1989b) withstand the antimicrobial actions of these phagocytic cells.

Previous studies concerned with the survival mechanisms adopted by *S. typhimurium* during its infection of macrophage cells, have tended to take the direct approach of screening large numbers of bacterial mutants for reduced survival *in vivo* (*ie* within macrophages) (Fields *et al*, 1986; Miller *et al*, 1989a). These studies have identified several genetic loci of specific importance in the intracellular survival of *S. typhimurium*. Of particular note has been the identification and characterisation of the *phoP* locus (Groisman *et al*, 1989; Miller *et al*, 1989a) and in later studies, the *pagC* locus (Miller *et al*, 1990a). Nevertheless, there are still thought to be a large number of, as yet, unidentified virulence loci that encode macrophage survival functions in *S. typhimurium* (Groisman and Saier, 1990).

An alternative to the method described above, but with equal potential to identify virulence loci in *S. typhimurium*, is to adopt an indirect approach for studying intracellular survival mechanisms. For example, if one can determine the precise bactericidal environment found within an activated macrophage, this information can be used to isolate

bacterial mutants *in vitro* (*ie* in the absence of macrophages), following exposure to equivalent types of stress, and such mutants can then be re-assessed for their response to macrophage interactions. This procedure can be greatly facilitated by using transposon generated mutants with the capacity to form transcriptional fusions and, therefore, to report gene expression (*eg* Mu*dlux* (Engebrecht *et al*, 1984)). These fusions not only provide a sensitive assay for recording macrophage-induced stresses, but also allow the degree of stress to be accurately and quantitatively assessed (*ie* by the level of light emission).

This chapter is mainly concerned with the isolation of Mu*dlux* fusions of the virulent *S. typhimurium* strain SL1344 which produce light in response to stresses which reflect those encountered during interaction and endocytosis by macrophages (*eg* H_2O_2, OCl^-, and low pH) (Groisman and Saier, 1990). In each case, these fusions were isolated from a pool of mutants constructed in a *galE* derivative of SL1344 (designated MPG202) (see chapter 2 for methodology and below) by screening for light production in response to exposure to a defined stress (*eg* H_2O_2) *in vitro*. Gene fusions of interest were subsequently transduced into a wildtype SL1344 background by phage P22 transduction where indicated. In such instances, the phenotypes of the fusion strains were re-examined to eliminate the possibility of transposition events.

3.2 Isolation of fusions induced by hydrogen peroxide.

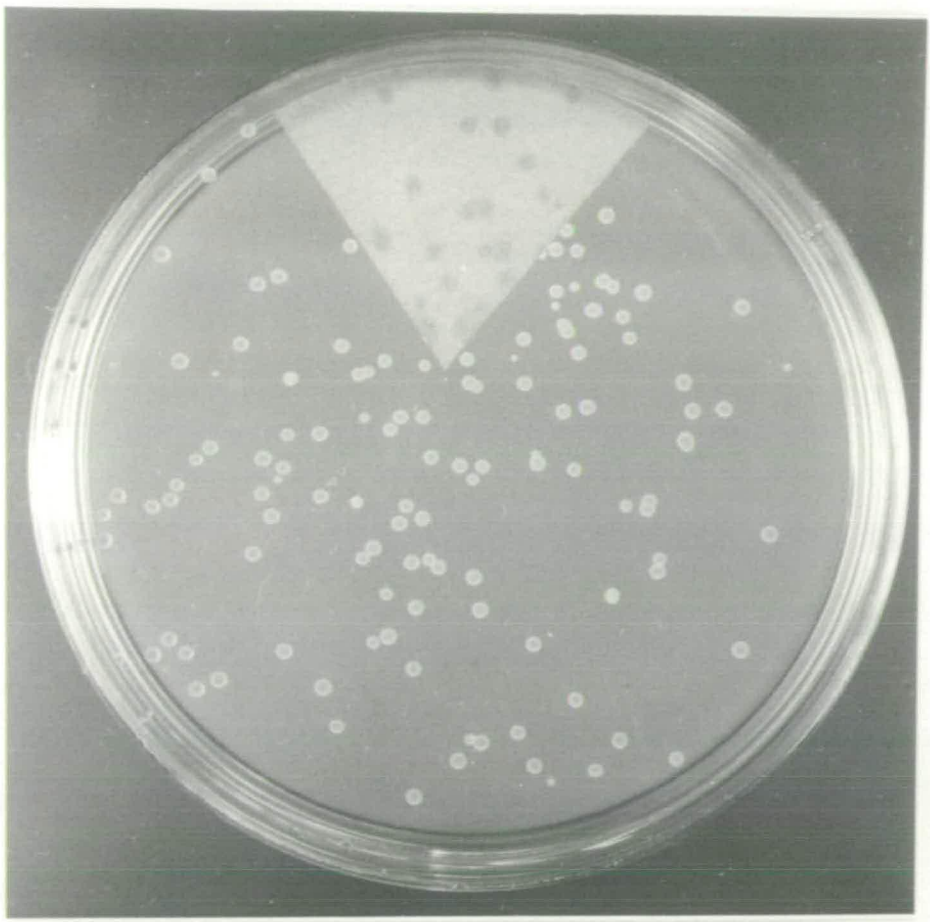
Approximately 65,000 colonies of the MPG202 Mu*dlux* pool were screened for the presence of fusions that produced light in response to hydrogen peroxide. LB plates containing approximately 1000 colonies per plate, grown overnight at 30°C, were initially screened at room temperature to allow constitutive light emitting fusions to be eliminated.

Each of these plates was then overlaid for approximately 2 - 3 hours with filter paper soaked in 90 mM concentrations of hydrogen peroxide. Fusions which were induced by this particular stress, and which produced light to a visible level, were colony purified and rescreened as before. This procedure resulted in the isolation of 34 hydrogen peroxide-inducible fusions. On subsequent analysis however, only 12 of these fusions were found to have acceptably low levels of background light when not induced by hydrogen peroxide (*eg* see figure 3.1). These 12 fusions were subsequently designated MPG278 to MPG289.

Characterization of these 12 fusions was initially continued on solid LB medium. They were again induced by overlaying pure cultures of each strain with hydrogen peroxide-soaked filters (90 mM H_2O_2). Each fusion was carefully observed by eye (both uninduced and induced by hydrogen peroxide) over a period of 3 hours from the initial time of induction, and again after 24 hours. This demonstrated that there was a large degree of variability between the patterns of light production by these fusions (see table 3.1 for data recorded during the initial 3 hour period of hydrogen peroxide induction). For example, the time between initial induction of a fusion by hydrogen peroxide and subsequent observation of light, was found to vary from approximately 40 minutes to 80 minutes. Four of the fusions (MPG279, MPG280, MPG281 and MPG289) were also found to only produce light on 2 of the 4 occasions that this procedure was conducted, indicating that the growth phase of these latter fusions or some other uncontrolled factor might be equally important for light induction. No light was observed from any of the fusions at 24 hour from the initial time of exposure to the stimulus. Further analysis of the 12 hydrogen peroxide inducible fusions was conducted to determine the effect of low pH on the light response of each of the strains. Pure cultures of each fusion, grown on LB plates overnight

Figure 3.1. Detection of light production by Mu*dlux* fusions in *S. typhimurium* in response to hydrogen peroxide.

The MPG202 Mu*dlux* pool was screened (approximately 65,000 colonies) for the presence of fusions that produced light in response to hydrogen peroxide by overlaying LB plates, containing approximately 1000 colonies per plate (grown overnight at 30°C), with filter paper soaked in 90 mM concentrations of this oxidant at room temperature. Colonies emitting light upon exposure to hydrogen peroxide were purified on LB plates and then retested as before. Shown is a typical plate screen of a purified culture (in this instance a pure culture of MPG203 (derived from MPG285)) overlaid with a filter soaked in hydrogen peroxide (A). Light emission was recorded from purified cultures after approximately 2 - 3 hours of exposure to hydrogen peroxide, both by visual examination and by exposing the plates to X-ray film for approximately 10 minutes at room temperature. Panel B shows the light response from a pure culture of MPG203 induced by exposure to hydrogen peroxide, as described above.



A

B

Table 3.1. Stress-induced light emissions from Mu*dlux* fusions originally isolated from the MPG202 pool using hydrogen peroxide as a stimulus.

The MPG202 Mu*dlux* pool was screened (approximately 65,000 colonies) for the presence of fusions that produced light in response to hydrogen peroxide (see text for details). Shown are the light emissions, recorded visually on LB plates at room temperature, for a number of these fusions when exposed to filters soaked in 90 mM hydrogen peroxide, or filters soaked in LB adjusted to pH 5.6 with citrate or acetate buffer (in each case light emissions were recorded after approximately 3 hours of exposure to each stress (H_2O_2 or low pH)), or when plates were placed at low temperature (8°C) overnight. Also shown are the light emissions from each of the fusions when bacteria were left untreated, both on LB plates (solid) and in LB media (liquid) during exponential growth. Data shown are representative of three such tests, repeated. V. High, High, Medium and Low refer to light intensities. Variable, indicates that the light intensity varied between repeat tests. V = visible, BV = barely visible and NV = not visible.

Table 3.2. Stress-induced light emissions from Mu*dlux* fusions originally isolated from the MPG202 pool using sodium hypochlorite as a stimulus.

The MPG202 Mu*dlux* pool was screened (approximately 40,000 colonies) for the presence of fusions that produced light in response to sodium hypochlorite (see text for details). A number of these fusions were then treated in an identical fashion to the hydrogen peroxide-induced fusions discribed in table 3.1. Again, data shown are representative of three such tests, repeated. See table 3.1 for key to abreviations.

Table 3.1

Fusion number	H_2O_2 90mM	pH↓ 5.6	Temp↓ 8°C	Controls Solid	Liquid
MPG278	Medium	NV	NV	NV	NV
MPG279	Variable	NV	High	NV	NV
MPG280	Variable	BV	NV	BV	V
MPG281	Variable	NV	High	NV	NV
MPG282	High	Low	NV	BV	V
MPG283	Low	High	NV	BV	V
MPG284	Medium	High	NV	BV	V
MPG285	High	NV	NV	NV	NV
MPG286	Low	V.High	NV	BV	V
MPG287	V.High	Medium	NV	BV	V
MPG288	High	NV	Medium	NV	Variable
MPG289	Variable	Medium	NV	BV	V

Table 3.2

Fusion number	$NaOCl^-$ 100mM	H_2O_2 90mM	pH↓ 5.6	Temp↓ 8°C	Controls Solid	Liquid
MPG290	High	NV	NV	Medium	NV	V
MPG291	High	NV	NV	Medium	NV	NV
MPG292	V.High	NV	Medium	High	BV	NV

at 30°C, were overlaid with filter paper soaked in LB medium adjusted to pH 5.6 with citric acid. Each of these fusions was then observed by eye for any subsequent light production over a 3 hour period at room temperature, and again after 24 hours. This pH treatment resulted in visible production of light by 3 (MPG283, MPG286 and MPG289) of the 12 fusions (see table 3.1). The times until light induction occurred at low pH corresponded well with those observed using hydrogen peroxide as a stimulant. However, both the magnitude and the duration of the low pH responses were significantly higher than observed for each individual sample when exposed to hydrogen peroxide. This experiment was repeated with LB medium which was adjusted to pH 5.6 with acetate in order to confirm that the response was really due to pH and not an artifact resulting from citrate metabolism. A similar result was observed using acetate as the stimulus.

During the course of this study, another stress was identified which also appeared to induce several of the hydrogen peroxide-inducible fusions. Purified cultures of each of the 12 hydrogen peroxide-induced fusions were routinely stored on LB plates in a cold room at approximately 6°C to 8°C. By chance, it was noted that 3 (MPG279, MPG281 and MPG288) of the 12 fusions produced high levels of light under these conditions, when examined visually, without any apparent stimulus having been applied. This phenomenon was further investigated by placing a number of LB plates, streaked with MPG279, MPG281 and MPG288 and grown overnight at 30°C, at temperatures of 30°C, 23°C (room temperature), or 8°C for durations of 2, 4, 6 and 12 hours. It was established that light from these 3 fusions could only be detected visually (see table 3.1) after at least 12 hours at 8°C. It was concluded that light induction in these 3 strains was due to prolonged temperature downshift - cold shock (this is discussed in more detail below).

In order to prepare more accurate light induction profiles of the 12 hydrogen peroxide-inducible fusions when subjected to stress, each fusion was tested in liquid culture (LB). Initial experiments were conducted to assess the level of background light in each of these fusions when uninduced. Overnight cultures of each fusion (grown in LB at 30°C), were diluted by 100-fold in fresh LB and incubated for 1 hour at 30°C. Each of these exponentially growing cultures (approximately 10^7 bacteria ml^{-1}) was then measured for its background light emission at room temperature in a single cell luminometer. Unfortunately, 7 of the 12 fusions were found to produce unacceptably high levels of background light (see table 3.1). The remaining 5 fusions (MPG278, MPG279, MPG281, MPG285, and MPG288) were deemed suitable for further investigation using this procedure (MPG279 is discussed in more detail in section 3.5 below). In particular, MPG285 was found to have the lowest levels of background light when uninduced and to have high levels of light when induced with hydrogen peroxide (see Chapter 4, figure 4.3). The specificity of this light induction suggested that MPG285 might be a suitable candidate for reporting oxidative stress upon interaction with macrophages (see Chapter 4).

3.3 Screening for fusions induced by low pH.

The MPG202 (Mu*dlux*) pool was also screened for fusions that produced light in response to low pH. Approximately 40,000 colonies, which were grown on LB plates overnight at 30°C, were initially screened at room temperature to eliminate constitutive light emitting fusions. Each plate containing approximately 1000 colonies was then overlaid with filter paper soaked in LB medium adjusted to pH 5.6 with citric acid. Fusions which were induced by this particular stress, and which produced light to

a visible level, were colony purified and rescreened as before. On rescreening purified cultures, however, it was noticed that all 15 pH-inducible fusions that had been isolated produced unacceptably high levels of background light (when uninduced) under normal growth conditions on LB plates.

The above test was repeated by overlaying plates of colonies from the MPG202 (Mu*dlux*) pool (approximately 10,000 colonies) with filter paper soaked in LB medium adjusted to pH 5.6 with acetic acid. Again, however, the 3 pH-inducible fusion that were isolated by this procedure were also found to produce unacceptably high levels of background light when rescreened as purified cultures. No further work was conducted upon any of these fusions.

3.4 Isolation of fusions induced by sodium hypochlorite.

Fusions producing light in response to sodium hypochlorite were isolated from the MPG202 (Mu*dlux*) pool in a manner similar to that described for hydrogen peroxide. Approximately 40,000 colonies, grown overnight at 30°C on LB, were initially screened to eliminate constitutive light emitting fusions, and then overlaid with filter paper soaked in 100 mM sodium hypochlorite. This resulted in the isolation of 12 fusions that produced visible light in response to this stimulus. However, as was found with the majority of the hydrogen peroxide inducible fusions, 9 of the sodium hypochlorite inducible fusions were found to produce unacceptably high levels of (uninduced) background light when they were re-tested as pure cultures on solid LB medium at room temperature. These 9 fusions were ignored and work was concentrated on the remaining 3 which had minimal levels of background light (designated MPG290, MPG291 and MPG292).

Subsequent analysis of these 3 sodium hypochlorite inducible fusions was again initially continued on solid LB medium. Pure culture of each of these fusion were overlaid with sodium hypochlorite-soaked filters (100 mM), and then observed by eye over a period of 3 hours from the initial time of induction, and again after 24 hours (control plates of uninduced fusions were observed simultaneously). This demonstrated that there was only a minor difference between the light intensities of each of the 3 fusions (see table 3.2). In addition, light emission was first observed after approximately 120 minutes in all three cases. Furthermore, once fully induced, each of these fusions emitted intense light for an extremely long period of time (still visible after 24 hours).

As with the hydrogen peroxide inducible fusions, each of the sodium hypochlorite inducible fusions was re-screened for inducibility by low pH, cold shock and hydrogen peroxide (see table 3.2). As before, plates of the 3 fusion were either overlaid with filters soaked in 90 mM hydrogen peroxide or LB adjusted to pH 5.6 with citric acid, or alternatively, placed at approximately 8°C for an overnight period. Surprisingly, all 3 fusions were seen to glow brightly after cold shock, with MPG292 additionally observed to produce a significant light response when induced by low pH (no low pH response was observed in MPG290 or MPG291). In contrast, no light at all could be seen from any of these three strains after induction with hydrogen peroxide. Furthermore, both MPG291 and MPG292 (but not MPG290) were found to produce very little background light in solution when not specifically induced. A more detailed investigation of these two fusions was conducted in liquid culture (see below).

3.5 Isolation of fusions induced by cold shock.

Fusions producing light in response to cold shock, were also isolated from the MPG202 (Mu*dlux*) pool. Approximately 40,000 colonies, which were grown on LB plates overnight at 30°C, were initially screened at room temperature (approximately 20°C) to eliminate constitutive light emitting fusions. The remaining fusions were then cold shocked by placing plates of these colonies at 8°C overnight. This resulted in the isolation of 46 cold shock-inducible fusions. However, 16 of these fusions were observed to produce unacceptably high levels of uninduced background light (constitutive) when they were re-tested as pure cultures on solid LB medium at room temperature. The remaining 30 fusions (designated MPG293-MPG322) were all found to have insignificant levels of background light, allowing their cold shock response on solid medium to be studied.

Observation of pure cultures of the 30 cold shock-inducible fusions on solid LB medium, both uninduced (room temperature) and induced by cold shock (8°C for a period of 24 hours), demonstrated that there was a large degree of variability between the light emissions from each of these strains (see table 3.3). This suggested that a number of different gene fusions had been isolated. As with the other types of stress inducible fusions, each of the 30 cold shock inducible fusions was subsequently tested for induction by alternative stresses (see table 3.3), including hydrogen peroxide, low pH, and sodium hypochlorite. The effect of nalidixic acid was also examined at a concentration of 10 mM. Again this was achieved by overlaying pure cultures of each fusion (grown overnight on LB plates at 30°C) with filter paper soaked in solutions of one of the aforementioned chemicals, and then observing each plate by eye over a subsequent 3 hours period. The results demonstrated that only 1 of the 30

fusions was induced by hydrogen peroxide (MPG293) and only 2 of the fusions were induced by low pH (MPG294 and MPG309). However, 15 fusions and 10 fusions were induced to some degree by exposure to either sodium hypochlorite or nalidixic acid, respectively (see table 3.3). One fusion (MPG309) was found to be induced to a reasonably high level by cold shock, sodium hypochlorite, low pH, and nalidixic acid, but showed no light induction whatsoever following exposure to hydrogen peroxide.

Further analysis of the 30 cold shock inducible fusions was conducted in liquid culture. As before, this was done to allow accurate light induction profiles for each of the fusions to be established in response to different stresses (cold shock, or exposure to sodium hypochlorite, or to low pH, or to nalidixic acid). Initial light measurements recorded in a liquid scintillation counter at room temperature, demonstrated that 8 of the 30 cold shock inducible fusions had unacceptably high levels of background light in liquid culture when uninduced. The remaining 22 fusions which displayed insignificant levels of background light (see table 3.3), plus 3 previously isolated cold shock inducible fusions (MPG279 - originally isolated as inducible by hydrogen peroxide - and MPG291 and MPG292 - originally isolated as inducible by sodium hypochlorite), were subsequently characterized by cold shocking exponential cultures (approximately 5 x 10^5 c.f.u ml^{-1}) at 10°C and recording light emissions at intervals over a period of 24 hours. Light responses for eight of the cold shock inducible fusions are shown for the first 6 hours in figure 3.2. As can be seen, there was a great deal of variation in light emission from the fusions examined, both in terms of magnitude and in terms of the profiles of light emission with time.

Throughout all of the above cold shock experiments, one fusion in particular (MPG300) was shown to give consistently minimal levels of background light at room temperature (23°C) and extremely high levels

Table 3.3. Stress-induced light emissions from Mu*dlux* fusions originally isolated from the MPG202 pool using cold shock as a stimulus.

The MPG202 Mu*dlux* pool was screened (approximately 40,000 colonies) for the presence of fusions that produced light in response to cold shock treatment (see text for details). Shown are the light emissions, recorded visually on LB plates at room temperature, for a number of these fusions when plates were placed at low temperature (8°C) overnight, or exposed to filters soaked in either 100 mM sodium hypochlorite, or 90 mM hydrogen peroxide, or LB adjusted to pH 5.6 with citrate or acetate buffer, or 10 mM nalidixic acid (NA) (in each case light emissions were recorded after approximately 2 - 3 hours of exposure to each stress ($NaOCl^-$, H_2O_2, low pH or NA)). Also shown are the light emissions from each of the fusions when bacteria were left untreated, both on LB plates (solid) and in LB media (liquid) during exponential growth. Data shown are representative of three such tests, repeated. E. High, V. High, High, Medium, Low and V. Low refer to light intensities. Vari, indicates that the light intensity varied between repeat tests. V = visible, BV = barely visible and NV = not visible.

Table 3.3

Fusion number	Temp↓ 8°C	pH↓ 5.6	H_2O_2 90mM	NaOCl 100mM	NA 10mM	Controls Solid	Liquid
MPG293	High	BV	Medium	NV	NV	NV	NV
MPG294	Low	Medium	BV	BV	BV	BV	V
MPG295	High	NV	NV	V.Low	NV	NV	NV
MPG296	Medium	NV	V.Low	NV	BV	NV	NV
MPG297	High	NV	NV	Low	NV	NV	V
MPG298	Low	NV	NV	V.Low	NV	NV	V
MPG299	Low	BV	BV	BV	BV	BV	V
MPG300	E.High	NV	NV	NV	Low	NV	NV
MPG301	High	NV	NV	NV	NV	NV	NV
MPG302	High	NV	NV	V.Low	NV	NV	NV
MPG303	V.High	BV	BV	Medium	BV	BV	BV
MPG304	High	NV	NV	NV	NV	NV	NV
MPG305	V.High	BV	BV	BV	BV	BV	NV
MPG306	Medium	BV	BV	BV	Low	BV	NV
MPG307	V.High	BV	BV	Medium	BV	BV	NV
MPG308	V.High	BV	BV	Medium	BV	BV	V
MPG309	V.High	Medium	BV	Medium	Low	BV	BV
MPG310	Medium	BV	Low	BV	BV	BV	NV
MPG311	V.High	BV	BV	Low	BV	BV	BV
MPG312	V.High	BV	BV	BV	BV	BV	V
MPG313	V.High	BV	BV	Medium	BV	BV	BV
MPG314	V.High	BV	BV	BV	BV	BV	V
MPG315	Medium	NV	NV	NV	NV	NV	NV
MPG316	Low	NV	NV	Medium	Medium	NV	NV
MPG317	High	BV	BV	BV	BV	BV	V
MPG318	Medium	NV	NV	NV	NV	NV	NV
MPG319	V.High	NV	NV	Low	Low	NV	NV
MPG320	Vari	NV	NV	NV	NV	NV	NV
MPG321	Vari	NV	NV	NV	NV	NV	NV
MPG322	Vari	NV	NV	NV	NV	NV	NV

of light at 10°C. When cold shocked, both on LB plates and in LB medium, this fusion was clearly distinguishable from all of the other cold shock inducible fusions due to its rapid rate of light induction (first to be visualised or recorded) and also because of its extremely high light intensity (consistently found to be the brightest cold shock fusion) at the peak of induction at 10°C (see figure 3.2). Furthermore, when an exponential culture (approximately 5 x 10^7) of this fusion was incubated at 8°C and monitored over an extended period, light emission was visually apparent for more than 4 months. Further characterization of this fusion is described in Chapter 6.

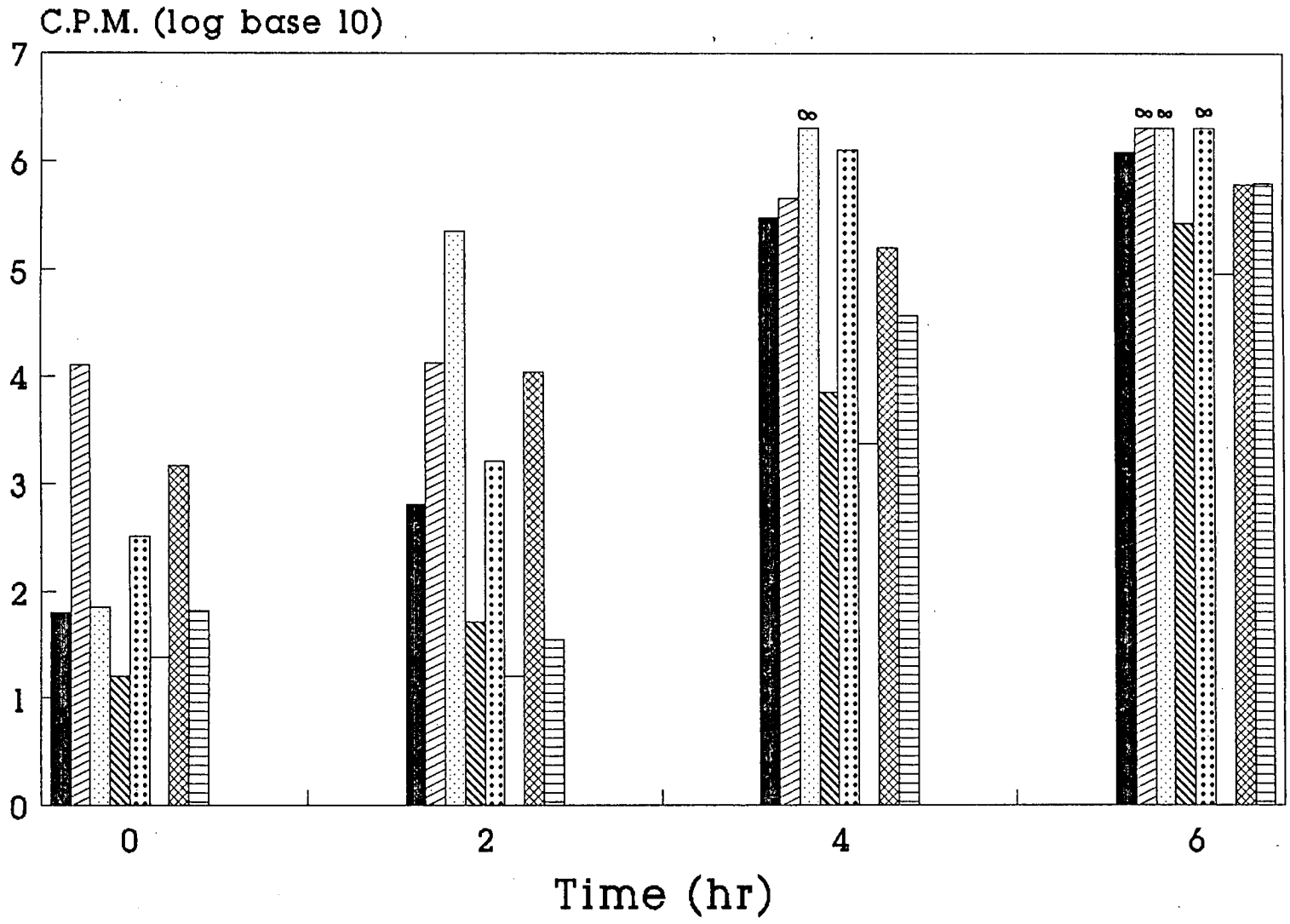
3.6 Discussion.

Treatment of *S. typhimurium* with hydrogen peroxide has been reported to induce the synthesis of at least 30 different proteins (Christman *et al*, 1985; Morgan *et al*, 1986). This feature, together with the fact that Mu inserts into the genome at random, indicate that the 34 hydrogen peroxide inducible fusions initially isolated during this study probably represent a selection of different gene fusions, rather than the repeated isolation of a single fusion. This is confirmed by the diversity seen in the stress-induced light responses of these fusions (see table 3.1).

Unfortunately, because many of the hydrogen peroxide-inducible fusions were found to have extremely high levels of uninduced background light, it was not possible to accurately determine the full range of stress-induced light responses in all 34 fusions by eye when examined on solid media. However, preliminary studies on the 12 fusions that did allow stress-induced light production to be detected on LB plates, demonstrated that a certain degree of overlap existed between the bacterial stress responses induced by hydrogen peroxide, pH and cold shock

Figure 3.2. Variation in light emissions between cold shock fusions.

Exponentially growing cultures (prepared by diluting overnight cultures by 10,000-fold in LB media and incubating for 1 hour at 30°C, to give approximately 5 x 10^5 c.f.u. ml^{-1}) of eight cold shock fusions (MPG295 (■), MPG296 (▨), MPG300 (⊡), MPG302 (▧), MPG316 (⊡), MPG291 (□), MPG292 (▩), MPG279 (▤)), were monitored in a liquid scintillation counter (set on the tritium channel) at room temperature for their initial light emissions (time 0). Each of these cultures was then placed in a 10°C water bath and monitored at regular intervals over a 24 hour period for bioluminescence, again in a liquid scintillation counter (set on the tritium channel) at room temperature. Light emissions recorded from the eight cultures prior to cold shock treatment and at 2, 4, and 6 hours post cold shock treatment are shown. Off scale values are show as infinity (∞).


C.P.M. (log base 10)
7
6
5
4
3
2
1
0
0
2
4
6
Time (hr)

(see table 3.1) (This same overlap in stress responses was also seen during the subsequent examination of a number of fusion originally isolated as emitting light in response to cold shock (see table 3.3).) Indeed, only 2 hydrogen peroxide inducible fusions (MPG278 and MPG285) were found which did not produce light in response to one or other of these alternative stresses when visually examined on solid media.

Previous work on the characterization of stress proteins in *S. typhimurium* has, to date, identified only a few genes that are induced by both low pH and hydrogen peroxide. In fact, of the 52 proteins reported to have their rates of synthesis altered following acid shock in *S. typhimurium* (Foster, 1991), only the heat shock protein DnaK has been confirmed as both low pH and hydrogen peroxide inducible (Foster, 1991; Morgan *et al*, 1986). Interestingly, Buchmeier and Heffron (1990) have reported that this protein is also significantly induced during the interaction of *S. typhimurium* with macrophage cells. Despite these findings, however, there is no cross-protection seen between these two stresses in *S. typhimurium* (*ie* acid adapted cells do not show increased survival when subjected to a subsequent challenge by hydrogen peroxide and *visa versa*) (Foster and Hall, 1990). Furthermore, in *E. coli*, VanBogelen and colleagues (1987) have shown that DnaK is induced by a range of stresses, including heat shock, hydrogen peroxide treatment, nalidixic acid treatment and ethanol treatment. Subsequent work has shown that this protein is also induced by menadione treatment (Greenberg and Demple, 1989), starvation (Matin, 1991), and ironically, during alkaline shift (Taglicht *et al*, 1987).

Initial studies aimed at isolating fusions from the MPG202 Mu*dlux* pool which responded solely to low pH, were relatively unsuccessful because of the high levels of background light that appeared in many of these strains. Difficulties in isolating specific pH inducible fusions have

also been reported in *E. coli* by Slonczewski and colleagues (1987). This group found that by replica plating a pool of Mu*dlacZ* fusions from X-gal plates at pH 7.4 to X-gal plates at pH 5.6 and selecting colonies that were white at pH 7.4 and blue at pH 5.6, only 2 strict low pH inducible fusions could be isolated from a total of 82,000 colonies. Unless the sensitivity of this latter assay was not sufficient to allow adequate detection of fusions which respond poorly to low pH (β-galactosidase assays are reported to be at least 10^2 to 10^3 times less sensitive than luminescence assays (Meighen, 1991)), it seems unlikely that a more extensive screening of the MPG202 (Mu*dlux*) pool would have resulted in any greater success. It may well be that, because of the increased sensitivity of the *lux* system, low pH-inducible fusions such as the two identified by Slonczewski and colleagues (1987) might even have been eliminated during the screening procedure due to high levels of background light.

In contrast to the high levels of background light associated with the majority of the pH inducible fusions isolated during this study, the three hydrogen peroxide inducible fusions that were also cold shock inducible, MPG279, MPG281 and MPG288, did not appear to have high levels of background light (see table 3.1). Unfortunately, two of these fusions, MPG279 and MPG281, were found to have unpredictable hydrogen peroxide inducible responses when rescreened (either producing moderately high levels of light or none at all). Nevertheless, the third of these fusions, MPG288, gave reproducible light responses to both of the aforementioned stresses. This latter fusion might prove to be useful as a candidate strain for future studies on the overlap that exists between the bacterial stress responses induced by hydrogen peroxide and cold shock. (Proof for this overlap has previously been shown by Jones and colleagues (1987), who demonstrated that cold shocking exponentially growing cultures of *E. coli* results in the induction of at least 13 different

proteins, including RecA, a protein that is also induced by hydrogen peroxide (VanBogelen *et al*, 1987)).

None of the fusions inducible by sodium hypochlorite was found to be inducible by hydrogen peroxide. This might be connected with the fact that a large number of the sites and target molecules damaged by hypochlorite exposure in bacterial cells are distinct from those effected by hydrogen peroxide exposure (Schraufstatter *et al*, 1990). Thus, it is possible that these different forms of cellular damage might induce separate stress responses and so lead to the induction of dissimilar stress proteins. Sodium hypochlorite was initially chosen as a stress to test fusions in this bacterium because it simulated analogous stresses in the phagocytic cells such as neutrophils (Foote *et al*, 1983). However, in view of the fact there have been no reports as to the bactericidal activity of this compound within macrophage cells, in which this intracellular parasite has been found to reside (Finlay and Falkow, 1989a), this choice of compound may have limited relevance.

In contrast to the lack of fusions inducible by both sodium hypochlorite and by hydrogen peroxide in *S. typhimurium*, a large number of fusions were found to produce light in response to hypochlorite injury and cold shock (see tables 3.2 and 3.3). Precisely what the connection is between these latter stresses in not known. However, further analysis of some of these hypochlorite-cold shock-inducible fusions may provide a greater insight into the phenomenon.

Characterization of the remaining cold shock-inducible fusions showed that, further to the identification of strains that produced light in response to hydrogen peroxide or sodium hypochlorite, some of these strains were also induced by low pH or nalidixic acid (see tables 3.2 and 3.3). The mechanism(s) by which light production was regulated in these latter fusions is not known. However, previous work by Jones and

colleagues (1992b) has shown that the α-subunit of DNA gyrase, a protein specifically inhibited by nalidixic acid, is induced during cold shock treatment of *E. coli*. This implies that the level of DNA supercoiling within the cell might play some role in the regulation of cold shock genes. This is substantiated the fact that the nucleoid protein H-NS, which has also been shown to be involved in DNA supercoiling (Higgins *et al*, 1988), is similarly induced during cold shock treatment of *E. coli* (La Teana *et al*, 1991). Furthermore, the level of DNA supercoiling has also been reported to change at low temperature in *E. coil* cells (Goldstein and Drlica, 1984; Wang and Syvanen, 1992).

Finally, a comparison of the light production from eight of the cold shock inducible fusions during a temperature downshift, indicated that there were a number of subtle differences between the responses of these isolates (see figure 3.2). Initial light recordings from each of these eight fusions taken at 30°C, demonstrated a large variation between the intensities of background light levels (see figure 3.2, time 0). This is consistent with work conducted by Jones and colleagues (1987) who showed by protein gel electrophoresis that only one cold shock protein was uniquely synthesized at 10°C (F10.6; subsequently designated CS7.4 by Goldstein *et al*, 1990), and that the remaining cold shock proteins were all induced to differing levels during growth at 37°C. Jones and colleagues (1987) also discovered that each of the proteins induced by cold shock was synthesized at different rates and to different levels. This corresponds well with the findings in this study. One cold shock inducible fusion, MPG300, was found to be induced quite rapidly and to a very high level (see figure 3.2). This fusion was selected for further study in the belief that it might be useful as an indicator strain which could be further manipulated genetically to facilitate analysis of the regulation of the cold shock response (see chapters 6 and 7).

The wide range of *S. typhimurium* stress-inducible fusions isolated during this study, and the ease with which the majority of these fusions were subsequently characterized, clearly shows the benefits of using a transcriptional light reporter system such as Mu*dlux*. In the following chapters, the majority of work will deal with the further characterization and identification of the hydrogen peroxide-inducible fusion MPG285 (see chapters 4 and 5) and the cold shock-inducible fusion MPG300 (see chapters 6 and 7).

CHAPTER 4

Chapter 4: Light emissions from *S. typhimurium* cells carrying a Mu*dlux* fusion, following interaction with macrophages of the cell line J774.2.

4.1 Introduction.

The role of the respiratory burst during microbial infections of professional phagocytes has been demonstrated to be fundamental for the full killing capacity of these cells. This is clearly demonstrated by the recurrent infections suffered by individuals with phagocytic cells in which this microbicidal capacity is absent (*eg* those suffering from Chronic Granulomatous Disease (Ganz *et al*, 1990)). Moreover, careful analysis of the respiratory burst of phagocytes has revealed that the most probable basis for the microbicidal activity of these cell is the generation of reactive oxygen species, including hydrogen peroxide (Forman and Thomas, 1986; Hassett and Cohen, 1989).

The precise roles played by hydrogen peroxide in microbial killing during phagocytosis are not fully known. Numerous *in vitro* studies (*ie* bacterial studies not involving phagocytes) have implicated hydrogen peroxide itself to be an extremely bactericidal compound (Watson, 1990; Storz *et al*, 1990; Demple, 1991). However, this oxidant has also been shown to be essential for the generation of other cytotoxic compounds such as hypochlorous acid (Albrich *et al*, 1981) and singlet oxygen (Badwey and Karnovsky, 1980; Noronha-Dutra, 1993) which have both been implicated as important factors in the intracellular killing of bacterial pathogens by phagocytic cells (Ganz *et al*, 1990; Noronha-Dutra, 1993). A better understanding of how an intracellular pathogen such as *S. typhimurium* is able to survive exposure to hydrogen peroxide might therefore serve to increase our knowledge of the various survival

mechanisms adopted by these parasites during the course of infection and lead to the development of better vaccines.

Previous work concerned with the identification of *S. typhimurium* proteins induced during infection of mouse macrophages (Buchmeier and Heffron, 1990), has shown that more than 34 different bacterial proteins were elevated. However, only one of these bacterial proteins, DnaK, was also amongst the 30 or so proteins that had formerly been identified as hydrogen peroxide-inducible in *S. typhimurium* (Christman *et al*, 1985, Morgan *et al*, 1986). (The information in the above reports was gained from analysis by two-dimensional gel electrophoresis, with more than 400 proteins examined in the former of these studies (Buchmeier and Heffron, 1990).) The finding, therefore, that mice inoculated with sublethal doses of *S. typhimurium* produced high levels of antibodies to another hydrogen peroxide-inducible bacterial protein, namely catalase II (the HPI-type catalase regulated by *oxyR*) (Kagaya *et al*, 1992) was somewhat surprising, since this data would indicate that catalase II might also be expected to be induced during *S. typhimurium* infections of macrophage cells.

The reason why macrophages might be expected to elicit an immune response leading to the generation of catalase II antibodies, is that these phagocytic cells have been reported to be responsible for triggering immunity to microbial infections in animals (Badwey and Karnovsky, 1980; Adams and Hamilton, 1984). Furthermore, macrophages are also, in theory, capable of inducing this enzyme in bacteria through their production of millimolar concentrations of hydrogen peroxide as part of the respiratory burst (Hassett and Cohen, 1989). It therefore seems likely that the detection of catalase II antibodies in the aforementioned mouse study was mediated, at least in part, *via* macrophage activation and the presence of processed peptide.

In view of the above results it seems obvious that accurately determining which bacterial proteins are induced during phagocytosis is extremely difficult and is unlikely to be adequately accomplished solely by biochemical techniques, such as two-dimensional gel electrophoresis. An alternative approach to solving the latter problem is not to identify directly the bacterial proteins induced during macrophage infection, but to tag and monitor the activation of their corresponding genes with the aid of a transcriptional reporter system such as Mu*dlux*. From the results in chapters 3, it is clear that it is possible to isolate a range of *S. typhimurium* strains containing transcriptional fusions which are capable of producing light specifically in response to stresses which are analogous to those found within an activated macrophages (*eg* hydrogen peroxide (Badwey and Karnovsky, 1980; Forman and Thomas, 1986), and possibly hypochlorite which is generated in other activated phagocytes, such as neutrophils and monocytes (Rosen *et al*, 1987)). This chapter will concern itself mainly with the further characterisation of such fusions, with regard to their light responses during the infection of macrophages, and will attempt to establish whether such an approach has the potential to identify those genes that are induced during the process of bacterium-macrophage interaction.

4.2 Preliminary analysis of Mu*dlux* fusions for production of light following macrophage interaction.

In chapter 3 a number of Mu*dlux* fusions of *S. typhimurium* were identified which produced light in response to exposure to hydrogen peroxide (MPG278, MPG285 and MPG288) or sodium hypochlorite (MPG291 and MPG292). These were transduced into clean SL1344 backgrounds with the aid of bacteriophage P22, and redesignated as the

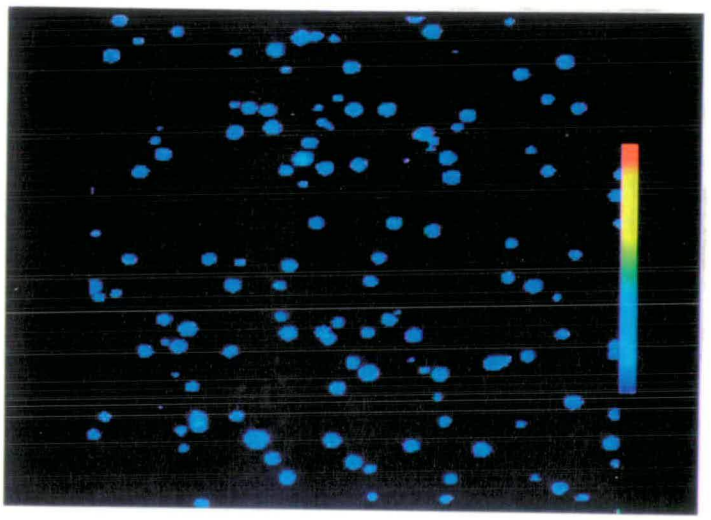
hydrogen peroxide-inducible fusions MPG354, MPG203, and MPG377, or the hypochlorite-inducible fusions MPG373 and MPG374, respectively. The phenotype of each fusion was re-tested (as previously described in chapter 3) and found to behave similarly to the donor strains in terms of light production (see tables 3.1 and 3.2).

The above fusions (MPG354, MPG203, MPG377, MPG373 and MPG374) were further tested for light production upon interaction with macrophages of the mouse cell line J774.2. Macrophages cells, at a density of approximately 10^6 cells ml^{-1}, were first coated on cavity slides overnight in tissue culture medium containing penicillin and streptomycin, at 37°C. Unbound macrophages and residual antibiotic were then removed by gently washing each slide several times in PBS, to leave a confluent layer of bound macrophages at a density of approximately 10^5 cells ml^{-1}. Each slide was then infected with an exponentially growing culture of one of the above strains (prepared by diluting an overnight culture of bacterial cells by 100-fold in antibiotic free tissue culture medium and incubating at 37°C for 1 hour to give approximately 10^7 bacteria ml^{-1}) at a ratio of approximately 100:1 (bacteria: macrophages). After 60 minutes of infection time at 37°C, each of the slides was removed from the incubator and examined for light emission at room temperature, using a Zeiss Axioskop microscope with a photon video camera attached.

Close examination of the macrophage cell sheets, demonstrated that the slide infected by the hydrogen peroxide-inducible fusion MPG203 produced a transient peak of light after approximately 20 minutes (*ie* 80-90 minutes from the initial time of infection). This response was relatively short lived and could only be monitored for approximately 15 to 20 minutes on each occasion. Nonetheless, the detection of light from this interaction indicated that it was indeed possible to monitor specific bacterial gene induction during the infection of macrophages. None of the

Figure 4.1. Preliminary analysis of MPG203 for production of light following macrophage interaction.

Macrophages cells, at a density of approximately 10^6 cells ml^{-1}, were coated on cavity slides overnight in tissue culture medium containing penicillin (200 U ml^{-1}) and streptomycin (100 µg ml^{-1}), in a 5% CO_2 incubator at 37°C. Unbound macrophages and residual antibiotic were then removed by gently washing each slide several times in phosphate-buffered saline, to leave a confluent layer of bound macrophages at a density of approximately 10^5 cells ml^{-1}. Each slide was then infected with an exponentially growing culture of a previously characterized Mu*dlux* fusion (prepared by diluting an overnight culture of bacterial cells by 100-fold in antibiotic free tissue culture medium and incubating at 37°C for 1 hour to give approximately 10^7 bacteria ml^{-1}) at a ratio of approximately 100:1 (bacteria: macrophages). After 60 minutes of infection time at 37°C, each of the slides was removed from the incubator and examined for light emission at room temperature, using a Zeiss Axioskop microscope (x40 magnification) with a photon video camera attached (set on photon counting mode). The light emission from an MPG203-J774.2 infection, monitored over a 5 minute period, is shown (C). Comparative light emissions from slides which contained only MPG203 cells (A) or MPG203 cells with 100 µM H_2O_2 added prior to incubation for 60 minutes at 37°C (B), both again monitored over a 5 minute period, are also shown. In all three cases the same exponential culture of MPG203 cells (prepared as above) was used. Light intensity is indicated by the scale bar (red represents peak intensity, *ie* the areas within the camera's field of view that had the highest number of photon strikes over the 5 minute period).



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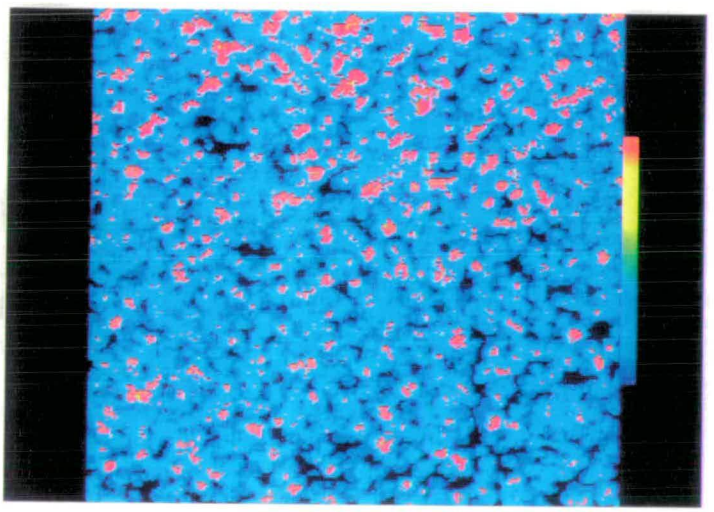

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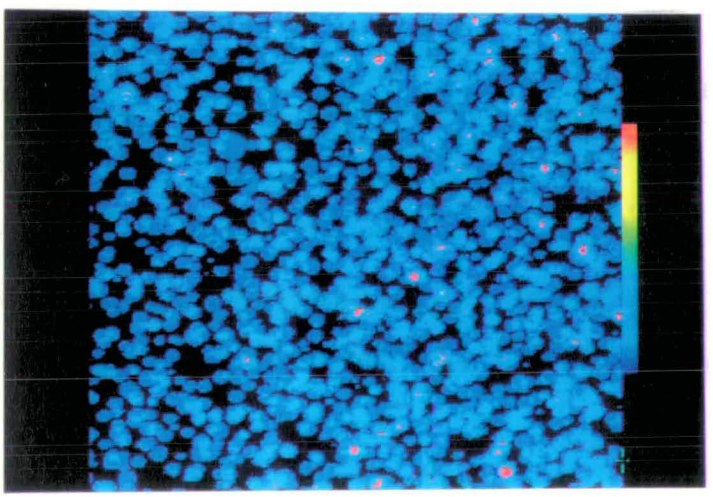

C

other fusions produced any significant light following macrophage infections in this preliminary trial.

Further to the above experiment, a crude attempt was made to compare the level of light produced by a culture of MPG203 cells during macrophage infection, to that produced by a proportional density of these bacteria exposed to hydrogen peroxide directly. This was accomplished by repeating the macrophage infection assay on slides, as described above (again using similar conditions and cell numbers), and comparing the light produced from this interaction with the light produced from slides which contained only bacterial cultures with or without 100 μM H_2O_2 added at time 0 (*ie* the time at which bacteria were added to the macrophage cell sheet). (In each case the same exponential culture of MPG203 cells, at approximately 10^7 bacteria ml^{-1}, was used). The results of this experiment are shown in figure 4.1. Only background light which was equivalent to the level detected from a blank slide (*ie* background noise from the photon video camera) could be observed from the culture of non-induced MPG203 cells (panel A). However, the cultures of bacterial cells induced by either hydrogen peroxide (panel B) or by interaction with macrophages (panel C), produced significantly more light. Furthermore, comparison of the light emissions seen in panels B and C, indicated that levels of light induced following macrophage interaction was not maximal, suggesting that the respiratory burst produced by the macrophages may be limited. (A more refined version of this experiment is discussed in section 4.10).

4.3 Effects of hydrogen peroxide concentration and bacterial cell density on light induction from MPG203.

As a result of the above experiment, further work was focused upon the hydrogen-peroxide inducible fusion MPG203. Experiments were

initially conducted to establish the concentration ranges over which hydrogen peroxide was able to induce light at different bacterial cell densities. Using a white 96-well microtitre plate, exponentially growing bacteria, at a density of approximately 10^8 bacteria ml^{-1} (prepared by diluting an overnight culture of MPG203 cells by 10-fold in LB medium and incubating at 30°C for 1 hour), were serially diluted in 0.3 log steps down all 8 rows of the plate and then decreasing concentrations of hydrogen peroxide (40 mM to 40 μM in 0.3 log steps) were added to 11 of the 12 columns of wells (column 12 served as a non-induced control). The plate was then incubated at room temperature for 1 hour before observing light emission visually and by exposing the plate to X-ray film for 10 minutes. From figure 4.2 it can be seen that, at density of 5×10^7 bacteria ml^{-1}, light induction was maximal between 100 - 300 μM H_2O_2, whereas a density of 5×10^6 bacteria ml^{-1} light induction was maximal between 20 - 80 μM H_2O_2. Further analysis of this data established the working concentration of hydrogen peroxide which resulted in light induction from cultures of MPG203 to be approximately 1 mM to 10 μM at bacterial densities of 10^8 to 10^5 bacteria ml^{-1}, respectively.

The kinetics of light induction in MPG203 were subsequently characterised more specifically by luminometry. As before, different densities of MPG203 cells, in exponential growth (prepared by diluting an overnight culture of MPG203 cells by 10-fold, 100-fold, 1000-fold, or 10,000-fold in LB medium and incubating at 30°C for 1 hour), were monitored for their light production in response to incubation at room temperature in the presence of varying concentrations of hydrogen peroxide (1 mM, 100 μM, 10 μM, or no H_2O_2). However, this time light readings were numerically quantified using a single cell luminometer. The results of this experiment (see figure 4.3) demonstrated that, at 10^8 bacteria ml^{-1} (panel A), substantial light production occurred in a transient

Figure 4.2. Determining the working concentration of hydrogen peroxide for inducing light in MPG203.

An overnight culture of MPG203 was diluted by a factor of ten in LB medium and incubated for one hour at 30°C to re-establish exponential growth. 100 µl volumes of this culture were diluted down all eight rows of a white 96-well microtitre plate (A) containing 100 µl volumes of LB (approximately 10^8 to 10^6 c.f.u. ml^{-1} diluted from rows A-H in 0.3 log steps). The first eleven of these bacterial dilution ranges (columns 1-11, rows A-H) were then overlaid with an equal volume of a H_2O_2 dilution range (40 mM to 40 µM H_2O_2 diluted from columns 1-11 in 0.3 log steps), the end column (12) acting as a control for background light. The plate was left at room temperature for one hour, and subsequent light emission observed by eye and recorded by exposing the plate to X-ray film for 10 minutes (B).


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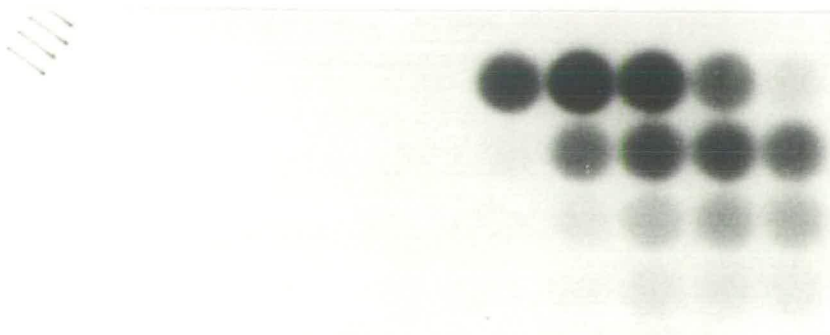
MADE IN U.S.A.
DYNATECH MicroFLUOR™

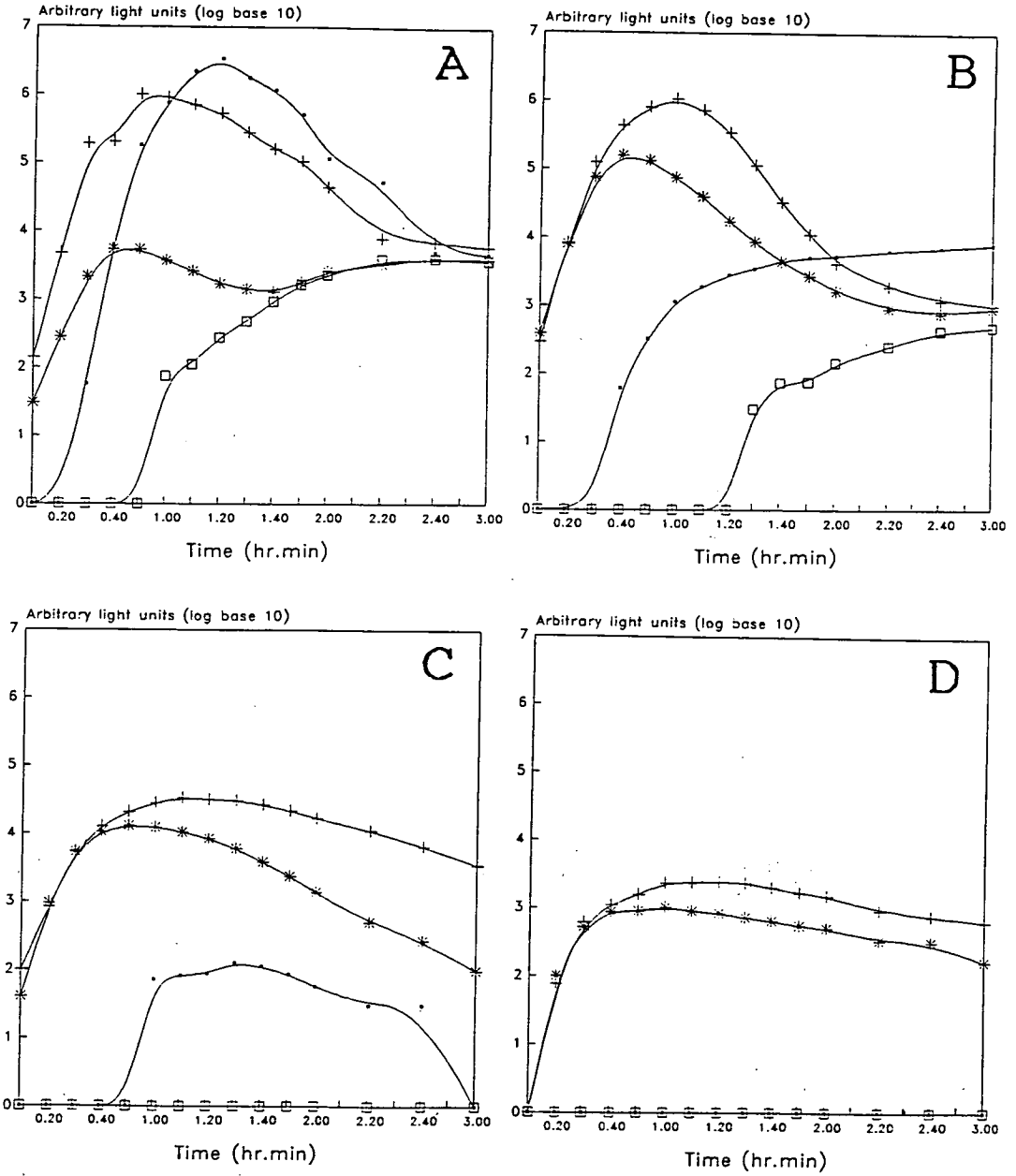
A



B

Figure 4.3. Light induction and suppression in MPG203 in response to hydrogen peroxide.

Exponential cultures of MPG203 (prepared by diluting an overnight culture of MPG203 cells by 10-fold, 100-fold, 1000-fold, or 10,000-fold in LB medium and incubating at 30°C for 1 hour) were treated with hydrogen peroxide to give a final concentration of 10 (-*-), 100 (-+-) or 1000 (-•-) μM, and light emission was immediately recorded from each sample over a period of 3 hours at room temperature using a single cell luminometer set on a sensitivity level of 6.0 mV and counting for 10 seconds. The response observed with 10^8 c.f.u. ml^{-1} (A), 10^7 c.f.u. ml^{-1} (B), 10^6 c.f.u. ml^{-1} (C), or 10^5 c.f.u. ml^{-1} (D) is shown (in each case a background (noise) value of approximately 120 arbitrary light units has been subtracted). No hydrogen peroxide was added to control samples (-□-).


Arbitrary light units (log base 10)
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Time (hr.min)
Arbitrary light units (log base 10)
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Time (hr.min)
Arbitrary light units (log base 10)
C
Time (hr.min)
Arbitrary light units (log base 10)
D
Time (hr.min)

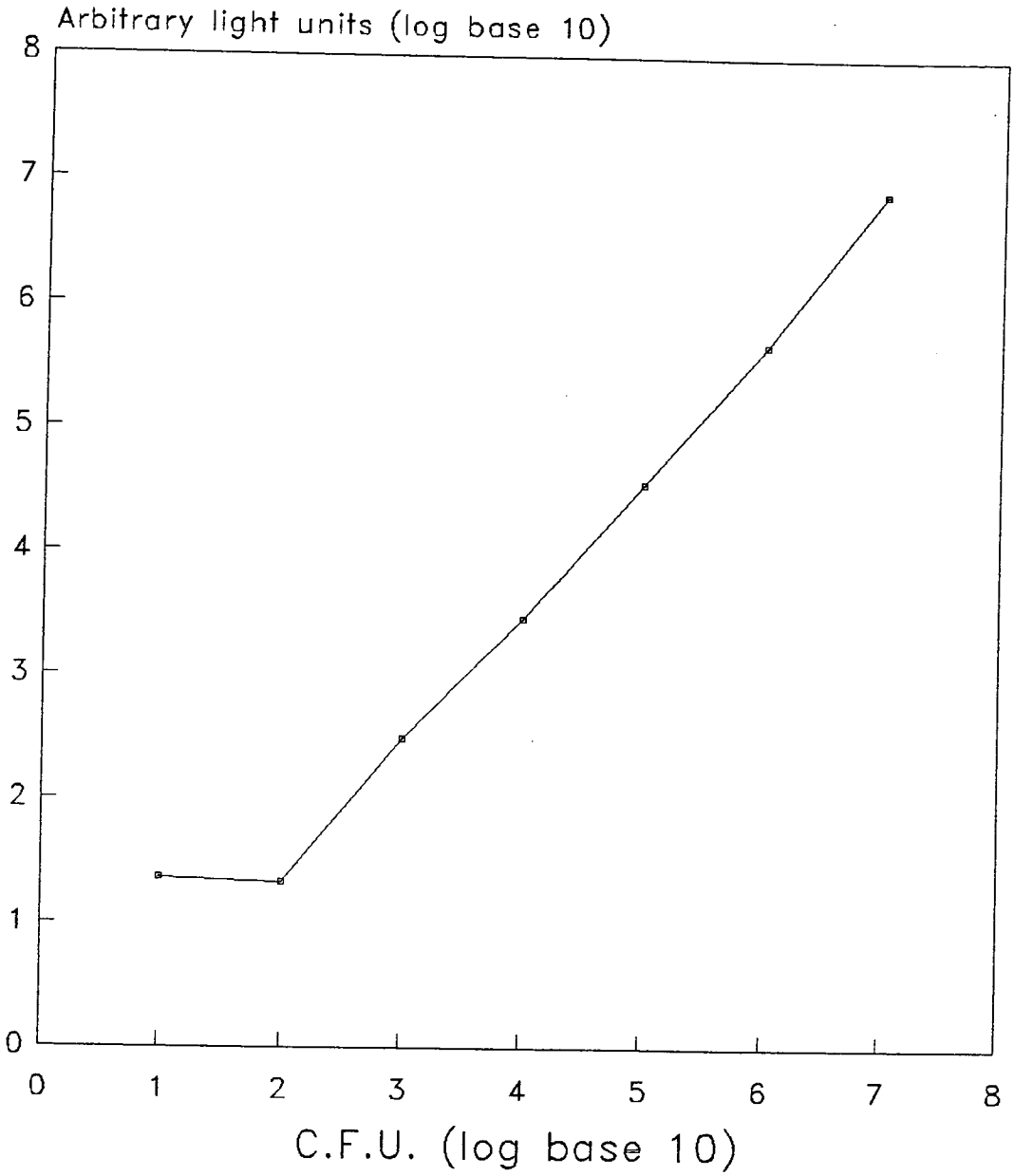
fashion at hydrogen peroxide concentrations of 1 mM and 100 μM (peak light emissions occurring approximately 80 minutes and 50 minutes after the addition of this oxidant, respectively), but at the higher concentration of H_2O_2, the peak of the light response was delayed by approximately 30 minutes compared to that induced by 100 μM H_2O_2. A delay was also seen in cultures at 10^7 bacteria ml^{-1} (panel B) when treated with 100 μM and 10 μM H_2O_2, with peak light emissions occurring approximately 60 minutes and 40 minutes after the addition of this oxidant, respectively. Exposure to 1 mM hydrogen peroxide again caused an approximate 20 minute delay in the peak of light response.

Comparison of the hydrogen peroxide light induction profiles of MPG203, at bacterial densities of 10^8 and 10^7 bacteria ml^{-1}, also demonstrated that decreasing the bacterial density by 10-fold had an extremely marked effect on the level of light produced from this fusion at high concentrations of hydrogen peroxide, with 1 mM H_2O_2 inducing approximately 1000-fold more light in cultures of 10^8 bacteria ml^{-1} than in 10^7 bacteria ml^{-1}, in the same time period. (Previous calibration of the luminometer, comparing light measurements between different densities of viable cells of the constitutive light producer MPG349, demonstrated light to be recorded in a linear fashion *ie* 10^5 bacteria ml^{-1} produced 10-fold more light than 10^4 bacteria ml^{-1} (see figure 4.4)). The poor light response produced at the lower bacterial density, upon exposure to high concentrations of H_2O_2, probably reflects a degree of cellular damage and also differences in the threshold of induction. This is also suggested by light measurements taken from hydrogen peroxide induced cultures of MPG203 at cell densities of 10^6 (panel C) and 10^5 (panel D) bacteria ml^{-1}.

Finally, it is worth noting that over the 3 hour time course of this experiment, the cell densities for each of the untreated control samples increased approximately 20-fold. This had no measurable effect on light

Figure 4.4. Calibration of luminometer.

An exponential cultures of the constitutive light emitting fusion MPG349 (prepared by diluting an overnight culture of MPG203 cells by 100-fold in LB medium and incubating at 30°C for 1 hour), was diluted from 10^7 to 10^1 c.f.u. ml^{-1} (in step of 1 log) in LB medium. 1 ml volumes of each dilution were then immediately monitored for light emission over a period of 20 minutes at room temperature using a single cell luminometer set on a sensitivity level of 6.0 mV and counting for 10 seconds. Shown are the arbitrary light units recorded from each of these samples after 15 minutes (in each case a background (noise) value of approximately 150 arbitrary light units has been subtracted).


Arbitrary light units (log base 10)
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C.F.U. (log base 10)

induction from cell densities originating at 10^6 or 10^5 bacteria ml^{-1}. However, in samples where cell densities originated at 10^8 or 10^7 bacteria ml^{-1}, a gradual increase in the level of background light was observed. This indicates that expression of the Mu*dlux* fusion in MPG203 may be affected by growth phase.

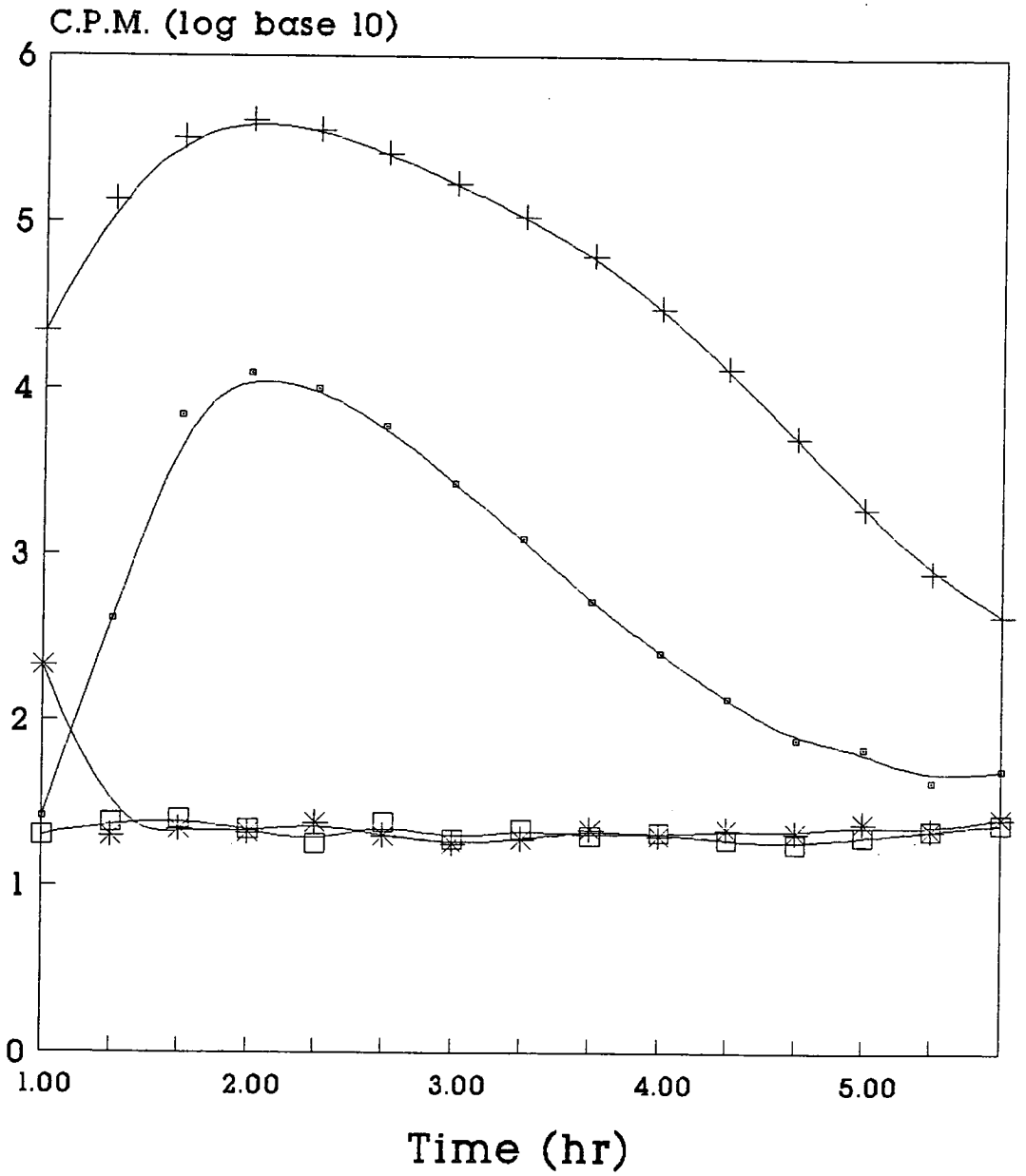
4.4 Effects of temperature and gentamicin on light production by MPG203.

In order to determine whether the production of light observed during infection of macrophages with MPG203 (see figure 4.1, panel C) was due to bacteria within or outside the macrophage cells, a series of experiments was conducted. These experiments exploited previous findings that the *Vibrio ficheri lux* system is inactivated slightly above 30°C (Friedland and Hastings, 1967; Sakharov *et al*, 1988), and also, that the aminoglycoside antibiotic gentamicin does not enter the phagosomal vacuole of macrophages and so can be used selectively to eliminate protein synthesis in extracellular bacteria, whilst bacterial protein synthesis within macrophages is unaffected (Vaudaux and Waldvogel, 1979; Lissner *et al*, 1983).

The effect of temperature on light production was examined in exponentially growing cultures of MPG203 cells. An overnight culture of this strain was diluted by 10,000-fold (*ie* 10^5 bacteria ml^{-1}) in tissue culture medium and incubating at 30°C for 1 hour. This culture was then divided into four 10 ml aliquots and two of these aliquots were incubated at either 30°C or 37°C for one hour in the presence of 100 μM H_2O_2 (the two remaining aliquots were treated as stated below). The two aliquots were then monitored at room temperature in a liquid scintillation counter. The results (see figure 4.5) indicated that although similar induction

Figure 4.5. High temperature or gentamicin inhibits light production by MPG203.

Exponential cultures of MPG203 (prepared by diluting an overnight culture of MPG203 cells by 10,000-fold in tissue culture medium and incubating for 1 hour at 30°C to give approximately 10^5 bacteria ml^{-1}), were incubated for 1 hour at 30°C or 37°C in the presence of hydrogen peroxide (100 µM) and subsequently monitored in a liquid scintillation counter (set on the tritium channe) at room temperature. The results show light production following the 1 hour incubation period. Gentamicin (100 µg ml^{-1}) was added to parallel samples after 40 minutes of incubation in the presence of hydrogen peroxide, and incubation was continued at 30°C or 37°C for a further 20 minutes, prior to analysis. Samples were treated as follows: 37°C (-•-), 30°C (-+-), 37°C plus gentamicin (-□-), 30°C plus gentamicin (-*-).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Time (hr)

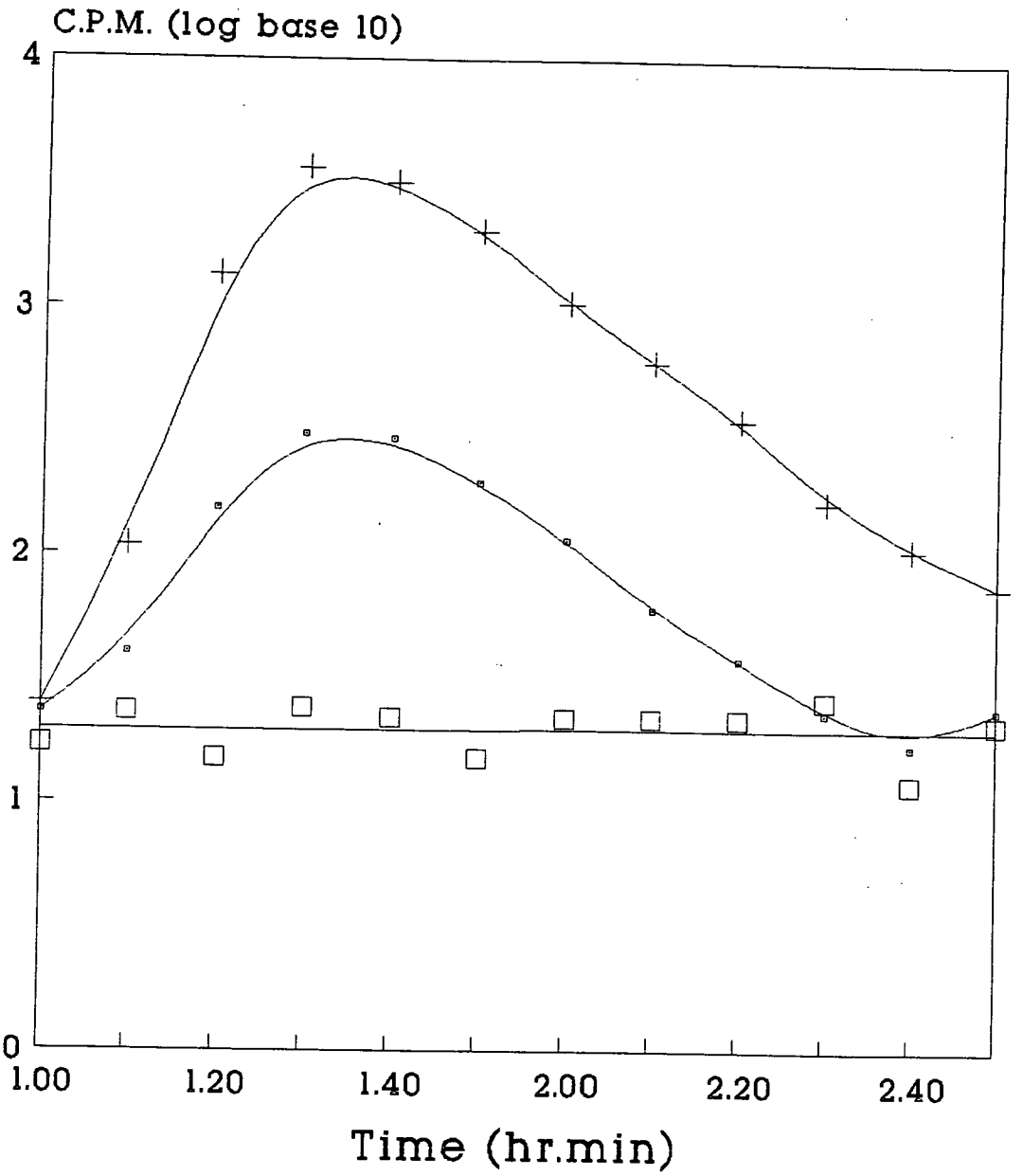
profiles were obtained at both 30°C and 37°C, light emission was significantly diminished when samples were initially incubated at 37°C as opposed to 30°C. This suggests that the light produced following initial incubation at 37°C represents translation of residual messenger RNA or renaturation of heat inactivated Lux proteins after the incubation temperature has been lowered to room temperature. When gentamicin was added to equivalent samples after 40 minutes of incubation at each temperature (in order to assess the effect of this compound on translation) and incubation was then continued for a further 20 minutes as before, light production at either temperature was observed to be abolished in response to hydrogen peroxide, even after 20 minutes of exposure to gentamicin. This indicates that *de novo* translation of Lux enzymes was necessary for light production in MPG203 cells and that bacteria in direct contact with gentamicin were unable to produce any significant light *via* this enzymatic reaction.

4.5 Establishing the source of MPG203 light emission following macrophage interaction.

Having shown in preliminary experiments that the fusion strain MPG203 resulted in light emission following interaction with cells of the mouse macrophage cell line J774.2 (see figure 4.1, panel C) and that, in theory, it was possible to eliminate light from non-endocytosed bacteria by temperature control and selective use of gentamicin (see figure 4.5), further experiments were conducted to establish the source of MPG203 light emission following macrophage interaction. Two confluent macrophage cell sheets (grown to a density of approximately 5 x 10^5 cell ml^{-1} in 250 ml tissue culture flasks containing 50 ml tissue culture medium supplemented with penicillin and streptomycin, at 37°C), were

Figure 4.6. Interaction of MPG203 with J774.2 cells results in light production.

Macrophage cell sheets (grown to a density of approximately 5 x 10^5 cell ml^{-1} in tissue culture medium containing penicillin (200 U ml^{-1}) and streptomycin (100 µg ml^{-1}) at 37°C) were gently washed in phosphate-buffered saline to remove residual antibiotic, and infected with exponentially growing cultures of MPG203 cells (prepared by diluting an overnight culture of bacterial cells by 100-fold in antibiotic-free tissue culture medium and incubating at 37°C for 1 hour) at a ratio of approximately 20:1 (bacteria-macrophages) for 40 minutes at 37°C. Cells were then washed extensively in PBS, to remove unbound bacteria. Fresh tissue culture medium, with (-•-) and without (-+-) gentamicin (100 µg ml^{-1}), was added to parallel samples and incubation was continued at 37°C for a further 20 minutes. Cell sheets were then stripped by vigrous hand agitation and monitored for light emission in a liquid scintillation counter (set on the tritium channel) at room temperature. The results show light production following the one hour incubation period. Control cultures of MPG203 were incubated for the same period of time at 37°C in the absence of macrophages. Gentamicin was then added to one of the cultures, 20 minutes before the end of the (1 hour) incubation period. MPG203 cells incubated in the absence of hydrogen peroxide and gentamicin are shown (-□-).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Time (hr.min)

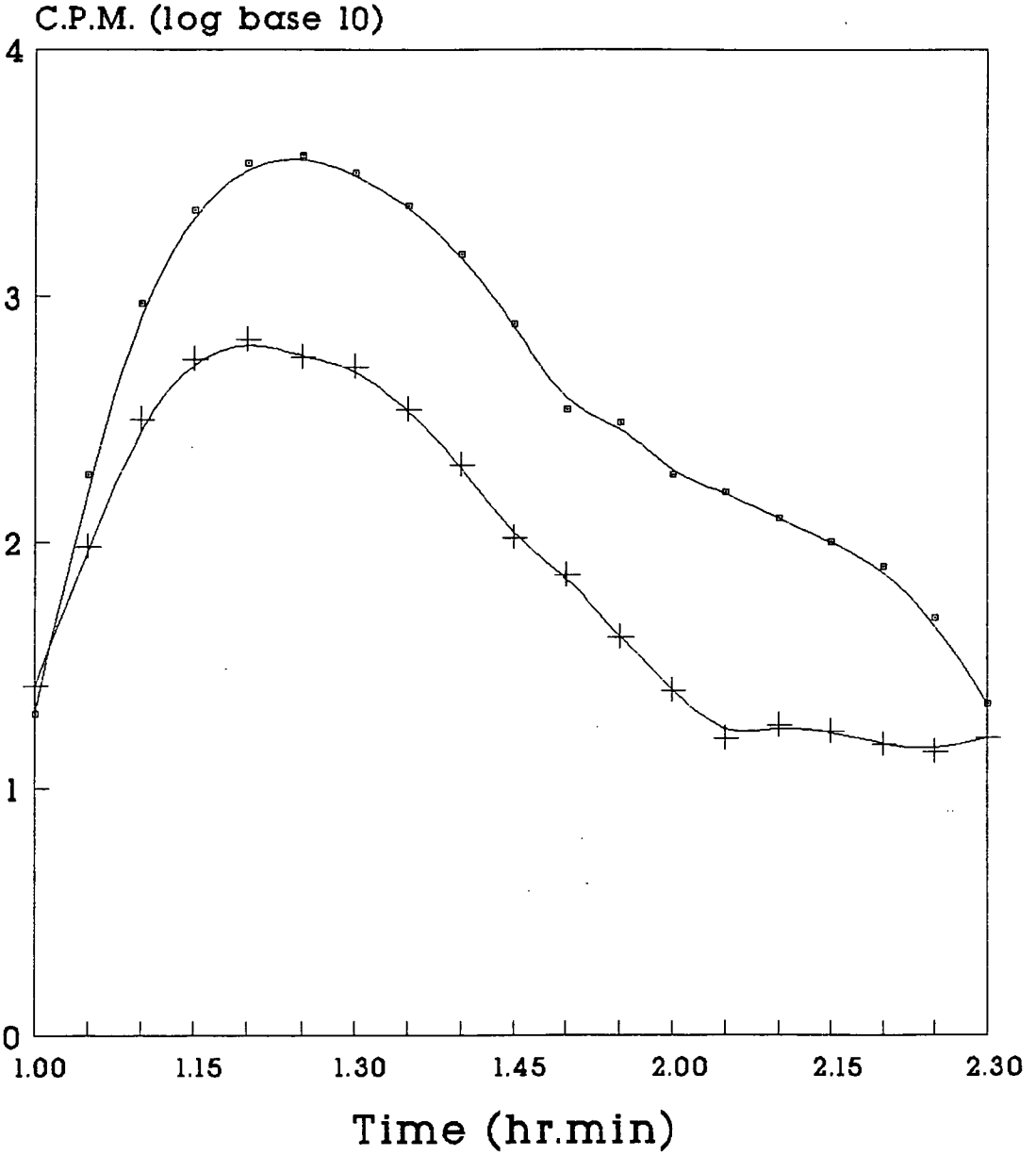
gently washed in PBS to remove residual antibiotic, and infected with exponentially growing cultures of MPG203 cells a a ratio of approximately 20:1 (bacteria-macrophages) for 40 minutes at 37°C. (The MPG203 cells had previously been prepared by diluting an overnight culture of bacterial cells by 100-fold in antibiotic-free tissue culture medium and incubating at 37°C for 1 hour) The macrophages were again gently washed in PBS to remove unbound bacteria, and each cell sheet was then overlaid with fresh tissue culture medium, with or without gentamicin, and incubation was continued at 37°C for a further 20 minutes. Both MPG203-infected macrophage cell sheets were then stripped by agitation and light production was subsequently monitored at room temperature in a liquid scintillation counter. The results are shown in figure 4.6. As before, transient light production was observed, reaching a maximum after about 80-90 minutes from the initial time of infection. However, the presence of gentamicin was found to reduce light production substantially, suggesting that a large proportion of the light detected resulted from bacteria bound to the macrophage surface. No significant light was seen from any of the control samples.

4.6 Effect of catalase on light production by macrophage-induced MPG203 cells.

In order to verify that the macrophage-induced light response of MPG203 (see figures 4.1C and 4.6) was elicited specifically by hydrogen peroxide, as opposed to any other stimulant which might be produced by these macrophage cells (Badwey and Karnovsky, 1980; Forman and Thomas, 1986), the microbe-phagocyte interaction was re-examined in the presence or absence of catalase. Parallel cultures of macrophages were infected with MPG203 cells (both macrophages and bacterial cells were

Figure 4.7. Catalase inhibits the light production which results from interaction of MPG203 with J774.2 cells.

Bacteria and macrophages were prepared as for figure 4.6. Macrophages were infected with MPG203 cells at 37°C in tissue culture flasks, in the presence or absence of catalase, and incubated for 40 minutes. Cells were then washed extensively in phosphate-buffered saline to remove unbound bacteria. Fresh tissue culture medium, with (-+-) and without (-•-) catalase (100 U per ml), was added to parallel samples and incubation was continued at 37°C for a further 20 minutes. Cell sheets were then stripped and monitored for light emission in a liquid scintillation counter (set on the tritium channel) at room temperature. The results show light production following the one hour incubation period.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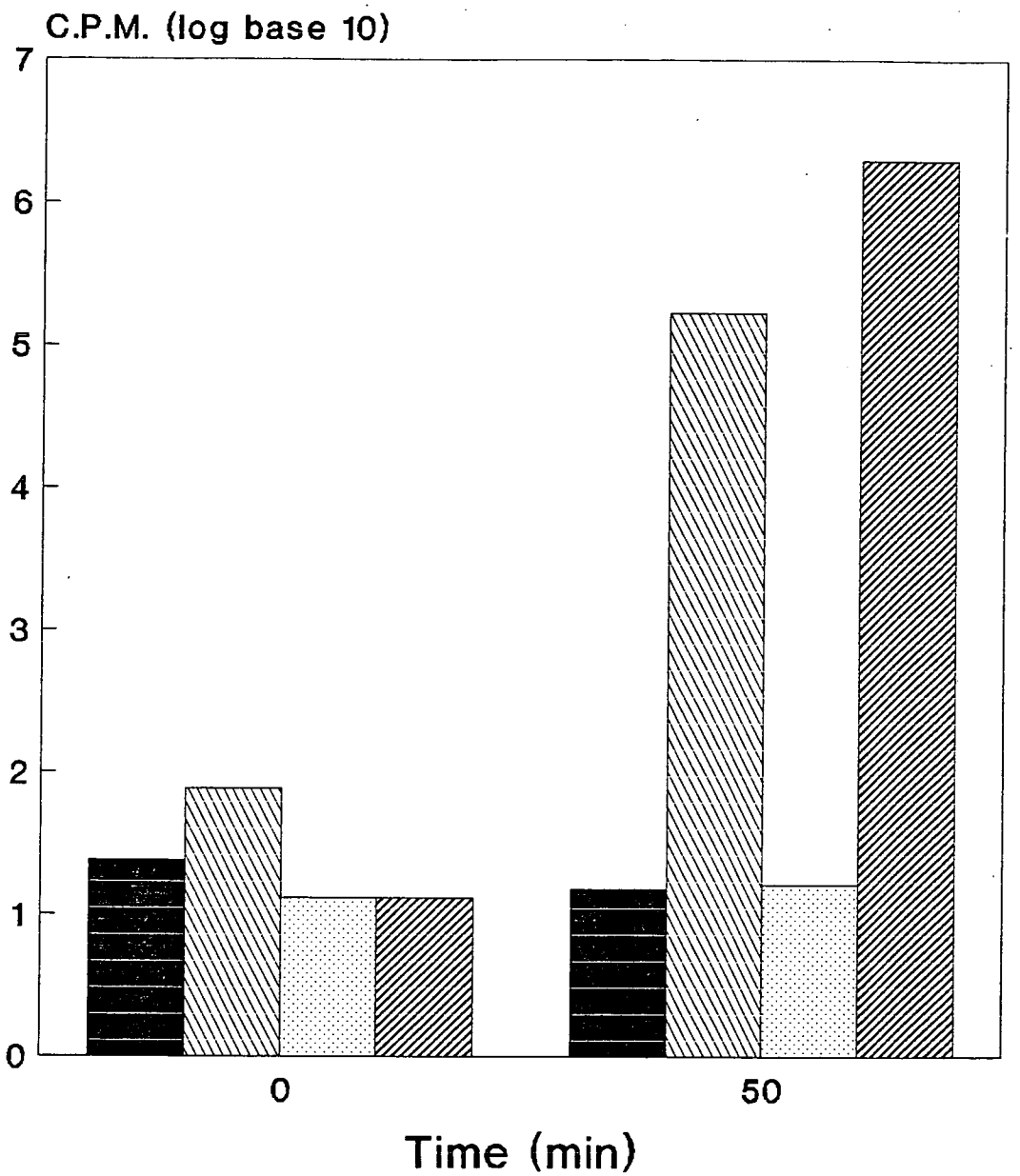
prepared as before) at a ratio of approximately 20:1 (bacteria-macrophages). Catalase was added simultaneously to one of the MPG203-infected macrophage cell sheets and then both cultures were incubated for 40 minutes at 37°C. The cell sheets were gently washed to remove unbound bacteria and fresh tissue culture medium was added, with and without catalase. Incubation was continued at 37°C for a further 20 minutes, after which cell sheets were stripped by vigorous hand agitation and light emission was subsequently recorded in a liquid scintillation counter at room temperature in the presence or absence of catalase. From figure 4.7 it can be seen that transient light production occurred, reaching a maximum at about 80-90 minutes from the time of microbe-macrophage interaction, as before. However, inclusion of catalase in the medium from the initial point of infection was observed to reduce light production to less than 20 per cent of the level recorded at peak emission. This confirmed that induction of the bacterial transcriptional reporter system occurred primarily in response to hydrogen peroxide produced by the macrophage cells.

4.7 Assessment of potential light induction by macrophage-bound MPG203 cells.

The result from section 4.5 indicated that light production by MPG203 could be monitored both extracellularly and intracellularly. However, it remained unclear whether the macrophage-bound bacteria were fully activated in response to the respiratory burst of the phagocyte and also, whether the response could be re-elicited. In order to resolve these questions, the bacterium-macrophage samples which had previously been tested for light production (see figure 4.6) were subjected to further analysis. Immediately after the macrophage infection assay described in

Figure 4.8. Macrophage-bound cells of MPG203 are capable of further light production.

Immediately after light had subsided, samples containing bacteria and macrophages from the experiment described in figure 4.6 were subjected to externally added hydrogen peroxide (100 μM) and further monitored in a liquid scintillation counter (set on the tritium channel) at room temperature. Shown are the light emissions monitored immediately after hydrogen peroxide addition (time 0) and following 50 minutes of exposure. Samples are indicated as follows: MPG203-J774.2 cells in the presence (■) or absence (▨) of gentamicin. MPG203 cells in the presence (▣) or absence (▨) of gentamicin are also shown.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section 4.5 was complete, hydrogen peroxide (100 μM) was added to each of the samples (MPG203-infected J774.2 cells in the presence or absence of gentamicin, and control samples of MPG203 in the presence or absence of gentamicin) at room temperature. The light responses were then monitored in a liquid scintillation counter at the initial time of H_2O_2 addition (time 0) and 50 minutes after this addition.

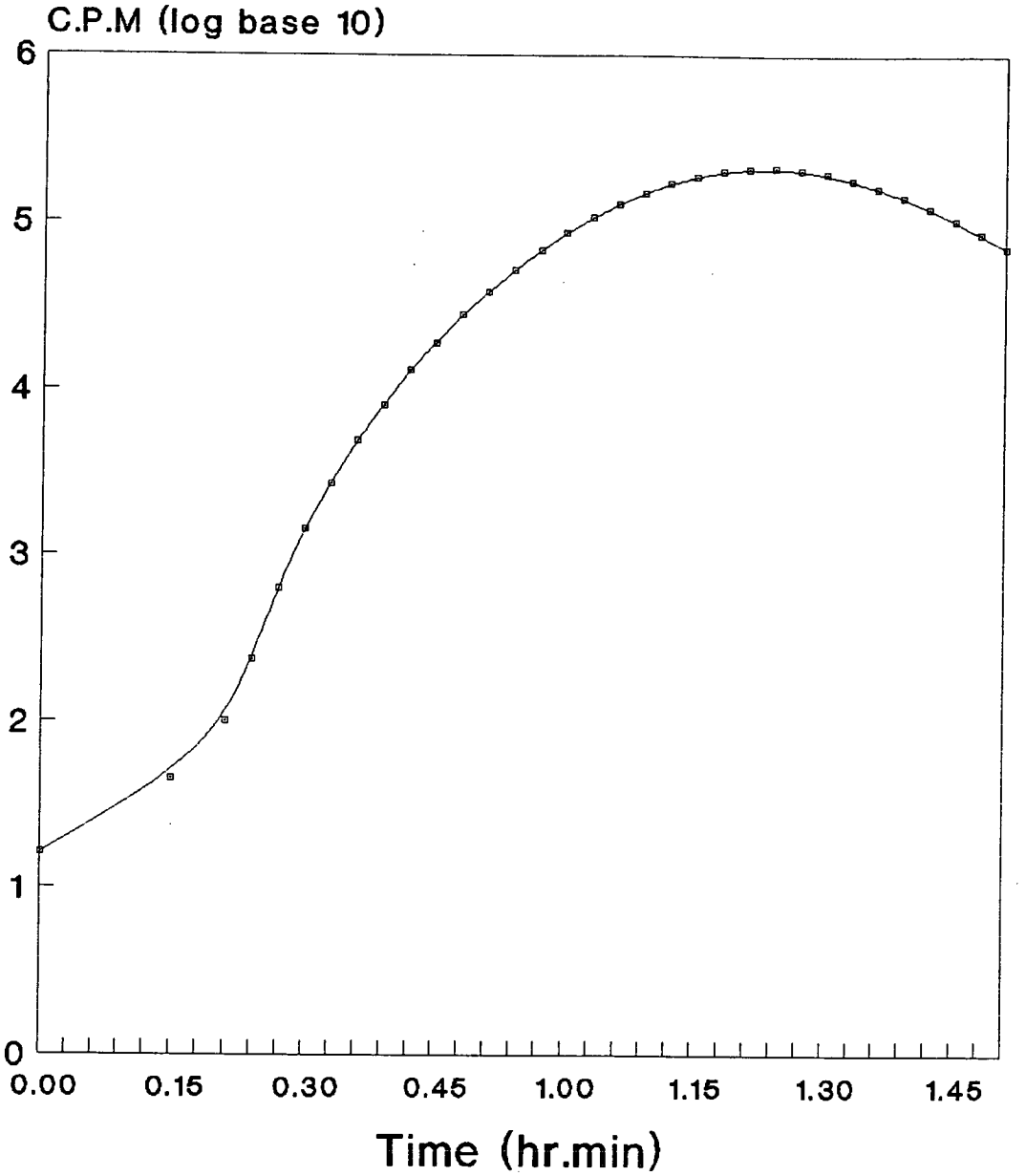
Light emissions from each of the samples at both time points are shown in figure 4.8. No significant light emission could be detected from any of the samples upon the addition of hydrogen peroxide at 0 minutes. However, after 50 minutes of exposure to this oxidant, a large burst of light production was observed from both the samples which lacked gentamicin, whereas, there was no further light response seen from either of the samples which contained gentamicin. This new light response from the MPG203-infected macrophage sample greatly surpassed that previously seen during the initial bacterial-macrophage interaction (see figure 4.6). This suggests that macrophage interaction had not elicited a full response from the bound bacteria. This finding is in agreement with the data from figure 4.1C, where bacteria which were stimulated with hydrogen peroxide directly produced a greater response than that observed when an equivalent number of bacteria were allowed to interact with macrophage cells.

4.8 Assessment of potential light induction by intracellular MPG203 cells.

The question of whether intracellular light production by phagocytosed bacteria (in the presence of extracellular gentamicin) was a true representation of the level of gene induction or whether some form of suppression had occurred as a result of the adverse phagosomal environment (*eg* excessive oxidant, low pH, bactericidal peptides (Forman

Figure 4.9. MPG203 cells from lysed macrophages are capable of substantial light production.

Macrophage cells, containing MPG203, which had been maintained in tissue culture medium, in the presence of gentamicin (approximately 4 hours from the initial time of bacterial infection (continued from figure 4.6)), were washed repeatedly by gentle centrifugation (600g for 5 minutes in a bench-top MSE Centaur 1 centifuge), and then disrupted by more rigorous centrifugation (3,000g for 10 minutes). Pellets were resuspended in fresh tissue culture medium (without gentamicin) and hydrogen peroxide (100 μM) was added. Light emission was immediately recorded in a liquid scintillation counter (set on the tritium channel) at room temperature. Samples of MPG203 which had also been maintained in gentamicin were treated similarly but no light was recorded above background.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and Thomas, 1986; Foster, 1992; Hiemstra *et al*, 1993), or low O_2 levels, factors which might result in the disruption of the bacterium's normal cellular metabolism or bioluminescent capacity), remained unanswered. The macrophage cells which had been continuously incubated (approximately 4 hours) in medium containing gentamicin (see figures 4.6 and 4.8) were therefore gently centrifuged (600 x g, 10 min.) and washed to remove the residual antibiotic, and subsequently lysed by more rigourous centrifugation (3,000 x g, 10 min.). Hydrogen peroxide (100 μM) was then added, and light production was recorded by liquid scintillation counting at room temperature. This procedure resulted in substantial bioluminescence (see figure 4.9), indicating that the majority of the bacteria had remained viable within the macrophages, although they had produced only low levels of light when phagocytosed (see figure 4.6). (This also confirmed that addition of hydrogen peroxide to the macrophages had not resulted in extensive cell lysis (see figure 4.8)).

Samples of the lysed macrophage solution, taken prior to re-activation by hydrogen peroxide, were plated to estimate viable bacteria. Approximately 10^6 bacteria were recovered from the total supernatant, indicating that the bacteria to macrophage ratio was approximately 0.1 at this point. This confirmed that the low level of light production in the presence of gentamicin was not solely a result of inefficient endocytosis of MPG203 but that the bioluminescent response of the bacteria was indeed markedly reduced within the phagosomal environment.

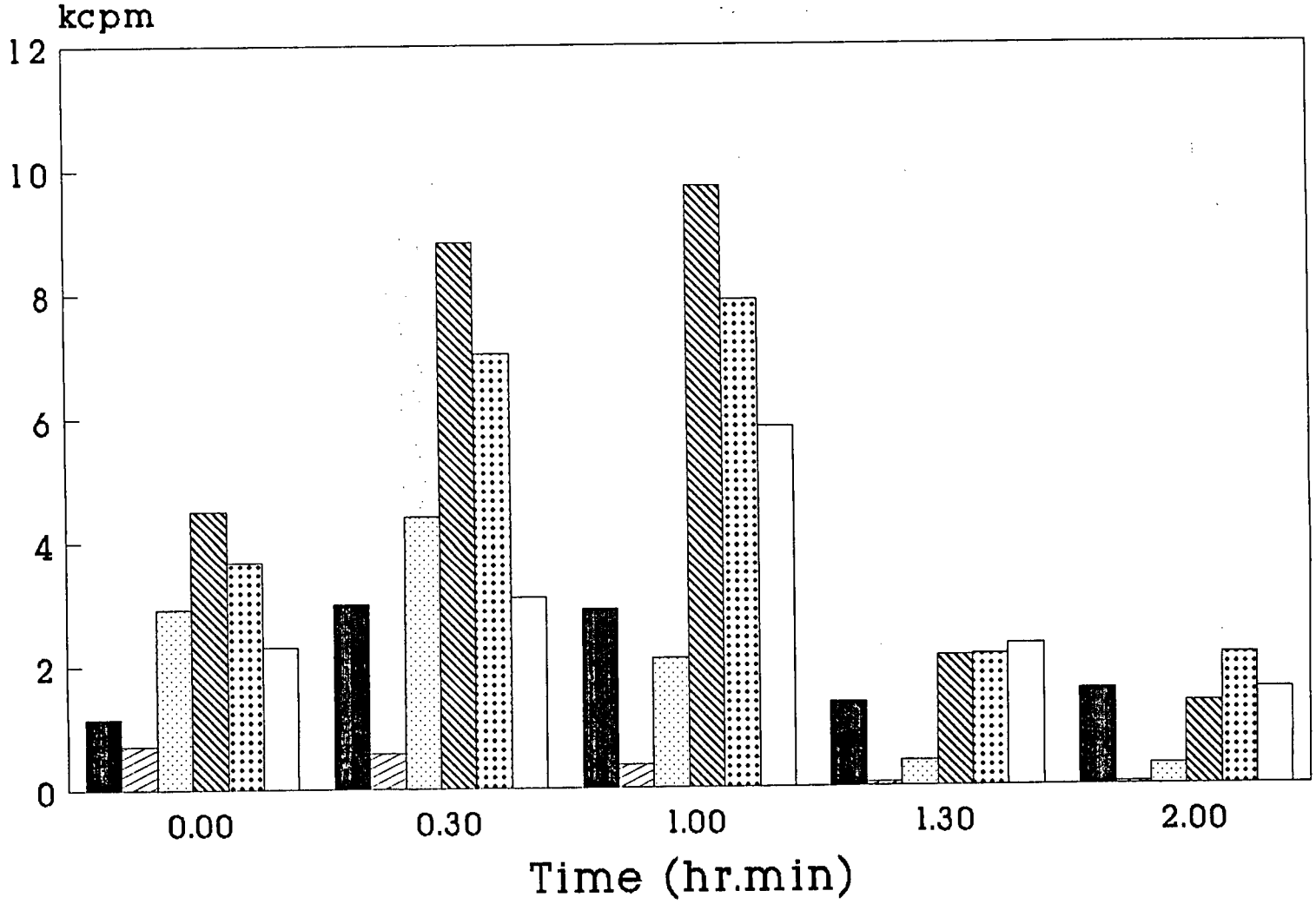
4.9 Effects of MPG203 cells upon the respiratory burst of macrophages.

Previous studies by Donowitz and colleagues (1990) reported that endocytosis of intracellular pathogens, such as *Legionella micdadei*, may result in abolition of the phagocytic respiratory burst following phorbol

myristate acetate (PMA) stimulation, in a manner which was dependent on the bacteria-macrophage ratio and the duration of incubation (*ie* period of infection). Such a situation might provide an explanation for the low level of intracellular light observed following phagocytosis of MPG203 cells (see figure 4.6). In order to investigate this possibility, J774.2 cells (approximately 5 x 10^4 ml^{-1}) were infected with SL1344 cells at different ratios at 37°C. (Bacterial cells, grown to late exponential/early stationary phase, were freshly prepared in antibiotic free tissue culture medium, at 37°C) At selected time intervals, the macrophages were then stimulated with PMA at a concentration of 500 ng ml^{-1} and the peak activity of the respiratory burst for each sample was determined with luminol by chemiluminescence in a liquid scintillation counter at room temperature. As can be seen from figure 4.10, a decline in the respiratory burst was observed, relative to the PMA response, over a two hour time period. This was particularly evident at very high infection ratios. However, substantial activity was still recorded over this time interval at the bacterium:macrophage ratios which had been used for bioluminescent studies (see figure 4.6). (No light was detected from any sample in the absence of luminol.) Since the peak light response from MPG203 occurred approximately 80-90 minutes after initial interaction with the macrophages, it seems unlikely that the low level of intracellular light observed in figure 4.6 can be fully explained by inhibition or abolition of the respiratory burst, following endocytosis. This suggests that some other factor(s) must be responsible, at least in part, for the low level of bioluminescence observed following phagocytosis.

Figure 4.10. Infection of J774.2 cells with SL1344 may reduce the respiratory burst.

J774.2 cells, grown in tissue culture medium containing penicillin (200 U ml^{-1}) and streptomycin (100 µg ml^{-1}), were washed extensively in phosphate-buffered saline to remove residual antibiotic, gently pelleted at 600 g for 5 minutes in a bench-top MSE Centaur 1 centifuge, and then resuspended in antibiotic-free tissue culture medium at an approximate density of 5 x 10^4 cells ml^{-1}. Freshly grown cells of SL1344 (grown to stationary phase in antibiotic-free tissue culture medium at 37°C) were mixed with the macrophages at approximately 10,000:1 (▨), 1,000:1 (▩), 100:1 (▧), 10:1 (▪) and 1:1 (□), respectively, and incubated at 37°C. At appropriate intervals, PMA and luminol were added and light production was recorded in a liquid scintillation counter at room temperature. The values shown represent peak light emission. The influence of PMA on light production by J774.2 in the absence of SL1344 is also shown over the same time period (■). No light was detected from any samples in the absence of luminol.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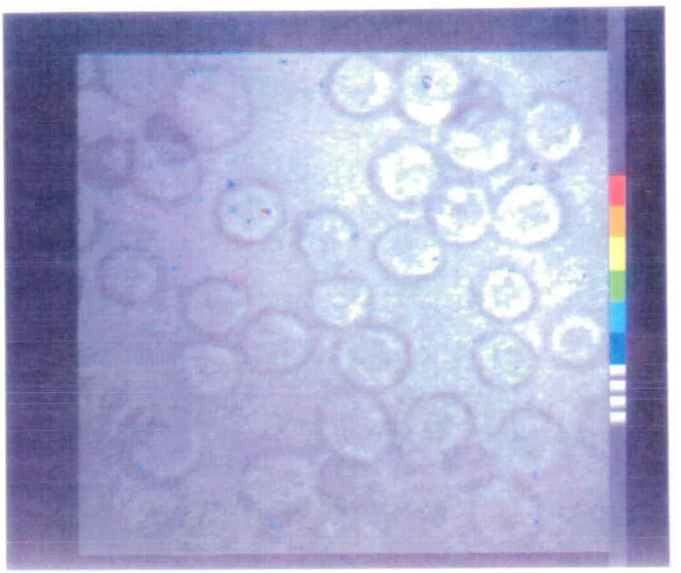
4.10 Visualisation of MPG203 interaction with J774.2 cells.

The above studies (see figures 4.1C and 4.6) revealed that the strain MPG203 responded to J774.2 cells with light production. However, the level of endocytosis observed suggested that all the cells of the macrophage population might not be behaving uniformly. To resolve this dilemma a procedure (see chapter 2 for fuller details) similar to that initially used to record light from MPG203 infections of macrophages on cavity slides (see section 4.2), was conducted to establish whether light production from internalised bacteria could be visualised.

As before, slides with confluent sheets of bound macrophage cells (prepared as in section 4.2), each at a density of approximately 10^5 cells ml^{-1}, were infected with an exponentially growing cultures of MPG203 (prepared by diluting an overnight culture of bacterial cells by 100-fold in antibiotic free tissue culture medium and incubating at 37°C for 1 hour to give approximately 10^7 bacteria ml^{-1}) at a ratio of approximately 100:1 (bacteria: macrophages). After 40 minutes of incubation at 37°C, unbound bacteria were removed by gentle washing in PBS, gentamicin was added in fresh tissue culture medium and incubation was continued for a further 20 minutes at 37°C. Following this, samples were examined on a Zeiss Axioskop microscope with a photon video camera attached. As observed previously (see figure 4.1C), a transient peak of light was observed after approximately 20 minutes of observation at room temperature. However, due to the presence of gentamicin, the overall light emission from this cell sheet was considerably lower than that seen in figure 4.1C. From figure 4.11, it can be seen that the intensity of light recorded from individual macrophages varied considerably and suggests that the number of bacteria endocytosed per macrophage or the level of induction of the respiratory burst may fluctuate significantly between individual cells or macrophage

Figure 4.11. Visualisation of light production by MPG203, internalized within J774.2 cells.

Macrophages cells, at a density of approximately 10^6 cells ml^{-1}, were coated on cavity slides overnight in tissue culture medium containing penicillin (200 U ml^{-1}) and streptomycin (100 µg ml^{-1}), in a 5% CO_2 incubator at 37°C. Unbound macrophages and residual antibiotic were then removed by gently washing each slide several times in phosphate-buffered saline, to leave a confluent layer of bound macrophages at a density of approximately 10^5 cells ml^{-1}. Each slide was infected with exponential MPG203 cells (prepared by diluting an overnight culture of bacterial cells by 100-fold in antibiotic-free tissue culture medium and incubating at 37°C for 1 hour to give approximately 10^7 bacteria ml^{-1}) at a ratio of approximately 100:1 (bacteria: macrophages) at 37°C for 40 minutes. Slides were then washed several times in PBS to remove unattached bacteria from the macrophage cell sheet, covered with fresh tissue culture medium containing gentamicin (100 µg ml^{-1}), and re-incubated at 37°C for a further 20 minutes. Each of the slides was removed intermittently from the incubator and examined for light emission at room temperature, using a Zeiss Axioskop microscope (x40 magnification) with a photon video camera attached (set on photon counting mode). Foci of light emission are indicated in colour and have been superimposed onto the MPG203-J774.2 infected cell sheet (same field of view with external illumination) using computer software (Hamamatsu). Intensity of light emission (bioluminescence) is indicated by the scale bar (red represents peak intensity, *ie* the areas within the camera's field of view that had the highest number of photon strikes over the 5 minute period).



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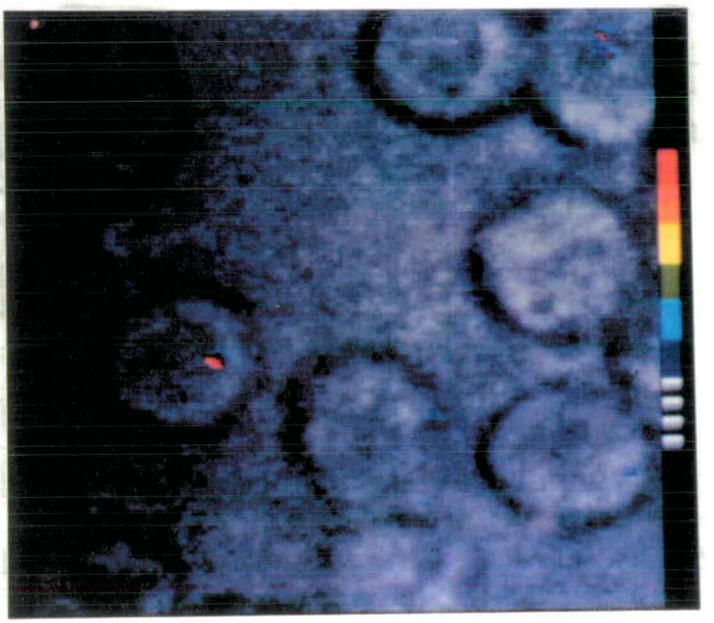


B

subpopulations. This is in accordance with reports from other phagocytic studies (Gentle and Thompson, 1990).

4.11 Discussion.

The ability to survive within macrophages is fundamental to the pathology of the enteroinvasive forms of salmonellae. However, the mechanisms by which these bacteria fully achieve this remains largely unknown due to the complexities of studying such interactions. As discussed in Chapter 1, extensive analysis of *S. typhimurium* mutants bearing transposon insertions, has led to the identification of a number of loci which are required for virulence and survival within phagocytic cells (Fields *et al*, 1986; Fields *et al*, 1989; Miller *et al*, 1989a; Gahring *et al*, 1990). Nevertheless, despite the immense value of the latter approach, direct characterisation of the response of the genes which affect virulence was not possible during macrophage interaction because of the nature of the genetic tag. Other studies, such as those by Buchmeier and Heffron (1990), have circumvented this problem by selectively labelling *de novo* synthesized proteins of endocytosed bacteria with radioactive amino acids in the presence of inhibitors of eukaryotic cell translation. However, as discussed in section 4.1, this procedure again could only provide a narrow range of information on the majority of the bacterial proteins induced during phagocytosis due to the limitations of the analytical techniques involved.

Using light as a means of reporting bacterial gene expression from within phagocytic cells has several advantages over both of the above procedures, with the main benefits being that light-based reporter systems are non-destructive and allow transient gene expression to be recorded continuously from bacterial cells (Carmi *et al*, 1987; Stewart *et al*, 1989).

Moreover, the absence of a requirement for inhibitors of eukaryotic translation results in minimal perturbation to the function of the phagocyte. Hence, the use of a light-based reporter system to tag genetic loci of *S. typhimurium* during this study, allowed for previously isolated fusions (found to be induced by analogous stresses to those found in activated macrophages) to be accurately characterized during the actual infection of the phagocytic cells, something that was not possible by either of the aforementioned methods.

Of the numerous stress-induced fusions isolated in chapter 3, only 5 fusions were chosen to test as potential candidates for reporting light upon interaction with macrophages. These fusions were selected because of their relatively high levels of stress-induced light and negligible levels of background light in liquid culture (see tables 3.1 and 3.2). In spite of this careful screening procedure however, only one of these five fusions, MPG203, was found to produce substantial light during infection of the macrophage cells under the conditions examined. Nevertheless, careful analysis of this hydrogen peroxide-inducible fusion clearly demonstrates the merits of this system, and provides direct genetic evidence for the involvement of a hydrogen peroxide-induced response in *S. typhimurium* following macrophage interaction. (A more extensive pilot study might also be productive for the other four remaining fusions.)

In vitro studies of MPG203 cells (*ie* bacterial studies not involving phagocytes) indicated that both the concentration of hydrogen peroxide and the bacterial density are influencing factors in the induction of a hydrogen peroxide-elicited bioluminescent response in *S. typhimurium* and that the cells exhibit a transient reaction to low or intermediate levels of the stimulus, as evidenced by light emissions (see figures 4.2 and 4.3). However, high levels of the toxic metabolite appear to result in suppression of light production *in vitro*, although higher bacterial densities

appear to exhibit a greater tolerance. The reason for this enhanced protection from hydrogen peroxide which occurs at higher bacterial numbers is not clear. Previous work by Smibert and colleagues (1978) has shown that the microaerophilic organism *Campylobacter jejuni* also tolerates higher levels of oxygen metabolites at high cell density. In addition, cultures of *E. coli* in the late exponential phase of growth have been reported to be more resistant to hydrogen peroxide (Jenkins *et al*, 1988; Ma and Eaton, 1992), although in this case, synthesis of a protective catalase is known to occur during this phase of growth (Loewen *et al*, 1985; Schellhorn and Hassan, 1988b).

Light was seen at high cell densities of MPG203 independent of hydrogen peroxide addition (see figures 4.3A and 4.3B), but this may simply reflect combined basal gene activity from a large number of cells. An alternative hypothesis, is that this fusion is induced during late exponential growth in a similar fashion to the protective catalase discussed above. Work by Schellhorn and Stones (1992), has shown that a significant increase in acid fermentation and the production of acetate occurs as *E. coli* enters stationary phase, and that weak acids such as acetate are able to induce the hydrogen peroxide inducible genes *katE* (encoding HPII catalase) and *katF* (encoding a sigma factor required for *katE* expression), as well as the *E. coli* hydrogen peroxide inducible catalase, HPI (encoded by *katG* which constitutes part of the OxyR regulon).

The precise mechanism by which *katE* and *katF* are induced during the stationary phase is not understood. However, acetate utilization is known to result in increased respiratory activity and, hence, causes an increase in the intracellular flux of reactive oxygen intermediates, including hydrogen peroxide (Schellhorn and Stone, 1992). It seems likely, therefore, that the induction of hydrogen peroxide-inducible genes

during the early stationary phase of growth in *E. coli*, could result from these cells producing acetate, so leading to the generation of reactive oxygen species. Similarly, this might also explain why the hydrogen peroxide-inducible fusion MPG203 was induced as a culture of this strain approached its stationary phase. (It is possible that this process might operate as part of a global genetic system which is specifically activated during the bacterium's entry into the stationary phase of growth.)

Further to the above hypothesis, it is also possible that the low levels of background light that were seen at high cell densities of MPG203 might alternatively be explained by a mechanism similar to that described by VanBogelen and colleagues (1987). This latter group have demonstrated that depletion of certain amino acids in cultures of *E. coli* cells (such as during starvation *ie* the stringent response), caused an increase in the accumulation of particular nucleotides (*eg* AppppA and ppGpp) within the cell. This same group also showed that these nucleotides, also called alarmones, were rapidly induced by hydrogen peroxide or the compound 6-amino-7-chloro-5,8-dioxoquinoline (ACDQ), which in turn have both been shown to induce the synthesis of *oxyR*-regulated proteins (Christman *et al*, 1985; Lee *et al*, 1983; VanBogelen *et al*, 1987). Consequently, an *oxyR*-regulated Mu*dlux* gene fusion might be expected to respond to high cell densities in a similar manner to MPG203 due to an increase in one or more of these nucleotides within the cell. Other interpretations are also possible however.

A large number of genetic mutations which result in attenuated virulence of *S. typhimurium in vivo* (*ie* within the host) have now been reported (Chatfield *et al*, 1991; Collins *et al*, 1991; Curtiss and Kelly, 1987; Hoiseth and Stocker, 1981; Johnson *et al*, 1991; Miller *et al*, 1989b). However, few examples have been directly correlated with a role

in macrophage interaction. The *pho* regulon is an exception to this (see chapter 1) and acts by mediating resistance to antibacterial polypeptides which are produced within the phagolysosome (Fields *et al*, 1989). The present study provides direct evidence that the multigenic, hydrogen peroxide-induced response also belongs in this category and plays a role in the primary events following bacterium-macrophage interaction.

Light production from MPG203 appears to follow adhesion of a proportion of the *S. typhimurium* cells to the macrophages, presumably indicating a response to the toxic oxygen metabolites produced at the phagocyte cell surface (see figures 4.1C and 4.6). The substantial reduction in luminescence which occurred in the presence of catalase (see figure 4.7) provides strong evidence that macrophage-produced hydrogen peroxide is the major stimulating agent in this interaction, lending further support to the *in vitro* phenotype of the gene fusion (see figures 4.1B and 4.3). The observation of bioluminescence from endocytosed bacteria (see figures 4.6 and 4.11) is consistent with other reports that the effects of the respiratory burst continue within the phagosome (Hassett and Cohen, 1989) and supports the view that the endocytic vacuole is an oxygenated environment. The fact that light can be detected within the endosomal environment indicates that the level of damage to the biochemical functions of the bacteria at this point is insufficient to abolish the supply of substrates which are required for the *lux* reporter system, namely oxygen, reduced flavin mononucleotide and aldehyde (Ziegler and Baldwin, 1981).

The reason for poor light production, following bacterial endocytosis (see figure 4.6) remains unclear. One possible explanation is that ingestion of *S. typhimurium* by macrophages might result in abolition or diminution of the respiratory burst, thus limiting the stimulus (hydrogen peroxide) for induction of the gene fusion within the host cell. Such a

situation has been reported with other intracellular pathogens, such as *Legionella micdadei* (Donowitz *et al*, 1990), in which it has been shown that the respiratory burst may be completely abolished over a period of 90 to 120 minutes, at infection ratios of 30:1 and 10:1 (bacteria:phagocytes), respectively. The results of our study indicate that a decline in the respiratory burst activity relative to the PMA-induced response does occur following ingestion of *S. typhimurium* at very high infection ratios (see figure 4.10). However, at the ratios of bacteria to macrophages which were used for the bioluminescence studies (see figures 4.6 and 4.11), the relative reduction in respiratory burst activity was found to be fairly moderate, and it would seem unlikely that this explanation alone could adequately account for the low intracellular bioluminescence which was observed.

An alternative view may be that the low level of intracellular light observed may reflect the influence of the harsh phagosomal environment on the bacterial cell. *In vitro* studies (see figure 4.2 and 4.3) have shown that millimolar concentrations of hydrogen peroxide can suppress bioluminescence at densities of 10^7 cells ml^{-1} or less, and such concentrations have been suggested to occur within phagolysosomal environments (Hassett and Cohen, 1989). Equally, Foster and Hall (1991) have shown that if *S. typhimurium* cells fail to adapt to the levels of acidity which can be found within macrophages, damage to intracellular enzymes may ensue. Genetic repression in *S. typhimurium* has also been reported to occur within macrophages, and Buchmeier and Heffron (1990) have shown that a large number of bacterial proteins (approximately 34%) exhibit a substantial reduction in synthesis, following phagocytosis. The possibility therefore remains that a combination of these and/or other factors may be responsible for the low level of intracellular light recorded.

The level of light recorded from surface-bound bacteria also appears submaximal (see figure 4.6), since subsequent addition of hydrogen peroxide results in almost a hundredfold increase in the detectable light response (see figure 4.8). Similarly, when an equal number of MPG203 were stimulated by hydrogen peroxide directly or by interaction with macrophages, substantially more light was produced by direct hydrogen peroxide induction (see figure 4.1B and 4.1C). The reason for such restricted light production from the MPG203 cells following macrophages interaction is not apparent, but may simply reflect a limited secretion of hydrogen peroxide at the macrophage cell surface. Alternatively, this sub-maximal light response may reflect variations in the levels of the respiratory burst produced by individual or sub-populations of macrophages. Further visualisation of light emission with the photon counting camera (see figure 4.11), has also suggested that substantial differences can occur in the levels of light produced from endocytosed bacteria within individual phagocytic cells and further suggests that the response of the macrophage population to the *S. typhimurium* cells is not entirely uniform. Significant differences may also exist in the phagocytic activity of individual macrophage cells and this might be expected to influence the level of the respiratory burst.

Further characterisation of the genetic locus disrupted in MPG203 is discussed in chapter 5. However, it has not yet been established whether this mutant is attenuated in mice, therefore, its potential for use as an attenuated vaccine strain is not yet known. Previous studies have shown that attenuated strains of *S. typhi* may be good candidates for live oral vaccines, and clinical trials show promise (Forrest *et al*, 1992; Tacket *et al*, 1992). Attenuated strains of other *Salmonella* species also hold potential for vaccination of farm animals (Johnston, 1991). The future design of live oral vaccines will require the rational attenuation of virulent

organisms with defined gene inactivation. The ability to identify bacterial genes which are involved in discrete stages of the infection process is an important aspect of such an approach. In this respect, use of a light-based reporter system may not only provide important insights into the infection process of *S. typhimurium* but may also reveal important clues about the stresses which are encountered by internalised pathogens within the phagocytic environment. Such stresses clearly must be surmountable by the bacteria in order to give rise to disease.

CHAPTER 5

Chapter 5: Identification of the Mu*dlux* target site in the hydrogen peroxide- and macrophage-inducible *S. typhimurium* strain MPG203.

5.1 Introduction.

The precise roles played by hydrogen peroxide-inducible proteins in the pathology of *S. typhimurium* infections in mice are not fully understood. However, several loci of this bacterium which are induced by hydrogen peroxide *in vitro* are known to be required for full virulence *in vivo*, these include the *htrA* locus (Johnson *et al*, 1991), encoding a homologue to the *E. coli* heat shock protein HtrA (Lipinska *et al*, 1988), a locus which encodes an outer membrane protein of unknown function (Stinavage *et al*, 1990), and the *recA* and *recBC* loci (Buchmeier *et al*, 1993), which encode proteins known to be essential for the repair of DNA damage (Walker, 1984). Furthermore, recent studies have reported that the H_2O_2-induced catalase of *S. typhimurium* plays a role in eliciting immunological protection against this bacterium in mice (Kagaya *et al*, 1992), indicating that some level of the enzyme must be present during the infection process.

The transient expression of light by MPG203 in response to hydrogen peroxide (see figures 3.1, 4.1, 4.2 and 4.3) and also upon infection of macrophages (see figures 4.1, 4.6 and 4.11), suggests that this bacterium rapidly induces self-protective functions in response to oxidative stress and that once the influence of the stimulus has subsided, expression of the gene fusion is again reduced. Unfortunately, this response is typical of that seen for the majority of the 30 proteins induced by hydrogen peroxide in *S. typhimurium* (Christman *et al*, 1985; Morgan *et al*, 1986) and provides no further clue to the identity of the target gene containing the Mu*dlux* insertion. The next logical step towards the

identification of the target locus therefore, seemed to be the establishment of what other factors affected the regulation of this gene fusion, and then comparing the overall profile with those reported for hydrogen peroxide regulated genes in other studies (for a review see Farr and Kogoma, 1991).

5.2 Characterization of the Mu*dlux* gene fusion transduced from MPG203 into TA4100 and TA4108 backgrounds.

Previous work by Christman and colleagues (1985) has demonstrated that the *oxyR* locus is responsible for regulating approximately 9 of the 30 or so hydrogen peroxide-inducible proteins of *S. typhimurium*. As discussed in Chapter 1, this was demonstrated by the isolation of an *S. typhimurium* mutant, TA4100 (*oxyR1*), in which the expression of these nine proteins was constitutively elevated. This group also used a Tn*10* mutagenesis and excision system to create an *S. typhimurium* mutant deleted for the *oxyR* locus, TA4108 (*oxyΔ2*), where the hydrogen peroxide inducibility of the nine above mentioned OxyR-regulated proteins was abolished.

In order to establish whether the gene fusion in MPG203 was *oxyR*-regulated, the Mu*dlux* element was first moved into the strains TA4100 and TA4108 by phage P22 transduction. A number of each of the transductants were then colony purified. Cultures of these were then grown in duplicate overnight on LB plates at 30°C, and one of these plates was overlaid with filter paper soaked in 90 mM H_2O_2 (the remaining plate was left as a control). Each of the plates was then examined for light production by eye over a period of approximately 2 hours.

None of the TA4108 transductants produced any visible light either in the presence or absence of hydrogen peroxide, and indicated that this fusion was probably *oxyR* regulated. However, this conclusion was not immediately obvious when the fusion was observed in an *oxyR1* background. Comparison of the TA4100 transductants in the presence and absence of the latter oxidant demonstrated that these transductants produced only basal levels of light (barely visible) without exposure to hydrogen peroxide, but substantial amounts of light in the presence of hydrogen peroxide (light emission was maximal after approximately 40 minutes of induction). A possible explanation of why the TA4100 transductants only produced low level of light in the absence of hydrogen peroxide lies in the previous findings of Christman and colleagues (1985). This group have reported that the relative abundance of some of the *oxyR* regulated proteins was lower in unstimulated TA4100 cells than in hydrogen peroxide-treated LT2. This implies that the *oxyR1* mutation alone might not fully induce all of the *oxyR* regulated proteins (see discussion).

To ensure that the transduced Mu*dlux* fusions in TA4100 and TA4108 were still in the same gene as that found in MPG203, one of each of these transductants (respectively designated as MPG351 and MPG352) was colony purified and P22 lysates were prepared on each and then used to transduce their respective gene fusions into clean SL1344 backgrounds. Each of the resulting transductants was tested for an MPG203-like phenotype and confirmed as being correct. It seemed very likely therefore that the gene fusion in MPG203 was *oxyR* regulated.

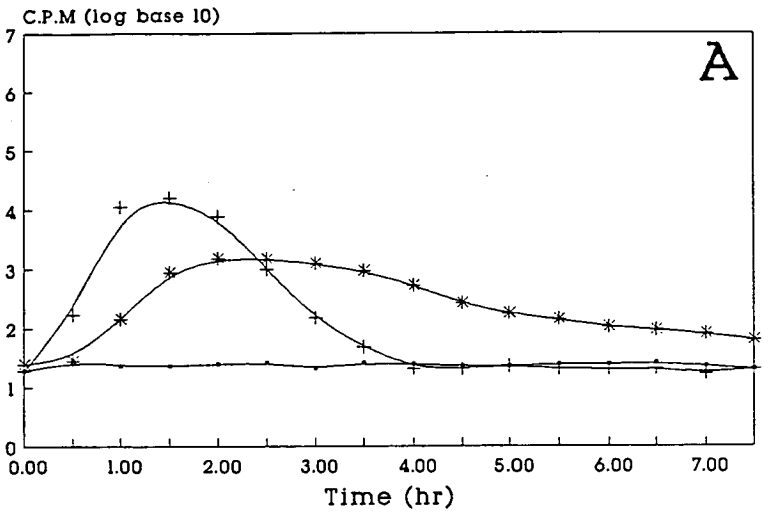
Both MPG351 and MPG352 were further characterised as previously described for MPG203. Essentially, exponentially growing cultures of MPG203, MPG351 and MPG352 (prepared by diluting overnight cultures of each strain by 10,000-fold in LB medium and

incubating for 1 hour at 30°C to give approximately 10^5 viable bacteria ml^{-1}) were each divided into 3 aliquots and treated with either 20 μM or 200 μM H_2O_2 (final concentration), or left untreated. These 9 cultures were then monitored for their light production over an 8 hour period at room temperature in a liquid scintillation counter (see figure 5.1). As before (see figure 4.3), a low density of MPG203 cells (see figure 5.1A) was found to emit no light when left untreated, but produced a transient light response which peaked at approximately 90 minutes after addition of 20 μM H_2O_2 and was repressed by 200 μM H_2O_2. In contrast, a low density of MPG351 cells (see figure 5.1B) was found to produce light constitutively, with a peak emission occurring after approximately 4 hours, regardless of the treatment. Comparison of the light intensities recorded for each of the 3 different treatments at this 4 hour time point, demonstrated there to be approximately 50-fold more light induced by MPG351 cells treated with 200 μM H_2O_2 than by the same density of cells left untreated. Furthermore, MPG351 appeared to be more able to tolerate higher concentrations of hydrogen peroxide than MPG203 at the density examined (approximately 10^5 viable bacteria ml^{-1}), with MPG351 cells producing approximately 4 to 5-fold more light in response to 200 μM H_2O_2 compared to that induced by 20 μM H_2O_2 (as opposed to the suppressed response of MPG203 at 200 μM H_2O_2).

Initial recordings of light production from cultures of MPG352 (see figure 5.1C), showed there to be proportionally very little light produced by this strain. However, comparison of the light profiles seen in the untreated culture of MPG352 and in that of the untreated culture of MPG203, showed there to be a small transient burst of light in MPG352. This unstimulated light response was confirmed by monitoring a slightly more dense culture of MPG352 cells over a long period of time. In this instance, an exponentially growing culture of MPG352 (prepared by

Figure 5.1. The Mu*dlux* gene fusion in MPG203 is *oxyR*-regulated.

Exponentially growing cultures of MPG203 (*oxyR*$^+$), MPG351 (*oxyR*^{c}) and MPG352 (*oxyR*$^-$), were prepared by diluting overnight cultures 10,000-fold in LB medium and incubating at 30°C for 1 hour to give approximately 10^5 c.f.u. ml^{-1}. Each sample was then divided into 3 aliquots and treated with 200 µM H_2O_2 (-*-), 20 µM H_2O_2 (-+-), or left untreated (-•-). Light emissions from each treatment of MPG203 cells (A), MPG351 cells (B) and MPG352 cells (C), monitored at room temperature in a liquid scintillation counter (set on the tritium channel), are shown.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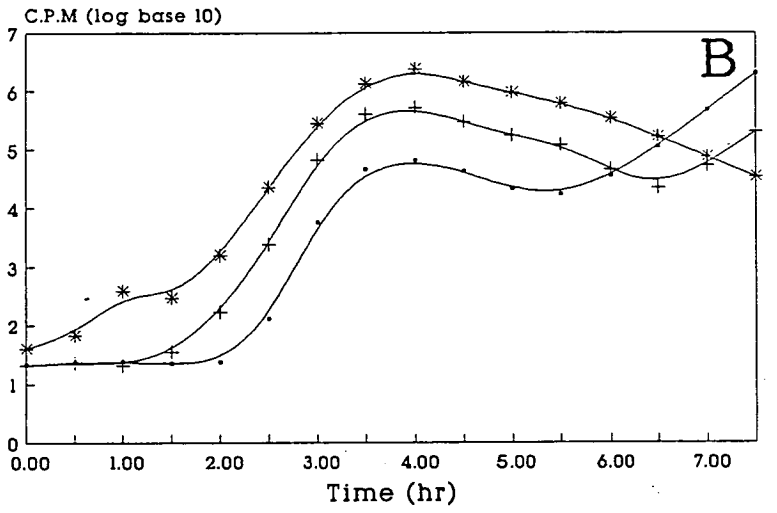
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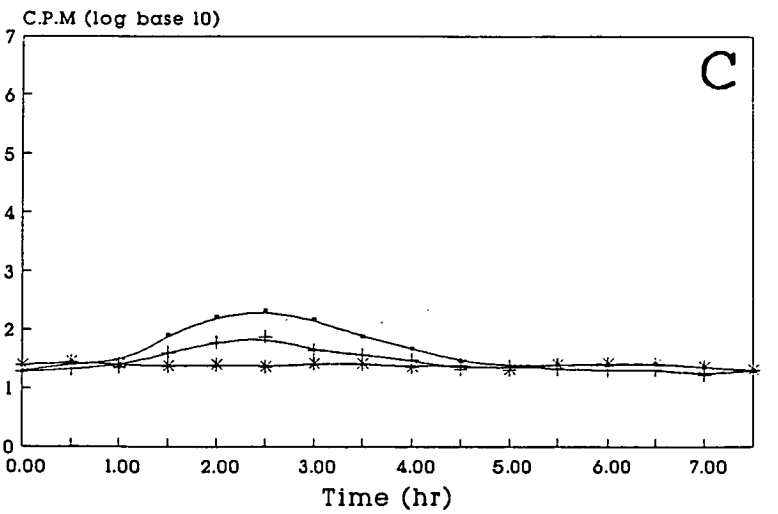
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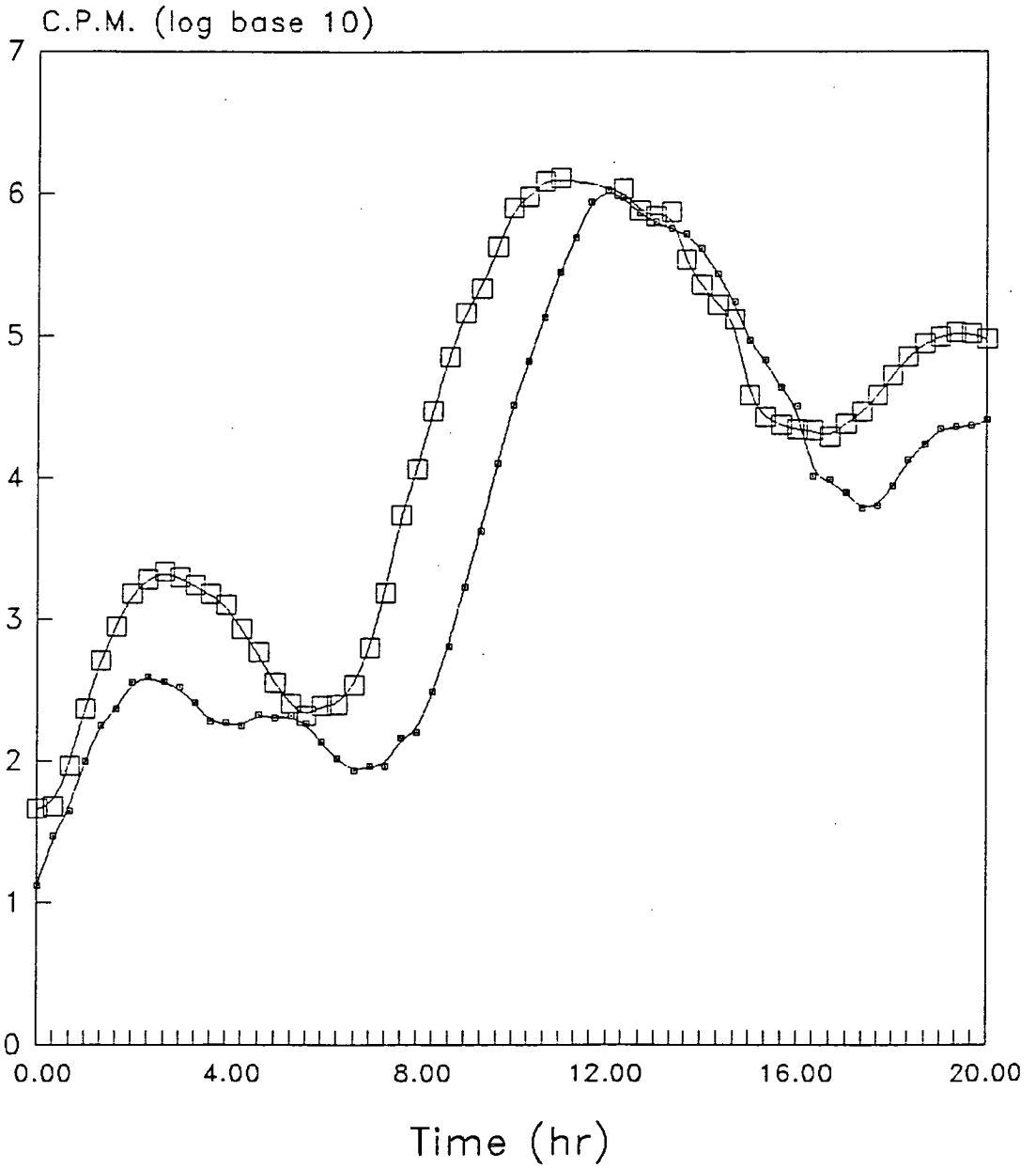
Figure 5.2. The Mu*dlux* gene fusion in MPG203 has basal gene activity in a mutant *oxyR* background at high density.

An exponentially growing culture of MPG352 (prepared by diluting an overnight culture 1000-fold in LB medium and incubating at 30°C for 1 hour to give approximately 10^6 c.f.u. ml^{-1}), was divided into two aliquots and subsequently treated with either 100 μM H_2O_2 (-•-) or left untreated (-□-). Light emissions, monitored at room temperature in a liquid scintillation counter, are shown.


C.P.M. (log base 10)
7
6
5
4
3
2
1
0
0.00
4.00
8.00
12.00
16.00
20.00
Time (hr)

diluting overnight cultures by 1000-fold in LB medium and incubating for 1 hour at 30°C to give approximately 10^6 viable bacteria ml^{-1}) was divided into two aliquots and subsequently treated with either 100 μM H_2O_2 or left untreated, and then monitored for light production over a 20 hours period at room temperature in a liquid scintillation counter. As with 10^5 viable bacteria ml^{-1} (see figure 5.1C), a small peak of light was detected from these cultures after approximately 140 to 160 minutes. However, 100 μM H_2O_2 was found to significantly quench this response (see figure 5.2). A second peak of light could again be recorded in each of the treated and untreated samples after 11 to 12 hours of monitoring at room temperature (approximately 9 to 10 hours after the first peak of light), indicating that this fusion might be regulated to a base level by some *oxyR*-independent mechanism. Alternatively, as discussed in chapter 4, it is possible that this fusion produces a low level of constitutive light, and that this light production is more obvious at high cell densities.

5.3 Effect of menadione on MPG203.

Of the nine proteins reported to be constitutively expressed at an elevated level in the *E. coli oxyR2* mutant, TA4110 (VanBogelen *et al*, 1987), only the enzymes catalase-hydroperoxidase I (HPI; encoded by *katG*) and alkylhydroperoxide reductase (Ahp; encoded by *ahpC* and *ahpF*) have been found to be additionally induced by superoxide generating compounds such as paraquat and menadione (Walkup and Kogoma, 1989; Greenberg and Demple, 1989). These two enzymes, in particular, have been demonstrated to be extremely important for effective protection against oxidative stress, with constitutive over-production of either Ahp or HPI reported to be sufficient to restore normal levels of oxidative resistance to *oxyR* deletion mutants when subjected to H_2O_2 or

either of the previously mentioned superoxide generating compounds (Greenberg and Demple, 1988). Since both HPI catalase and alkylhydroperoxide reductase have also been found to be constitutively elevated in the *S. typhimurium* mutants, TA4100 (Christman *et al*, 1985), it seems very likely that treatment of an *S. typhimurium* $oxyR^+$ strain with either paraquat or menadione would each result in the induction of these same two enzymes.

By utilizing the above information it was possible to distinguish between the number of potential *oxyR*-regulated genes which might serve as the target site for Mu*dlux* in MPG203. As before, colonies of MPG203 (approximately 1000 per plate, grown overnight at 30°C on LB) were monitored for light production at room temperature when overlaid with Whatman no. 3 filter paper soaked in 100 mM menadione. After approximately 90 minutes of this treatment the majority of colonies were observed to glow brightly. These results indicate that the gene fusion in MPG203 was highly induced by superoxide radicals, indicating that the Mu*dlux* was associated with either the *katG* or *ahp* loci.

5.4 Comparative sensitivity of MPG203 to killing by hydrogen peroxide and cumene hydroperoxide.

Previous work on both *E. coli* and *S. typhimurium* has demonstrated that Tn*10*-mediated deletions in the *ahp* locus resulted in hypersensitivity to killing by cumene hydroperoxide, but did not affect sensitivity to killing by hydrogen peroxide (Morgan *et al*, 1986; Storz *et al*, 1989). Furthermore, *katG* mutants of both these bacterial species have been shown to be hypersensitive to killing by hydrogen peroxide (Loewen *et al*, 1985a; Morgan *et al*, 1986). Thus, by combining this information and utilizing it in a peroxide inhibition assay, it is possible to discriminate

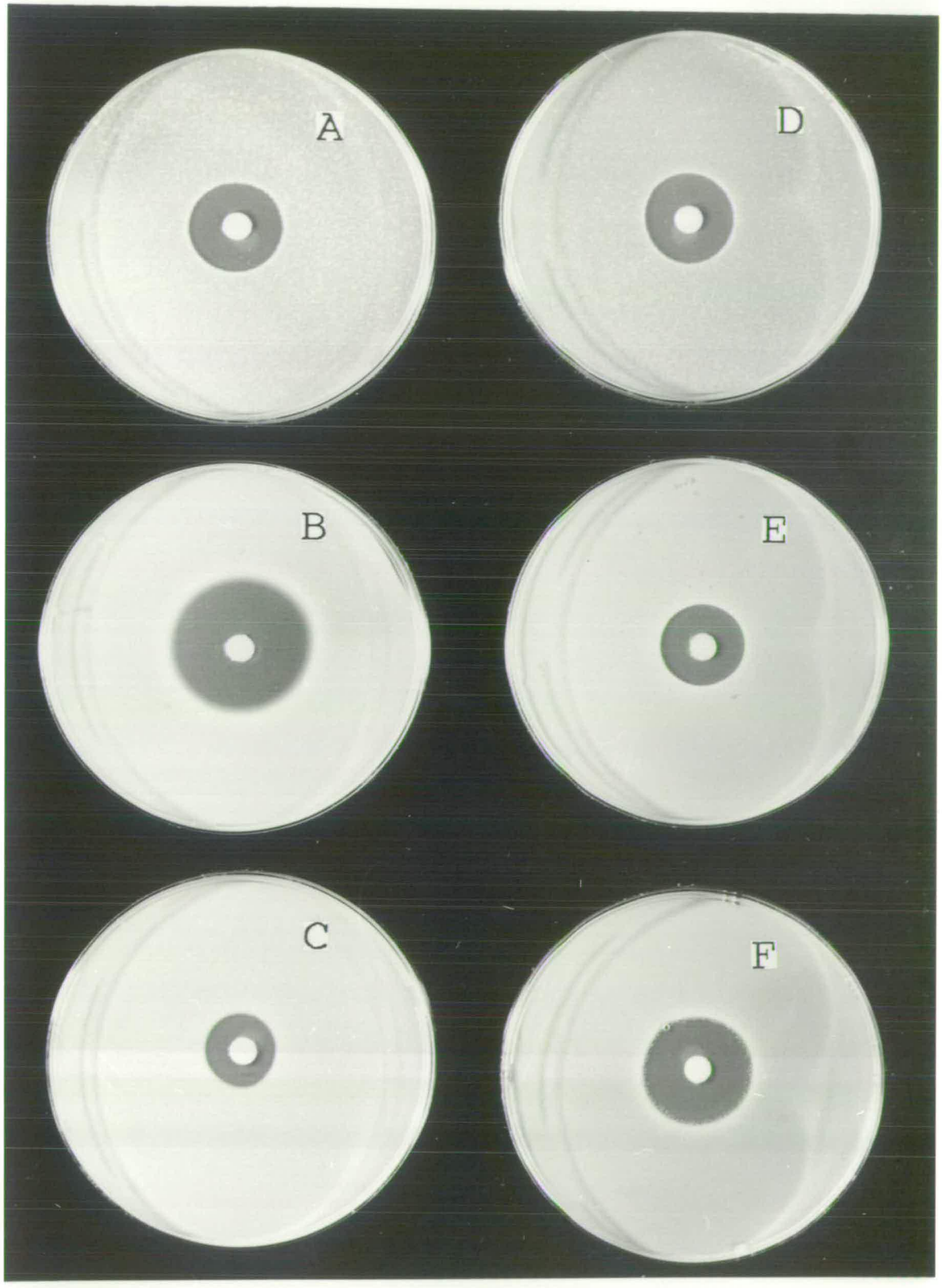
between the two loci that have been proposed as candidates for the Mu*dlux* insertion in MPG203, namely *katG* and *ahp*.

Overnight cultures of MPG203 and its parental strain, SL1344, were therefore mixed with LB top agar and plated on LB plates. Cumene hydroperoxide or hydrogen peroxide (both at 10 µl of a 3% solution) was then added to a 6 mm disk of filter paper which had been placed in the middle of each plate. Plates were then incubated overnight at 30°C. Treatment of SL1344 with either of these peroxides resulted in equivalent zones of inhibition (see figures 5.3A and 5.3D), with the average diameters of clearing being 21 mm in both cases. In contrast, identical treatment of MPG203 showed that cumene hydroperoxide (see figure 5.3B) resulted in an average zone of clearing of 34 mm, compared to an average 20 mm zone with hydrogen peroxide (see figure 5.3E). MPG203 was therefore, more sensitive to killing by cumene hydroperoxide than hydrogen peroxide, indicating the *ahp* locus to be the most likely target for the Mu*dlux* element in MPG203.

To provide further evidence for the above interpretation, a plasmid (pDSA23) carrying the *ahp* operon from *E. coli* (Smillie *et al*, 1992) was transformed into MPG203, and the peroxide inhibition assay was repeated for this strain (designated as MPG353) in conjunction with MPG203 and SL1344. The effects of this plasmid in MPG353 can be seen in figures 5.3C and 5.3F for treatment with cumene hydroperoxide and hydrogen peroxide, respectively. The average zone of inhibition for this strain when treated with cumene hydroperoxide was 10 mm (compared to 34 mm in MPG203 and 21 mm in SL1344), indicating that pDSA23 not only abolished sensitivity to this compound, but in fact provided higher levels of resistance than seen in the wildtype strain SL1344. Surprisingly, however, this same plasmid was found to increase the sensitivity of MPG353 to hydrogen peroxide (with the zone of inhibition for this

Figure 5.3. MPG203 is more sensitive to killing by cumene hydroperoxide than by hydrogen peroxide.

100 µl volumes of overnight cultures of SL1344, MPG203, or MPG353, prepared in LB medium at 30°C, were added to 3 ml of soft agar and overlaid on LB plates. On the centre of each plate was placed a 6 mm disc of Whatman No.3 cellulose, and 10 µl solutions containing 3% hydrogen peroxide (dissolved in water) or 3% cumene hydroperoxide (dissolved in dimethyl sulfoxide) were carefully pipetted onto each. Plates were then incubated overnight at 30°C. Shown are the zones of killing for SL1344 treated with 3% cumene hydroperoxide (A) or 3% hydrogen peroxide (D), MPG203 treated with 3% cumene hydroperoxide (B) or 3% hydrogen peroxide (E), and MPG353 treated with 3% cumene hydroperoxide (C) or 3% hydrogen peroxide (F).


A
D
B
E
C
F

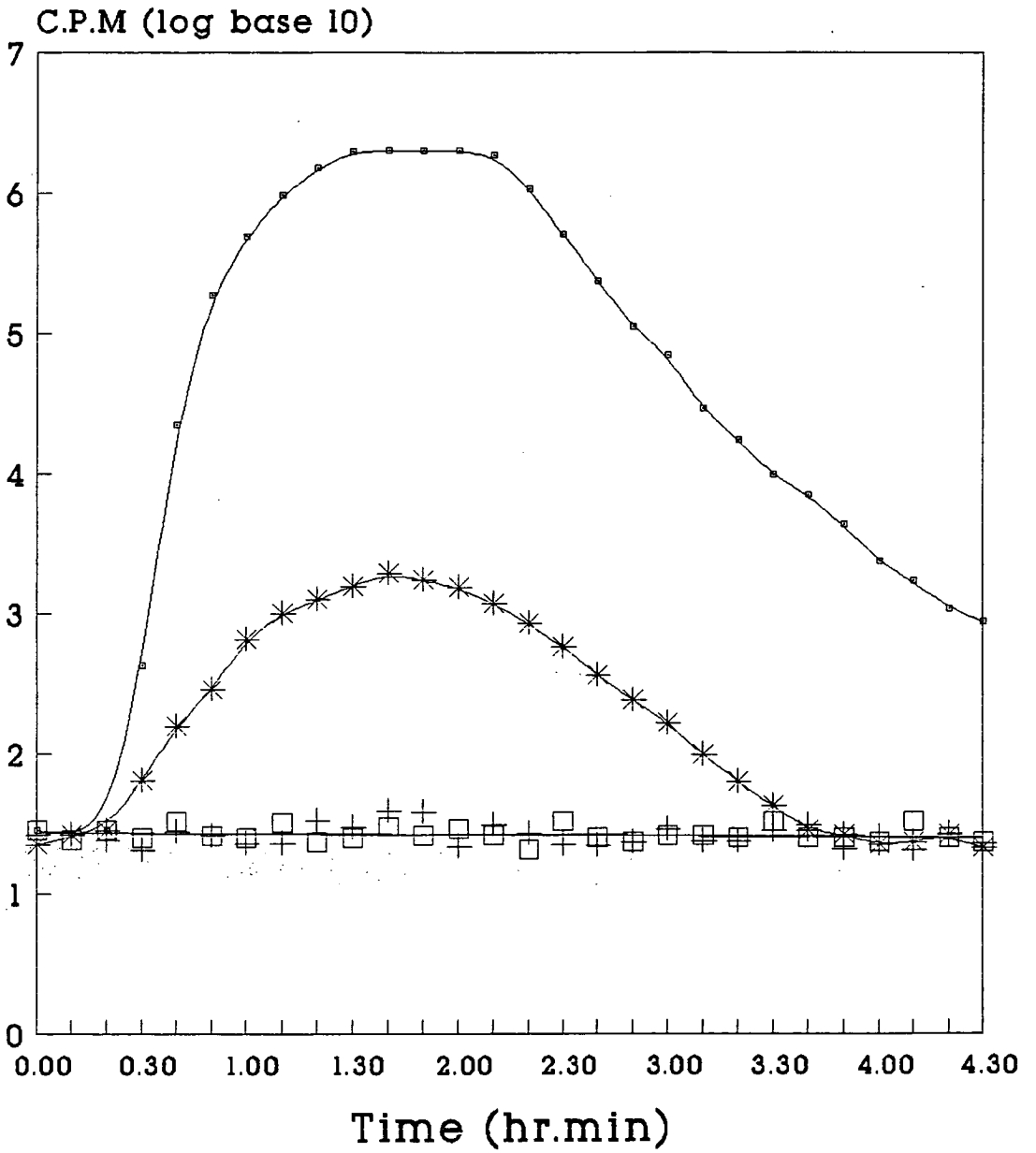
treatment averaging 26 mm in MPG353, compared to 20 mm in MPG203 and 21 mm in SL1344). This agrees with work previously conducted by Storz and colleagues (1989), who explained this phenomenon as being due to either some form of cellular stress, as a result of hydrogen peroxide inducing over-production of alkyl hydroperoxide reductase, or the presence of the *ahp* promoter region on the multi-copy plasmid causing the OxyR protein to be titrated away from other *oxyR*-regulated genes which normally play a role in providing resistance to hydrogen peroxide, such as the *katG* gene which encodes a catalase.

5.5 Effect of pDSA23 on hydrogen peroxide induced light production in an MPG203 background (designated as MPG353).

To determine the effect that pDSA23 might have upon the hydrogen peroxide induced light response of the MPG203 gene fusion, exponentially growing cultures of MPG203 and MPG353 (prepared by diluting overnight cultures of each strain by 1000-fold in LB medium and incubating for 1 hour at 30°C to give approximately 10^6 viable bacteria ml^{-1}) were treated with hydrogen peroxide (100 μM final concentration) and their light emissions were recorded in a liquid scintillation counter over a period of approximately 4 hours at room temperature (see figure 5.4). MPG353 was observed to produce a peak of light which was over a 1000-fold less intense than that generated by an equivalent density of MPG203 cells. Over-production of the enzyme alkyl hydroperoxide reductase therefore appeared to reduce the level of induction of the *lux* genes of the Mu*dlux* fusion located on the *S. typhimurium* chromosome.

Figure 5.4 Introduction of pDSA23 into MPG203 reduces hydrogen peroxide-induced light emission from this Mu*dlux* fusion.

Exponential cultures of MPG203 and the equivalent strain carrying pDSA23, MPG353, were prepared by diluting overnight cultures of these bacteria 1000-fold in LB medium and incubating at 30°C for 1 hour to give approximately 10^6 c.f.u ml^{-1}. Both culture were then divided into two volumes and treated with 100 μM H_2O_2 or left untreated, and each sample was immediately monitored in a liquid scintillation counter at room temperature. Light emissions were as shown: MPG203 treated with 100 μM H_2O_2 (-•-), MPG203 untreated (-+-), MPG353 treated with 100 μM H_2O_2 (-*-), MPG353 untreated (-□-)


C.P.M (log base 10)
7
6
5
4
3
2
1
0
0.00
0.30
1.00
1.30
2.00
2.30
3.00
3.30
4.00
4.30
Time (hr.min)

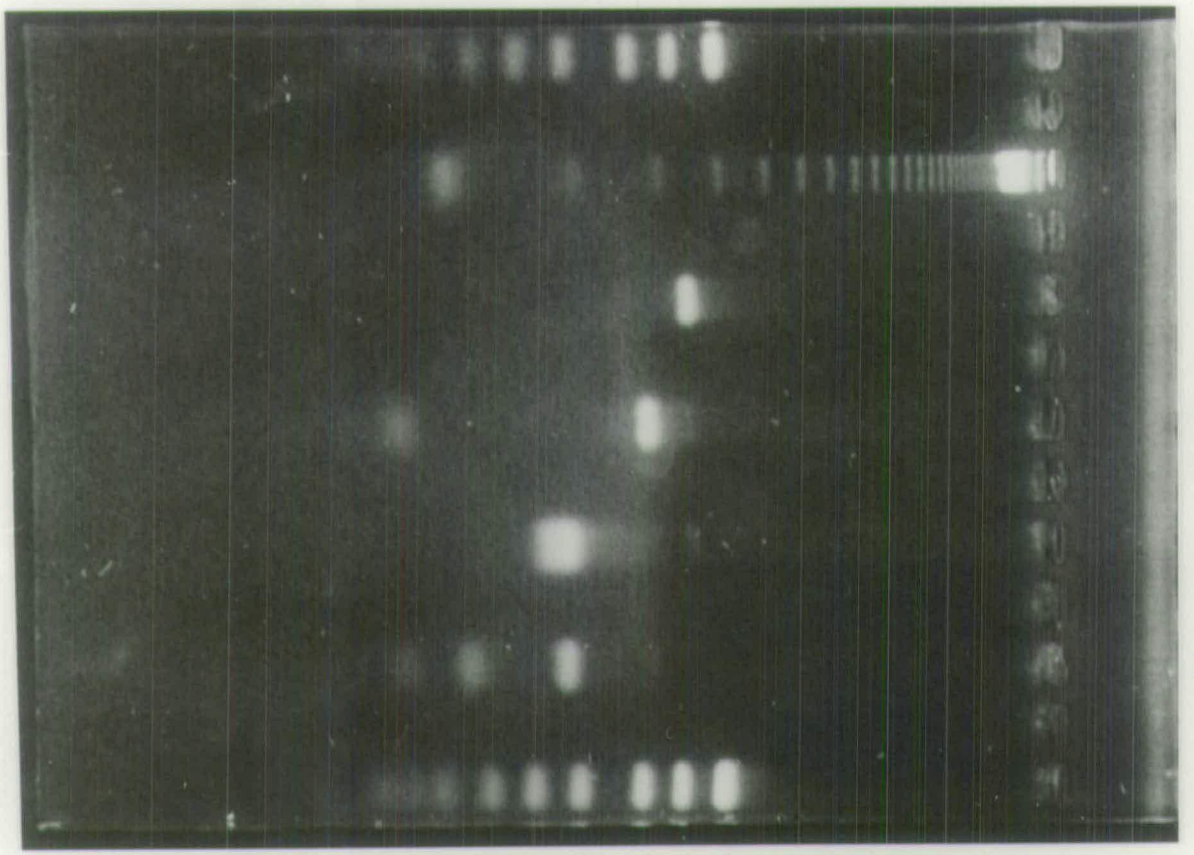
5.6 PCR of the junction between Mu*dlux* and the target gene of MPG203

In order to confirm that the MPG203 gene fusion was in fact located within the *ahp* locus, and additionally, to determine which of the two genes of this operon (*ahpC* or *ahpF*) contained the Mu*dlux* insertion, further analysis of this fusion was conducted at the molecular level. To achieve this the DNA at the fusion junction of MPG203 was amplified using PCR. Since all of the above evidence indicated that this fusion occurred within one of the genes of the *ahp* operon, an oligonucleotide primer (an eighteenmer designated 823W; see table 2.1) was synthesized which was equivalent to the sense strand from the start of the *ahpC* coding region (the proximal gene of the operon) (Tartaglia *et al*, 1990), whilst the antisense primer (a twentymer designated 404X; see table 2.1) was taken from a sequence of Mu DNA situated near the fusion junction (Kahmann and Kamp, 1979). PCR was performed over 30 cycles, using both of the above primers and chromosomal DNA taken from MPG203 (see chapter 2 for full methodology). The product was run on a 2% agarose gel to reveal a DNA fragment of approximately 400 base pairs (see figure 5.5). This inferred that, following subtraction of the Mu DNA fragment, the fusion junction in MPG203 could be estimated as lying approximately 300 bases downstream from the start of the *ahpC* coding region.

By studying the DNA sequence of the *S. typhimurium ahp* operon previously reported by Tartaglia and colleagues (1990), two unique restriction sites, *EcoRI* and *PstI*, were identified, situated within this same 300 base region of *ahpC*. In order, to check that this PCR product was not an artifact, these two restriction enzymes were therefore used to digest the PCR product, and the fragments were evaluated by electrophoresis on a 2% agarose gel (see figure 5.5). The restriction patterns confirmed that the

Figure 5.5 PCR of the junction between Mu*dlux* and the target gene of MPG203 produces a DNA fragment with restriction sites characteristic of *ahpC*.

PCR was performed upon chromosomal DNA from the fusion containing strain MPG203 using a sense primer (an eighteenmer designated 823W; see table 2.1) taken from the start of the *ahpC* coding region (Tartaglia *et al*, 1990) and an antisense primer (a twentymer designated 404X; see table 2.1) taken from a sequence of Mu DNA situated near the fusion junction (Kahmann and Kamp, 1979). Approximately 1 µg quantities of PCR product were cut with the restriction enzymes *EcoRI* and/or *PstI*, and run on a 2% agarose gel with similar quantities of uncut PCR product and molecular weight marker DNA (pUC cut with *Hpa II* and 123 base ladder). The resulting DNA band patterns are shown. Samples in each lane are indicated.


pUC HpaII
123 base ladder
PCR product Uncut
PCR product EcoRI
PCR product PstI
PCR product EcoRI/PstI
pUC HpaII
bases
501/489
404
328
242
190
147
110
89
67

PCR product had indeed been cut by both *EcoRI* and *PstI*, and that the sizes of the resulting fragments corresponded well with those predicted. In particular, a double digest of this PCR product was seen to give three DNA fragments, with two of these fragments of between 110 - 147 base pairs and 67 - 89 base pairs. This agreed well with the 123 base pair and 75 base pair fragments calculated as the actual sizes expected.

5.7 Sequencing of the PCR product derived from the junction between Mu*dlux* and the target gene of MPG203.

In order to determine the precise DNA junction of the *ahp*::Mu*dlux* gene fusion in MPG203 the PCR product obtained in section 5.6 was cloned and then sequenced. Restriction of the amplified DNA had already demonstrated that it contained a unique *EcoRI* site within the *ahpC* region (see figure 5.5) and this allowed the fragment to be directionally cloned into an appropriate plasmid vector (pBluescript KS; see table 2.3). The PCR product was blunt ended first with Klenow polymerase and then cut with *EcoRI* before being cloned into the *SmaI/EcoRI* sites of pBluescript. The DNA was then transformed into JM83 cells of *E. coli.* Plasmid DNA was then isolated from transformants and the insert was subsequently sequenced by the dideoxy-DNA sequencing method of Sanger and colleagues (1977) using double stranded DNA as the template and the T7 bluescript primer or the Mu 404X primer to initiate the polymerisation (see table 2.1). Sequenase version 2.0 was used for the reactions. The results confirmed that the Mu*dlux* insertion was indeed located within the *ahpC* gene, with the DNA fusion junction occurring in frame after the 318 th base from the start (ATG) of the *ahpC* coding region (Tartaglia *et al*, 1990) (see figure 5.6A).

Figure 5.6 The locus disrupted by the Mu*dlux* element in MPG203 is *ahpC*, a gene encoding a protein (C22) with a possible role in the pathogenicity of *S. typhimurium*.

Panel A

The PCR product which contains the fusion junction of MPG203 (see figure 5.5) was cloned into Bluescript KS by blunt end-*EcoR1* ligation and transformed into *E. coli* JM83 cells. Plasmid DNA was then isolated and sequenced using both the T7 bluescript primer (see table 2.1) and the Mu 404X primer (see table 2.1). The sequence of the cloned *ahpC*::Mu fragment is shown (A). The Mu sequence has joined to the 318 th base from the start of the *ahpC* coding region (Tartaglia *et al*, 1990). *EcoRI* and *PstI* restriction sites are underlined. An additional base not shown in the reported sequence of Mu (Kahmann and Kamp, 1979) is indicated by an overlying dot (.). The first and last three bases shown in the sequence (tcg and ggg) represent pBluescript KS sequence.

Panel B

The comparative amino acid sequence of AhpC (*S.t* C22) (Tartaglia *et al*, 1990) and a number of other proteins from a diverse range of pathogenic organisms, including *Entamoeba histolytica* (*E.h*) (Torian *et al*, 1990), *Mycobacterium avium* (*M.a*) (Yamaguchi *et al*, 1992), *Mycobacterium leprae* (*M.l*) (Smith, unpublished), and *Helicobacter (Campylobacter) pylori* (*H.p*) (O'Toole *et al*, 1991), are shown (B). The number next to the initials of each of these organisms refers to the molecular weight (kDa) of the corresponding protein, with the number of residues from each of these proteins given at the end of each sequence. Conserved amino acids are in bold type and marked with an asterisk (*), with a 13 amino acid region that is boxed, repressing a possible common functional domain (Flores *et al*, 1993).

A

KS *ahpC*
tcg-AATTCATTGAAGTCACCGAGAAAGATACCGAAGGCCGCTGGAGCGTCTTCTTC

TTCTACCCGGCCGATTTTACCTTTGTTTGCCCGACTGAACTGGGTGACGTTGCCGAC

CATTACGAAGAACTGCAGAAGCTGGGCGTAGACGTTTATTCCGTCTCAACCGATACT

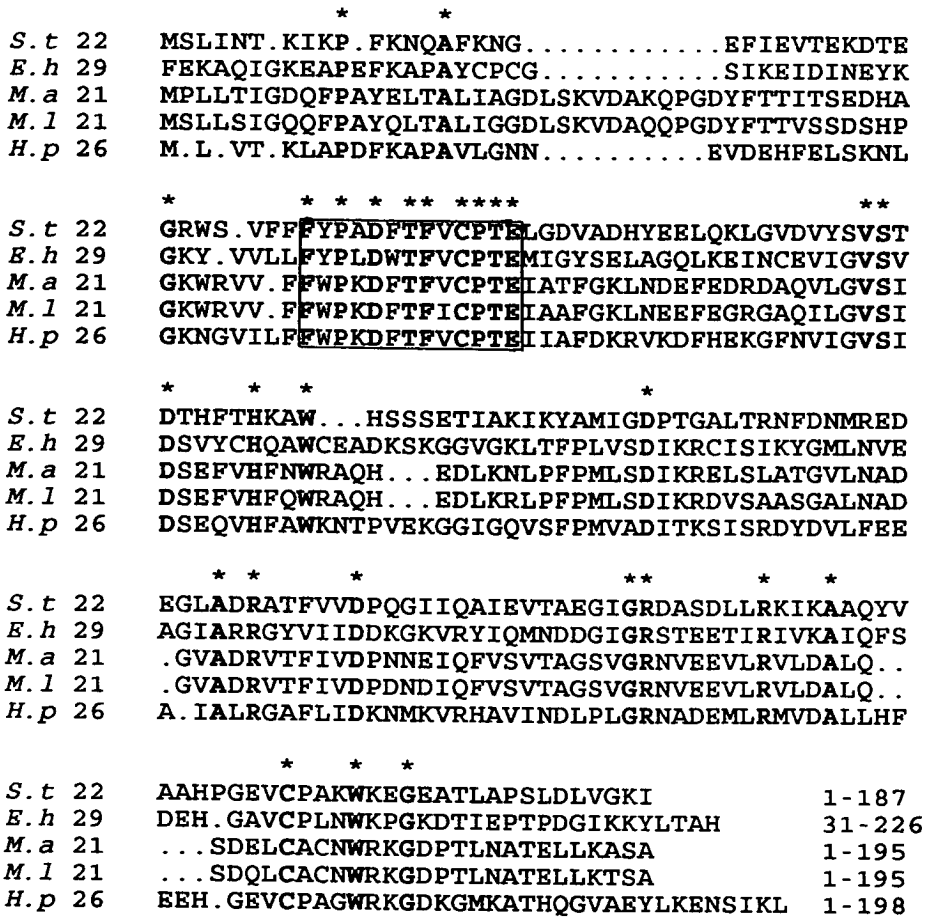
CACTTCACGCACAAAGCATGGCACAGCAGCTCTGAAACTATCGCAAAAATCAAATAT

ahpC Mu
GCGATGATCGGCGACCCGACTGGCGCCCTGACCCGT::tgaagcggcgcacgaaaaa

cgcgaaagcgtttcacgataaatgcgaaaactttagctttcgcgcttcaaatgaaac

Mu KS
agatgtat-ggg

B



5.8 Discussion.

The identity of the *S. typhimurium* locus in which the hydrogen peroxide- and macrophage-inducible Mu*dlux* gene fusion has inserted in MPG203, has been established as *ahpC*, the gene encoding the smaller subunit (C22) of the heterodimeric enzyme alkyl hydroperoxide reductase (Tartaglia *et al*, 1990). This gene has previously been demonstrated to be part of the OxyR regulon (Christman *et al*, 1985), and is essential in the reduction of organic hydroperoxides which cause extensive cellular damage (Jacobson *et al*, 1989).

The availability of two *oxyR* mutants, TA4100 (*oxyR1*) and TA4108 (*oxyΔ2*) originally isolated by Christman and colleagues (1985), proved to be extremely useful in analysing the regulation of this *ahpC*::Mu*dlux* fusion (see figure 5.1). Interestingly, this fusion did not appear to be fully induced in a TA4100 background (MPG351; see figure 5.1B), since hydrogen peroxide treatment of MPG351 cultures resulted in significant increases in light production over a culture that had not been stressed by this oxidant. This was surprising in view of the previous work by Christman and colleagues (1985) which demonstrated that *oxyR1* mutants constitutively over-produce *oxyR*-regulated proteins, including *ahpC*.

The reason why it is possible to further induce cultures of MPG351 is not fully known. However, elucidation of the nature of the *oxyR1* mutation allows for a possible explanation. Christman and colleagues (1989) have demonstrated that an analogous *oxyR1* mutation (termed *oxyR2*) isolated in *E.coli* (Christman *et al*, 1985), resulted from a missense mutation (C.G to T.A transition) that changed alanine to valine at amino acid position 234 of the OxyR protein, rather than from an increase in the expression of this protein or from increased levels of *oxyR*

mRNA. Since *oxyR1* and *oxyR2* were both prepared by the same procedure (chemical mutagenesis (Christman *et al*, 1985)) and exhibit a similar phenotype, it seems reasonable that the constitutive over-production of *oxyR*-regulated proteins observed in the *S. typhimurium* strain TA4100 (*oxyR1*) may also represent a comparable missense mutation. If it is assumed that oxidation of OxyR normally causes a conformational change in the tertiary structure of this protein (Storz *et al*, 1990), allowing it to activate *oxyR*-regulated promoters, then it seems likely that the constitutive nature of the *oxyR1* mutant may similarly be the result of a conformational change in OxyR, but that the properties of this altered protein are such that it is not fully active. Further oxidation may therefore result in an OxyR polypeptide which has adopted a more active state. Such a situation would result in enhanced synthesis of the *ahp::lux* fusion without a requirement for increased synthesis of OxyR.

In addition to the unexpected increase in light production observed from cultures of MPG351 when stressed by exposure to hydrogen peroxide, monitoring of the *ahpC*::Mu*dlux* construct in a TA4108 (*oxyΔ2*) background (MPG352) also gave unexpected results (see figure 5.1C). Cultures of these cells (uninduced by hydrogen peroxide) were found to produce a very small and transient peak of light that was not observable in an equivalent culture of MPG203. Since TA4108 mutants are deleted for *oxyR* (Christman *et al*, 1985), this activity must be *oxyR*-independent. Furthermore, brief, low level induction would probably preclude detection in studies which employed alternative techniques for monitoring, such as the analysis of two-dimentional protein gels (*eg* Christman *et al*, 1985). This emphasizes one of the benefits of using a light based reporter system. In addition to the initial transient peak of light observed at lower cell densities (see figure 5.1C), a second much larger peak of light was

observed as this culture became more turbid and went into its late exponential-early stationary phase of growth (see figure 5.2).

Light emissions observed in cultures of MPG352 might simply reflect low level constitutive expression of this gene fusion, which is particularly obvious at high cell densities. (This theory is substantiated by the fact that alkyl hydroperoxide reductase activity is present at wild-type (uninduced) levels in TA4108 mutants (Christman *et al*, 1985).) However, this theory does not take into account the transience of these light emissions. An alternative theory, is that these light emissions reflect normal fluctuations in basal gene expression during growth, possibly as a result of local changes in the state of the chromosomal copy number. A second alternative, is that these transient peaks of light delineate a discrete growth phase phenomenon (*eg* stationary phase gene expression). This latter theory has already been expounded in the preceding chapter (chapter 4) in which two possible mechanisms for stationary phase induction of stress-inducible genes were discussed based on acetate fermentation or the accumulation of alarmones. These features remain to be further determined.

Previous work on alkyl hydroperoxide reductase has demonstrated that the genes encoding the two subunits of this enzyme are actually part of the same operon (Tartaglia *et al*, 1990), and that a single transposon insertion within the *ahpC* locus is sufficient to eliminates both the basal and OxyR-stimulated levels of both protein subunits (Storz *et al*, 1989). Therefore, it is very likely that the *ahpC* fusion isolated in this study also will have resulted in the elimination of the activities of both Ahp subunits. Jacobson and colleagues (1990) have shown that the larger subunit of this enzyme, AhpF (F52a), is capable of reducing a number of substrates even in the absence of the smaller AhpC (C22) protein. However, this group further demonstrated that the smaller subunit of Ahp is required for the

reduction of organic hydroperoxides. Thus, it is not clear whether the zone of death observed following the treatment of MPG203 with cumene hydroperoxide (see figure 5.3) results from abolishing one or both enzyme subunits. In either case the same dramatic killing would be seen. Interestingly, additional work by Jacobson and colleagues (1990) has shown that *in vitro* activity of both Ahp subunits combined could only be achieved in the presence of protein albumins such as bovine serum albumin. However, the relevance of this finding, and whether it might have any bearing on the pathogenicity of *S. typhimurium* remains unclear.

The importance of alkyl hydroperoxide reductase in the reduction of lipid peroxides and a variety of other organic peroxides to their corresponding alcohols has been clearly demonstrated for survival of oxidatively stressed cells of *S. typhimurium* (Christman *et al*, 1985; Storz *et al*, 1989; Jacobson *et al*, 1989). The exact mechanisms involved in this process however, are not known. Claiborne and colleagues (1992) have suggested that, on the basis of the structural similarities between F52a (Tartaglia *et al*, 1990) and the *E. coli* thioredoxin reductase (TR) (Russel and Model, 1988), Ahp may function in a similar manner to the thioredoxin-thioredoxin reductase system. This group have therefore suggested that the most probable mechanism by which Ahp functions is by the transfer of electrons from NADPH, via an FAD cofactor and a redox active disulphide on F52a, to a second redox active disulphide on the C22 protein which, in turn, acts to reduce the substrate alkylhydroperoxide. This would imply that the regions containing these redox active disulphides are probably essential to the activity of these proteins.

Whether the *S. typhimurium* Ahp protein exhibits an additional function during this bacterium's interaction with macrophages has not yet been determined. However, recent work by Flores and colleagues (1993)

and Smillie and Haywood (personal communication) has shown that C22 exhibits sequence similarity to a number of other proteins from a diverse range of pathogens, including *Entamoeba histolytica* (Torian *et al*, 1990), *Cryptosporidium parvum* (Flores *et al*, 1993), *Helicobacter (Campylobacter) pylori* (O'Toole *et al*, 1991), and *Mycobacterium avium* (Yamaguchi *et al*, 1992)). Furthermore, Flores and colleagues (1993) have speculated that one particular 13 amino acid region (see figure 5.6B) within these proteins may represent a common functional enzymatic domain. This region contains an invarient Cys residue which may be responsible for the reported redox properties of C22 seen in *E. coli* and *S. typhimurium* (Jacobson *et al*, 1989).

Previous work on *E. histolytica* has shown that the protein identified as containing this same 13 amino acid region (Flores *et al*, 1993), is present as the major free thiol containing protein found on the surface of this amoeba during extraintestinal infection (Torian, *et al*, 1990). Furthermore, Reed and colleagues (1992) have demonstrated that this same 29 kDa protein can be used to differentiate between pathogenic and non-pathogenic clinical isolates of *E. histolytica.* This has led to speculation that this protein must be required for this parasite to survive and tolerate extraintestinal aerobic environments, possibly through the action of redox active cysteines (Flores *et al*, 1993). It therefore seems possible that C22 may also play a similar role in enhancing the pathogenicity of *S. typhimurium* but in a different environment, such as by conveying a level of protection during the respiratory burst, following macrophage interaction.

CHAPTER 6

Chapter 6: Identification of the Mu*dlux* target site in the cold shock-inducible *S. typhimurium* strain MPG361.

6.1 Introduction.

The initial objective of this study was to isolate Mu*dlux* gene fusions in *S. typhimurium* which produced light in response to defined stress conditions similar to those found within activated macrophages (*eg* H_2O_2, OCl^-, and low pH (Groisman and Saier, 1990)). It was hoped that such mutants might lead to the identification of genetic loci that were important to this pathogen's survival inside phagocytic cells (see chapters 4 and 5). The initial discovery that some of these particular stress-inducible fusions also produced light in response to cold shock (see tables 3.1, 3.2 and 3.3) was therefore somewhat unexpected, especially since this latter phenomenon did not at first appear to logically correspond with any of the other stress conditions that had been examined.

A literature search of the cold shock response in *E. coli* revealed that at least one of the identified cold shock inducible genes, *recA* (Jones *et al*, 1987), was also induced by hydrogen peroxide, a stress condition that had previously been tested for in this study. Furthermore, this search revealed that the cold shock response was one of the most poorly characterized stress responses so far identified. Since the present study had already established that the Mu*dlux* reporter system was capable of accurately monitoring the latter response (see figure 3.2), it was thought that this system might also be of value in unravelling how the cold shock response was co-ordinately controlled.

In order to accomplish the above goal, the Mu*dlux* fusion from MPG300 was firstly transduced into SL1344 with phage P22 and redesignated as MPG361. This fusion was chosen as the most suitable

candidate to act as a reporter of the cold shock response, because it appeared to be selectively induced to a high level by a temperature downshift and showed no significant background light at room temperature or above (see table 3.3 and figure 3.2). Furthermore, prolonged storage of MPG361 at 10°C demonstrated that this mutation was not overly detrimental to the bacterium's survival at low temperature (assuming that the Mu*dlux* resulted in complete loss of gene function).

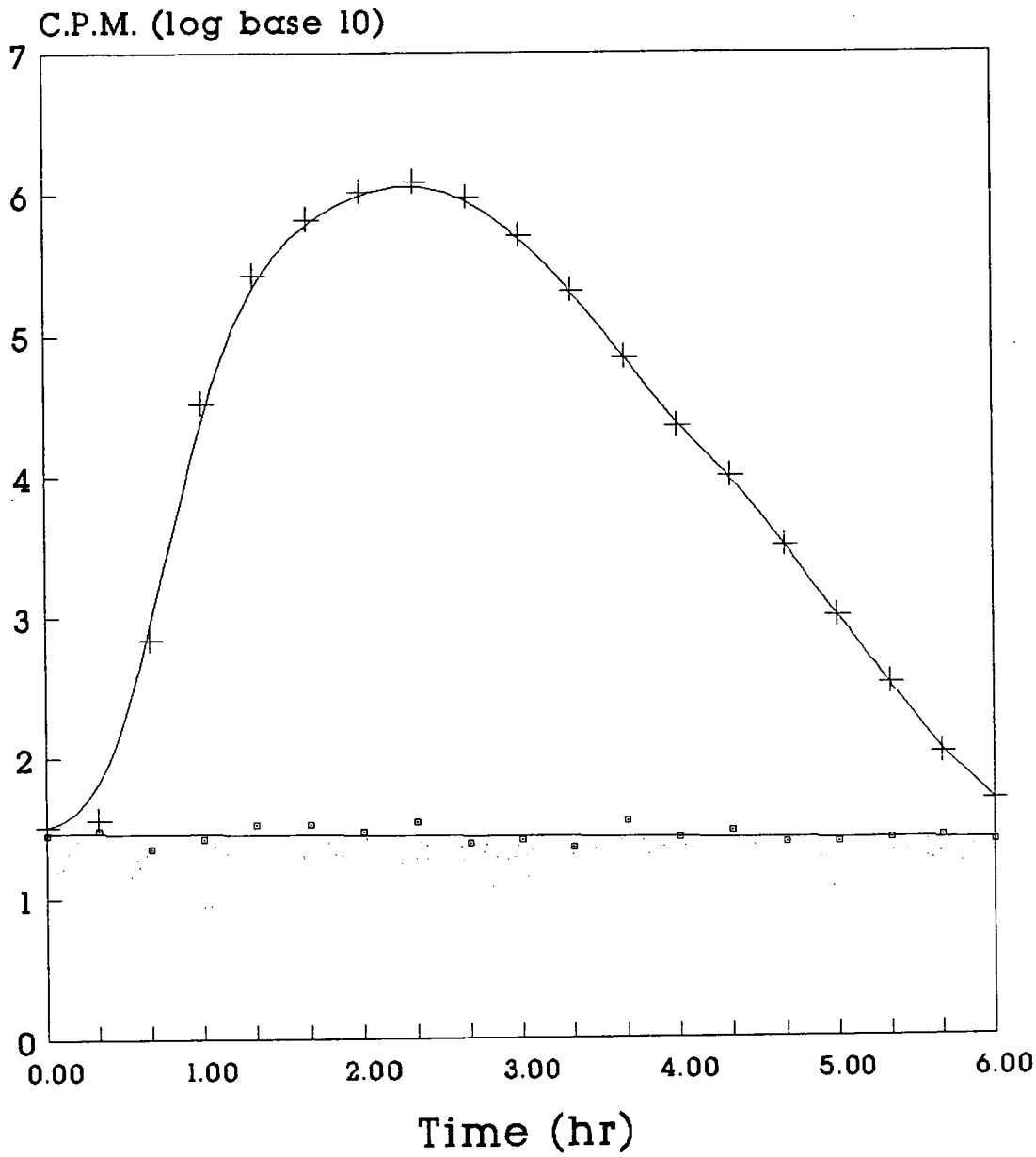
This chapter will concern itself solely with the further characterisation and identification of the cold shock-inducible gene fusion of MPG361. The characterisation of potential cold shock regulatory loci will be discussed in the next chapter.

6.2 Effect of tetracycline on the light production of MPG361.

Previous studies have shown that a large number of stress conditions are capable of inducing the major heat shock proteins in the cells of a great many organisms (Lindquist and Craig, 1988). This has led VanBogelen and Neidhardt (1990) to hypothesize that the induction of such proteins in *E. coli* was due to changes in the translational capacity of the cell. These workers subsequently tested this theory by treating exponentially growing cultures of *E. coli* with low concentrations of antibiotics that were known to affect ribosomes and alter the rates of protein synthesis. By examining 2D-gels of the proteins produced by the bacteria following this treatment, the latter group demonstrated that antibiotics such as kanamycin, puromycin, and streptomycin, were capable of inducing the synthesis of all the major heat shock proteins whereas, in contrast, antibiotics such as chloramphenicol, tetracycline, erythromycin, spiramycin, and fusidic acid acted to induce the majority of the cold

Figure 6.1. Tetracycline induces light emission from MPG361 when this cold shock-inducible fusion is at room temperature.

An exponential culture of MPG361 (prepared by diluting an overnight culture 100-fold in LB media and incubating for 1 hour at 28°C to give approximately 10^7 c.f.u ml^{-1}) was divided into seven aliquots and treated with low concentrations (1 μg ml^{-1} or 10 μg ml^{-1}) of tetracycline, chloramphenicol, or streptomycin, or left untreated. Each sample was then monitored for light production at room temperature (24°C) in a liquid scintillation counter (set on the tritium cannel). Light emission from a culture treated with tetracycline at a concentration of 1 μg ml^{-1} is shown (-+-). No light above background was detected from cultures treated with a higher concentration of tetracycline, or with either concentration of chloramphenicol or streptomycin (data not shown). Similarly, no light was detected from an untreated culture (-•-).


C.P.M. (log base 10)
7
6
5
4
3
2
1
0
0.00
1.00
2.00
3.00
4.00
5.00
6.00
Time (hr)

shock proteins, whilst the synthesis of most other proteins, including the heat shock proteins, diminished.

In order to determine whether the above phenomenon could be reproduced in MPG361, exponentially growing cultures of these cells (prepared by diluting an overnight culture of MPG361 cells by 100-fold in LB media and incubating at 28°C for 1 hour to give approximately 10^7 bacteria ml^{-1}) were treated with low concentrations (1 μg ml^{-1} or 10 μg ml^{-1}) of tetracycline, chloramphenicol, or streptomycin (negative control), and then immediately monitored for light production at room temperature (24°C) in a liquid scintillation counter. The results of this experiment (see figure 6.1) demonstrated that tetracycline at a concentration of 1 μg ml^{-1} was capable of inducing a substantial transient light response from MPG361, and that the peak of this response occurred approximately 140 minutes from the time of antibiotic addition. No light was recorded from bacterial cultures treated with a higher concentration of tetracycline, or with either concentration of chloramphenicol or streptomycin. Though not in complete agreement with the observations of VanBogelen and Neidhardt (1990), with *E. coli*, this experiment clearly demonstrated that MPG361 was capable of producing an analogous response at least to tetracycline.

6.3 Mapping the location of the Mu*dlux* gene fusion on the chromosome of the *S. typhimurium* strain MPG361.

The analysis of previous light data pertaining to MPG361 (see table 3.3 and figure 3.2), indicated that this fusion might lie within an *S. typhimurium* gene analogous to the *cspA* gene (encoding the major cold shock protein CS7.4) of *E. coli* (Goldstein *et al*, 1990), since this gene (of all the reported cold shock genes) seemed to match the induction profile

of MPG361 most closely. As evidence towards this hypothesis, the chromosomal map location of the Mu*dlux* fusion was investigated to determine if it was located at an equivalent position on the genome of *S. typhimurium* to that reported for *cspA* in *E. coli.* The close genetic relationship of *S. typhimurium* and *E. coli* might predict this.

Goldstein and colleagues (1990) have previously shown that *cspA* maps at 79 minutes on the *E. coli* chromosome, therefore, *S. typhimurium* strains with Tn*10* insertions at known locations around 79 minutes (Kukral *et al*, 1987) were infected with phage P22 and the transposon was transduced into MPG361 (selecting for tetracycline resistance encoded by the Tn*10*). The ability of transductants to abolish light induction in MPG361 was then examined. The results showed that the Tn*10* obtained from strain AK3040, which mapped at approximately 78 minutes on the *S. typhimurium* chromosome, was capable of abolishing light in 100% of the 1000 or so transductants to grow on LB tetracycline plates. Furthermore, none of the 50 transductants that were patched from these plates onto LB kanamycin plates were able to grow, confirming that this Tn*10* insertion had replaced the Mu*dlux* fusion in MPG361. The other Tn*10*s, mapped at 79 minutes (from strain AK3312) and 80 minutes (from strain AK3294), both tested as negative for abolishing light in MPG361. Tn*10*s which mapped immediately upstream (anti-clockwise) of the 78 minutes region in *S. typhimurium* were not available for testing

6.4 Effect of Mu*dlux* on the cold shock-induced protein profile of MPG361.

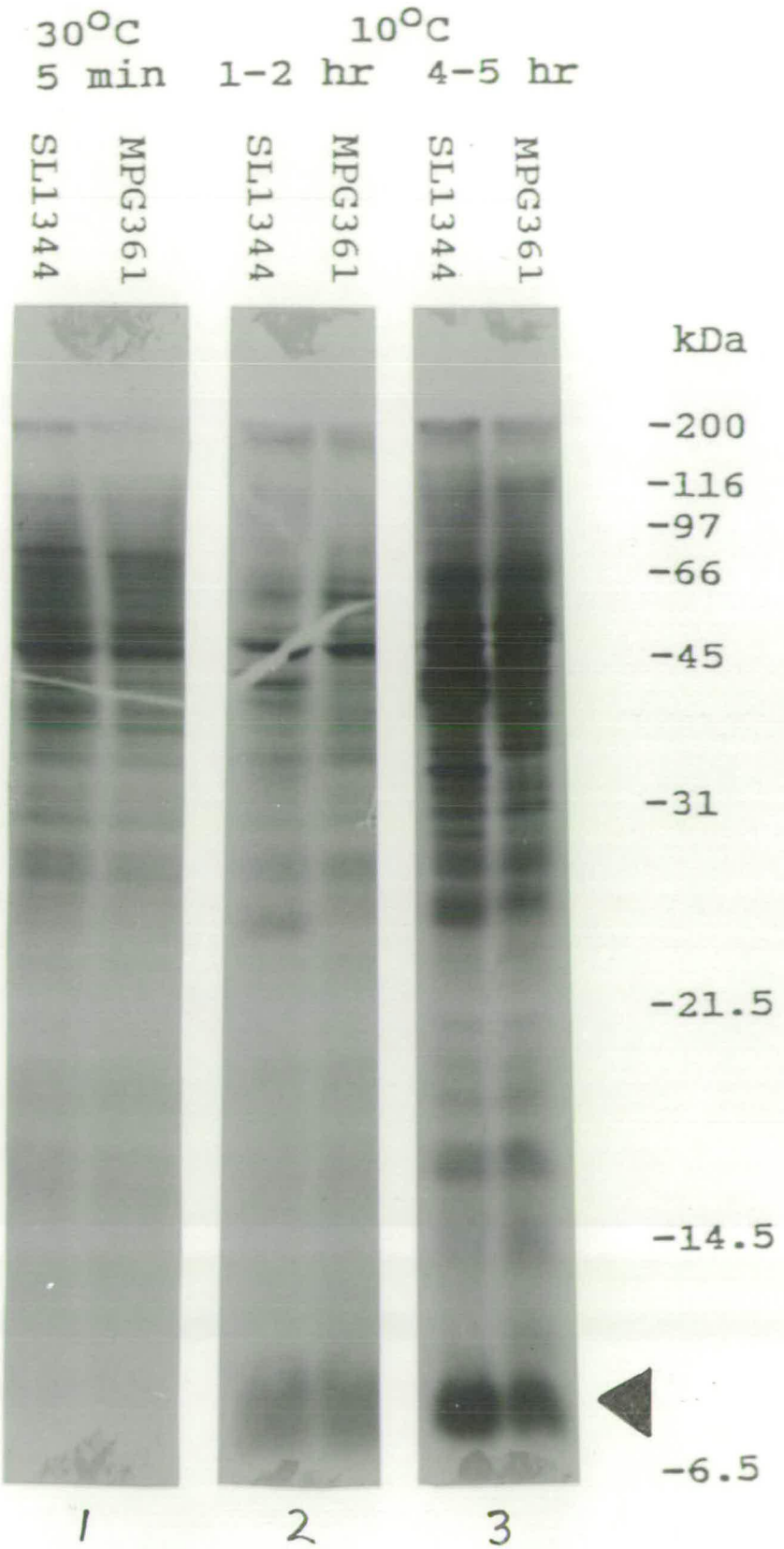
Previous work concerned with the characterization of CS7.4 in *E. coli* (Jones *et al*, 1987; Goldstein *et al*, 1990) demonstrated that this protein was easily recognised on both one- and two-dimentional protein

gels by pulse-labelling with radioactive methionine, due to a combination of its high level of induction during cold shock and its relatively small size. It was decided that the former of these two techniques should be used to allow a comparative analysis to be made between the protein profiles of MPG361 and its parental strain SL1344, both before and during cold shock treatment.

Exponentially growing cultures of SL1344 and MPG361 were prepared by diluting overnight cultures of these bacteria by 100-fold in minimal medium supplemented with 0.4% glucose and all of the common amino acids (1 mM) except methionine and incubating at 30°C until an optical density at 420 nm of approximately 0.3 - 0.5 was reached. Both cultures were then aliquoted into 3 equal volumes and pulse-labelled with ^{35}S-methionine at 30°C for 5 minutes or incubated at 10°C, and then labelled after 1 hour and 4 hours of cold shock (the latter two samples were each labelled for a period of 1 hours). Each sample was chased with 1 mM nonradioactive methionine for a further 5 minutes, and then denatured by boiling in Laemmli sample buffer and run on a 15% SDS-polyacrylamide gel. Subsequent comparison of the protein profiles (see figure 6.2) from SL1344 and MPG361 at each of these time points, demonstrated the presence of a highly induced protein band situated just above the 6.5 kDa protein marker in both SL1344 and MPG361 samples after 1 hour and 4 hours of cold shock treatment but which was absent in samples which were labelled at 30°C. This corresponded well with the previously reported position of CS7.4 in *E. coli* (Goldstein *et al*, 1990). From this data it was concluded that either the Mu*dlux* had not inserted within the *S. typhimurium* equivalent of the *cspA* gene, or that insertion of the Mu*dlux* element had resulted in the formation of a translational fusion of similar size to the CS7.4 protein.

Figure 6.2. MPG361 synthesizes a polypeptide comparable to CS7.4 during cold shock treatment.

Exponential cultures of MPG361 and SL1344 were grown to an optical density at 600 nm of approximately 0.3 - 0.5 in minimal medium supplemented with 0.4% glucose and all of the common amino acids (1 mM) except methionine. Each culture was then divided into 1 ml aliquots and pulse-labelled with 10 μCi of ^{35}S methionine for 5 minutes at 30°C or for 1 hour at 10°C after 1 and 4 hours of incubation at this latter temperature. Each sample was chased with 1 mM nonradioactive methionine for a further 5 minutes, and then denatured and run on a 15% SDS-polyacrylamide gel with an appropriate molecular weight protein marker (as described in methods). One-dimentional protein profiles from SL1344 and MPG361 prior to cold shock treatment (1), and 1-2 hours (2) and 4-5 (3) hours post-cold shock treatment are shown, with the position of a cold shock-induced protein indicated by an arrow (◂). The corresponding positions of the molecular weight protein marker after Coomassie staining are also shown.


30°C
5 min
10°C
1-2 hr
4-5 hr
SL1344
MPG361
SL1344
MPG361
SL1344
MPG361
kDa
-200
-116
-97
-66
-45
-31
-21.5
-14.5
-6.5
1
2
3

6.5 PCR of junction between Mu*dlux* and the target gene of MPG361.

Since it was still possible that the MPG361 fusion was situated within an *S. typhimurium* homologue of the *E. coli cspA* gene, an oligonucleotide primer (an eighteenmer designated A926; see table 2.1) was synthesized which was equivalent to the sense strand from the start of the *E.coli cspA* coding region (Goldstein *et al*, 1990). This *cspA* primer was then used, in conjunction with an oligonucleotide (a twentymer, designated 404X; see table 2.1) taken from a sequence of Mu DNA situated near the fusion junction (Kahmann and Kamp, 1979), to perform PCR over 30 cycles using chromosomal DNA taken from MPG361 (see chapter 2 for full methodology). The product was run on a 2% agarose gel to reveal a DNA fragment of approximately 200 base pairs. This suggested that the Mu*dlux* element had inserted into MPG361 approximately 100 bases downstream from the start of the *S. typhimurium cspA* coding region.

6.6 Sequencing of the PCR product derived from the junction between Mu*dlux* and the target gene of MPG361.

The precise DNA junction of the MPG361 gene fusion and the nature of the resulting translational product were determined by cloning and sequencing the PCR product corresponding to this region. The amplified DNA fragment was first blunt ended with Klenow polymerase and then ligated into an appropriate plasmid vector (pBluescript SK; see table 2.3) cut with *smaI*. The DNA was then transformed into NM522 cells of *E. coli*. Plasmid DNA was isolated from transformants and the insert was subsequently sequenced by the dideoxy-DNA sequencing method of Sanger and colleagues (1977), using double stranded DNA as

Figure 6.3. The Mu*dlux* element in MPG361 lies within a homologue of the *E. coli cspA* gene and results in a fusion protein of approximately the same size as CS7.4.

Panel A

The PCR product derived from MPG361, using a sense primer (an eighteenmer designated A926; see table 2.1) taken from the start of the *E. coli cspA* coding region (Goldstein *et al*, 1990) and an antisense primer (a twentymer designated 404X; see table 2.1) taken from a sequence of Mu DNA situated near the fusion junction (Kahmann and Kamp, 1979), was first blunt ended with Klenow polymerase and then cloned into Bluescript SK by blunt end ligation. The DNA was transformed into NM522 cells, and then sequenced using both the T7 bluescript primer (see table 2.1) and the Mu 404X primer (see table 2.1). The sequence of the cloned *cspS*::Mu fragment is shown (A). An additional base not shown in the reported sequence of Mu (Kahmann and Kamp, 1979) is indicated by an overlying dot (.). The first and last three bases shown in the sequence (ccc and ggg) represent pBluescript SK sequence.

Panel B

The location of the Mu*dlux* insert in *cspS* results in a polypeptide of 71 amino acids in length (B). The DNA fusion junction is denoted by an asterisk (*) with the termination codon TGA shown in bold. A potential putative nucleic acid binding domain (Schindelin *et al*, 1993b) is underlined.

A

SK *cspS*
ccc-ATGTCCGGTAAAATGACTGGTATCGTAAAATGGTTTAACCCTGAAAAGGGCTT
cspS Mu
TGGTTTCATTACGCCTA::tgaagcggcgcacgaaaaacgcgaaagcgtttcacgat
Mu SK
aaatgcgaaaactttagctttcgcgcttcaaatgaaacagatgtat-ggg

B

ATG TCC GGT AAA ATG ACT GGT ATC GTA AAA TGG TTT AAC
Met Ser Gly Lys Met Thr Gly Ile Val Lys Trp Phe Asn

*
CCT GAA AAG GGC TTT GGT TTC ATT ACG CCT ATG AAG CGG
Pro Glu **Lys Gly Phe Gly Phe Ile** Thr Pro Met Lys Arg

CGC ACG AAA AAC GCG AAA GCG TTT CAC GAT AAA TGC GAA
Arg Thr Lys Asn Ala Lys Ala Phe His Asp Lys Cys Glu

AAC TTT AGC TTT CGC GCT TCA AAT GAA ACA GAT GTA TTA
Asn Phe Ser Phe Arg Ala Ser Asn Glu Thr Asp Val Leu

ATT ACT GCT TTT TAT TCA TTA CAT GGG GAT CAC TTA GGG
Ile Thr Ala Phe Tyr Ser Leu His Gly Asp His Leu Gly

AAG GTG CGA ACA AGT CCC **TGA**
Lys Val Arg Thr Ser Pro

the template and the T7 bluescript primers or the Mu 404X primer to initiate the polymerisation (see table 2.1). Sequenase version 2.0 was used for the reactions. This confirmed that the MPG361 fusion was indeed located within a gene (subsequently designated *cspS*) (see figure 6.3A) that appeared very similar to the *E. coli cspA* gene (Goldstein *et al*, 1990), with the DNA fusion junction occurring to the 70th base from the start (ATG) of the *S. typhimurium cspS* coding region.

Comparison of this short region of *S. typhimurium* DNA with the corresponding 52 coding bases of the *E. coli cspA* gene (excluding the first 18 bases found in the oligonucleotide A926, which would automatically be identical), demonstrated these sequences to be 83% identical at the nucleotide level, with the corresponding predicted 17 amino acids (amino acids 7 - 23) encoded by both these regions of DNA sharing 88% identity (see figure 6.4). The open reading frame was found to continue translation across the fusion junction of MPG361 into the Mu sequence and resulted in a polypeptide of 71 amino acids in length (see figure 6.3B). As the *E. coli cspA* gene encodes a polypeptide (CS7.4) of 70 amino acids (Goldstein *et al*, 1990), it seems likely that these two gene products would be indistinguishable on a one-dimentional protein gel. This might explain the anomalous result observed in figure 6.2, where pulse-labelling of MPG361 cells at 10°C still resulted in the labelling of a protein of approximately 7 kDa, the predicted size of CspS.

6.7 Discussion.

Previous characterization of the Mu*dlux* gene fusion found in the *S. typhimurium* strain MPG300 (see chapter 3), demonstrated that the pattern of light induction (see figure 3.2) seen during temperature downshift of a culture of *S. typhimurium* carrying this mutation, was

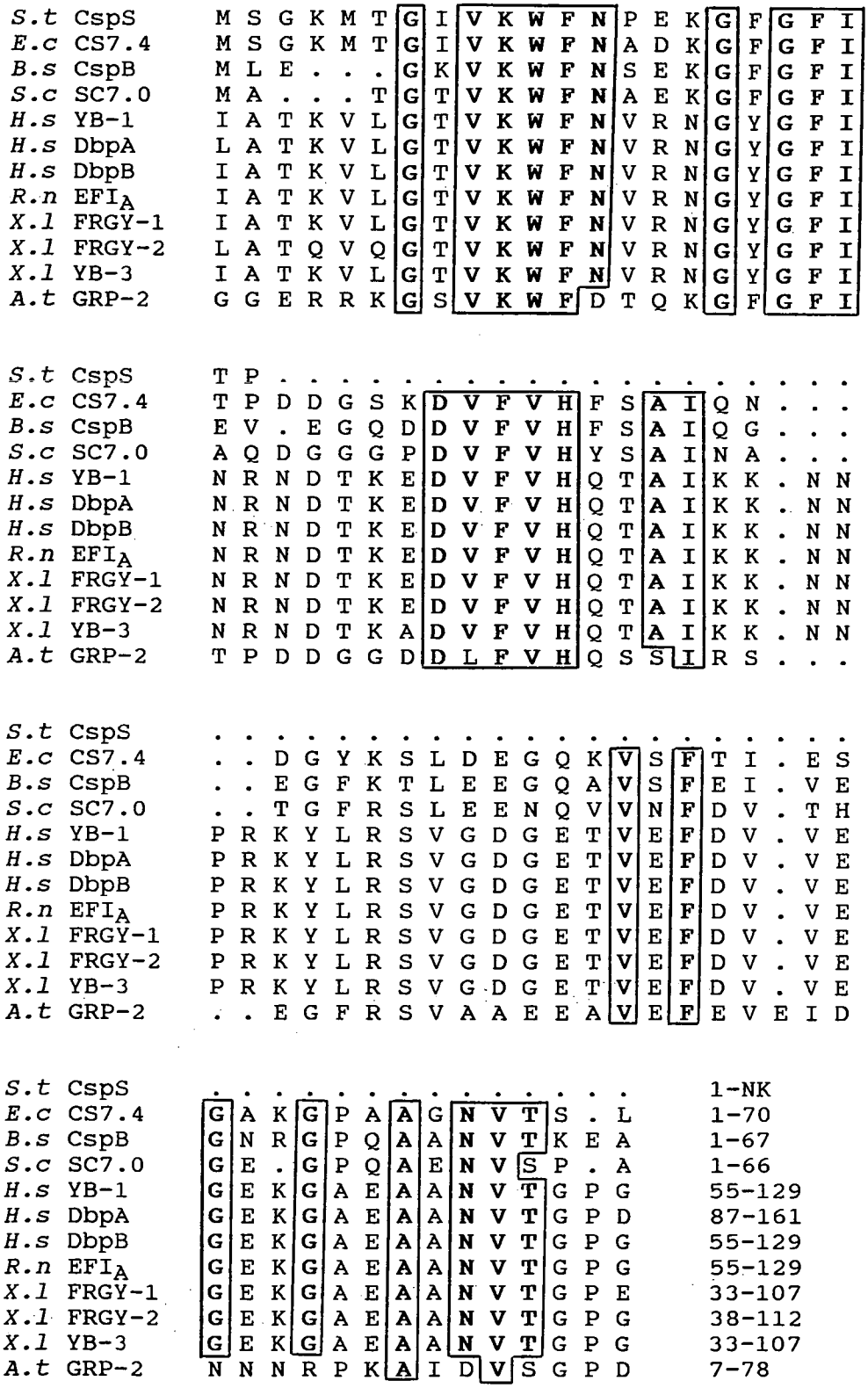
analogous to the induction profile observed for CS7.4 in *E. coli* following cold shock induction (Jones *et al*, 1987; Goldstein *et al*, 1990). Subsequent studies have now confirmed that the above fusion was indeed situated within an *S. typhimurium* gene (*cspS*) which is highly related to *cspA* of *E. coli*. This further attests to the ability of this light reporter system to monitor accurately gene induction from stress-response systems.

To date, an abundant cold shock protein homologous to CS7.4 has also been identified in *B. subtilis* (Willimsky *et al*, 1992) and *S. clavuligerus* (Av-Gay *et al*, 1992) following a temperature downshift. Comparative analysis of these related proteins demonstrated the general characteristics of this polypeptide to be small (approximately 7 kDa), hydrophilic, and to show a significant level of sequence identity to each other, and also (Wistow, 1990), to one of the domains of the human DNA-binding proteins DbpA, DbpB (Sakura *et al*, 1988) and YB-1 (Didier *et al*, 1988) (see figure 6.4). Since the region of *S. typhimurium* DNA identified from the MPG361 gene fusion was found to encode a cold shock protein with nearly 90% identity over 17 amino acids to the *cspA* gene of *E. coli* (see figure 6.4) and since *cspS* maps to approximately the same chromosomal region of DNA as *cspA* (see section 6.3), it seems probable that the complete protein (CspS) encoded by this *Salmonella* gene would also possess the characteristics associated with the aforementioned major cold shock proteins (*ie* small, hydrophilic, and showing a DNA-binding capacity).

Despite the above findings regarding the structural characteristics of these cold shock proteins, their functional importance to the cell during temperature downshift is still not known. Two potential roles for these proteins, however, have been suggested. The first was proposed by Goldstein and co-workers (1990) who anticipated that, based on its small size and its considerable proportion of hydrophilic residues, CS7.4 might

Figure 6.4. CspS shows some of the characteristics of a DNA-binding protein.

Shown is the alignment of the partially deduced amino acid sequence of the major cold shock protein of *S. typhimurium* (CspS, this work), and the full amino acid sequences of the CS7.4 protein from *E. coli* (Goldstein *et al*, 1990), and homologues from *B. subtilis* (CspB, Willimsky *et al*, 1992) and *S. clavuligerus* (SC7.0, Av-Gay *et al*, 1992), with the conserved regions of a number of eukaryotic DNA-binding proteins from *Homo sapiens* (YB-1, Didier *et al*, 1988; DbpA and DbpB, Sakura *et al*, 1988), *Rattus norvegicus* (EFI_A, Ozer *et al*, 1990), *Xenopus laevis* (FRGY-1 and FRGY-2, Tafuri and Wolffe, 1990; YB-3, Cohen and Reynolds, 1991) and *Arabidopsis thaliana* (GRP-2, De Oliveira *et al*, 1990). Identical amino acid residues are shown boxed and in bold, with dots (.) representing spaces in alignments. The position of aligned residues from each of these proteins are given at the end of each sequence.


S.t CspS M S G K M T G I V K W F N P E K G F G F I
E.c CS7.4 M S G K M T G I V K W F N A D K G F G F I
B.s CspB M L E . . . G K V K W F N S E K G F G F I
S.c SC7.0 M A . . . T G T V K W F N A E K G F G F I
H.s YB-1 I A T K V L G T V K W F N V R N G Y G F I
H.s DbpA L A T K V L G T V K W F N V R N G Y G F I
H.s DbpB I A T K V L G T V K W F N V R N G Y G F I
R.n EFI$_A$ I A T K V L G T V K W F N V R N G Y G F I
X.l FRGY-1 I A T K V L G T V K W F N V R N G Y G F I
X.l FRGY-2 L A T Q V Q G T V K W F N V R N G Y G F I
X.l YB-3 I A T K V L G T V K W F N V R N G Y G F I
A.t GRP-2 G G E R R K G S V K W F D T Q K G F G F I
S.t CspS T P . . . . . .
E.c CS7.4 T P D D G S K D V F V H F S A I Q N . . .
B.s CspB E V . E G Q D D V F V H F S A I Q G . . .
S.c SC7.0 A Q D G G G P D V F V H Y S A I N A . . .
H.s YB-1 N R N D T K E D V F V H Q T A I K K . N N
H.s DbpA N R N D T K E D V F V H Q T A I K K . N N
H.s DbpB N R N D T K E D V F V H Q T A I K K . N N
R.n EFI$_A$ N R N D T K E D V F V H Q T A I K K . N N
X.l FRGY-1 N R N D T K E D V F V H Q T A I K K . N N
X.l FRGY-2 N R N D T K E D V F V H Q T A I K K . N N
X.l YB-3 N R N D T K A D V F V H Q T A I K K . N N
A.t GRP-2 T P D D G G D D L F V H Q S S I R S . . .
S.t CspS . . . . . .
E.c CS7.4 . . D G Y K S L D E G Q K V S F T I . E S
B.s CspB . . E G F K T L E E G Q A V S F E I . V E
S.c SC7.0 . . T G F R S L E E N Q V V N F D V . T H
H.s YB-1 P R K Y L R S V G D G E T V E F D V . V E
H.s DbpA P R K Y L R S V G D G E T V E F D V . V E
H.s DbpB P R K Y L R S V G D G E T V E F D V . V E
R.n EFI$_A$ P R K Y L R S V G D G E T V E F D V . V E
X.l FRGY-1 P R K Y L R S V G D G E T V E F D V . V E
X.l FRGY-2 P R K Y L R S V G D G E T V E F D V . V E
X.l YB-3 P R K Y L R S V G D G E T V E F D V . V E
A.t GRP-2 . . E G F R S V A A E E A V E F E V E I D
S.t CspS 1-NK
E.c CS7.4 G A K G P A A G N V T S . L 1-70
B.s CspB G N R G P Q A A N V T K E A 1-67
S.c SC7.0 G E . G P Q A E N V S P . A 1-66
H.s YB-1 G E K G A E A A N V T G P G 55-129
H.s DbpA G E K G A E A A N V T G P D 87-161
H.s DbpB G E K G A E A A N V T G P G 55-129
R.n EFI$_A$ G E K G A E A A N V T G P G 55-129
X.l FRGY-1 G E K G A E A A N V T G P E 33-107
X.l FRGY-2 G E K G A E A A N V T G P G 38-112
X.l YB-3 G E K G A E A A N V T G P G 33-107
A.t GRP-2 N N N R P K A I D V S G P D 7-78

serve as an antifreeze protein in *E. coli*. This hypothesis has recently been given more credence by the comparison of the crystal structure of CspB from *B. subtilis* (Schindelin *et al*, 1993a) with that of the type III antifreeze protein (AFP) produced by fishes of the sub-order *Zoarcoidei* (Sonnichsen *et al*, 1993). These proteins show a considerable level of structural similarity, although no extensive similarity is obvious at the primary sequence level. Both polypeptides are small (66 and 67 residues respectively), are composed entirely of antiparallel β-sheets with connecting turns and loops, and have a globular structure of approximately 30 Å in diameter. Furthermore, the folding pattern of both proteins conceal an unusually high proportion of hydrophobic residues in the core, meaning that these proteins are artificially hydrophilic through not presenting extended hydrophobic areas to the solvent. Based on the structural similarities between CspB and this antifreeze protein, there is some logic in suggesting that one of the possible roles of the major cold shock protein of *B. subtilus*, and indeed the related major cold shock proteins from other bacterial species, is to protect their respective bacteria from damage by ice crystal formation.

In support of the above hypothesis, Goldstein and co-workers (1990) have shown that when cell cultures of *E. coli*, grown at 37°C, were frozen and thawed after preincubation at 10°C for 6 hours, there was as much as a 70-fold increase in survival compared to cells frozen and thawed without the 10°C preincubation. This suggests that either CS7.4 or another activity induced at low temperature was protecting *E. coli* cells from damage inflicted during subsequent freezing and thawing. Similarly, Willimsky and colleagues (1992) demonstrated that the insertional inactivation of *cspB* in *B. subtilis* rendered these cells more susceptible to killing by freezing at - 80°C and then thawing. Surprisingly, however, this group also found that this effect could be essentially nullified by the

preadaption of these cells at 10°C for 2 hours, suggesting that the major cold shock protein encoded by this gene was not the only factor involved in cold shock survival of this bacterium (or that the truncated polypeptide which resulted from insertional inactivation retained some functional activity).

The ability of MPG361 to survive freeze-thawing was not tested during the present study. Nevertheless, this mutant was shown to remain extremely tolerant to long term storage at low temperature (light could be detected from a culture of these cell for more than 4 months at approximately 8°C), indicating that this fusion did not have any major deleterious effects on the survival of *S. typhimurium* at low temperature. However, since characterization of the *cspS*::Mu protein fusion showed that approximately one third of the original cold shock protein remained unaltered, enough structural similarity may still remain to permit some degree of function, thereby resulting in a wildtype phenotype.

In contrast to the theory that bacterial cold shock proteins, such as CS7.4 and CspB, might function as antifreeze proteins, some workers (including La Teana *et al*, 1991; Jones *et al*, 1992; and Schindelin *et al*, 1993a and 1993b) have suggested that these proteins, like the human-DNA binding protein YB-1 (Didier *et al*, 1988), function as transcriptional regulators that specifically recognise direct or inverted CCAAT boxes (ATTGG). However, unlike YB-1, which is reported to be a negative regulatory factor that represses expression of the major histocompatibility complex class II genes in man, CS7.4 has been shown to be a positive regulatory factor, inducing synthesis of the *E. coli* H-NS protein (La Teana *et al*, 1991). NusA and GyrA have also been shown to contain CCAAT sequences within their promoter regions, and synthetic oligonucleotide copies of these sequences have been shown to bind purified CS7.4 in gel retardation assays (Jones *et al*, 1992b). Equally,

expression of CS7.4 under a controlled promoter *in vivo* in *E. coli* was show to result in enhanced levels of both NusA and GyrA (Jones *et al*, 1992b). The promoter region of *cspA* has also been demonstrated to possess a CCAAT sequence (Goldstein *et al*, 1990), although it has not yet been shown whether CS7.4 is capable of positive or negative autoregulation.

Further to the above findings, Schindelin and colleagues (1993a and 1993b) have reported that CspB preferentially binds to single stranded DNA and may be additionally capable of binding RNA. This more liberal nucleic acid binding ability suggests that this cold shock protein may have a wider function within bacterial cells than just acting as a transcriptional activator of certain cold shock genes. This would perhaps help to explain why this protein is induced to such high levels during a temperature downshift.

Interestingly, work by Almiron and colleagues (1992) has demonstrated that another DNA binding protein (designated Dps) that has been shown to regulate the synthesis of a number of proteins during its stress induction in *E. coli*, has the additional ability to protect cells from DNA damage. It therefore seems possible that CS7.4 and it contemporaries in other bacterial species may also have a dual function, perhaps by acting as specific antifreeze proteins capable of protecting cellular functions whilst also serving as a transcriptional activator. Alternatively, this protein species might act in a similar fashion to the cold shock inducible protein H-NS (La Teana *et al*, 1991). This DNA binding protein has been demonstrated to affect *in vitro* rRNA synthesis in *E. coli* systems by increasing the transition temperature between the open and closed forms of the rRNA promoter (Travers and Cukier-Kahn, 1974). Perhaps the binding of a major cold shock protein at or in the vicinity of a promoter region of a cold shock gene has a similar effect (*ie*

decreasing that promoter's transition temperature so allowing transcription). Another possibility, is that this cold shock protein might allow bacteria to continue the synthesis of proteins essential for their survival at low temperature by stabilizing, or possibly even altering, the topology of certain nucleic acids. A similar regulatory function has recently been attributed to H-NS during its interaction with certain DNA molecules (Tupper *et al*, 1994).

Close examination of the proposed protein fusion found in MPG361 (see figure 6.4) demonstrated that a chimeric polypeptide may still be produced (also indicated by figure 6.2) and that this might be capable of functioning as a nucleic acid binding protein. This was suggested by a comparison of the amino acid sequence of this protein with that of CspB (Willimsky *et al*, 1992), which has been reported to contain its putative nucleic acid binding domain within the central β-strand on subdomain 1 (Schindelin *et al*, 1993a and 1993b). This region of CspB, known as RNP-1, consists of the residues Lys, Gly, Phe, Gly, Phe, and Ile (Schindelin *et al*, 1993b), and is still present in the *S. typhimurium* MPG361 fusion protein (see figure 6.3B).

Work by Tanabe and colleagues (1992) has shown that CS7.4 is regulated at the level of transcription in *E. coli*, and that this regulation is probably initiated by a protein(s) which binds to the transcription initiation site during cold shock. This was deduced from *in vivo* footprinting experiments which showed that this site was protected at 14°C but not at 37°C, indicating that a specific cold shock factor was bound to this site during temperature downshift. This group has however, also shown that the site at which this unknown regulatory protein binds is different to the potential CCAAT binding site for CS7.4 that has been reported by Jones and co-workers (1992b). Gel retardation experiments conducted with purified CS7.4 and a DNA fragment from *cspA* containing

cspA containing both of the above protein binding sites, have demonstrated that the CS7.4 polypeptide itself showed no cold shock-specific binding (Tanabe *et al*, 1992). These findings suggest that, although CS7.4 may be involved in self-regulation at some level, this protein is probably not the major factor responsible for the transcriptional regulation of *cspA* following temperature downshift. (A potential candidate for the gene(s) encoding this unknown regulatory protein in *S. typhimurium* is discussed in chapter 7).

Despite the above findings, the underlying mechanisms of how a temperature fluctuation is sensed by the cell are still largely unknown. VanBogelen and Neidhardt (1990) have proposed that one possible mechanism for this temperature regulation in *E. coli* is *via* the ribosomes. This hypothesis is attractive because the major regulatory molecule for the heat shock response in this bacterium is σ^{32} (Strauss *et al*, 1987) and a significant proportion of its increase after a temperature upshift occurs at some post-transcriptional step (*eg* by increased translation of pre-existing mRNA for σ^{32}) (Strauss *et al*, 1987). The discovery therefore, that a low concentration of tetracycline (an antibiotic known to block the A site of bacterial ribosomes (Gale *et al*, 1981)) could induce light in an exponentially growing cultures of MPG361 (see figure 6.1), indicated that the synthesis of the major cold shock protein of *S. typhimurium* was also affected by the state of the ribosome, albeit in a minor way.

Furthermore, the fact that tetracycline-induced light production from the *cspS*::Mu*dlux* fusion was transient at room temperature (24°C) (see figure 6.1), whereas cold shock-induced light production from this fusion was maintained over a long period of time at low temperatures (see section 3.5), indicates that either the inducing signal produced by the addition of this antibiotic was different to that induced by cold shock treatment, or that the factor responsible for this induction was unstable or

inhibited at room temperature. Differences regarding the effect of low temperature or the addition of C-group antibiotics (VanBogelen and Neidhardt, 1990) on the regulation of the major cold shock protein have been reported by Jiang and colleagues (1993). This group showed that the transient induction of CS7.4 in *E. coli* at 15°C became constitutive upon the addition of low concentrations of chloramphenicol (an antibiotic shown to have a similar effects on protein induction in *E. coli* to tetracycline (VanBogelen and Neidhardt, 1990)). This again indicated that the latter antibiotic was capable of artificially transmitting a cold shock signal that was not deregulated by the normal mechanisms.

The reason why chloramphenicol did not induce light in MPG361 is not known. However, Jiang and colleagues (1993) also found that this antibiotic could not induce *E. coli* CS7.4 at 37°C (although the author does state that this latter response may have been due to the slightly higher levels of antibiotic used in this study compared to that previously used by VanBogelen and Neidhardt (1990)). An explanation might simply be that chloramphenicol is not as effective at inducing a cold shock response in *E. coli* or *S. typhimurium* cells as tetracycline.

How a bacterium would sense subtle environmental change in temperature reduction is not entirely known. However, it is conceivable that these organisms possesses a regulatory protein(s) (*eg* the ribosome or a sigma subunit) that is activated by a temperature induced conformational change, and that this new structure allows the molecule to regulate transcription of cold shock genes directly or indirectly, much in the same way as the oxidation of OxyR enables it to bind more specifically to the promoter regions of *oxyR*-regulated genes and induce proteins such as alkylhydroperoxide reductase (Storz *et al*, 1990a). Alternatively, it is possible that this latter hypothesis could apply to a molecule of nucleic acid. Nagai and colleagues (1991) have proposed that regulation of the

heat shock response is due to increased temperature in the cell causing a conformational change in the secondary structure of the *rpoH* mRNA. This in turn may lead to the synthesis of σ^{32} which mediates the synthesis of the heat shock proteins. Other possible regulatory mechanisms are discussed in the following chapter.

CHAPTER 7

Chapter 7: Characterization of chromosomal loci responsible for regulating cold shock induced light production in a range of *S. typhimurium* Mu*dlux* fusions.

7.1 Introduction.

Tanabe and colleagues (1992) have shown that the major cold shock protein in *E. coli*, CS7.4, is regulated at the level of transcription and that this regulation was probably initiated by a protein(s), other than itself, which bound upstream from the transcription initiation site of *cspA* during cold shock treatment. However, despite initial evidence for the existence of this *cspA* regulatory protein(s), no other information has been published as yet.

Probably one of the main reasons that the regulatory protein of *cspA* has not yet been fully characterized in *E. coli*, is that it has not been possible to identify its corresponding genetic locus. Previous studies directed at characterizing other cold shock proteins, such as CS7.4 (Jones *et al*, 1987; Goldstein *et al*, 1990; Tanabe *et al*, 1992), have resolved this problem by using approaches such as reverse genetics (using knowledge of the amino acid sequence of the protein under investigation to construct degenerate oligonucleotides which can be used to probe for the gene encoding the protein of interest). However, this technique is not always possible, especially when the protein is only synthesized in minor quantities and is difficult to detect.

Tanabe and colleagues (1992) have shown that the protein which bound near the transcription initiation site of *cspA* during cold shock was only synthesized during growth at low temperature. Previous studies by Jones and colleagues (1987) on the cold shock response of *E. coli* however, demonstrated that CS7.4 (F10.6) was the only detectable protein

to be synthesized exclusively during cold shock. This suggests that Tanabe's protein must be synthesized at a much lower level during cold shock than CS7.4, making the task of identifying the protein by reverse genetics extremely difficult.

An alternative method for resolving the problem would be to adopt a genetic approach. For example, if a culture of *E. coli* cells were randomly mutagenised, the gene(s) encoding the regulatory protein for *cspA* could then, theoretically, be isolated by screening for mutants that were unable to induce CS7.4 during cold shock. It would of course be essential to first tag *cspA* with a reporter system that would allow expression of this gene to be monitored. The *S. typhimurium* strain MPG361, which contained a Mu*dlux* insertion within an analogous gene to the *E. coli cspA* gene, therefore provided an ideal candidate for such an approach.

7.2 Isolation of mutants which influence cold shock-induced light production in MPG361.

The *S. typhimurium* cold shock fusion strain MPG361 was randomly mutagenised by Tn*10* mutagenesis. This resulted in the generation of approximately 55,000 independent transposon insertion mutants. Initial screening demonstrated that none of these mutants produced visible light at room temperature (23°C) on LB plates. These mutants were subsequently cold shocked overnight at 10°C and then carefully rescreened by eye for colonies that were no longer capable of producing light in response to the temperature downshift. A total of 23 dark colonies were isolated (designated MPG378 to MPG400). Each of these 23 colonies was purified and retested for their response to

temperature downshift. All were confirmed as no longer being cold shock inducible.

Colonies which retained their cold shock inducibility (the bulk of those screened in the above experiment) were returned to room temperature and stored overnight. These were then rescreened for loss of ability to inactivate the cold shock response *ie* continued (constitutive) light production at room temperature. Four colonies were found to produce a significant level of light under these conditions, whilst no light whatsoever was observed from any of the other colonies. However, only 2 of these mutants (designated MPG401 and MPG402), retested as positive and continued to produce post-cold shock light at room temperature for more than 4 days, after which time light from the overgrown colonies began to diminish. These were termed 'bright mutants'.

All 25 of the Tn*10* (dark and bright) mutants were further examined for their ability to co-transduce with the Mu*dlux* element by using phage P22 to transduce each of the Tn*10* elements into a wildtype SL1344 background. Transductants were selected on LB tetracycline plates, and then patched onto LB kanamycin. However, only one of the 23 Tn*10*s originally isolated from the dark mutants was found to transduce independently of the Mu*dlux* element. This Tn*10*, isolated from MPG386, was designated as Tn*10* D12. The colony morphology of the SL1344 strain bearing this Tn*10* (subsequently designated as MPG407) was distinctively flat and translucent, and had a reduced growth rate (as indicated by small colony size) - features which were also associated with the original isolate, MPG386. The remaining 22 Tn*10*s were all found to co-transduce with the Mu*dlux* element, indicating that the abolition of cold shock-induced light in these cases was probably due to the Tn*10* disrupting some part of the Mu*dlux* fusion or an upstream regulator *in cis*.

In contrast, both of the Tn*10* elements originally isolated from the bright mutants MPG401 and MPG402 (designated as Tn*10* C1 and Tn*10* C4, respectively), were found to transduce independently of their corresponding Mu*dlux* element. The resulting SL1344 Tn*10* transductants were subsequently designated as MPG408 and MPG409, respectively. Reintroduction of each of the three Tn*10* elements (Tn*10* D12, Tn*10* C1 and Tn*10* C4) into MPG361 by phage P22 transduction, resulted in the predicted dark or bright phenotypes, further confirming that the corresponding mutant characteristics resulted from the Tn*10* insertions.

7.3 Specificity of the Tn*10* mutations affecting regulation of the *cspS*::Mu*dlux* fusion.

Having established that the Tn*10* insertions in MPG386, MPG401 and MPG402 were responsible for either loss or constitutive expression of the *cspS*::Mu*dlux* fusion, it was decided to test if the effects of these Tn*10*s were specific to this one Mu*dlux* fusion or whether they were also capable of regulating other cold shock inducible fusions. A number of fusions, previously isolated as producing light in response to a temperature downshift (see tables 3.1, 3.2 and 3.3), were transduced with phage P22 from their *galE* background into wildtype SL1344 backgrounds. Each of these transductants was colony purified and then checked for its original cold shock phenotype (see tables 7.1 and 7.2 for strain designation). Next the Tn*10* elements from strains MPG407, MPG408 and MPG409 were transduced into each of the cold shock strains under study. The Tn*10* D12 element from MPG407 was also transduced into two control strains which were known to emit light constitutively (designated MPG349 and MPG419). These control strains were included to ensure that the elimination of light by Tn*10* D12 was

Table 7.1. Effect of Tn*10* D12 on the light emissions from cold shock-inducible fusions.

Shown are the light emissions (as detected visually on solid LB media) from a range of cold shock-inducible Mu*dlux* fusion strains, with and without the Tn*10* D12 insertion, both at room temperature (23°C) and after an overnight incubation at 10°C. Fusion numbers in brackets refer to the strain that the Mu*dlux* element was originally isolated in (*ie* prior to transduction into a clean background). MPG419 is a constitutive light-emitting fusion that was treated in an identical fashion to the cold shock-inducible fusions and acted as a control. E. High (extremely high), V. High, High, Medium and Low refer to light intensities. BV = barely visible and NV = not visible.

Table 7.1

Fusion number	Without Tn*10* D12 23°C	Without Tn*10* D12 10°C	With Tn*10* D12 23°C	With Tn*10* D12 10°C
MPG356 (MPG293)	NV	High	NV	NV
MPG357 (MPG295)	NV	High	NV	NV
MPG358 (MPG296)	NV	Medium	NV	NV
MPG359 (MPG297)	NV	High	NV	NV
MPG360 (MPG298)	NV	Low	NV	NV
MPG361 (MPG300)	NV	E.High	NV	NV
MPG362 (MPG303)	NV	V.High	NV	NV
MPG363 (MPG305)	NV	V.High	NV	NV
MPG365 (MPG307)	BV	V.High	BV	NV
MPG366 (MPG309)	NV	V.High	BV	NV
MPG367 (MPG310)	BV	Medium	BV	NV
MPG368 (MPG311)	NV	V.High	BV	NV
MPG369 (MPG313)	NV	V.High	NV	NV
MPG370 (MPG316)	NV	Low	NV	NV
MPG371 (MPG319)	NV	V.High	NV	NV
MPG372 (MPG322)	NV	Low	NV	NV
MPG373 (MPG291)	NV	Medium	NV	NV
MPG374 (MPG292)	NV	High	NV	NV
MPG375 (MPG279)	NV	High	NV	NV
MPG376 (MPG281)	NV	High	BV	NV
MPG377 (MPG288)	BV	Medium	BV	BV
MPG415 (MPG301)	NV	High	NV	NV
MPG416 (MPG302)	NV	High	NV	NV
MPG417 (MPG308)	NV	V.High	NV	NV
MPG418 (MPG290)	NV	Medium	NV	NV
MPG419	E.High	E.High	E.High	E.High

Table 7.2. Effect of Tn*10* C1 on the light emissions from cold shock-inducible fusions.

Shown are the light emissions (as detected visually on solid LB media) from a range of cold shock-inducible Mu*dlux* fusion strains, with and without the Tn*10* C1 insertion, at room temperature before cold shock treatment (23°C[1]), after cold shock treatment (10°C), and at room temperature 24 hours after cold shock treatment (23°C[2]). Fusion numbers in brackets refer to the strain that the Mu*dlux* element was originally isolated in (*ie* prior to transduction into a clean background). E. High (extremely high), V. High, High, Medium and Low refer to light intensities. BV = barely visible and NV = not visible.

Table 7.2

Fusion number	Without Tn*10* Cl			With Tn*10* Cl		
	23°C[1]	10°C	23°C[2]	23°C[1]	10°C	23°C[2]
MPG356 (MPG293)	NV	High	NV	Medium	High	NV
MPG357 (MPG295)	NV	High	NV	NV	BV	NV
MPG358 (MPG296)	NV	Medium	NV	NV	BV	NV
MPG359 (MPG297)	NV	High	NV	Low	Low	Medium
MPG360 (MPG298)	NV	Low	NV	Medium	High	NV
MPG361 (MPG300)	NV	E.High	NV	NV	E.High	High
MPG362 (MPG303)	NV	V.High	NV	Low	Medium	Low
MPG363 (MPG305)	NV	V.High	NV	Medium	Medium	High
MPG365 (MPG307)	NV	V.High	BV	Low	Low	High
MPG366 (MPG309)	NV	V.High	BV	Medium	High	Low
MPG367 (MPG310)	NV	Medium	BV	Low	Low	Low
MPG368 (MPG311)	NV	V.High	BV	Low	Medium	High
MPG369 (MPG313)	NV	V.High	NV	Low	Medium	Medium
MPG370 (MPG316)	NV	Low	NV	NV	BV	NV
MPG371 (MPG319)	NV	V.High	NV	Low	High	High
MPG372 (MPG322)	NV	Low	NV	NV	Low	NV
MPG373 (MPG291)	NV	Medium	NV	Low	Medium	Medium
MPG374 (MPG292)	NV	High	NV	BV	NV	Low
MPG375 (MPG279)	NV	High	NV	NV	Medium	Medium
MPG376 (MPG281)	NV	High	BV	Medium	Medium	Low
MPG377 (MPG288)	BV	Medium	NV	Medium	Low	Medium
MPG415 (MPG301)	NV	High	NV	Low	Medium	Medium
MPG416 (MPG302)	NV	High	NV	Low	High	Medium
MPG417 (MPG308)	NV	V.High	NV	NV	Medium	NV
MPG418 (MPG290)	NV	Medium	BV	Medium	Low	Medium

Table 7.3. Effect of Tn*10* C4 on the light emissions from cold shock-inducible fusions.

Shown are the light emissions (as detected visually on solid LB media) from a range of cold shock-inducible Mu*dlux* fusion strains, with and without the Tn*10* C4 insertion, at room temperature before cold shock treatment (23°C[1]), after cold shock treatment (10°C), and at room temperature 24 hours after cold shock treatment (23°C[2]). Fusion numbers in brackets refer to the strain that the Mu*dlux* element was originally isolated in (*ie* prior to transduction into a clean background). E. High (extremely high), V. High, High, Medium and Low refer to light intensities. BV = barely visible and NV = not visible.

Table 7.3

Fusion number	Without Tn*10* C4			With Tn*10* C4		
	23°C[1]	10°C	23°C[2]	23°C[1]	10°C	23°C[2]
MPG356 (MPG293)	NV	High	NV	Medium	High	NV
MPG357 (MPG295)	NV	High	NV	NV	Low	NV
MPG358 (MPG296)	NV	Medium	NV	Low	Medium	NV
MPG359 (MPG297)	NV	High	NV	High	V.High	NV
MPG360 (MPG298)	NV	Low	NV	High	High	NV
MPG361 (MPG300)	NV	E.High	NV	NV	E.High	High
MPG362 (MPG303)	NV	V.High	NV	Medium	High	Low
MPG363 (MPG305)	NV	V.High	NV	High	High	Low
MPG365 (MPG307)	NV	V.High	BV	Medium	Medium	Low
MPG366 (MPG309)	NV	V.High	BV	Medium	Medium	NV
MPG367 (MPG310)	NV	Medium	BV	Low	Low	Low
MPG368 (MPG311)	NV	V.High	BV	Medium	Medium	Medium
MPG369 (MPG313)	NV	V.High	NV	Medium	High	Low
MPG370 (MPG316)	NV	Low	NV	Low	Medium	NV
MPG371 (MPG319)	NV	V.High	NV	NV	Low	High
MPG372 (MPG322)	NV	Low	NV	NV	Low	Low
MPG373 (MPG291)	NV	Medium	NV	NV	Low	Medium
MPG374 (MPG292)	NV	High	NV	Low	Low	NV
MPG375 (MPG279)	NV	High	NV	NV	Medium	Medium
MPG376 (MPG281)	NV	High	BV	Medium	High	NV
MPG377 (MPG288)	BV	Medium	Low	Low	Low	Low
MPG415 (MPG301)	NV	High	NV	Low	Medium	Medium
MPG416 (MPG302)	NV	High	NV	Low	Medium	Medium
MPG417 (MPG308)	NV	V.High	NV	NV	Medium	NV
MPG418 (MPG290)	NV	Medium	BV	Low	Low	Low

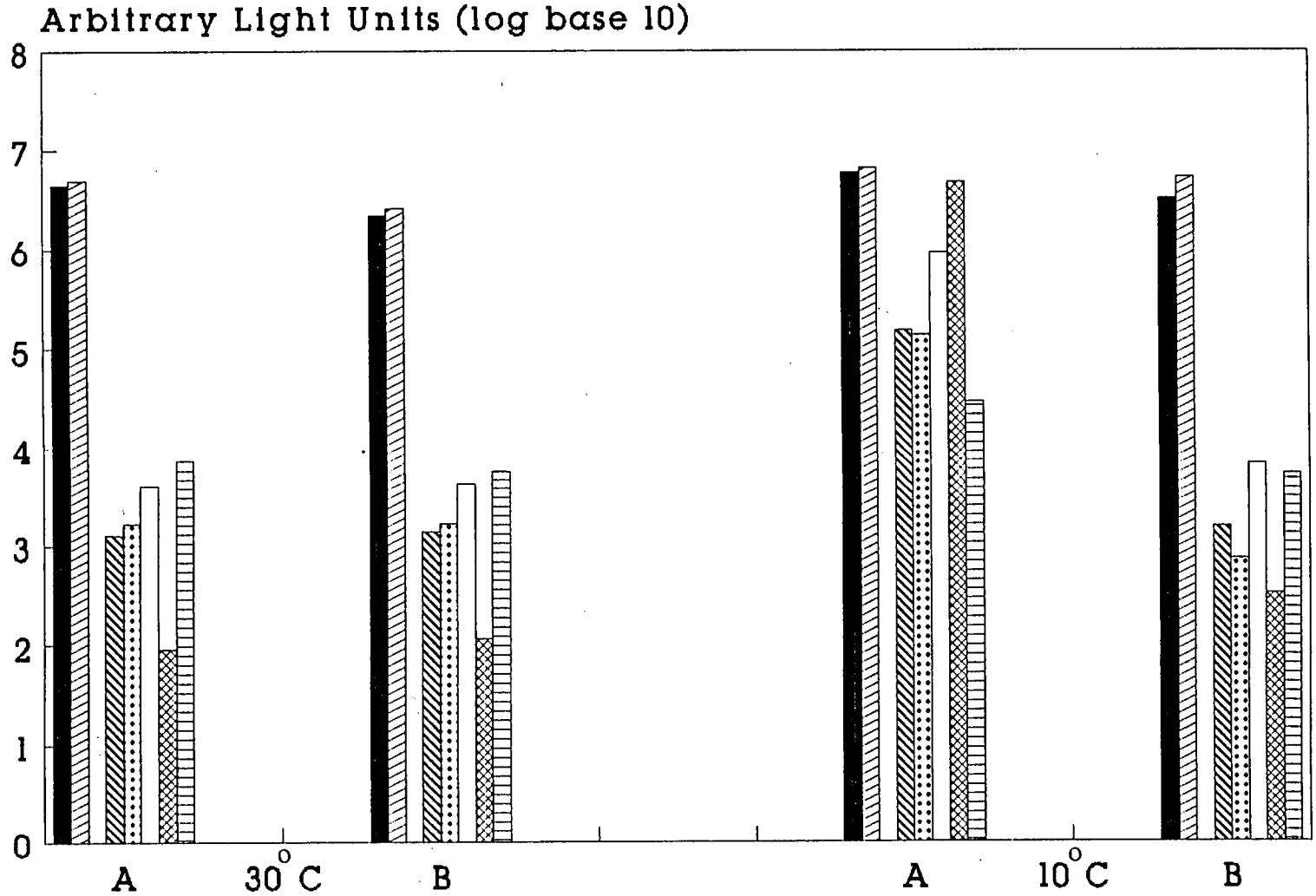
cold shock specific and did not represent a pleiotropic phenomenon. Transductants were then visually monitored for their light production at room temperature and after overnight incubation at 10°C on solid LB media (see tables 7.1, 7.2 and 7.3). A number of the transductants containing Tn*10* D12 were also tested for light emission in liquid culture (each prepared by diluting an overnight culture 100-fold in LB medium and incubating at 30°C for 1 hour to give approximately 10^7 c.f.u. ml^{-1}) at 30°C and after 4 hours at 10°C (see figure 7.1), using a liquid scintillation counter.

The results of the above experiments clearly showed that, on solid media, Tn*10* D12 was not only responsible for abolishing cold shock induced light in MPG361 at 10°C, but could also completely eliminate this light in all of the other cold shock inducible fusions under the same conditions (A basal level of light could be seen from the fusion MPG377 on solid media following cold shock. However, this emission was only of the same magnitude as that seen at room temperature prior to cold shock.) (see table 7.1). Furthermore, as previously observed for MPG386, all of the strains which received the Tn*10* D12 insertion by transduction formed colonies that were morphologically abnormal (*ie* flat and translucent) and slower growing than their parental strains. In contrast, the constitutive light emitting fusion MPG419, which also had it's morphology and growth rate altered when this Tn*10* was introduced, appeared relatively unaffected in its light production and still produced extremely high emissions on solid media, both at room temperature and during cold shock treatment.

A similar outcome to the solid media study was also observed with liquid culture (see figure 7.1). This experiment demonstrated that, whilst the induction of light following cold shock treatment was eliminated in each of these strains in the presence of Tn*10* D12, the basal levels of light observed at 23°C for each of the five cold shock fusions examined was

Figure 7.1. Tn*10* D12 has a universal effect on light production from cold shock fusions following a temperature downshift.

Five cold shock-inducible fusions (MPG376 (▨), MPG373 (▥), MPG371 (□), MPG361 (▩) and MPG377 (▤)) and two constitutive light emitting fusions (MPG349 (■) and MPG419 (▧)) were phage P22 transduced with the Tn*10* D12 element from MPG407 and colony purified. Light emissions from exponential cultures (prepared by diluting overnight cultures 100-fold in LB medium and incubating at 30°C for 1 hour to give approximately 10^7 c.f.u. ml^{-1}) of these transductants (B) and their parental strains (A) are shown at 30°C and after 4 hours at 10°C. Bioluminescence was recorded at room temperature using a single cell luminometer set on a sensitivity level of 6.0 mV and counting for 10 seconds (in each case a background (noise) value of approximately 90 arbitrary light units has been subtracted).


Arbitrary Light Units (log base 10)
8
7
6
5
4
3
2
1
0
A
30° C
B
A
10° C
B

present at roughly the same level following cold shock treatment in the presence of the Tn*10*. This indicated that the locus disrupted by this transposon was not responsible for regulating basal gene expression in the strains studied.

In comparison to the clear influence on regulation demonstrated by Tn*10* D12, neither of the two Tn*10*s which were shown to cause constitutive, post-cold shock light induction in MPG361 (*ie* Tn*10* C1 and Tn*10* C4) gave such unequivocal results when tested on the other cold shock-inducible fusions (see tables 7.2 and 7.3). In general, the latter two Tn*10*s acted to increase the overall level of background light at room temperature, both before and after cold shock treatment. A certain degree of variation was observed when the effects of these two Tn*10*s were compared in terms of the levels of light production from individual fusions (*ie* MPG359 and MPG365; tables 7.2 and 7.3). However, these differences were generally small. It could only be concluded that Tn*10* C1 and Tn*10* C4 might have a general regulatory effect on the expression of cold shock-inducible fusions.

7.4 Complementation of the cold shock regulatory locus abolished by insertion of Tn*10* D12.

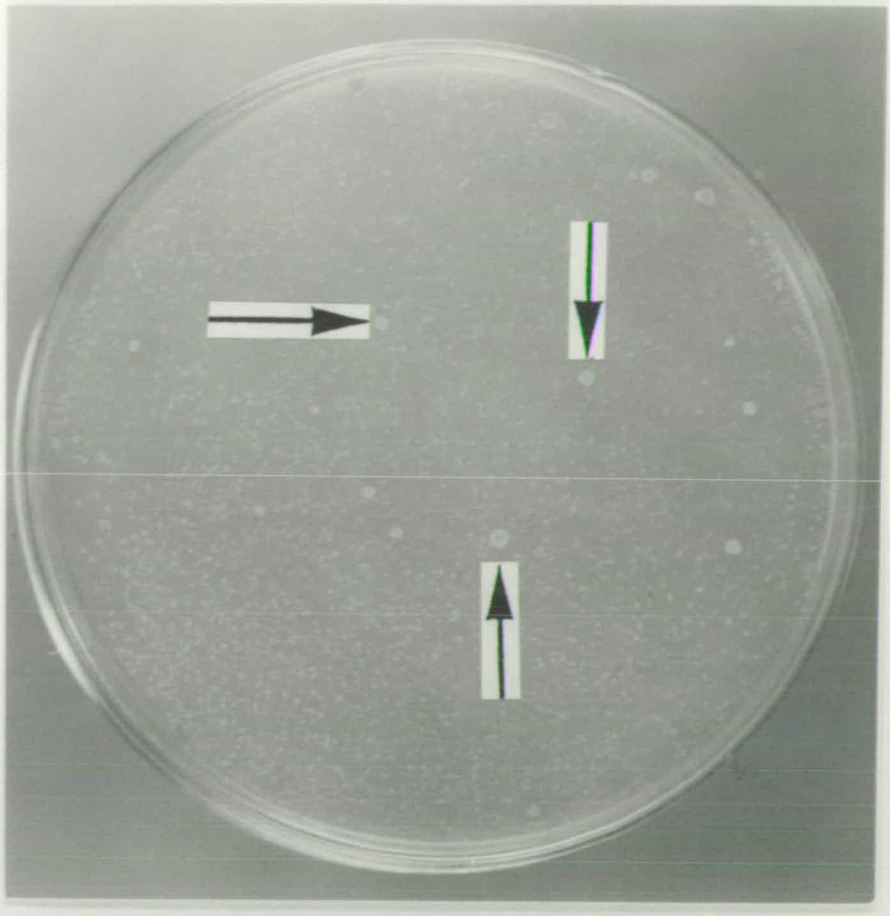
The initial characterization of the Tn*10* D12 Mu*dlux* fusions described above, established that the abolition of the cold shock-inducible light and the morphological changes observed in these strains were probably due to a single mutation. It was therefore predicted that complementation of both these phenotypes should be possible by introducing a single wildtype locus. To test this theory, a pBR325 plasmid library (see chapter 2 for details), containing random fragments of wildtype *S. typhimurium* DNA, was transduced into an overnight culture

of MPG386 with the aid of phage P22, and bacteria containing plasmid DNA were then selected on LB plates containing chloramphenicol. From figure 7.2A it can be seen that a number of the transductants (grown overnight at 30°C) regained wildtype growth rates and colony morphology, suggesting that these colonies had been complemented by a plasmid carrying an *S. typhimurium* locus corresponding to that disrupted by Tn*10* D12 or alternatively, a locus which was capable of suppressing the mutant phenotype. Furthermore, by subsequently placing this plate at 10°C overnight, it was shown that these faster growing transductants were also now capable of producing cold shock-induced light (see figure 7.2B). This supports the theory that these two phenotypic changes (altered morphology and the elimination of light production following cold shock) were due to disruption of a single genetic locus.

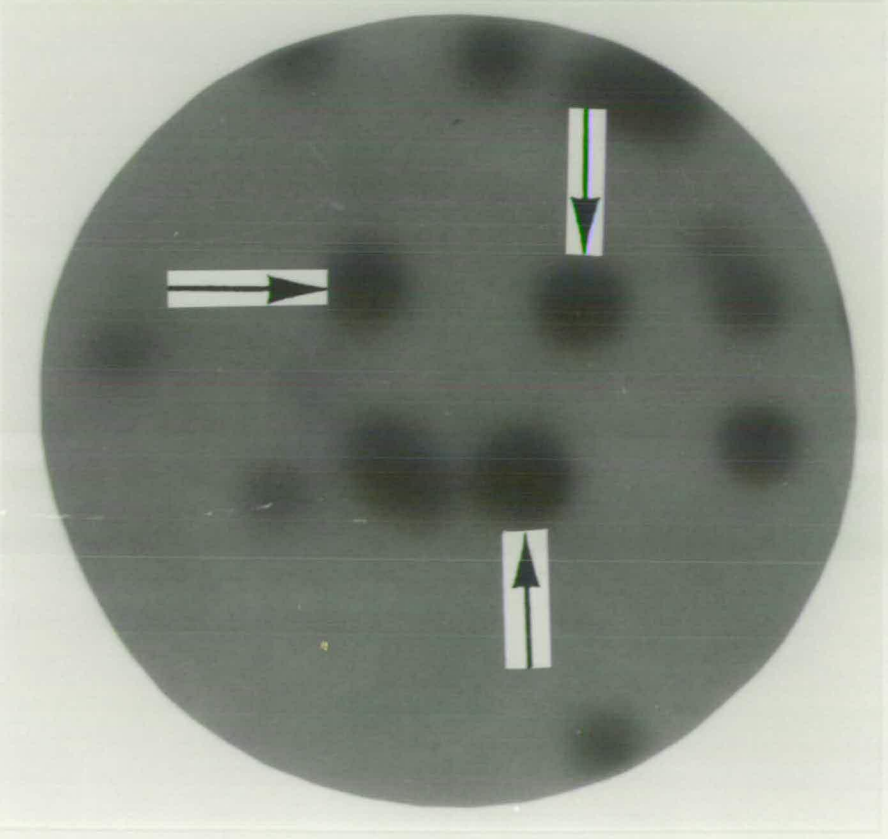
To confirm that the complementation seen in certain of the MPG386 transductants was plasmid borne and not due to some form of reversion, plasmid DNA (designated pKPF12) from a purified, light emitting, transductants was re-transduced into two independent Tn*10* D12 backgrounds (MPG386 (equivalent to SL1344 Mu*dlux*::*cspS* Tn*10* D12) and MPG407 (equivalent to SL1344 Tn*10* D12)). The resulting transductants (respectively designated MPG404 and MPG410) were both found to fully complement the mutant phenotypes associated with the Tn*10* D12 insertion (*ie* restoring normal colony morphology and growth rate in both MPG404 and MPG410 and, additionally, cold shock-induced light production in MPG404).

Figure 7.2. Phenotypic complementation of a Tn*10* D12 mutant.

An overnight culture of MPG386 was phage P22 transduced with a plasmid pBR325 library (see chapter 2 for details) containing random fragments of wildtype *S. typhimurium* DNA, and bacteria containing plasmid DNA were selected on LB chloramphenicol plates. Shown are a plate of such transductants (A) and their corresponding light emission (B) subsequent to this plate being place at 10°C overnight. Arrows are directed at three of these colonies which exhibit phenotypic complementation.



A



B

7.5 Comparing cold shock induced light production from the strains MPG361 with that from its derivatives MPG386 (MPG361 Tn*10* D12) and MPG404 (MPG361 Tn*10* D12 pKPF12).

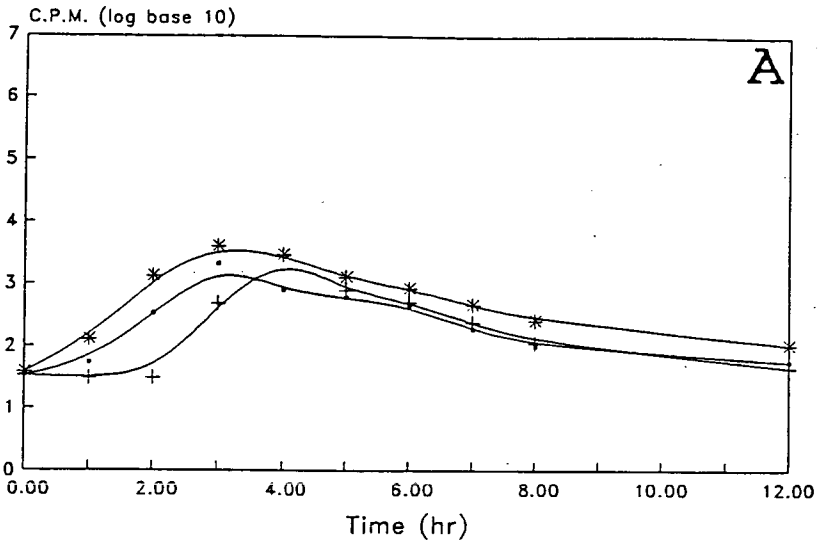
Light emissions from the cold shock inducible fusion MPG361 were compared with those of its derivatives, MPG386 (equivalent to MPG361 carrying Tn*10* D12) and MPG404 (equivalent to MPG361 carrying Tn*10* D12 and pKPF12). Exponentially growing cultures of these strains were prepared by diluting overnight cultures 100-fold in LB media and incubating at 30°C for 1 hour. Each culture was then aliquoted into 5 x 10 ml volumes and incubation was continued at either 24°C, 22°C, 15°C, 10°C, or 5°C for a further 12 - 48 hours, while being monitored at regular intervals in a liquid scintillation counter at room temperature. In each case relatively high densities of bacteria were used (approximately 5 x 10^7 c.f.u ml^{-1}) to ensure that any background light could be recorded.

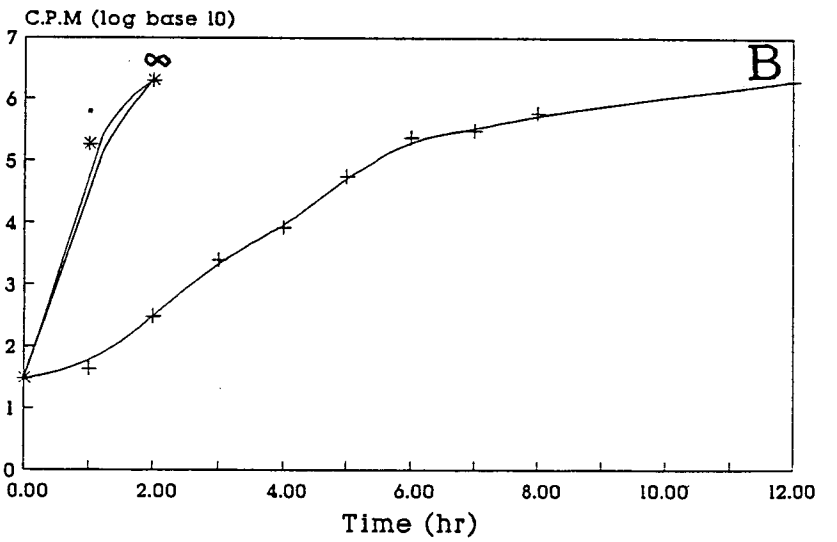
The results of this experiment (see figure 7.3) demonstrated that none of the strains was capable of producing substantial light emission at 24°C (see panel A). Nevertheless, it was possible to observe a small transient peak of light from each of these cultures approximately 3 - 4 hours after the initial temperature downshift (a drop of 6°C). Since this light emission was transient however, it is likely that the *cspS*::Mu*dlux* fusion was induced as a result of the sudden temperature decrease and was not due to specific cold shock regulation occurring at 24°C. Similar results have been observed for the induction of *cspA* in *E. coli* (Jones *et al*, 1992), though following a slightly larger temperature decrease.

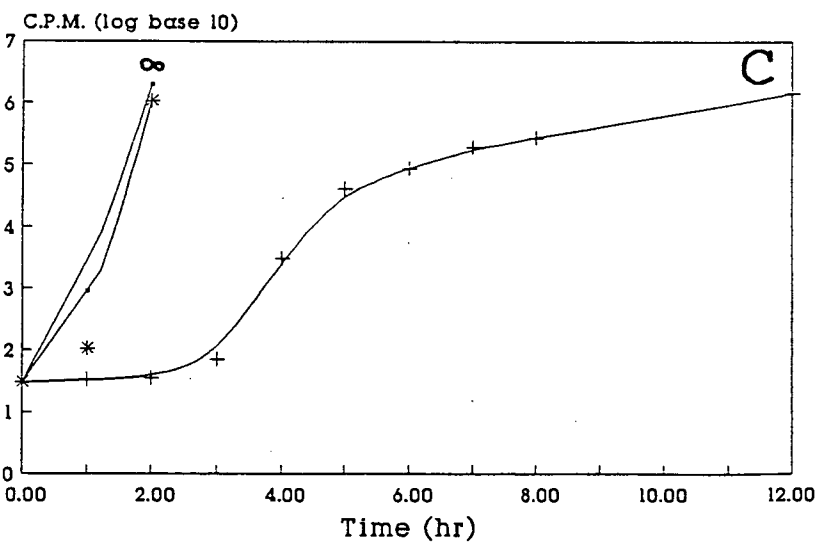
In contrast to the minimal and transient levels of light seen from each of the above cultures at 24°C, the patterns of light induction seen below this temperature were quite different. Light emissions recorded from cultures of the strain MPG361 at 22°C (see panel B), 15°C

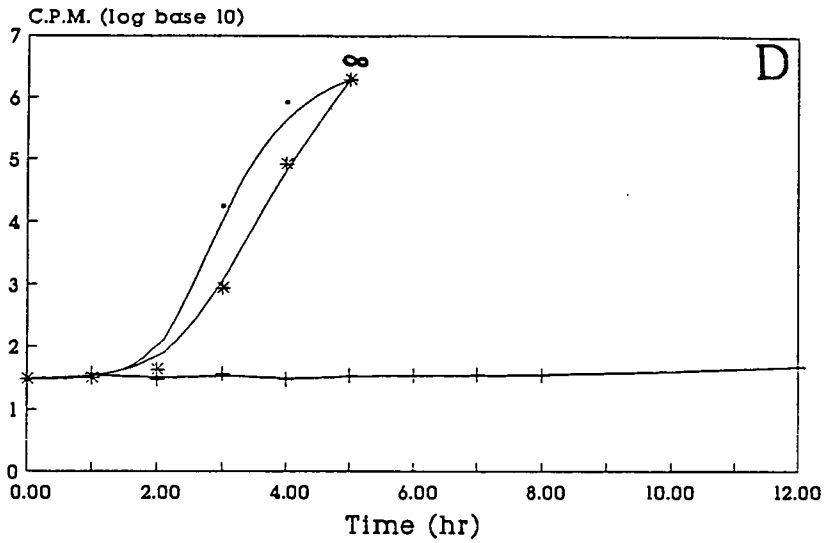
Figure 7.3. Tn*10* D12 efficiently abolishes light from the *cspS*::Mu*dlux* fusion at low temperature.

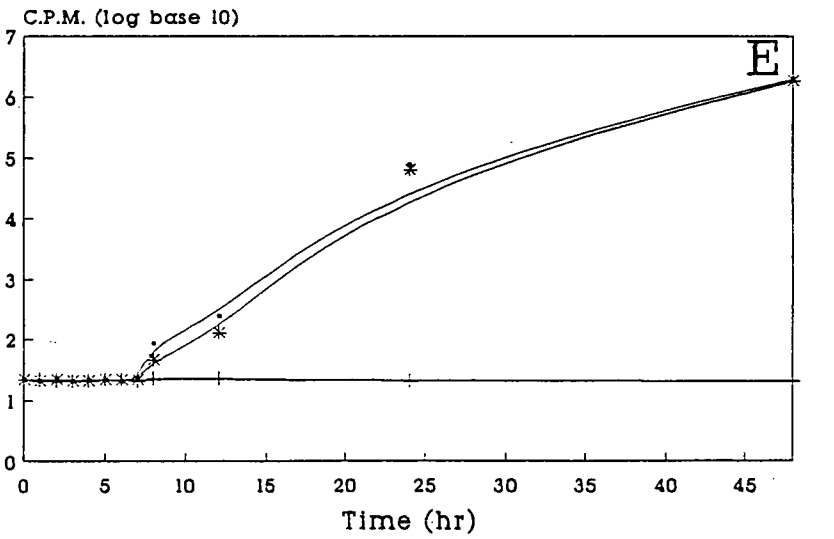
Exponential cultures of MPG361, MPG386 (equivalent to MPG361 carrying Tn*10* D12) and MPG404 (equivalent to MPG361 carrying Tn*10* D12 and pKPF12), were prepared by diluting overnight cultures 100-fold in LB medium and incubating for 1 hour at 30°C to give approximately 5 x 10^7 c.f.u ml^{-1}. The cultures were then divided into 5 aliquots and one of each was incubated at a different temperature. Shown are the light emissions from MPG361 (-•-), MPG386 (-*-) and MPG404 (-+-) at 24°C (A), 22°C (B), 15°C (C), 10°C (D) and 5°C (E), recorded in a liquid scintillation counter at room temperature. Values off scale are show as infinity (∞).


C.P.M. (log base 10)
A
0
1
2
3
4
5
6
7
0.00
2.00
4.00
6.00
8.00
10.00
12.00
Time (hr)


C.P.M (log base 10)
B
0
1
2
3
4
5
6
7
0.00
2.00
4.00
6.00
8.00
10.00
12.00
Time (hr)


C.P.M. (log base 10)
C
0
1
2
3
4
5
6
7
0.00
2.00
4.00
6.00
8.00
10.00
12.00
Time (hr)


C.P.M. (log base 10)
D
7
6
5
4
3
2
1
0
0.00
2.00
4.00
6.00
8.00
10.00
12.00
Time (hr)


C.P.M. (log base 10)
E
7
6
5
4
3
2
1
0
0
5
10
15
20
25
30
35
40
45
Time (hr)

(see panel C) and 10°C (see panel D) were extremely intense, and rapidly rose above the upper detectable limits of the scintillation counter. The remaining culture of this strain, incubated at 5°C, also produced substantial amounts of light, but this did not occur until approximately 24 hours had elapsed (see panel E). Very little light was registered from analogous cultures of the Tn*10* D12 containing strain MPG386, especially at 10°C and 5°C (see panels D and E). At the two intermediate temperatures of 22°C and 15°C a low level of background light was recorded which increased very slowly with time (see panels B and C). This is largely in agreement with previously recorded light data from cultures of MPG386 (see figure 7.1). Finally, the light profiles recorded from MPG404 (see panels B, C, D and E) demonstrated that the *S. typhimurium* locus present on the plasmid pKPF12 was capable of fully complementing the disruption caused by the Tn*10* D12 insertion at all temperatures.

7.6 Effect of Tn*10* D12 on the low temperature survival of *S. typhimurium* cells.

The above results demonstrated that the transposon insertion, Tn*10* D12, had a very dramatic effect on the regulation of all the cold shock-inducible *S. typhimurium* Mu*dlux* fusions isolated during this study. It therefore followed that the locus disrupted by this transposon might also be important for the survival of this bacterium in liquid culture following temperature downshift. In order to investigate this, an experiment was conducted comparing the long term survival of MPG407 at 10°C with that of the strains SL1344, MPG361 and MPG410 at this same temperature. Exponentially growing cultures of each of the above strains were prepared by diluting overnight cultures 10,000-fold in LB media and incubating at

30°C for 1 hour. Each culture, containing approximately 2 x 10^5 c.f.u. ml^{-1} (as determined by plating), was then placed in a 10°C waterbath and bacterial counts taken at regular intervals over a 6 day period.

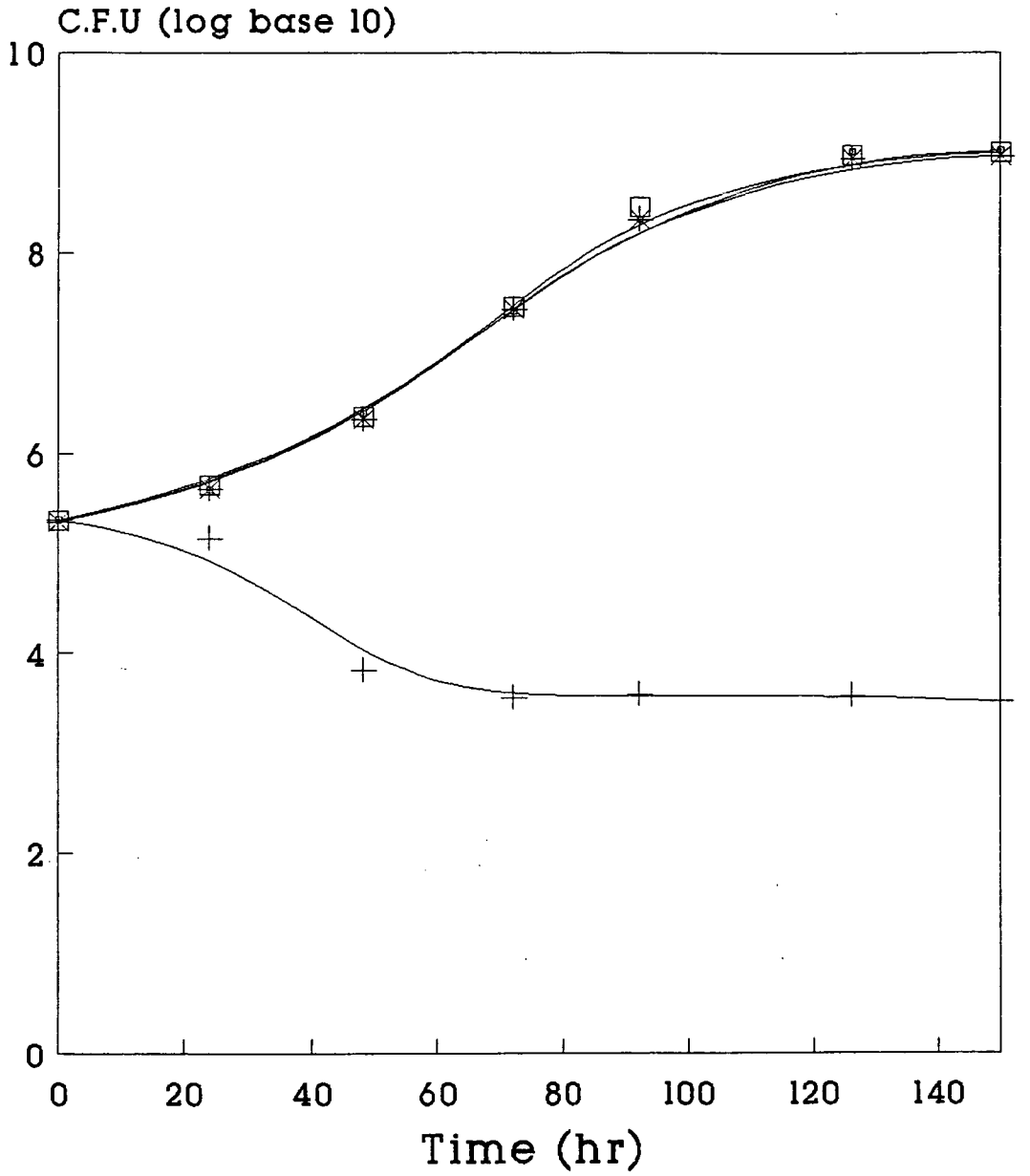
Figure 7.4 shows that the Tn*10* D12 mutation did indeed have an extremely detrimental effect on low temperature survival of MPG407, especially during the first 48 hours of this temperature downshift where there was almost a 100-fold decrease in the number of viable bacteria. After this time period, the rate of bacterial death became almost static. At each successive plating however, it was noted that the colony size became progressively smaller indicating perhaps, the viability of these bacteria was still decreasing..

In contrast to MPG407, both MPG361 and MPG410 were found to respond to low temperature in a similar fashion to the wildtype strain SL1344, indicating that neither of these strains was greatly altered in their cold sensitivity. In the case of MPG361, this confirmed previous findings that the disruption caused by this particular Mu*dlux* insertion was not overly detrimental to the low temperature survival of *S. typhimurium*, despite the fact that the element had inserted within the *cspS* gene which encodes the major cold shock protein of this bacterium (see chapter 6). Whether the fusion protein has retained any functional activity remains to be determined.

Finally, the wildtype survival patterns demonstrated by MPG410 (see figure 7.4) endorses previous results which showed that the plasmid pKPF12 fully complements the defects resulting from insertion of Tn*10* D12 (see figures 7.2 and 7.3). These data suggest that the control of cold shock-induced light production and the restoration of growth rate, colony morphology, and low temperature survival, are all co-ordinately regulated *via* a locus carried on pKPF12.

Figure 7.4. Tn*10* D12 has a detrimental effect on the survival of *S. typhimurium* at low temperature.

Overnight cultures (grown in LB) of SL1344, MPG361 (equivalent to SL1344 carrying *cspS*::Mu*dlux*), MPG407 (equivalent to SL1344 carrying Tn*10* D12) and MPG410 (equivalent to SL1344 carrying Tn*10* D12 and pKPF12), were diluted by 10,000-fold in fresh LB medium and grown to a density of approximately 2 x 10^5 c.f.u. ml^{-1} at 30°C. Each of these exponential cultures was then placed in a 10°C water bath and bacterial counts were taken at regular intervals over a 6 day period as shown: SL1344 (-•-), MPG361 (-*-), MPG407 (-+-) and MPG410 (-□-).


C.F.U (log base 10)
10
8
6
4
2
0
0
20
40
60
80
100
120
140
Time (hr)

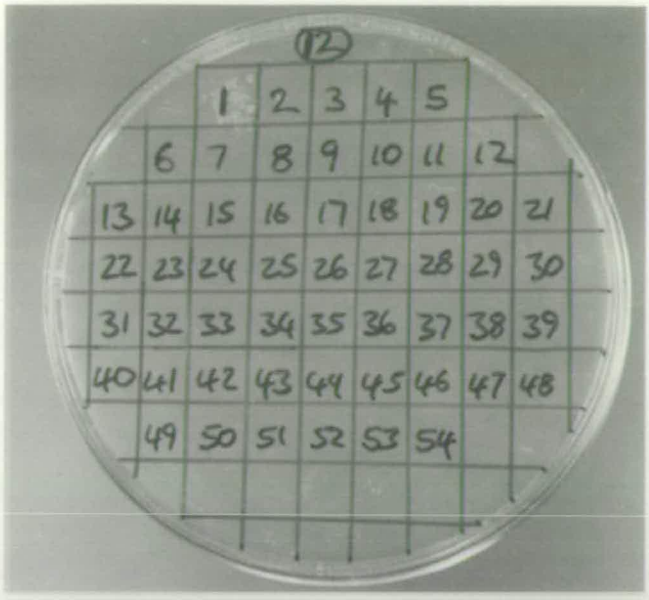
7.7 Mapping the chromosomal location of the three *S. typhimurium* loci disrupted by the transposons Tn*10* D12, Tn*10* C1 and Tn*10* C4.

The three transposon mutations Tn*10* D12, Tn*10* C1 and Tn*10* C4, previously characterized as causing alterations in the regulation of light production from cold shock-inducible Mu*dlux* fusions of *S. typhimurium* (see tables 7.1, 7.2 and 7.3), were genetically mapped on the *S. typhimurium* chromosome using a procedure developed by Benson and Goldman (1992). In brief, the strains MPG407 (containing Tn*10* D12), MPG408 (containing Tn*10* C1) and MPG409 (containing Tn*10* C4) were each grown to approximately 5 x 10^8 bacteria ml^{-1} in LB media containing tetracycline, at 37°C. The cells were washed in PBS, diluted 10-fold in the latter solution, and 100 µl of the suspension was plated on fresh Bochner plates. Each plate was then spotted with 5 µl volumes of each of a set of 54 Mu*d*-P22 bacteriophage lysates. (Each lysate is enriched for a defined region, approximately 3 minutes, of *S. typhimurium* chromosomal DNA from a range of genetic locations) (see table 7.4) Finally, the plates were incubated at 42°C for 24 to 48 h.

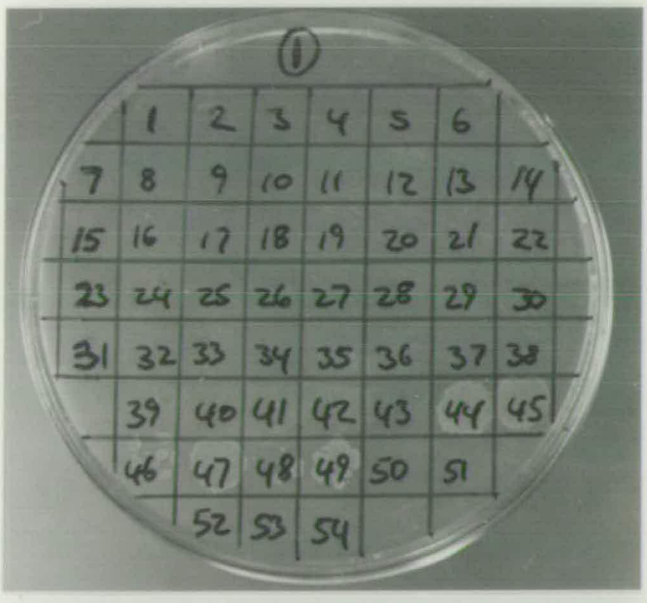
The results of this experiment are shown in figure 7.5, with the approximate map positions of the *S. typhimurium* chromosomal DNA contained within each Mu*d*-P22 lysates provided in table 7.4. Typically, the Mu*d*-P22 insertion close to the site of the Tn*10* insertion gave a confluent spot of tetracycline sensitive transductants, while the remaining spots appeared similar to the background of cells that did not receive any phage. As can be seen from the comparative region of bacterial growth on each of these plates, Tn*10* C1 (see figure 7.5B) and Tn*10* C4 (see figure 7.5C) were both eliminated by transduction with a number of Mu*d*-P22 lysates (numbers 44, 45, 46, 47, and 49), indicating that these two transposons mapped to approximately the same region on the

Figure 7.5. The chromosomal locations on *S. typhimurium* of the loci disrupted by the transposons Tn*10* D12, Tn*10* C1 and Tn*10* C4.

Tn*10* insertions within *S. typhimurium* strains were mapped using Mu*d*-P22 lysates (Benson and Goldman, 1992). Cultures of MPG407 (carrying Tn*10* D12), MPG408 (carrying Tn*10* C1) and MPG409 (carrying Tn*10* C4) were grown to a density of approximately 5 x 10^8 c.f.u. ml^{-1} in LB media. The cells from each culture were then washed once in phosphate-buffered saline, diluted 10-fold in the same solution, and 0.1 ml of each suspension was plated on fresh Bochner plates. The complete set of 54 Mu*d*-P22 transducing lysates were each diluted 10-fold, and 5 µl volumes were spotted onto the pre-spread Bochner plates, marked as shown. The plates were then incubated at 42°C for 24 to 48 hours. Confluent patches of growth represent tetracycline sensitive transductants resulting from the elimination of the Tn*10* by insertion of a Mu*d*-P22 in MPG407 (A), MPG408 (B) or MPG409 (C). Corresponding *S. typhimurium* chromosomal map locations are shown in table 7.4.


12
1 2 3 4 5
6 7 8 9 10 11 12
13 14 15 16 17 18 19 20 21
22 23 24 25 26 27 28 29 30
31 32 33 34 35 36 37 38 39
40 41 42 43 44 45 46 47 48
49 50 51 52 53 54

A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7 8 9 10 11 12 13 14
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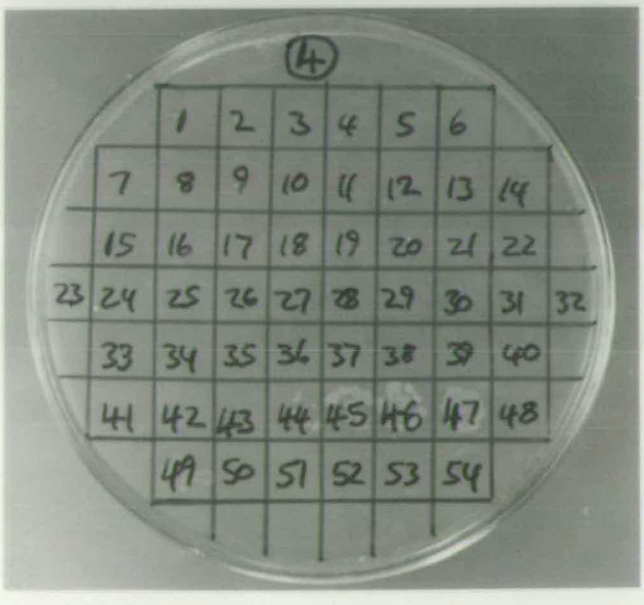
23 24 25 26 27 28 29 30
31 32 33 34 35 36 37 38
39 40 41 42 43 44 45
46 47 48 49 50 51
52 53 54

B


4
1 2 3 4 5 6
7 8 9 10 11 12 13 14
15 16 17 18 19 20 21 22
23 24 25 26 27 28 29 30 31 32
33 34 35 36 37 38 39 40
41 42 43 44 45 46 47 48
49 50 51 52 53 54

C

Table 7.4. The chromosomal locations of *S. typhimurium* loci involved in cold shock, mapped using Mu*d*-P22.

Shown are the chromosomal map positions (in minutes) of a number of the genetic loci found to be involved in the cold shock response in *S. typhimurium*. These were mapped using a set of 54 Mu*d*-P22 lysates (Benson and Goldman, 1992). The letters anoted after each minute region refers to phage packaging of chromosomal DNA in a clockwise (A) or anti-clockwise (B) direction. Plus (+) and minus (-) symbols indicate the degree of homology between the locus being mapped and the DNA present in the corresponding lysate.

Table 7.4

Mud-P22 lysogen	Minutes	Tn*10* C1	Tn*10* C4	Tn*10* D12	pKPF 12	*cspA*
1	0.00 (A)	-	-	+++	+++	-
2	0.00 (B)	-	-	-	-	-
3	3.5 (A)	-	-	-	-	-
4	3.5 (B)	-	-	-	++	-
5	7 (A)	-	-	-	-	-
6	7 (B)	-	-	-	-	-
7	8.5 (A)	-	-	-	-	-
8	12 (A)	-	-	-	-	-
9	12 (B)	-	-	-	-	-
10	14 (A)	-	-	-	-	-
11	14 (B)	-	-	-	-	-
12	17 (A)	-	-	-	-	-
13	17 (B)	-	-	-	-	-
14	21.5 (A)	-	-	-	-	-
15	21.5 (B)	-	-	-	-	-
16	28.5 (A)	-	-	-	-	-
17	28.5 (B)	-	-	-	-	-
18	33 (A)	-	-	-	-	-
19	30 (B)	-	-	-	-	-
20	36 (A)	-	-	-	-	-
21	36 (B)	-	-	-	-	-
22	40.5 (A)	-	-	-	-	-
23	40.5 (B)	-	-	-	-	-
24	50 (A)	-	-	-	-	-
25	50 (B)	-	-	-	-	-
26	52 (A)	-	-	-	-	-
27	52 (B)	-	-	-	-	-
28	54 (A)	-	-	-	-	-
29	54 (B)	-	-	-	-	-
30	57 (A)	-	-	-	-	-
31	57 (B)	-	-	-	-	-
32	60 (B)	-	-	-	-	-
33	62.7 (A)	-	-	-	-	-
34	62.7 (B)	-	-	-	-	-
35	65 (A)	-	-	-	-	-
36	65 (B)	-	-	-	-	-
37	68.5 (A)	-	-	-	-	-
38	68.5 (B)	-	-	-	-	-
39	72 (B)	-	-	-	-	-
40	73.6 (A)	-	-	-	-	-
41	73.6 (B)	-	-	-	-	-
42	79.7 (A)	-	-	-	-	-
43	79.7 (B)	-	-	-	-	++
44	83 (A)	+++	+	-	-	-
45	83 (B)	+++	++	-	-	-
46	84 (A)	+	++	-	-	-
47	84 (B)	+++	+++	-	-	-
48	86.7 (A)	+	-	-	-	-
49	86.7 (B)	++	+	-	-	-
50	93 (A)	-	-	-	-	-
51	93 (B)	-	-	-	-	-
52	96.1 (A)	-	-	-	+	-
53	97 (A)	-	-	-	++	-
54	97 (B)	-	-	-	-	-

S. typhimurium chromosome (around 83 minutes). (The reason why so many of these lysates were seen to abolish both Tn*10* C1 and Tn*10* C4 was due to overlapping regions of bacterial DNA (see table 7.4)). In contrast, Tn*10* D12 (see figure 7.5A) was eliminated by transduction with only one lysate (number 1) which contained *S. typhimurium* DNA that maps to a completely different region of this bacterium's chromosome (approximately 0 - 3 minutes).

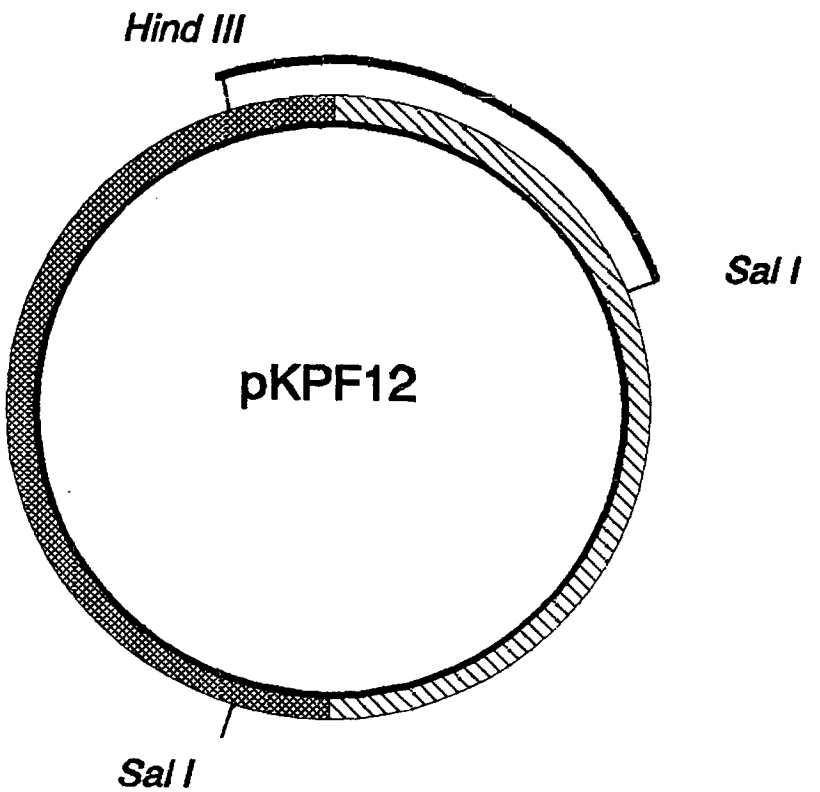
7.8 Mapping the chromosomal location of the pKPF12 *S. typhimurium* locus found to complement the disruption caused by the transposon Tn*10* D12.

The Mu*d*-P22 mapping set described above (Benson and Goldman, 1992) was also used to identify the chromosomal origin of the *S. typhimurium* DNA insert in pKPF12 that had previously been found to complement the defect which resulted from insertion of Tn*10* D12 in MPG386 and its derivatives. To achieve this a DNA dot blotting procedure was used (see chapter 2 for full methodology). This entailed radiolabelling a *HindIII/SalI* fragment (approximately 3 kb) of pKPF12 (see figure 7.6) by random priming with ^{32}P, and then hybridizing this to a nylon filter upon which denatured DNA from the 54 Mu*d*-P22 bacteriophage lysates had been bound along with a number of DNA controls (see legend from figure 7.7).

As can be seen in figure 7.7A, autoradiography of this filter demonstrated that the DNA probe from pKPF12 hybridized most strongly to the same lysate (#1) which was shown to result in exclusion of the Tn*10* D12 from MPG407 (see figure 7.5A). However, this DNA probe was also found to hybridize less efficiently to a number of other lysates (#4, #52 and #53). These lysates map from 96.1 minutes to 3.5 minutes

Figure 7.6. Restriction map of pKPF12 cut with *HindIII/SalI*.

The plasmid, pKPF12 (pBR325 with a *Sau3a* fragment of *S. typhimurium* DNA inserted at the *BamHI* site), which was found to phenotypically complement *S. typhimurium* mutants carrying the Tn*10* D12 insertion (see figure 7.2), was cut with the a range of restriction enzymes, including *HindIII* and/or *SalI*. A restriction map of pKPF12 cut with *HindIII/SalI* was derived, as shown. The approximate position of the pKPF12 *HindIII/SalI* DNA fragment used as a probe is shown as the region marked with a bold outlying line, this consist of approximately 0.5 kb of pBR325 DNA and 2.5 kb of *S. typhimurium* DNA.



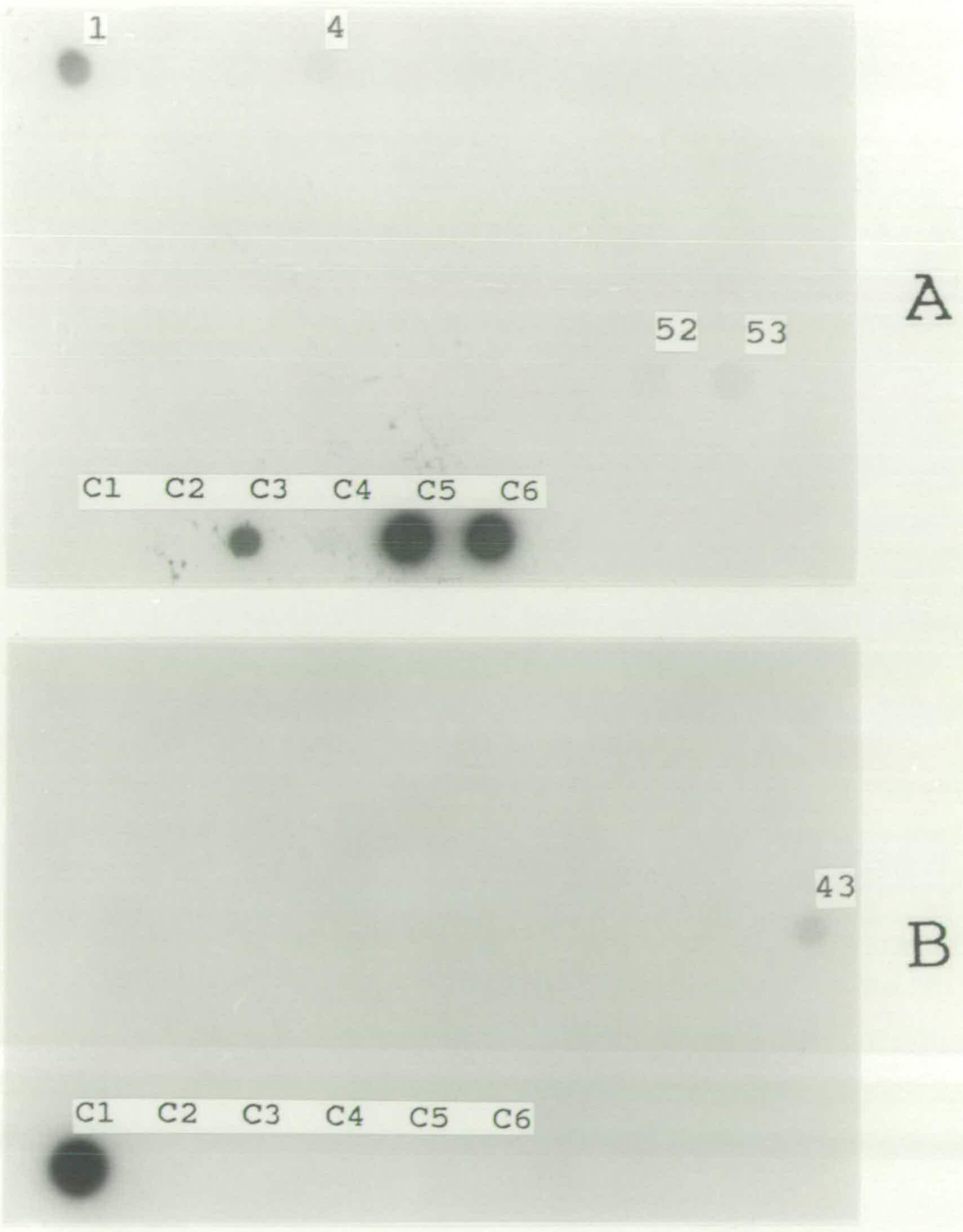
pBR325 DNA (approx. 6 kb)

S. typhimurium DNA (approx. 6 kb)

DNA fragment used as probe (approx. 3 kb)

Figure 7.7. The region of *S. typhimurium* DNA found on the plasmid pKPF12 maps to the same chromosomal locus as the transposon Tn*10* D12.

The chromosomal location of the *S. typhimurium* DNA inserted in pKPF12 was mapped by hybridization to Mu*d*-P22 lysates (Benson and Goldman, 1992). Denatured DNA from the 54 Mu*d*-P22 lysates (100 µl volumes) was bound to a nylon filter using a BioRad 96 well microfiltration apparatus (see methods), starting with lysate #1 in the top left corner well (A1) and adding subsequent lysates across the plate (left to right) from top to bottom. DNA controls (1 µg made up to 100 µl with H_2O) were also added as follows: PCR product of *cspA* from *E. coli* (C1), arbitrary PCR product (C2), pBR325 (C3), pBluescript (C4), pKPF12 from DH1 (C5), pKPF12 from MPG404 (C6). Shown are the autoradiographs from this filter probed with a ^{32}P random primed DNA fragments of pKPF12 cut with *HindIII/SalI* (panel A) and a PCR product of *cspA* from *E. coli* (panel B). Corresponding *S. typhimurium* chromosomal map locations are show in table 7.4.

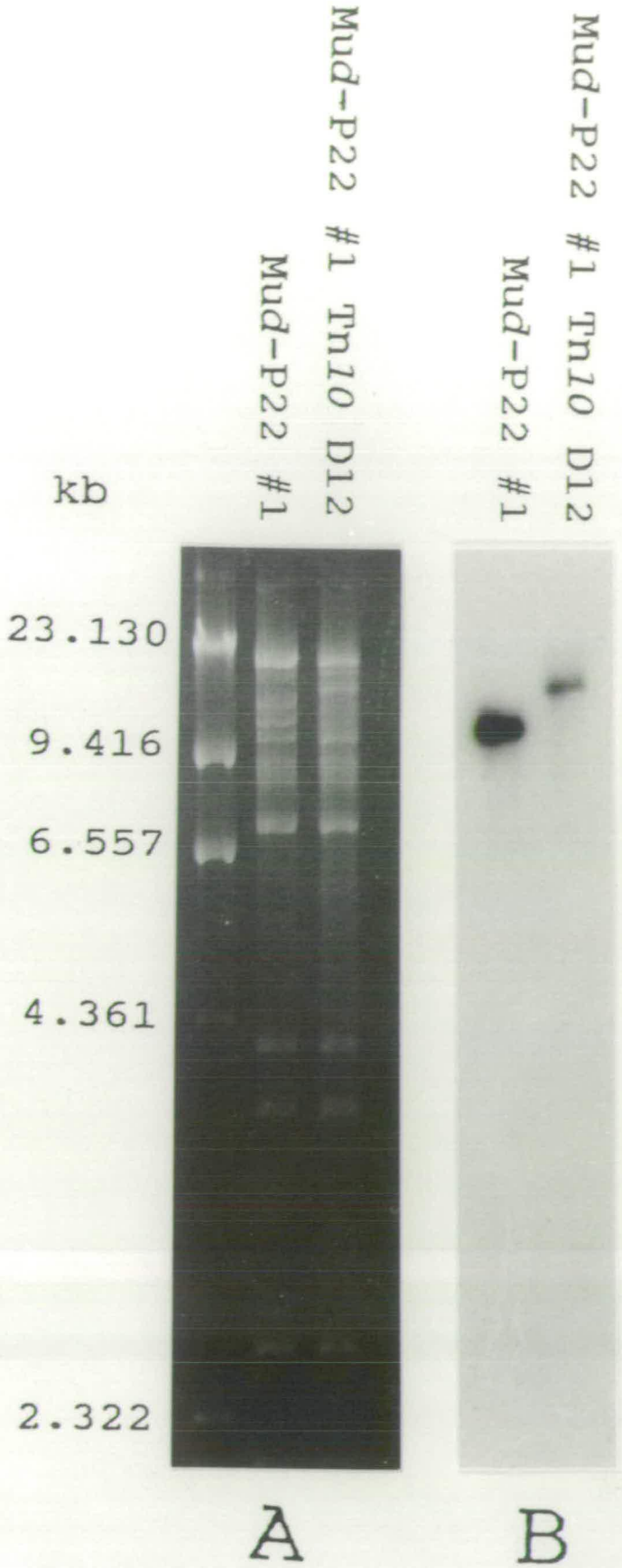

1
4
A
52
53
C1
C2
C3
C4
C5
C6
43
B
C1
C2
C3
C4
C5
C6

on the *S. typhimurium* chromosome and, taken together, place the insert found on pKPF12 at approximately 0 - 1 minutes (see table 7.4). This suggests that pKPF12 is likely to carry a copy of the wildtype locus disrupted by Tn*10* D12, rather than an unlinked locus which is capable of suppressing the defect (perhaps as a result of expression from a multicopy plasmid). Hybridization of this probe was also shown to occur strongly with purified pKPF12 (C5 and C6) and to the vector DNA (pBR325) without the insert (C3), indicating that the probe consisted of both pBR325 DNA and *S. typhimurium* DNA. No hybridization was observed between the probe and DNA corresponding to a PCR product of the *E. coli cspA* gene (A; see below).

The same procedure was also used to confirm the map location of the *S. typhimurium* homologue of the *E. coli cspA* gene by using a radiolabelled fragment of this latter gene to probe a second filter analogous to that described above. (The *E. coli cspA* probe used in this procedure was prepared through PCR of *E. coli* DNA from the strain W3110, amplified using the *cspA* primers A211 and A212 (see table 2.1) (Goldstein *et al*, 1990)). Hybridization of the *cspA* fragment (see figure 7.7B) was found to occur only with the DNA from the Mu*d*-P22 lysate number 43 which is enriched with genomic DNA from approximately 76.7 - 79.7 minutes. This is in good agreement with the 78 minutes recorded from mapping the MPG361 Mu*dlux* fusion by Tn*10* transduction (see chapter 6), and confirms the accuracy of this mapping procedure. As expected, the *E. coli cspA* probe was found to hybridize strongly to itself (C1), but none of the above pKPF12 controls (*ie* C5 and C6).

Figure 7.8. The *S. typhimurium* locus present on plasmid pKPF12 is the same as that disrupted by the transposon Tn*10* D12.

Mu*d*-P22 lysates were prepared from the *S. typhimurium* strains TT15223 (the strain used to produce Mu*d*-P22 lysate number 1 to which both Tn*10* D12 and pKPF12 had been found to map (see figures 7.5 and 7.6, and table 7.4)) and this same strain carrying Tn*10* D12. DNA was then extracted from the phage in each of these lysates (1 ml), and approximately 5 μg of each type of was DNA digested with *BglII*, and run on a 0.8% agarose gel with a set of DNA molecular weight markers (panel A). The gels was then Southern blotted and probed with a *HindIII/SalI* fragment from pKPF12, radiolabelled with ^{32}P by random priming (panel B) (exposed to X-ray film overnight at room temperature).


Mud-P22 #1
Mud-P22 #1 Tn10 D12
Mud-P22 #1
Mud-P22 #1 Tn10 D12
kb
23.130
9.416
6.557
4.361
2.322
A
B

7.9 Confirmation that the *S. typhimurium* locus present on plasmid pKPF12 is the same as that disrupted by the transposon Tn*10* D12.

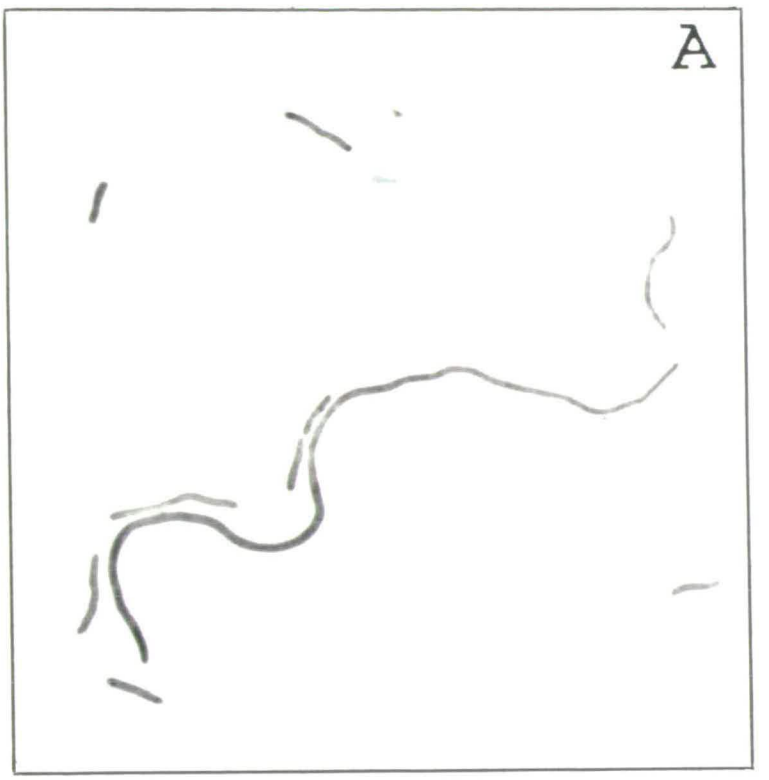
As further evidence that the plasmid pKPF12 carried a copy of the genetic locus disrupted by the transposon Tn*10* D12, Southern blotting analysis was undertaken. In order to do this, Tn*10* D12 was first of all transduced into the strain TT15223 which had been used to produce Mu*d*-P22 lysate number 1 to which both Tn*10* D12 and pKPF12 had been found to map. A lysate was then prepared from one of the resulting transductants and the phage DNA was restricted with *BglII* and run on a 0.8% agarose gel alongside similarly restricted phage DNA derived from the parental strain (see figure 7.8A). The gel was then Southern blotted to transfer denatured DNA to a solid support (nylon membrane), and this was probed with a *HindIII/SalI* fragment from pKPF12 (see figure 7.6), radiolabelled with ^{32}P by random priming. The results of this experiment (see figure 7.8B) clearly showed that in the lysate from TT15223, a DNA band of approximately 10 to 15 kb strongly hybridized to the DNA probe derived from pKPF12, but that the latter band had been significantly shifted by insertion of the transposon Tn*10* D12. This confirmed that the DNA fragment found on pKPF12 and that disrupted by Tn*10* D12 lay within the same 10 to 15 kb segment.

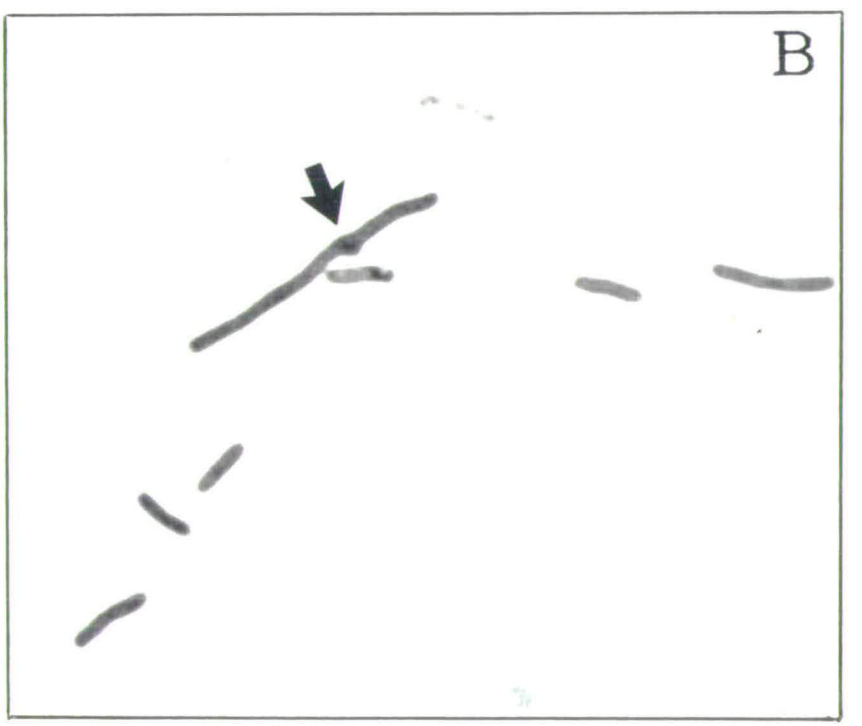
7.10 Microscopic examination of SL1344 cells containing Tn*10* D12.

The phenotypic characteristics associated with insertion of Tn*10* D12 within *S. typhimurium ie* slow growth rate, abnormal colony morphology, and cold sensitivity, suggested that this mutation was extremely deleterious to this bacterium. To determine whether Tn*10* D12 had any obvious morphological effects on the bacterial cells themselves,

Figure 7.9. *S. typhimurium* cells carrying Tn*10* D12 are highly filamented.

An overnight culture of MPG407 (equivalent to SL1344 carrying Tn*10* D12) was added to an equal volume of a 20% formalin solution made up in phosphate-buffered saline. A 10 µl volume of this mixture was then pipetted onto an agar block (approximately 1 cm x 1 cm x 2 mm composed of LB agar), premounted on a glass slide. This slide was examined by phase contrast microscopy (magnification x100). Panel A shows a highly filamented cell with a number of shorter filaments. Panel B shows a short filament with a raised surface bleb (indicated by the arrow).


A


B

cultures of the strain MPG407 (SL1344 Tn*10* D12) and the wildtype strain SL1344 were compared microscopically. Cultures of MPG407 and SL1344 were grown overnight at 30°C in LB media and then fixed in a 20% formalin solution made up in phosphate-buffered saline. Figure 7.9A shows that a significant proportion (approximately 20%) of MPG407 cells were highly filamentous (the majority of the remaining cells were composed of shorter filaments), and that some of these filamentous cells had raised surface blebs (see figure 7.9B and discussion). Comparative analysis of the wildtype SL1344 cells showed only normal, short, rod-shaped cells.

7.11 Discussion.

The *S. typhimurium* Mu*dlux*::*cspS* fusion strain, MPG361, proved to be ideal for the identification of mutants containing Tn*10* insertions capable of altering cold shock-induced light production, since these mutants were easily distinguished from the bulk of the transductants due to the characteristic light pattern normally produced by this fusion (see table 3.3 and figures 3.2) *ie* intense light emissions induced during cold shock treatment (making dark mutants easy to identify), and negligible background light at room temperature (making bright mutants easy to identify). Screening a pool of 55,000 Tn*10* mutants of MPG361 only produced three mutants with Tn*10* insertions which both specifically affected the regulation of light production in cold shock-inducible fusions (see tables 7.1, 7.2 and 7.3, and figure 7.1), and which mapped at different loci to *cspS* (see figure 7.5 and table 7.4). This demonstrated that the cold shock response is probably quite tightly regulated in this bacterium, especially with respect to the synthesis of its major cold shock protein.

The most characterized of the three cold shock-specific Tn*10* mutations, Tn*10* D12, was found to be due to an insertion which lay at approximately 0 - 1 minutes on the *S. typhimurium* chromosome (see figure 7.5A). This mutation not only abolished cold shock-inducible light in a range of Mu*dlux* fusions (see figure 7.1 and table 7.1), but was also observed to cause a number of additional phenotypic changes. For example, *S. typhimurium* cells that contained this latter mutation were found to be cold sensitive at 10°C (see figure 7.4), and to be slow growing (see figure 7.2), highly filamentous (see figure 7.9), and to form characteristically flat, translucent colonies at 30°C. Together these data indicate that the locus disrupted by Tn*10* D12 plays an important role during the growth of *S. typhimurium* at 30°C, but is essential for the survival of this bacterium approaching the lower limits of growth temperature.

Disrupted regulation of the Mu*dlux*::*cspS* gene fusion by Tn*10* D12 was shown to occur at all temperatures at which this fusion was capable of effectively functioning *ie* 22°C and below (see figure 7.3). However, this regulation was more dramatic at temperatures of 10°C and below (see figure 7.1 and figure 7.3D and 7.3E), again indicating that the locus disrupted by Tn*10* D12 is more essential at extreme growth temperatures. Further characterization of Tn*10* D12 demonstrated that the function(s) disrupted by this transposon could be fully complemented by the plasmid, pKPF12 (see figures 7.2, 7.3, and 7.4). This plasmid was shown to carry an *S. typhimurium* locus which mapped at approximately 0 - 1 minutes (see figure 7.7A and table 7.4) and which lay within 10 - 15 kb of the site at which Tn*10* D12 was found to insert (see figure 7.8), indicating that the region of *S. typhimurium* DNA carried by pKPF12 (see figure 7.6) was most likely the same as that disrupted by Tn*10* D12.

A possible candidate for the locus disrupted by Tn*10* D12 in *S. typhimurium* is *dnaK*, a gene which maps at approximately 0.3 minutes on the chromosome of the closely related bacterium *E. coli* (Georgopoulos, 1977). Previous work by Bukau and Walker (1989a) has shown that a particular mutant allele of *dnaK* in *E. coli*, designated Δ*dnaK52*, exhibited a number of mutant phenotypes that were found in the present study to be associated with *S. typhimurium* mutants containing Tn*10* D12 insertions. For example, Δ*dnaK52* mutants were reported to form abnormally flat and translucent colonies at 30°C. These mutant were also seen to be slower growing than wildtype *E. coli* cells at 30°C, were unable to grow or survive at temperatures of 11°C or 42°C, and under microscopic examination, were observed as filamentous at 30°C. (Certain *dnaK* mutants also produce filaments with raised surface blebs (personal communication, K. Begg)). Further work by Shi and colleagues (1992) also showed that DnaK was required for flagellum synthesis at 30°C, and that certain *E. coli dnaK* mutants were nonmotile at this temperature.

The precise mechanism by which the heat shock protein DnaK (Hsp70) might regulate light production from cold shock-inducible Mu*dlux* fusions in *S. typhimurium* is not known. However, close scrutiny of the proposed mechanism for this protein's modulation of the heat shock response in *E. coli* (Straus *et al*, 1990; Bukau, 1993), might help to elucidate this. Under normal, non-stressful conditions within the cell, DnaK directly interacts with σ^{32} (the heat shock specific sigma subunit of RNA polymerase). This interaction stops σ^{32} from inducing expression of the heat shock proteins, including DnaK (Grossman *et al*, 1984; Cowing *et al*, 1985). However, during heat shock, DnaK has been demonstrated to bind to denatured proteins (Goff and Goldberg, 1985; Ito *et al*, 1986) to mediates their DnaK-dependent repair or degradation (Gething and Sambrook, 1992; Schroder *et al*, 1993). This sequestering of DnaK by

damaged proteins prevents it from interacting with σ^{32}, which in turn allows activation of heat shock gene transcription. Once the damage proteins have been eliminated, DnaK is again free to interact with σ^{32} and to down-regulate the heat shock response. Thus, the heat shock response is self-limiting because the over-production of heat shock proteins, combined with the repair of protein damage by heat shock proteins (also involving DnaJ and GrpE), restores repression (Straus *et al*, 1990; Craig and Gross, 1991).

Interestingly, Bukau and Walker (1990) found that the repression of DnaK in Δ*dnaK52* mutants gave a significant increase in the cellular concentration of σ^{32}. This finding led these latter workers to speculate that the over-production of this sigma subunit was the most probable reason for the deleterious effect seen on these cell. Evidence for this hypothesis was subsequently shown during further investigation of Δ*dnaK52*, for these workers found that these mutants frequently gave rise to fast growing, normal shaped colonies (Bukau and Walker, 1989a) that were no longer cold sensitive (Bukau and Walker, 1990). Subsequent investigation found that these phenotypic revertents were due to a secondary mutation that had occurred within the *rpoH* gene, which resulted in a significant reduction in the cellular concentration of σ^{32} (Bukau and Walker, 1990). Whether similar suppressor mutations can be obtained in MPG407 remains to be determined.

How over-production of σ^{32} might have such a dramatic effect upon *E. coli* cells is not known. However, it is possible that, at low temperatures (*ie* < 30°C), higher than normal concentrations of σ^{32} in the cell are able to out-compete other sigma subunits (*eg* σ^{70}) for RNA polymerase (*eg* by titration and/or increased affinity). (Competition between σ^{32} and σ^{70} has been previously proposed by Grossman and colleagues (1985)). This would mean that promoters reliant upon other

sigma factors for transcription at this temperature would have a reduced capacity for expression, and may even be inoperable. Alternatively, the over-production of certain heat shock proteins at low temperatures might also be deleterious to the cells (Paek and Walker, 1987; Bukau and Walker, 1990).

A similar hypothesis that involves DnaK in the regulation of both heat shock- and cold shock proteins in *E. coli*, is dependent on this protein's control of cellular levels of free alarmones: a set of polyphosphorylated nucleotides (*eg* ppGpp, pppGpp, AppppA, ApppGpp, AppppG, ApppG, and ApppA) that have been reported to be responsible for sensing stress within the cell (Stephens *et al*, 1975; Bochner *et al*, 1984; Jones *et al*, 1992). Bochner and colleagues (1986) have speculated that the phosphorylase and nucleotidase activity of DnaK are effected by AppppA (a nucleotide that is not degraded by DnaK), and that these former activities of DnaK are responsible for regulating the heat shock response in *E. coli* (hypothesized on the finding that certain *dnaK* mutants were defective in turning off the heat shock response due to their mutated, though still functionally active, DnaK protein being desensitized to AppppA inhibition), possibly by phosphorylating regulatory proteins such as the sigma subunits or by degrading the precursors of the alarmones.

As discussed in Chapter 1, Mackow and Change (1983) have shown that there is an inverse relationship between ppGpp content and the total rate of RNA synthesis in *E. coli*, and have speculated that levels of ppGpp during temperature shift in this bacterium are responsible for regulation of the heat shock- and cold shock proteins. For example, shifting *E. coli* from 23°C to 40°C results in an immediate temperature-dependent increase in the synthesis of RNA followed by an increase in the ppGpp level and a corresponding decrease in the rate of synthesis of RNA. Conversely, a temperature downshift from 40°C to

23°C results in an immediate decrease in the synthesis of RNA followed by a decrease in the ppGpp level and a corresponding increase in the rate of synthesis of RNA (Mackow and Change, 1983). Furthermore, mutant *E. coli* (*relA spoT*) which cannot synthesis (p)ppGpp, have been shown to increase the synthesis of cold shock proteins and decrease the synthesis of heat shock proteins during a temperature downshift from 24°C to 10°C (Jones *et al*, 1992). Therefore, if DnaK is responsible for indirectly regulating the level of this nucleotide (Bochner *et al*, 1986), the disruption of *dnaK* would have very dramatic effects on the synthesis of temperature inducible proteins.

Despite a large proportion of the accumulated information obtained during this study pointing towards the identity of the Tn*10* D12 mutation as being due to the disruption of the *dnaK* gene (and consequentially the reduction or elimination of DnaK within a respective mutant *S. typhimurium* cells), it is equally possible that the disruption of another gene by this transposon might also lead to a number of the above phenotypic changes in this species of bacteria. Thus, each of the scenarios involving *dnaK* that have been discussed above are at present only hypothetical.

In contrast to the large amount of information gathered on the mutation Tn*10* D12, not enough data was available with respect to either Tn*10* C1 or Tn*10* C4, to allow allocation of a particular candidate gene to either of these mutations. Nevertheless, since both of these transposons acted to alter cold shock regulation in a number of those cold shock fusions tested (see tables 5 and 6), it is possible that these mutations may have disrupted loci encoding certain degradative enzymes affecting the stability of either the mRNA or the Lux proteins. Alternatively, it might also be speculated (by the same premise discussed for the possible actions of Tn*10* D12) that these Tn*10*s (Tn*10* C1 and Tn*10* C4) acted by altering

the regulation of alarmones. Interestingly, *E. coli spoT* (encoding ppGpp synthetase II) (Hernandez and Bremer, 1991; Xiao *et al*, 1991) has been shown to map (An *et al*, 1979) to approximately the same region as Tn*10* C1 and Tn*10* C4 (*spoT* has not been mapped in *S. typhimurium*).

An alternative mechanism by which Tn*10* C1 and Tn*10* C4 might alter cold shock regulation, is by disruption of a gene affecting DNA supercoiling. This phenomenon has been speculated to be involved with the regulation of a range of stress conditions within the cell, including regulation during temperature shift (Goldstein and Drlica, 1984; Wang and Syvanen, 1992). One of the major proteins influencing DNA supercoiling in the cell is DNA gyrase, a protein whose A subunit is encoded by the cold shock inducible gene *gyrA* (Jones *et al*, 1992), and whose B subunit is encoded by *gyrB*, a gene which has also been mapped (in *E. coli*)(Hansen and Meyenburg, 1979) to approximately the same region as both Tn*10* C1 and Tn*10* C4.

CHAPTER 8

This study has clearly shown the benefits of using the Mu*dlux* transcriptional reporter system for the identification and characterization of stress-inducible gene fusions in *S. typhimurium*. The unique ability of this light reporter to allow gene induction to be monitored in real-time and in a non-disruptive manner (Carmi *et al*, 1987), has not only provided a rapid and reliable screening method for monitoring bacterial stress responses under normal laboratory growth conditions (see tables 1, 2 and 3), but has also allowed the further characterisation of one of these stress-inducible gene fusions (MPG203, *ahpC*::Mu*dlux*) within the harsh intracellular environment of macrophage cells, *ie* in its true pathogenic environment (see figures 4.6 and 4.11), during infection. This versatility in reporting gene induction has shown the advantages of using a light based reporter system over other transcriptional reporters systems (*eg* β-galactosidase), especially in studies concerned with pathogen-host cell interactions.

Understanding how a bacterial pathogen such as *S. typhimurium* is capable of surviving stress in different habitats is of great importance in combating the human and animal diseases caused by this, and related organisms. Without this knowledge advancements in food hygiene and vaccine development would be almost at a standstill. For this reason, the major focus of this study was to identify loci responsible for allowing *S. typhimurium* to combat different environmental stresses, and more importantly, to determine which of these stress-inducible loci allowed this organism to survive and proliferate within macrophage cells.

Previous studies by Buchmeier and Heffron (1990), demonstrated that the interaction of *S. typhimurium* with macrophages specifically induced a minimum of 34 bacterial proteins. This data indicated that

survival of the latter pathogen during this phagocytic process was probably dependent on a number of different factors. This corresponded well with work by Fields and colleagues (1986) and Gahring and colleagues (1990), who discovered that an equally large number of discrete Tn*10* insertional mutations were capable of reducing this organism's ability to survive during macrophage infections. In both of the above studies however, the degree of information regarding this pathogenic process was extremely limited. This was mainly because of the complexities involved in reverse genetics, and in studying bacterial survival mechanisms *in vivo*, in macrophages. Furthermore, in many cases the disruption of a single locus would not have necessarily resulted in an observable reduction is the virulence of this bacterium. (An obvious exception to this is when the disruption is located within a major regulatory locus, such as *phoP* (Fields *et al*, 1986)).

The reason why the disruption of a seemingly important stress-inducible gene might not be overly deleterious to the bacterium's intracellular survival in macrophages, is that there is often a second gene encoding an analogous function which will enable the pathogen to compensate for this disruption, and so diminish any harmful effects. For example, in both *E. coli* and *S. typhimurium*, hydrogen peroxide and superoxide are both respectively dismutated by at least two different forms of the enzymes catalase and superoxide dismutase (reviewed by Farr and Kogoma, 1991). The proof that these latter enzyme homologues can compensate for one another is demonstrated by the fact that *E. coli* double mutants that are unable to produce either form of catalase (*katG katE*), or double mutants that are unable to produce either form of superoxide dismutase (*sodA sodB*), are considerably more sensitive to killing by chemical oxidants than are single mutants with only one of these loci disrupted (Farr *et al*, 1986; Schellhorn and Hassan, 1988).

Using a transcriptional reporter system such as Mu*dlux* for the identification of bacterial loci involved in virulence (predominantly involving the survival of stress within macrophage cell), circumvents the need for a disrupted locus to be deleterious to the intracellular survival of a bacterium. This is because in the latter case, the viability of the organism is no longer the measurable phenotype (although this can still be tested for), with the mutant's response to the macrophage cells now being recorded *via* its light induction. The present study has demonstrated that it would be feasible, and probably extremely beneficial, to repeat the above Tn*10* *S. typhimurium*-macrophage studies (Fields *et al*, 1986; Gahring *et al*, 1990) using the Mu*dlux* reporter system instead of a non-reporting transposon such as Tn*10*. Theoretically, this approach would enable many of the loci encoding the 34 of the macrophage-inducible proteins (Buchmeir and Heffron, 1990) to be identified (and characterised) *via* their light production, without having to perform the same degree of additional work involved in reverse genetics.

One possible drawback to using the Mu*dlux* reporter system in the hypothetical study discussed above, is that a number of the macrophage-inducible fusions might already be induced to a background level, as was seen with the majority of stress-inducible fusions characterised during the present study (see tables 3.1, 3.2 and 3.3). This latter phenomenon would make it extremely difficult to initially discriminate between these light emitting fusions and constitutive light emitting fusions, such as those found within housekeeping genes. To give an example of this, two of the proteins known to be induced in *S. typhimurium* during its infection of macrophage cells, are DnaK and GroEL (Buchmeir and Heffron, 1990). Both of these proteins have also been shown to be synthesized to a basal level under normal growth conditions (reviewed by Bukau, 1993). This would mean that induction

of a *dnaK*::Mu*dlux* or *groEL*::Mu*dlux* fusion during an infection of macrophage cells would be extremely difficult to see unless the most stringent of control conditions were employed.

Although the genetic loci encoding a number of the more general ('global') stress-inducible proteins might be difficult to isolate from *S. typhimurium*, due to basal level of gene induction, it is unlikely that more selectively regulated loci, such as those encoding specific virulence proteins, will present such a problem. This has clearly been demonstrated using the hydrogen peroxide-inducible fusion MPG203 (*ahpC*::Mu*dlux*). This particular fusion was found to have almost negligible background light emission and could be accurately monitored for gene induction within macrophage cells (see figures 4.6 and 4.11). Furthermore, since the use of this fusion demonstrated that it was possible to report light from within what could be judged as the harshest of intracellular environments within eukaryotic cells *ie* macrophages, it is likely that pathogens carrying this reporter system could be used to infect and report gene expression from a variety of different host cells, including epithelial cells.

One major advantage of being able to study the regulation of a particular bacterial stress-inducible gene fusion in a range of different host cell lines, is that it enables the researcher to identify which genes are essential to that pathogen during the different stages of the infective process. For example, *S. typhimurium* and pathogenic *E. coli* both infect and survive within epithelial cells (Moulder, 1985; Finlay and Falkow 1989b; Falkow, 1991; Falkow *et al*, 1992). However, only *S. typhimurium* is able to survive after being phagocytosed by macrophage cells (Finlay and Falkow 1989a; Falkow *et al*, 1992). As discussed above, the ability for this latter pathogen to survive within these professional phagocytic cells is probably encoded by more than one gene. Using *S. typhimurium* with specifically characterized Mu*dlux* fusions might enable such genes

to be identified by discriminating between the light emissions recorded during infection of the two different cell lines, such as epithelial cells and macrophages.

The identification of *S. typhimurium* genes that are specifically induced during this bacterium's infection of macrophage cells, might also prove to be extremely beneficial in the development of more effective vaccine strains. At present many of live attenuated vaccine strains of *S. typhimurium* are auxotrophic mutants, such as *aroA* mutants (Hoiseth and Stocker, 1981). Decreased virulence in the mutants is due to disruption of the aromatic amino acid biosynthetic pathway which means that this mutant cannot survive in nutrient poor environments such as those found within host tissues (Edwards and Stocker, 1988). This would imply that *aroA* mutants are attenuated due to a general impaired ability to survive within the host, and would explain why these mutants are still capable of causing disease in mice at high dosage (Hormaeche *et al*, 1990; Miller *et al*, 1990b)).

In contrast to the approach taken above, the development of an *S. typhimurium* strain that was selectively attenuated during its infection of macrophages, and not before, would probably provide a safer and more effective vaccine strain than the auxotrophic mutants discussed previously. The reason for this would be two-fold. Firstly, a mutation specifically affecting the bacterium's survival during phagocytosis by macrophages would enable a much larger number of bacteria to reach this latter stage of the infective process. (Theoretically, this would mean that the vaccine could contain a lower density of bacteria and would ensure that a full immune response was induced in the animal without necessarily compromising safety.) Secondly, since the majority of bacteria reaching this stage of the infection would have already passed through a number of other types of host cell *eg* epithelial cells (assuming oral vaccination),

the bacteria responsible for infecting the macrophages would be fully adapted and expressing those antigenic determinants induced by the prior infective process.

Previous work concerned with the isolation of *S. typhimurium* mutants (containing Tn*10* insertions) with reduced survival in macrophages (Fields *et al*, 1986; Fields *et al*, 1989; Miller *et al*, 1989a; Gahring *et al*, 1990; Miller *et al*, 1990a) has led to the development of several extremely effective vaccine strains, in particular, *phoP* mutants (Galan and Curtiss, 1989; Miller *et al*, 1990b). Whether *S. typhimurium ahp* mutants will also be effective as vaccine strains has still to be determined. However, this study has demonstrated that this locus was significantly induced during the infection of macrophages (see figures 4.6 and 4.11). Thus, even if *ahp* mutants prove not to be fully attenuated in subsequent mouse studies, this locus can still be used as an expression site for genes encoding a range of antigenic determinants, possibly even from other pathogens (*ie* to form a chimeric vaccine). This will hopefully lead to the development of vaccine strains where a specific antigenic determinant is only expressed where it is immunologically most usefully *ie* within the macrophage cells where it can be used for presentation to- and activation of T cells (Reeves, 1987).

In addition to the more devastating diseases that certain *Salmonella* species may induce in a susceptible host (*eg* typhoid fever, induced by *S. typhi* in man and *S. typhimurium* in mice (Finlay and Falkow, 1989a; Hsu, 1989)), strains such as *S. typhimurium* are also capable of causing a range of costly, though less severe disease symptoms (*eg* gastroenteritis) in a much broader host range. The major cause of these latter, non-systemic infections of the intestinal tract, is through food poisoning (Jay, 1992), usually as a result of improper storage or preparation of food. Indeed, it is estimated that the annual cost of *Salmonella* food poisoning

in the USA is approximately $50,000,000 (Cohen and Tauxe, 1986). This is hardly surprising however, considering estimates predict approximately 57% of cattle and 70% of chickens in the USA are carriers of *Salmonella* (Jay, 1992). This means that not only are their carcasses contaminated, but that a high percentage of their respective food products (*eg* milk and eggs) are also at risk of contamination. Again, however, these statistics are not unexpected in view of the fact that in 1989 the industry-wide incidence rates of salmonellae in animal feed stocks in the USA were greater than 49% (Jay, 1992).

In relation to disease arising from contaminated food stored at low temperatures (*eg* refrigeration at 4-7°C), species of *Salmonella* are much less of a problem than phychrotrophic pathogens such as *L. monocytogenes*. Nevertheless, an extensive study by Mossel and colleagues (1981) has shown that some foodbourne species of *Salmonella* are capable of slow growth at temperatures of 6°C or less, these include *S. typhimurium* (6.2°C), *S. heidelberg* (5.3°C) and *S. panama* (4°C). Thus, in contrast to *E. coli* which is reported to be a strict mesophile that is only capable of growth above 8°C (Ng *et al*, 1962), it appears that some salmonellae have the potential to proliferate at refrigeration temperatures. These data may reflect a fundamental difference between these latter genera of bacteria.

Since the completion of this study, Lee and colleagues (1994) and Hiraga and Yamanaka (personal communication to Jones and Inouye (1994)) have found that *E. coli* possesses a family of CspA homologues. In all, four additional *cspA* homologues were identified, designated *cspB*, *cspC*, *cspD*, *cspE*. These genes were mapped to 35, 40, 19 and 14 minutes on the *E. coli* chromosome, respectively, in contrast to 79 minutes for *cspA*. The genes encode for proteins of 71, 69, 74 and 69 residues, respectively, with 79, 70, 45 and 70% identity to CspA, respectively.

Furthermore, using translational *lacZ* fusions *E. coli cspB*, like *cspA*, was found to be cold shock inducible at the level of transcription, while *cspC* and *cspD* were not (Lee *et al*, 1994), the mechanism for the regulation of *cspE* has not as yet been determined.

Owing to the close relationship between *Salmonella* strains and *E. coli*, it is extremely likely that *S. typhimurium* will also contain a family of proteins homologous to the *E. coli* CspA family. The fact that the genes encoding these latter proteins (*ie* homologues of *S. typhimurium cspB*, *cspC*, *cspD*, *cspE*) were not detected during this study, by probing the *S. typhimurium* Mu*d*-P22 mapping set (Benson and Goldman, 1992) with a random primed PCR product of the *E. coli cspA* gene, was probably due to poor hybridization. This hypothesis is substantiated by the fact that even the lysate (#43) containing DNA predicted to be most similar to *cspA* (*ie cspS*) gave an extremely weak signal upon hybridization with this *E. coli* probe (see figure 7.7B). Perhaps a more successful approach to determine whether homologues of the above *csp* genes exist in *S. typhimurium*, would be to lower the stringency temperature during hybridization and to re-probe the set of Mu*d*-P22 lysates with the coding region of the *S. typhimurium cspS* gene.

The implications of finding the CspA family in *E. coli* are considerable. Jones and Inouye (1994) have implied that, since *B. subtilis* CspB has been shown to form dimers (Schindelin and colleagues, 1993a), it is possible that the proteins of the *E. coli* CspA family might also function as dimers and that different combinations of these subunits (*eg* CspA + CspB or CspA + CspC) may be capable of binding to, or effecting the regulation of, different molecules of nucleic acid. This hypothesis provides an alternative explanation as to why the disruption of the *S. typhimurium cspA* gene in MPG361 was not, as perhaps expected, overly deleterious to the long term survival of this bacterium during its

storage at low temperature. Additional work will have to be conducted to determine whether these proteins are also capable of providing cross-protection during cold shock treatment and a range of other stress conditions.

Providing genes which are homologous to *E. coli cspB*, *cspC*, *cspD*, *cspE* can be identified in *S. typhimurium*, the next logical step would be to determine whether the regulation of these genes is disrupted by the transposon Tn*10* D12, as it was for *cspS* (see figures 7.1 and 7.3). This would be of particular interest regarding the genes *cspC* and *cspD*, which are not cold shock inducible at the level of transcription (Lee *et al*, 1994). Furthermore, since *cspE* was actually found as a result of isolating multicopy suppressors of an *E. coli* mutant, *mukB106*, which was temperature sensitive and defective in chromosomal partitioning (Hiraga and Yamanaka (personal communication to Jones and Inouye (1994)), it would be interesting to see what effect, if any, the over-production of this protein would have on Tn*10* D12 mutants.

Finally, the identity of the loci disrupted by the transposons Tn*10* D12, Tn*10* C1 and Tn*10* C4 have still to be determined. However, the supposition that Tn*10* D12 might lie within the *dnaK* gene has recently been strengthened indirectly by the findings of Alix and Guerin (1993). These workers demonstrated that mutant DnaK chaperons cause ribosome assembly defects in *E. coli*, and suggest that this finding unifies the role of DnaK as a cellular thermometer as proposed by Craig and Gross (1991) and by McCarty and Walker (1991), and that of ribosomes as sensors of heat shock and cold shock as first suggested by VanBogelen and Neidhardt (1990). This finding is in agreement with previous work conducted by Nelson and colleagues (1992) in *Saccharomyces cerevisiae*, where it was shown that Hsp70 is also associated with the ribosomes, and that this relationship is essential for protein synthesis.

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Light Emission from a Mu*dlux* Transcriptional Fusion in *Salmonella typhimurium* Is Stimulated by Hydrogen Peroxide and by Interaction with the Mouse Macrophage Cell Line J774.2

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Hydrogen peroxide is known to induce a multigenic response in *Salmonella typhimurium* cells. We have used a Mu*dlux* transcriptional reporter system to identify and isolate fusions in the virulent strain SL1344 which respond to hydrogen peroxide in vitro by light production, and one of these fusions, MPG203, has been further characterized. Transient light production was observed from MPG203 at levels of hydrogen peroxide as low as 10 μM. However, high levels of this toxic oxidizing agent resulted in light suppression, particularly at low bacterial densities. This fusion was also shown to produce light following adhesion to cells of the mouse macrophage cell line J774.2. Furthermore, the response was greatly reduced in the presence of catalase, directly implicating hydrogen peroxide as the eliciting agent and suggesting the involvement of the hydrogen peroxide-induced bacterial stress response in the infection process. Chemiluminescence studies also indicated that inhibition of the respiratory burst may occur as the infection ratio is increased. In addition, the level of light produced from bacteria within individual macrophage cells was shown to vary.

Salmonella species are gram-negative bacteria which cause a wide spectrum of disease in humans and animals, including typhoid fever, bacteremia, and gastroenteritis (33). *Salmonella typhimurium* is a pathogen of mice and results in a typhoid-like disease similar to that observed in humans infected with *Salmonella typhi* (6). A central feature in the infection process which leads to enteric fever is the ability of the bacterium to enter and survive within macrophages (13). The latter cells may then act as an immunological shield and enhance bodily dissemination of the invading organism.

Phagocytic cells provide a primary line of defense against invading pathogens and normally result in their rapid destruction. Macrophages have developed an arsenal of oxygen-dependent and -independent mechanisms to effect killing. These include production of toxic oxygen derivatives via the respiratory burst, such as hydrogen peroxide, superoxide anions, and hydroxyl radicals (20). In addition, the internalized organisms are also exposed to a variety of esterases and antimicrobial peptides (16) and vacuolar acidification (17).

Active oxygen metabolites have been shown to cause damage to DNA, RNA, proteins, and lipids (20), and *S. typhimurium* is known to mount a coordinated stress response to excesses of hydrogen peroxide (3, 30). This metabolite results in the induction of approximately 30 bacterial proteins, and the regulatory locus, *oxyR*, plays an important role in controlling a subsection of this response (4). The related enteric organism *Escherichia coli* is also known to mount a coordinated, multigenic response to superoxide-generating compounds, with induction of a further subset of proteins (19, 38). The *soxRS* locus plays a key role in modulating this response (39).

In this study, we addressed whether evidence could be obtained to link the hydrogen peroxide-induced response of *S. typhimurium* directly to interaction with macrophage cells. To address this situation, we exploited a light-based bacterial transcriptional reporter system (11) to monitor cell-cell interaction. The paper describes the isolation of *S. typhimurium* fusions which respond to hydrogen peroxide with light production. One of these fusions is further characterized with regard to the effects of different levels of hydrogen peroxide exposure and also during interaction with cells of the mouse macrophage cell line J774.2.

MATERIALS AND METHODS

Bacterial strains, tissue culture cells, and growth media. All strains used in this study are listed in Table 1. For routine purposes, bacteria were grown in Luria broth containing kanamycin (50 μg ml^{-1}) where appropriate. All other experiments were carried out using RPMI medium containing feta calf serum (10%)—from here on known as tissue culture medium. Gentamicin (100 μg ml^{-1}) was used where stated. The mouse macrophage cell line, J774.2 (31), was obtained from the Sir William Dunn School of Pathology (Oxford, United Kingdom), and cells were routinely subcultured in tissue culture medium containing penicillin (200 U ml^{-1}) and streptomycin (100 μg ml^{-1}) in a carbon dioxide incubator at 37°C. Transductions in *S. typhimurium* were carried out with a high-transducing derivative of bacteriophage P22-*int* as described by Roth (32).

Construction of a Mu*dlux* pool in MPG202. The bacteriophage Mu derivative Mu*dlux*(Km), when inserted into a gene in the correct orientation, results in a transcriptional fusion that directs the synthesis of Lux enzymes necessary for light production (11). *S. typhimurium* is normally resistant to phage Mu. However, in previous studies, we have circumvented this restriction by using a *galE* derivative for infection (21). It has been shown that phage P1-sensitive (*galE*) derivatives of *S. typhimurium* can be infected with Mu that has been given a P1 host range and that the P1 host range ca

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TABLE 1. Bacterial strains[a]

Strain	Genotype	Source
HB101 (Mu*dlux*)	*hsdS20*(r_B^- m_B^-) *supE44 recA13 ara14 proA2 rpsL20 xyl-5 mlt-5 supE44*	M. Silverman (11)
CH424	*his-6165 galE496 ilv-452 hsdL16 hsdSA29 metE55 trpB2 xyl-404 rpsL120 flaA66 ompD1012*	C. F. Higgins (21)
MPG200	*his-6165 galE496 ilv-452 hsdL16 hsdSA29 metE55 trpB2 xyl-404 rpsL120 flaA66 ompD1012* Mu*dlux*	This work
CH463	*galE503 bio-561*/MuP1	C. F. Higgins (21)
MPG201	*galE503 bio-561*/MuP1 Mu*dlux*	This work
MPG202	*galE503 bio203*::Tn*10 his*	SL1344 derivative; this work
MPG203	*his* Mu*dlux*	SL1344 derivative; this work
SL1344	*his*	B. A. D. Stocker (22)

[a] All strains except HB101 and SL1344 are derivatives of *S. typhimurium* LT2. Mu*dlux*, Mud1 (Km^r *lux cts*62).

be conferred by the helper phage Mu*cts*62hP1 (abbreviated MuP1), which directs the synthesis of P1 tail fibers (7). We therefore constructed a dilysogen containing Mu*dlux* and MuP1 in a sequential manner. Mu*dlux* was transduced from the *E. coli* strain HB101 (Mu*dlux*) to a restriction-deficient P1-sensitive *S. typhimurium* strain (CH424) by P1-mediated transduction. A P22 lysate of the resultant Mu*dlux* lysogen (MPG200) was then used to transduce CH463 to kanamycin resistance (Km^r). The resultant dilysogen, MPG201, when temperature induced, gave high-titer Mu lysates that could efficiently transduce a *galE* derivative of virulent *S. typhimurium* strain, SL1344 (designated MPG202); during this transduction, Mu*dlux* is able to transpose and insert more or less at random into the recipient chromosome. A random pool of Mu*dlux* insertions was therefore obtained, as previously described (21). Approximately 65,000 kanamycin-resistant colonies were pooled and washed before screening for fusions which exhibited light induction following hydrogen peroxide exposure.

Isolation of fusions which exhibit hydrogen peroxide-induced light production. Cells from the random bank of MPG202 (Mu*dlux*) fusions, prepared as described above, were plated on Luria broth-kanamycin plates at a density of 10^3 CFU per plate and were incubated overnight at 30°C. Colonies were examined by eye to eliminate fusions which produced light prior to hydrogen peroxide stimulation. Plates were then overlaid with Whatman no. 3 cellulose discs (9-cm diameter), soaked in 90 mM hydrogen peroxide, and left at room temperature for 1 h. Insertions exhibiting light induction were identified, colony purified, and retested for light production following hydrogen peroxide exposure. The Mu*dlux* marker was then transduced into a clean background with phage P22 and retested for the correct phenotype prior to subsequent characterization.

Effects of cell density and hydrogen peroxide concentration on light production by MPG203. Overnight cultures of MPG203 were grown in Luria broth medium, and viable counts were determined. Cells were subsequently diluted in tissue culture medium to the appropriate density, and samples were incubated for 1 h at 30°C to allow for medium readjustment and to reestablish exponential-phase growth. Hydrogen peroxide was then added, and light production was recorded at appropriate intervals with a single-tube luminometer.

Influence of temperature and gentamicin on light production. The viability of overnight cultures was established by plating. Cultures were diluted in tissue culture medium to approximately 10^5 CFU/ml and incubated for 1 h at 30°C prior to the experiment. Samples were then incubated at 30 or 37°C for an hour in the presence of 100 μM hydrogen peroxide. Gentamicin was added to relevant samples after 40 min, and incubation was then continued as before for the remaining samples. After this period, samples were directly counted in a Beckman liquid scintillation counter (model LS1701) on the tritium channel at room temperature to assess (nonradioactive) light emission.

Interaction of MPG203 with J774.2 cells. In order to examine the interaction of MPG203 with macrophages, J774.2 cells were seeded in two flasks (250 ml), at a density of 5×10^6 cells per flask, in tissue culture medium containing penicillin and streptomycin, and cells were allowed to adhere until the next day. Overnight cultures of MPG203 were diluted to approximately 10^7 cells ml^{-1} in tissue culture medium and grown at 30°C for 1 h with aeration. Macrophage cell sheets were washed repeatedly in phosphate-buffered saline (pH 7.4) (PBS) and subsequently infected with the *S. typhimurium* cells at 37°C in tissue culture medium at a multiplicity of infection of approximately 20:1 (bacteria-cells). Incubation was continued for 40 min at 37°C, and both cell sheets were gently but extensively washed in PBS to remove the majority of unbound bacteria. Fresh tissue culture medium, with or without gentamicin, was added, and incubation was continued at 37°C for a further 20 min. After this period, cell sheets were stripped by vigorous hand agitation, and light emission was subsequently recorded by liquid scintillation counting at room temperature. (The effectiveness of macrophage release was assessed microscopically.) As a control, duplicate cultures of MPG203 alone were grown for 40 min at 37°C and then gentamicin was added to one of the two cultures to equate with conditions for macrophage interaction. Incubation was continued for a further 20 min at 37°C prior to liquid scintillation counting at room temperature.

After a 3-h interval from the initial point of infection, hydrogen peroxide (100 μM) was added to both macrophage and bacterial samples and light production was again recorded for approximately 1 h. Macrophage samples containing gentamicin were subsequently washed three times by low-speed centrifugation ($600 \times g$, 5 min) in tissue culture medium to remove residual gentamicin. High-speed centrifugation ($6,000 \times g$, 10 min) was then carried out to lyse macrophages, and the pellets were resuspended in fresh tissue culture medium. Hydrogen peroxide was added as before, and samples were measured by liquid scintillation counting. (Bacterial samples originally exposed to gentamicin produced no further light following this extended processing, indicating that cell death had occurred [data not shown].)

Influence of catalase on light production. In order to assess whether J774.2 cells elicited light induction in MPG203 cells by producing hydrogen peroxide, macrophage cells (seeded at approximately 5×10^6 cells per flask) and cultures of MPG203 were grown overnight and prepared for infection studies as described above. Two flasks (250 ml) containing J774.2 cell sheets were washed repeatedly in PBS (pH 7.4) as before, and each was then infected at 37°C with *S. typhimurium* cells in tissue culture medium at a multiplicity of infection of approximately 20:1 (bacteria-cells). Catalase (Sigma Chemical Co., Dorset, United Kingdom) was added simultaneously to one of the flasks (final activity, 100 U/ml of tissue culture medium), and both were incubated for a further 40 min at 37°C. The cell sheets were then washed extensively to remove unbound bacteria, and fresh tissue culture medium was added with and without catalase (100 U/ml). Incubation was continued at 37°C for a further 20 min, after which cell sheets were stripped by vigorous hand agitation and light emission was recorded subsequently in a liquid scintillation counter at room temperature.

Assay of respiratory burst activity in J774.2 cells. The respiratory burst was monitored by reaction with luminol (Sigma Chemical Co.) as previously described (18). Fresh tissue culture medium was added to overnight cell sheets of J774.2 cells (prepared as described above) which were then stripped by vigorous hand agitation and resuspended at a density of approximately 6×10^4 cells per ml. Overnight cultures of SL1344 cells were diluted in tissue culture medium to approximately 10^7 cells per ml and grown at 30°C to approximately 4×10^9 bacteria per ml prior to the experiment. Samples were then diluted, mixed with J774.2 cells at appropriate ratios, and further incubated at 37°C. (Bacterial densities were verified by plating.) At appropriate intervals, phorbol myristate acetate (PMA) (Sigma Chemical Co.; 500-ng/ml final concentration) and luminol (0.1 mM final concentration) were added. Thereafter, light production was recorded by monitoring chemiluminescence in a liquid scintillation counter at room temperature. Peak light emission occurred approximately 20 min after PMA addition.

Visualization of bacterium-macrophage interaction. J774.2 cells grown in tissue culture medium containing gentamicin were detached from flasks by gentle agitation in disodium EDTA (prepared in PBS [pH 7.4]) (23) and then resuspended in fresh tissue culture medium containing gentamicin at a density of approximately 10^7 cells ml^{-1}. Sterile cavity slides placed in dry petri dishes were coated with 200 μl of this suspension for 4 h at 37°C in a carbon dioxide incubator. Slides were subsequently washed with PBS and submerged in fresh tissue culture medium without gentamicin, and incubation was continued until the following morning. An overnight culture of MPG203 was then diluted to approximately 10^7 cells ml^{-1} in tissue culture medium, and exponential-phase growth was reestablished for 1 h at 30°C. Following this, macrophage-coated cavity slides (washed in PBS) were coated with 1 ml of bacterial suspension, and slides were incubated at 37°C for 40 min to enhance interaction. Unbound bacteria were subsequently removed by extensive washing in PBS, and fresh tissue culture medium containing gentamicin was added. Incubation was continued for a further 20 min at 37°C in a carbon dioxide incubator. Cavity slides were then examined at intermittent intervals for light emission, with the aid of a photon video camera (model 2400; Hamamatsu Photonics Ltd.) attached to a Zeiss Axioskop microscope.

RESULTS

Isolation of Mu*dlux* fusions of SL1344 which are induced in response to hydrogen peroxide. We attempted to identify fusions in the virulent *S. typhimurium* strain SL1344 which responded to hydrogen peroxide by genetically tagging the cells with the Mu*dlux* reporter system described by Engebrecht et al. (11). A pool of mutants containing random Mu*dlux* insertions was then constructed in a *galE* derivative of SL1344 (designated MPG202) (see Materials and Methods), and individual colonies were screened for light production when overlaid with Whatman no. 3 filter paper soaked in hydrogen peroxide (90 mM). Fusions were colony purified and rescreened for their response to hydrogen peroxide. Approximately 65,000 colonies were screened, and 34 fusions were obtained. One such fusion was chosen for further analysis following phage P22 transduction of the Mu*dlux* reporter into an SL1344 background (designated MPG203). A typical response of this isolate to hydrogen peroxide is shown in Fig. 1.

Light induction in MPG203 is dependent on bacterial density and hydrogen peroxide concentration. Use of a real-time reporter, such as Mu*dlux*, allowed us to examine the kinetics of gene induction in response to hydrogen peroxide. The level of light production from MPG203 in response to incubation in the presence of different concentrations of hydrogen peroxide was monitored by luminometry. The results showed that with 10^6 viable bacteria per ml, substantial light production occurred in a transient fashion at levels of hydrogen peroxide as low as 10 μM. However, at a concentration of 1 mM hydrogen peroxide, light production was diminished and delayed (Fig. 2A). We interpreted this to mean that exposure to high concentrations of hydrogen peroxide results in suppression of light production, possibly as a result of cellular stress.

The threshold of light induction and suppression was also examined in relation to bacterial density. Hydrogen peroxide at 10 μM also resulted in light induction at a lower bacterial density. However, light production at higher concentrations of hydrogen peroxide (1 mM) was completely abolished (Fig. 2B), indicating that higher bacterial densities of *S. typhimurium* cells are capable of tolerating higher levels of the toxic oxidizing agent.

Effect of temperature and gentamicin on light production by MPG203. Previous studies have reported that bacterial translation is rapidly abolished in the presence of gentamicin and that this feature can be exploited to highlight bacterial gene expression following endocytosis, since gentamicin does not inactivate bacterial protein synthesis within phagocytes (28). Furthermore, studies of the Lux proteins of *Vibrio fischeri* have reported that light production can be measured at 30°C but is lost at higher temperatures (10). We therefore examined the effect of temperature on light production from MPG203 cells which were initially incubated at 30 or 37°C for 1 h in the presence of hydrogen peroxide and subsequently monitored at room temperature in a liquid scintillation counter. Light emission was significantly diminished following prior incubation at 37°C compared with the level of light recorded after incubation at 30°C, although similar induction profiles were obtained in both cases (Fig. 3). This suggests that the light produced following incubation at 37°C represents translation of residual mRNA after the incubation temperature has been lowered to room temperature and is in good agreement with the view that enzyme synthesized at 37°C is irreversibly inactivated.

Gentamicin was also added to parallel samples at each

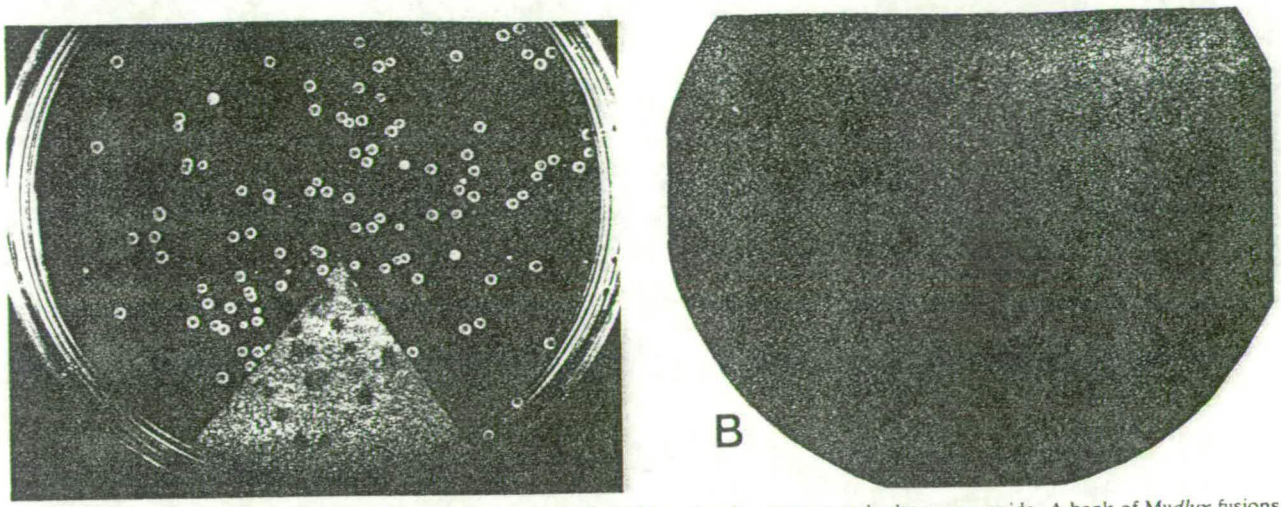

FIG. 1. Detection of light production by Mu*dlux* fusions in *S. typhimurium* in response to hydrogen peroxide. A bank of Mu*dlux* fusions of *S. typhimurium* was screened for light production in response to hydrogen peroxide. (A) Typical plate screen in which colonies are overlaid with filter paper soaked in hydrogen peroxide (90 mM). In this instance, a pure culture of MPG203 is shown with a section of the filter disc. (B) Light emission is recorded on X-ray film.

temperature, after 40 min of incubation in the presence of hydrogen peroxide, in order to assess the effect of this antibiotic on translation. Incubation was then continued at 30 or 37°C, as before, for a further 20 min, and samples were subsequently monitored in a liquid scintillation counter at room temperature (Fig. 3). Addition of gentamicin effectively abolished light production in response to hydrogen peroxide after 20 min at either temperature, reflecting both the combined effect of this compound on translation de novo and the turnover of Lux enzymes within MPG203.

Induction of light from MPG203 following macrophage interaction. Having shown that strain MPG203 resulted in

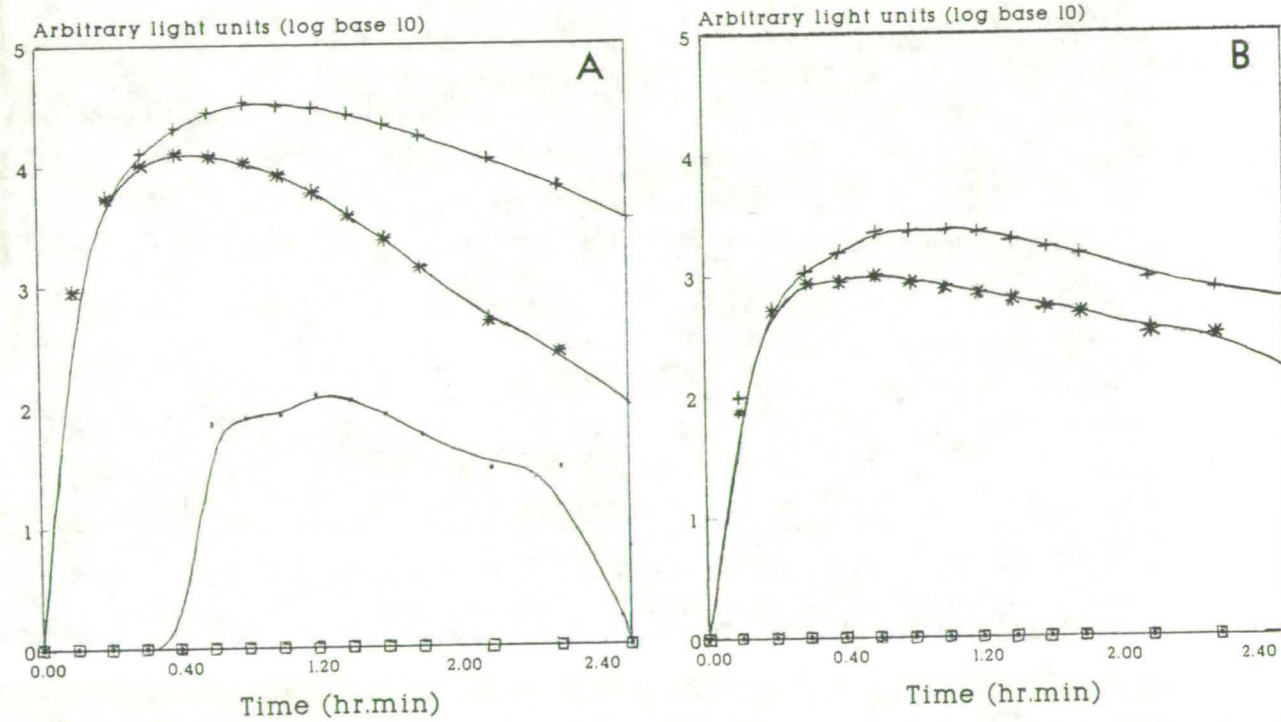

FIG. 2. Light induction and suppression in MPG203 in response to hydrogen peroxide. Hydrogen peroxide was added to cultures of MPG203 (prepared as described in Materials and Methods) to a final concentration of 10 (*), 100 (+), or 1,000 (·) μM, and light emission was immediately recorded by luminometry. The response observed with 10^6 CFU ml^{-1} (A) or 10^5 CFU ml^{-1} (B) is shown. No hydrogen peroxide was added to control samples (□).

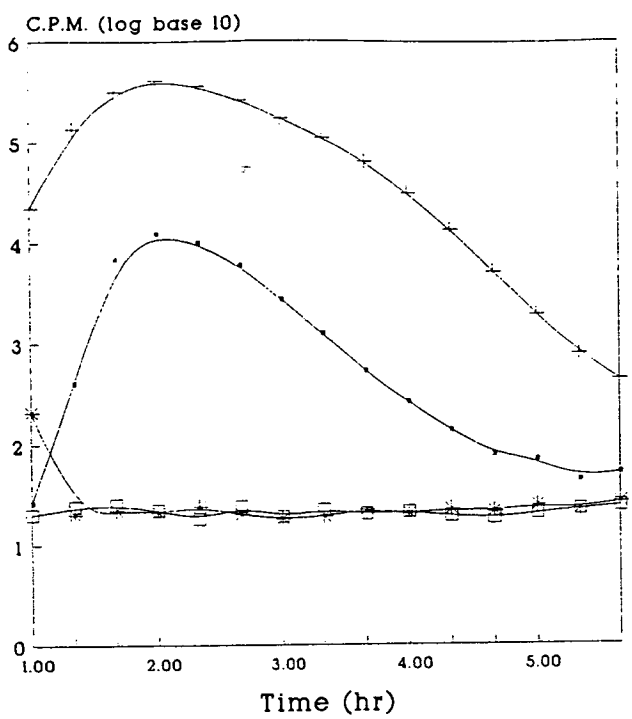

FIG. 3. High temperature or gentamicin inhibits light production by MPG203. Cultures were incubated for 1 h at 30 or 37°C in the presence of hydrogen peroxide (100 μM) and subsequently monitored by liquid scintillation counting at room temperature. The results show light production following the 1-h incubation period. Gentamicin (100 μg ml^{-1}) was added to parallel samples after 40 min, and incubation was continued for a further 20 min prior to analysis. Samples were treated as follows: 37°C (·), 30°C (+), 37°C plus gentamicin (□), or 30°C plus gentamicin (*).

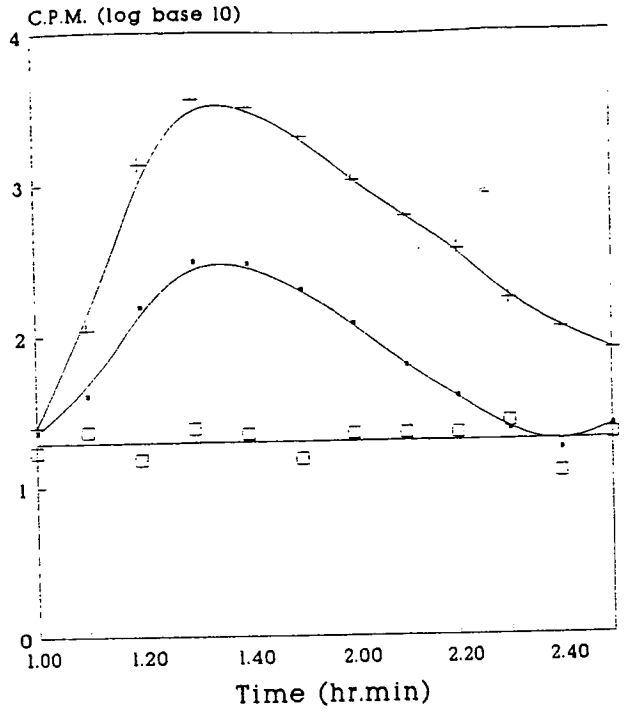

FIG. 4. Interaction of MPG203 with J774.2 results in light production. Bacteria and macrophage cells were prepared as described in Materials and Methods. Macrophages were infected with MPG203 cells at 37°C in tissue culture flasks and incubated for 40 min. Cells were then washed extensively in PBS to remove unbound bacteria. Fresh tissue culture medium with (·) and without (+) gentamicin (100 μg ml^{-1}) was added to parallel samples, and incubation was continued at 37°C for a further 20 min. Cell sheets were then stripped and monitored for light emission at room temperature. The results show light production following the 1-h incubation period. Control cultures of MPG203 were incubated for the same period of time at 37°C. Gentamicin was then added to one of the cultures 20 min before the end of the (1-h) incubation period. Results for MPG203 cells incubated in the absence of hydrogen peroxide and gentamicin are shown (□). (The gentamicin-containing sample result is not shown.)

light emission in vitro in response to hydrogen peroxide, we examined the interaction of this isolate with cells of the mouse macrophage line J774.2 to address whether the respiratory burst from these phagocytic cells would result in reporter gene expression. Macrophages were infected with cells of MPG203 for 1 h at 37°C, and light production was then monitored at room temperature in a liquid scintillation counter. The results are shown in Fig. 4. Transient light production was indeed observed, reaching a maximum after about 80 to 90 min from the initial point of infection. Furthermore, the presence of gentamicin was found to reduce light production substantially, suggesting that a large proportion of the light detected resulted from bacteria, bound to the macrophage surface, which were responding to hydrogen peroxide produced at the surface of the phagocyte plasma membrane.

Light production by macrophage-bound MPG203 cells is substantially reduced in the presence of catalase. The data from Fig. 4 indicated that MPG203 cells resulted in light production following interaction with cells of the mouse macrophage line, J774.2. In order to verify that the response was elicited by hydrogen peroxide, as opposed to any other stimulant which might be produced by these macrophage cells, the microbe-phagocyte interaction was reexamined in the presence or absence of catalase. Parallel cultures of macrophages were infected with MPG203 cells for 1 h at 37°C, and light emission was subsequently recorded in a liquid scintillation counter at room temperature. From Fig. 5, it can be seen that transient light production occurred, reaching a maximum at about 80 to 90 min, as before (Fig. 4). However, addition of catalase to the medium at the initial point of infection was observed to reduce light production to less than 20% of the level recorded at peak emission, thereby confirming that bacterial gene induction was occurring primarily in response to hydrogen peroxide produced by the macrophage cells.

Light production by macrophage-bound MPG203 is not maximally induced. The result from Fig. 4 indicated that light production by MPG203 could be monitored both extracellularly and intracellularly. However, it remained unclear whether the macrophage-bound bacteria were fully activated in response to the respiratory burst of the phagocyte and also whether the response could be reelicited. Bacterium-macrophage samples which had previously been shown to result in light production (Fig. 4) were therefore subjected to further analysis. Hydrogen peroxide (100 μM) was added to the samples at room temperature, and the response was

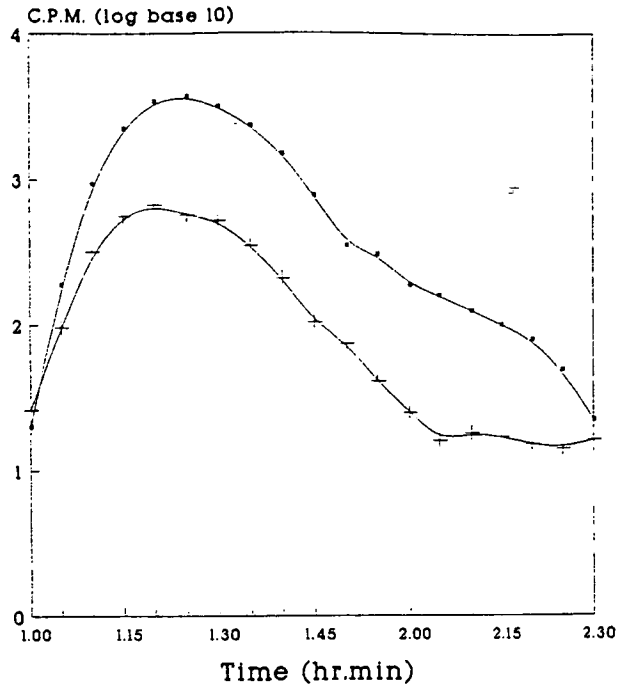

FIG. 5. Catalase inhibits light production which results from interaction of MPG203 with J774.2. Bacteria and macrophages were prepared as for Fig. 4. Macrophages were infected with MPG203 cells at 37°C in tissue culture flasks in the presence or absence of catalase and incubated for 40 min. Cells were then washed extensively in PBS to remove unbound bacteria. Fresh tissue culture medium with (+) and without (·) catalase (100 U/ml) was added to parallel samples, and incubation was continued at 37°C for a further 20 min. Cell sheets were then stripped and monitored for light emission at room temperature. The results show light production following the 1-h incubation period.

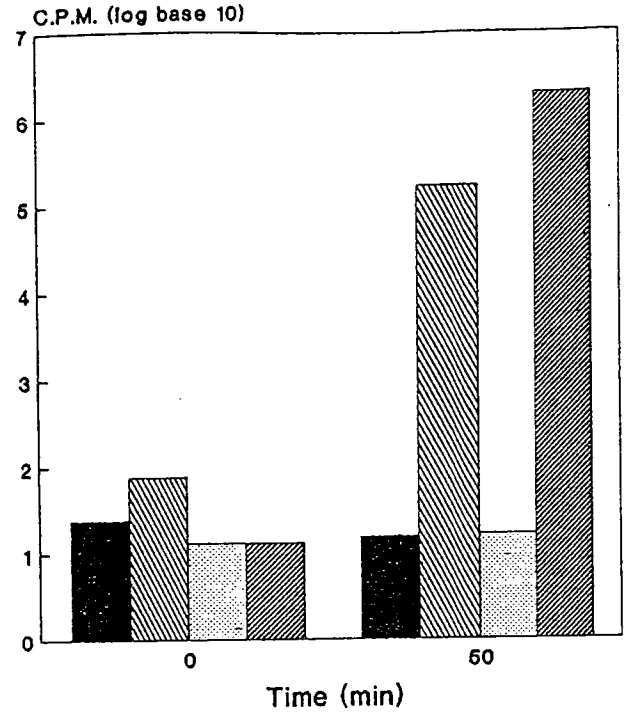

FIG. 6.. Macrophage-bound cells of MPG203 are capable of further light production. After light had subsided, samples containing bacteria and macrophages from the experiment described in the legend to Fig. 4 were subjected to externally added hydrogen peroxide (100 μM) and further monitored by liquid scintillation counting. Light emission is shown immediately after hydrogen peroxide addition and following 50 min of exposure. Shown are results for MPG203-infected J774.2 cells in the presence (■) or absence (▨) of gentamicin. Results for MPG203 cells in the presence (▥) or absence (▨) of gentamicin are also shown.

monitored. A large burst of light production (Fig. 6) which greatly surpassed that produced following macrophage interaction was clearly observed. This suggested that macrophage interaction had not elicited a full response from the bound bacteria. No further response was seen from samples which originally contained gentamicin.

Internalized MPG203 cells exhibit poor light production. The question of whether intracellular light production by phagocytosed bacteria (in the presence of extracellular gentamicin) was a true representation of the level of gene induction or whether some form of suppression had occurred remained unanswered. The macrophage cells which had continuously been incubated in medium containing gentamicin (Fig. 4 and 6) were therefore gently centrifuged and washed to remove the antibiotic and subsequently lysed by more rigorous centrifugation (6,000 × *g*, 10 min). Hydrogen peroxide was then added. Substantial light production (Fig. 7) was recovered, indicating that the internalized bacteria were still viable, although they had produced only low levels of light when phagocytosed (Fig. 4). (This also confirmed that addition of hydrogen peroxide to the macrophages had not resulted in cell lysis [Fig. 6].)

Samples of the lysed macrophage solution were plated to estimate viable bacteria. Approximately 10^6 bacteria were recovered from the total supernatant, indicating that the bacterium-to-macrophage ratio was approximately 0.1 at this point. This confirmed that the low level of light production in the presence of gentamicin was not solely a result of inefficient endocytosis of MPG203 but that the response of the bacteria was indeed markedly reduced within the phagosomal environment.

Infection with high levels of MPG203 reduces the respiratory burst in J774.2 cells. Previous studies (9) have reported that endocytosis of intracellular pathogens, such as *Legionella micdadei*, may result in abolition of the phagocytic respiratory burst following PMA stimulation, in a manner which is dependent on the number of organisms used and the duration of incubation. Such a situation might provide an alternative explanation for the low level of intracellular light observed following phagocytosis of MPG203 cells (Fig. 4). In order to address this possibility, J774.2 cells were infected at different ratios with SL1344 cells and incubated at 37°C. At selected time intervals, the macrophages were then stimulated with PMA and the peak activity of the respiratory burst for each sample was determined at room temperature with luminol by chemiluminescence. As can be seen from Fig. 8, a decline in the respiratory burst was observed, relative to the PMA response, over a 2-h time period. This was particularly evident at very high infection ratios. However, substantial activity was still recorded over this time interval at the bacterium-macrophage ratios used for bioluminescence

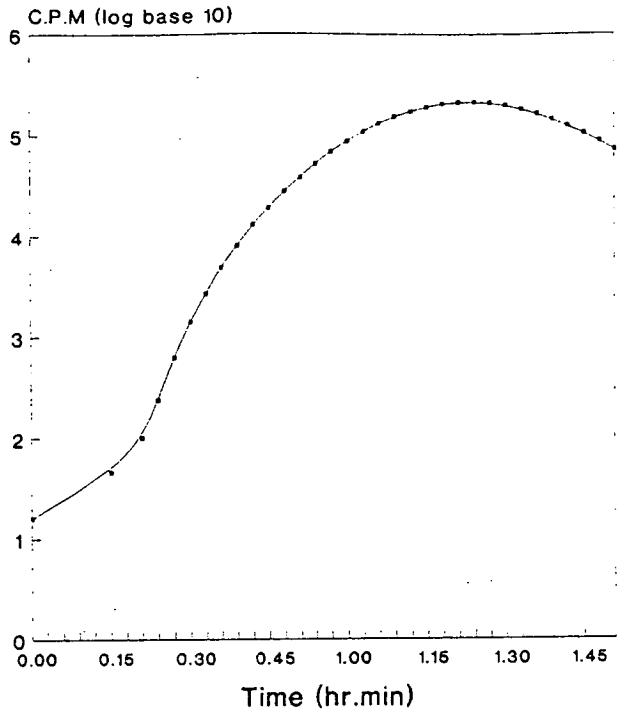

FIG. 7. MPG203 cells from lysed macrophages are capable of substantial light production. Macrophage cells containing MPG203 which had been maintained in tissue culture medium in the presence of gentamicin (Fig. 6) were washed repeatedly by gentle centrifugation (600 × *g*, 5 min) and then disrupted by more rigorous centrifugation (6,000 × *g*, 10 min). Pellets were resuspended in fresh tissue culture medium (without gentamicin), and hydrogen peroxide (100 μM) was added. Light emission was immediately recorded by liquid scintillation counting. Samples of MPG203 which had also been maintained in gentamicin were treated similarly, but no light above the background was recorded (data not shown).

studies (Fig. 4 and 9). Since the peak of bioluminescence from MPG203 occurred approximately 80 to 90 min after initial interaction with the macrophages (Fig. 4), it seems unlikely that the low level of light produced by the internalized bacteria can be fully explained by inhibition or abolition of the respiratory burst following endocytosis. This suggests that some other factor(s) may be responsible, at least in part, for the low level of bioluminescence observed following phagocytosis.

Visualization of MPG203 interaction with J774.2 cells. The above studies had revealed that MPG203 responded to J774.2 cells with light production. However, the level of endocytosis suggested that all the cells of the macrophage population might not be behaving uniformly. We therefore decided to examine whether light production from internalized bacteria could be visualized. Macrophage cells of J774.2 were coated on a cavity slide overnight in tissue culture medium, as described in Materials and Methods, and subsequently inoculated with MPG203 at a ratio of approximately 100:1 (bacterium-macrophage) at 37°C. After 40 min, unbound bacteria were removed, gentamicin was added in fresh tissue culture medium, and incubation was continued for a further 20 min. Following this, samples were examined on a Zeiss Axioskop microscope with a photon video camera attached. A transient peak of light was observed approximately 20 min later. From Fig. 9, it can be seen that the intensity of light recorded from individual macrophages varied considerably and suggests that the number of bacteria endocytosed per macrophage or the level of induction of the respiratory burst may fluctuate significantly between individual cells or macrophage subpopulations. This is in accordance with reports from other phagocytic studies (18).

DISCUSSION

The ability to survive within macrophages is fundamental to the pathology of the enteroinvasive forms of salmonellae, and an extensive study by Fields and colleagues (13) of mutants bearing Tn*10* insertions has provided important insights into factors which are required for virulence and survival within phagocytic cells. However, despite the immense value of the latter approach, direct characterization of the response of the genetic loci which affected virulence was not possible during macrophage interaction because of the nature of the genetic tag. Other studies (1) have circumvented this problem by selectively labelling de novo-synthesized proteins of endocytosed bacteria with radioactive amino acids in the presence of inhibitors of eukaryotic cell translation. While this approach revealed the induction of well-characterized bacterial stress proteins, such as GroEL, following two-dimensional gel electrophoresis and also showed that the cellular protein profiles of attenuated mutants differed from that of virulent strains when within the phagocyte, only limited information could be obtained concerning the nature of the other proteins induced or the genetic loci responsible.

In the present study, we report the value of a light-based reporter system as a means of tagging genetic loci of *S. typhimurium* cells which are induced in response to exposure to hydrogen peroxide in vitro. In addition, we have shown that one of these fusions also results in light emission following macrophage interaction. The turnover rate of the Lux enzymes in *S. typhimurium* provides an added advantage in this system, allowing both bacterial gene induction and repression to be recorded as they occur throughout the interaction. Furthermore, the absence of a requirement for inhibitors of eukaryotic translation results in minimal perturbation to the function of the phagocyte.

Macrophages elicit a respiratory burst following bacterial stimulation which begins at the cell surface and is mediated, in part, by an NADPH oxidase (34). One of the outcomes of this reaction is the transient production of hydrogen peroxide. *S. typhimurium* cells are known to mount a coordinated genetic response to hydrogen peroxide in vitro, with increased synthesis of approximately 30 proteins (3, 30). These are thought to function in the protection from and the repair of hydrogen peroxide-induced damage. The present study provides direct genetic evidence for the involvement of a hydrogen peroxide-induced response in *S. typhimurium* following macrophage interaction.

In vitro studies of MPG203 indicate that both the concentration of hydrogen peroxide and the bacterial density are influencing factors in the induction of a hydrogen peroxide-elicited stress response in *S. typhimurium* and that the cells exhibit a transient reaction to low or intermediate levels of the stimulus, as evidence by light emission (Fig. 2). However, high levels of the toxic metabolite appear to result in suppression of light production in vitro, although this effect is reduced at higher bacterial densities. The reason for this cross-protection from hydrogen peroxide which occurs at higher bacterial numbers is unclear. However, the mi-

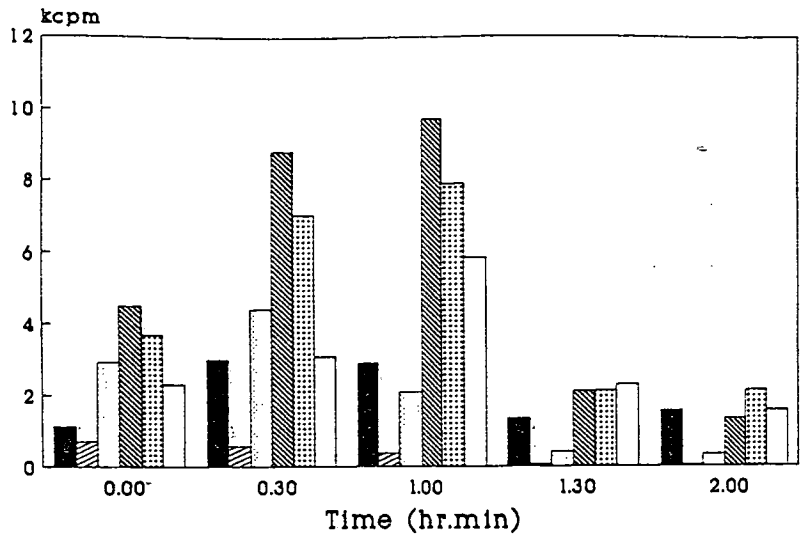

FIG. 8. Infection of J774.2 cells with SL1344 may reduce the respiratory burst. J774.2 cells (prepared as described in Materials and Methods) were suspended in tissue culture medium at an approximate density of 5×10^4 cells per ml. Freshly grown cells of SL1344 were mixed with the macrophages at approximately 10,000:1 (▨), 1,000:1 (□), 100:1 (▧), 10:1 (□), and 1:1 (□), respectively, and incubated at 37°C. At appropriate intervals, PMA and luminol were added and light production was then recorded in a liquid scintillation counter at room temperature. The value shown represents peak light emission. The influence of PMA on light production by J774.2 in the absence of SL1344 is also shown over the same time period (■). No light was detected from any samples in the absence of luminol (data not shown).

croaerophilic organism *Campylobacter jejuni* has also been reported to tolerate higher levels of oxygen metabolites at high cell density (35). In addition, late-log-phase cultures of *E. coli* have been reported to be more resistant to hydrogen peroxide (24), although in this case synthesis of a protective catalase is known to occur.

A large number of genetic mutations which result in attenuated virulence of *S. typhimurium* in vivo have now been reported (2, 5, 8, 22, 25, 29). However, few examples have been directly correlated with a role in macrophage interaction. The *pho* regulon is an exception to this situation and has been reported to mediate resistance to a class of antimicrobial polypeptides called defensins which are produced within the phagolysosome (12). The present study provides direct evidence that the multigenic, hydrogen peroxide-induced response also belongs in this category and plays a role in the primary events following bacterium-macrophage interaction. Light production appears to be elicited following adhesion of the *S. typhimurium* cells to the macrophages, presumably indicating a response to the toxic oxygen metabolites produced at the phagocyte cell surface. The substantial reduction in luminescence which occurred in the presence of catalase (Fig. 5) provides strong evidence that macrophage-produced hydrogen peroxide is the eliciting agent in this interaction, lending further support to our in vitro findings (Fig. 2). The observation of intracellular light is consistent with other reports that the effects of the respiratory burst continue within the phagosome (20) and supports the view that the endocytic vacuole is an oxygenated environment. The fact that intracellular light can be detected also indicates that the level of damage to the biochemical functions of the bacteria is insufficient to abolish the supply of substrates which are required for the *lux* reporter system, namely, oxygen, reduced flavin mononucleotide, and aldehyde.

The reason for poor light production following bacterial endocytosis (Fig. 4) remains unclear. One possible explanation is that ingestion of *S. typhimurium* might result in abolition of the respiratory burst, thus limiting the stimulus (hydrogen peroxide) for light induction intracellularly. Such a situation has been reported with other intracellular pathogens, such as *L. micdadei* (9), in which it has been shown that the respiratory burst may be completely abolished over a period of 90 to 120 min at infection ratios of 30:1 and 10:1 (bacterium-phagocyte), respectively. Our results indicate that a decline in the respiratory burst activity relative to the PMA-induced response does occur following ingestion of *S. typhimurium* at very high infection ratios (Fig. 8). However, at the ratios of bacteria to macrophages which were used for our bioluminescence studies (Fig. 4 and 9), the relative reduction in respiratory burst activity was found to be fairly moderate, and it seems unlikely that this explanation alone could adequately account for the low intracellular bioluminescence which we observed.

An alternative view may be that the low level of intracellular light observed reflects the influence of the harsh phagosomal environment on the bacterial cell. Our in vitro studies (Fig. 2) have shown that millimolar concentrations of hydrogen peroxide can suppress bioluminescence at densities of 10^6 cells per ml or less, and such concentrations have been reported to occur within phagolysosomal environments (20). Equally, Foster and Hall have shown that if *S. typhimurium* cells fail to adapt to the levels of acidity which can be found within macrophages, damage to intracellular enzymes may ensue (15). Genetic repression in *S. typhimurium* has also been reported to occur within macrophages, and Buchmeier and Heffron (1) have shown that a large number of bacterial proteins (approximately 34%) exhibit a substantial reduction in synthesis following phagocytosis. The possibility therefore remains that a combination of these or other factors is responsible for the low level of intracellular light recorded.

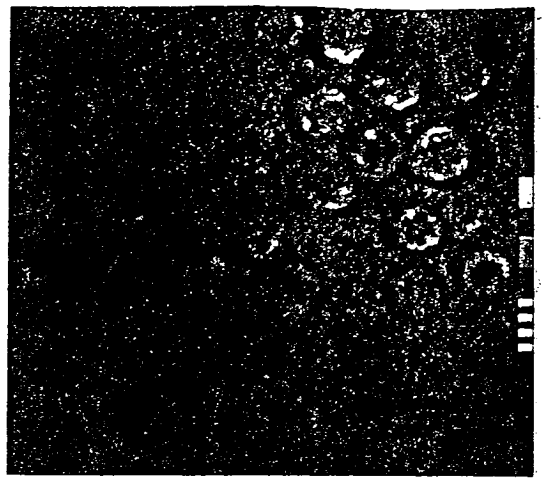

FIG. 9. Visualization of light production by MPG203 internalized within J774.2 cells. J774.2 cells were coated on cavity slides and infected (as described in Materials and Methods) at 37°C for 40 min. Samples were then washed, covered with fresh tissue culture medium containing gentamicin, and reincubated at 37°C for a further 20 min. Slides were then viewed intermittently with a microscope-attached photon video camera. Foci of light emission are indicated in color, and the intensity is indicated by the scale bar (red represents peak intensity).

The level of light recorded from surface-bound bacteria also appears suboptimal (Fig. 4), since subsequent addition of hydrogen peroxide results in an almost 100-fold increase in the detectable light response (Fig. 6). The reason for this is not apparent, but the phenomenon may simply reflect limited secretion of hydrogen peroxide at the macrophage cell surface. Alternatively, this submaximal light response may reflect variations in the levels of the respiratory burst produced by different subpopulations of macrophages. Visualization of light emission with a photon-counting camera (Fig. 9) has also suggested that substantial differences can occur in the levels of light produced from endocytosed bacteria within individual phagocytic cells and further suggests that the response of the macrophage population to the *S. typhimurium* cells is not uniform. Significant differences may also exist in the phagocytic activity of individual macrophage cells.

The function of the disrupted genetic locus in MPG203 has not been established yet. However, several loci which are induced by hydrogen peroxide in vitro in *S. typhimurium* cells are known to be required for virulence in vivo, including the *htrA* locus (25) and a locus which encodes an outer membrane protein (36). Furthermore, recent studies have reported that the OxyR-regulated catalase (HP-I) plays a role in eliciting immunological protection against *S. typhimurium* in mice (27), indicating that expression must occur during the infection process. The transient expression of light by MPG203 suggests that the cell rapidly elicits protective functions in response to hydrogen peroxide and that once the influence of the stimulus has subsided, expression of the gene fusion is again reduced. HP-I may well constitute part of such a multigenic protective strategy.

Attenuated strains of *S. typhi* are good candidates for live oral vaccination, and clinical trials show promise (14, 37). Crippled strains of other *Salmonella* species also hold potential for vaccination of farm animals (26). The future design of live oral vaccines will require the rational attenuation of virulent organisms with defined gene inactivation. The ability to identify bacterial genes which are involved in discrete stages of the infection process is an important aspect of such an approach. In this respect, use of a light-based reporter system not only may provide important insights into the infection process of *S. typhimurium* but also may reveal important clues about the stresses which are encountered by internalized pathogens when within the phagocytic environment. These clearly must be overcome in order to give rise to disease.

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