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Virulence gene regulation by nucleoid associated proteins in *Salmonella typhimurium*: A role for Fis in *Salmonella* pathogenicity island 2 gene regulation.

A dissertation presented for the degree of Doctor of Philosophy, in the Faculty of Science, Trinity College Dublin

by

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DECLARATIONS

I, Ronan Carroll, am the sole author of this thesis. The work presented herein represents my own work, except where duly acknowledged in the text, and has not been previously presented for a higher degree at this or any other University.
Some parts of this work have been included in the following paper:

Kelly, A.E., Goldberg, M.D., Carroll, R.K., Hinton, J.C., and Dorman, C.J. The Fis regulon of *Salmonella enterica* serovar Typhimurium: a role for Fis in the transcriptional control of virulence and flagella genes.

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SUMMARY

Nucleoid associated proteins are involved in many diverse biological functions in *S. typhimurium*. In addition to their structural role within the genome they have been implicated in transcriptional regulation of a variety of genes, including those involved in virulence. In this study the role of nucleoid-associated proteins in *S. typhimurium* virulence gene regulation was investigated.

The role of integration host factor (IHF) in regulating the *Salmonella* plasmid virulence (*spv*) genes was investigated. Mutational analysis of the IHF binding site revealed that a triplet of residues are required for IHF-mediated activation of the *spvR* promoter.

In this study, a role for the nucleoid-associated protein Fis (factor for inversion stimulation) in *Salmonella* pathogenicity island 2 (SPI 2) gene regulation was identified. Analysis of SPI 2 promoter activity demonstrated that Fis is required for activation of the SPI 2 genes during growth in LB broth, MM5.8 and during macrophage infection. The Fis protein can bind to the DNA throughout SPI 2 and Western analysis demonstrated that inside macrophages Fis is induced and its levels remain high throughout infection.

The effect of various environmental and physiological conditions on SPI 2 gene expression was also studied. Temperature, oxygen levels and supercoiling were demonstrated to influence expression from SPI 2 promoters. Furthermore, evidence suggests that the role of Fis in SPI 2 gene regulation may be through an effect on supercoiling.

To examine further the role of Fis in *S. typhimurium* gene regulation, microarray analysis was carried out. Following growth in a low-pH minimal medium that had previously been shown to induce SPI 2 genes, the genome expression profiles of wild type and *fis* mutant *S. typhimurium* were compared. The analysis confirmed that Fis is required for SPI 2 activation. Microarray analysis also identified a role for Fis in regulating expression of genes from integrated bacteriophage. These

bacteriophages carry virulence determinants and so this represents another link between Fis and virulence gene regulation.

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I guess there's only one person left to thank and she's the most important. Eimear. Your love and support have kept me going. You have been there for me at every step of the way. I couldn't have done it without you. Thank you.

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ABBREVIATIONS

AMV	Avian myeloblastosis virus
BSA	Bovine serum albumin
CDS	Coding DNA sequence
CRP	Cyclic AMP receptor protein
DCE	1,2-dichloroethane
DEPC	Diethylpyrocarbonate
DTT	Dithiothretol
EAEC	Enteroaggregative *Escherichia coli*
EPEC	Enteropathogenic *Escherichia coli*
H-NS	Histone-like nucleoid structuring protein
HTH	Helix-turn-helix
HU	Heat unstable protein
IHF	Integration host factor
MM5.8	Minimal medium 5.8
MUG	4-methylumbelliferyl-β-D-galactopyranoside
SCV	*Salmonella* containing vacuole
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SPI	*Salmonella* pathogenicity island
TCM	Tissue culture medium
TTSS	Type III secretion system

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"When you look back on your life you will regret the things you didn't do much more than the things you did"

1.1 *Salmonella* infection

1.1.1 *Salmonella*

Salmonella are Gram-negative facultative intracellular pathogens. The genus *Salmonella* is divided into two species, *Salmonella bongori* and *Salmonella enterica*, which are then further divided into subspecies or serovars. Serovars of *S. enterica* are associated with infection of a wide variety of animal hosts including poultry, cattle, pigs, mice and humans (Ohl and Miller, 2001). Acquired by ingestion of contaminated food or water, the disease caused by *Salmonella* varies depending on host organism and serovar of infecting bacteria (Kingsley and Baumler, 2000). Certain serovars have adapted to cause infection only in specific hosts, while others display a broader host range and can cause diseases of different severity in different host organisms. In humans, *Salmonella* infection causes two types of disease. Infection with *S. enterica* serovar Typhi (*S. typhi*) results in a systemic infection called typhoid fever, while *S. enterica* serovar Typhimurium (*S. typhimurium*) infection causes a limited gastroenteritis (Ohl and Miller, 2001). *S. typhimurium* is an example of a *Salmonella* species that has a broad host range and as such it has become the best studied *Salmonella* species. It causes a systemic, typhoid fever-like disease in mice that serves as a good model for human typhoid fever as well as inflammatory diarrhoea in calves, which is studied as a model for human gastroenteritis. In this study strains of *S. typhimurium* were used throughout.

1.1.2 *Salmonella* infection

As mentioned previously, all *Salmonella* infections occur following the ingestion of contaminated food or water. The acidic environment of the stomach is the first significant barrier to infection and only if a high inoculum is taken will the bacteria go on to cause infection (Giannella *et al.*, 1972). Once inside the small intestine the bacteria traverse the intestinal mucus layer and attach to intestinal epithelial cells. *S. typhimurium* attaches to the intestinal epithelium using surface expressed appendages called fimbriae (Baumler *et al.*, 1996). Shortly after adhering to the epithelium *S. typhimurium* invades the cells by a process known as bacterial-mediated endocytosis (Francis *et al.*, 1992). Massive cytoskeletal rearrangements

occur in the host cell, disrupting the normal brush border epithelium, and producing membrane ruffles that engulf the invading bacteria enclosing them in a large vacuole. As the name suggests, bacterial-mediated endocytosis is caused by the invading bacterium. Bacterial effector proteins that induce actin cytoskeleton rearrangements and membrane ruffling are injected into the host cell via a type III secretion system (Galan, 1996). These effectors are responsible for the uptake of the bacteria by the normally non-phagocytic cells. In mice it has been reported that *S. typhimurium* preferentially adheres to and invades through M cells, although invasion through non-phagocytic cells also occurs (Jones *et al.*, 1994). M cells are specialised epithelial cells, found in the Peyer's patches that sample intestinal antigens by pinocytosis and pass them on to the lymphatic system (Brandtzaeg, 1989). While it is reported that *S. typhimurium* preferentially binds to murine M cells, there is no evidence suggesting that they preferentially bind to bovine M cells, which raises questions about the relative role of M cells in *S. typhimurium* invasion (Watson *et al.*, 1995). Recently it has also been suggested that *S. typhimurium* can passively cross the intestinal barrier following phagocytosis by migrating CD18-positive phagocytes (Vazquez-Torres *et al.*, 1999).

Following bacterial internalization, the epithelial brush border reforms while the *S. typhimurium* bacteria reside within a vacuole where they survive and replicate. Strains of *Salmonella* that cause localised gastroenteritis can infect adjacent epithelial cells and secrete effector proteins into the host cell cytosol (Galyov *et al.*, 1997). These proteins cause cytokine release, inflammation and fluid secretion, which results in the gastroenteritis disease. Strains of *Salmonella* that cause systemic infection exit at the baso-lateral surface of the epithelial cells where they encounter the submucosal macrophage. Bacteria enter the macrophage and reside within vacuoles where they survive and eventually replicate (Alpuche-Aranda *et al.*, 1994). The ability to do so is dependent on several virulence mechanisms that allow evasion of the antimicrobial functions of the macrophage (Cirillo *et al.*, 1998; Hensel, 2000; Vazquez-Torres *et al.*, 2000). Infected macrophages migrate throughout the body resulting in bacterial dissemination and colonization of organs such as the liver and spleen.

1.2 Genetics of *Salmonella* virulence

1.2.1 Pathogenicity islands

It has been estimated that 4% of the *S. typhimurium* genome, corresponding to approximately 200 genes, is required for lethal infection of mice (Bowe *et al.*, 1998). Bacterial survival inside the host and the ability to mount successful infection is dependent on the activities of these genes and their products at the correct time and in the correct place. Virulence genes are distributed throughout the *S. typhimurium* chromosome and are also located on the 90 kb *Salmonella* large virulence plasmid (pSLV). Analysis of the *S. typhimurium* genome reveals that virulence genes are often found clustered together in groups. Small groups consisting of a few genes are called pathogenicity islets while large groups containing many genes organised into operons are called pathogenicity islands (Groisman and Ochman, 1996). Pathogenicity islands are usually associated with a particular virulence phenotype (e.g. invasion) and in many cases are sufficient to confer that phenotype on non-pathogenic bacteria (McDaniel *et al.*, 1995).

There are several lines of evidence that suggest that pathogenicity islands are acquired by horizontal gene transfer. Pathogenicity islands generally have a different G + C content from the rest of the bacterial chromosome (Hensel *et al.*, 1999), they are often flanked by genes that are contiguous in closely related species that lack the island, and they are often inserted into tRNA genes or contain the remnants of bacteriophages or transposons at their borders, which indicate that they were acquired by horizontal transfer (Groisman and Ochman, 1996).

To date a total of five pathogenicity islands have been characterised in *S. typhimurium* (Marcus *et al.*, 2000). Mutations in any of these islands result in attenuation of virulence. Two of these islands, SPI 1 and SPI 2, encode specialised devices for the delivery of virulence proteins into the host cell. These devices are called type III secretion systems.

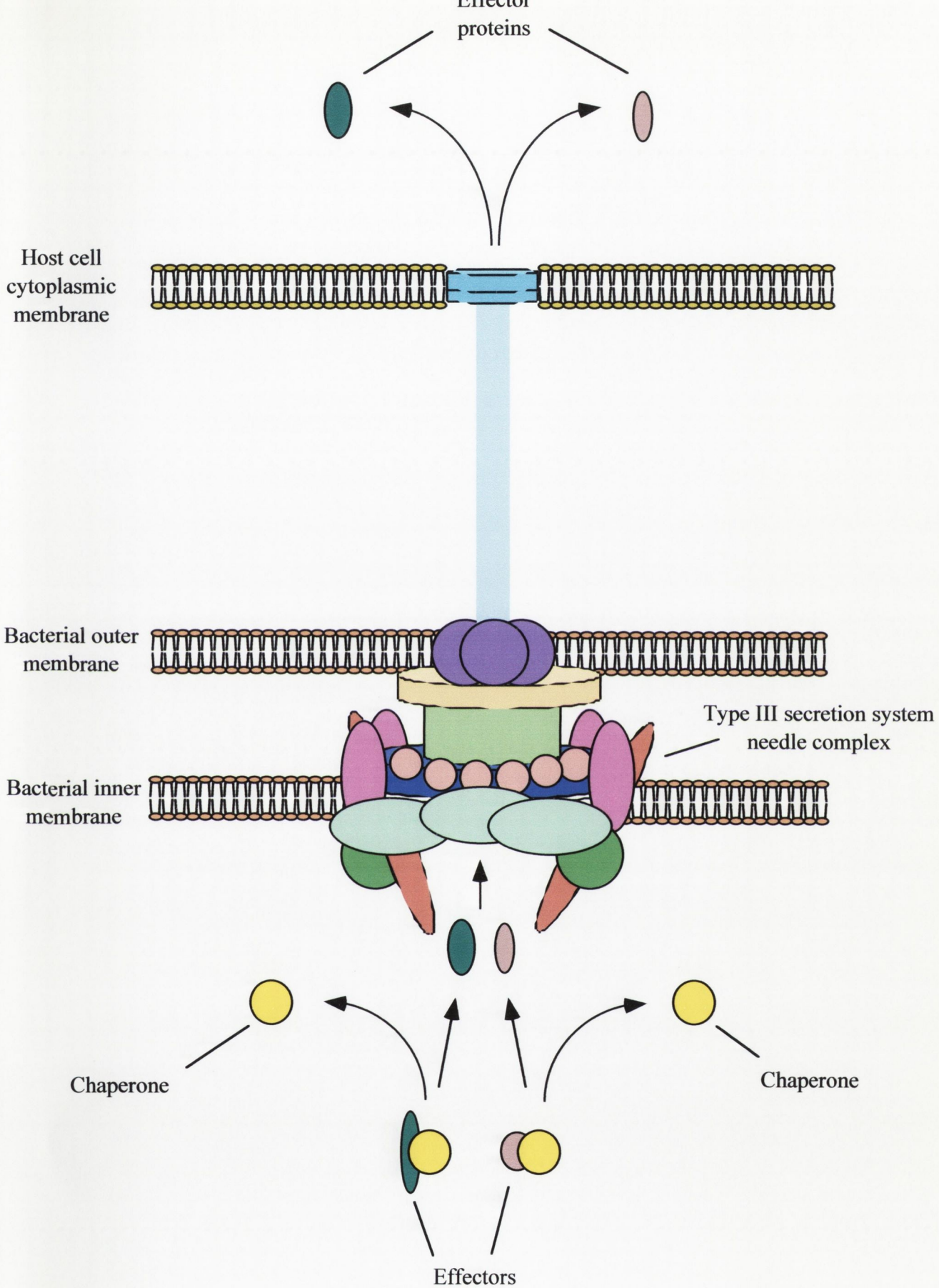
1.2.2 Type III secretion systems

Type III secretion systems (TTSSs) are found in a variety of animal and plant pathogens. They are specialised devices that allow bacteria to secrete proteins and inject them into the host cell cytoplasm (Fig. 1.1) (Hueck, 1998). This is a complex task in which the bacterial effector protein must traverse the bacterial inner and outer membranes as well as the host cell cytoplasmic membrane. The TTSS forms a channel in the bacterial inner and outer membranes using proteins that are homologous to those involved in bacterial flagellar assembly. This channel is associated on the outside of the cell with translocon proteins, which form a pore in the host cell membrane. This results in one continuous channel through which the bacterial effectors are inserted into the host cell (Hueck, 1998). Many effector proteins are associated with chaperones prior to secretion through the TTSS. These chaperones prevent incorrect folding and premature degradation of the effectors. TTSSs represent a large metabolic load on the cell and therefore their expression is tightly regulated. Many TTSSs encode their own regulatory proteins and their expression is frequently influenced by bacterial global regulatory proteins such as nucleoid-associated proteins (Schechter *et al.*, 2003). In this way the cell can sense environmental conditions in which expression of the TTSS is favourable and activate expression of the genes (Hueck, 1998).

While there is a great deal of homology between TTSSs from different bacteria, the effector proteins of each system are unique. These effectors are responsible for the virulence phenotype of the TTSS. Genes encoding effector proteins can be found within the same pathogenicity island as the TTSS or they can be found elsewhere on the chromosome (Knodler *et al.*, 2002). Recently it has been shown that many *S. typhimurium* effector proteins, secreted through both TTSS, are encoded within integrated bacteriophages (Figueroa-Bossi and Bossi, 1999). This suggests that after the acquisition of a TTSS, horizontal gene transfer continues to provide a repertoire of virulence proteins which "fine tunes" the virulence phenotype of that system (Figueroa-Bossi *et al.*, 2001; Miao and Miller, 2000).

Fig. 1.1. Type III secretion system needle complex.

The type III secretion system needle complex forms a continuous channel between the bacteria and host cell through which bacterial effector proteins travel. The needle complex traverses the bacterial inner and outer membranes and connects to translocon proteins which form a pore in the host cell cytoplasmic membrane. The effector proteins are associated with chaperones while inside the bacterial cell. When secreted they detach from the chaperones and enter the host cell.


Effector
proteins
Host cell
cytoplasmic
membrane
Bacterial outer
membrane
Type III secretion system
needle complex
Bacterial inner
membrane
Chaperone
Chaperone
Effectors

1.2.3 The PhoP/PhoQ two-component signal transduction system

The PhoP and PhoQ proteins form a two-component signal transduction system responsible for the regulation of over 40 genes in *S. typhimurium* (Miller *et al.*, 1989). Two-component signal transduction systems often consist of a membrane bound sensor/kinase (in this case PhoQ) and a cytoplasmic transcriptional regulator (PhoP). Upon receiving specific environmental signals the sensor/kinase phosphorylates the cytoplasmic regulator, activating it (Perraud *et al.*, 1999). The PhoP/PhoQ system is responsible for activating *pag* genes (PhoP-activated genes) and repressing *prg* genes (PhoP-repressed genes) (Miller *et al.*, 1989). Expression of PhoP/PhoQ is induced inside macrophages and the environmental signal sensed by PhoQ is thought to be low Mg^{2+}. PhoP-activated genes are switched on inside macrophages and many of them are required for intracellular survival. Interestingly PhoP does not regulate SPI 2 genes (Miao *et al.*, 2002). PhoP-repressed genes, including SPI 1, are switched off inside macrophages (Pegues *et al.*, 1995). Both *phoP* null and *phoP* constitutive mutants are avirulent indicating that the timing of PhoP activation and repression is crucial during infection (Miller *et al.*, 1989; Miller and Mekalanos, 1990).

1.2.4 *Salmonella* pathogenicity island 1

Salmonella pathogenicity island 1 (SPI 1) is the best characterised of the five pathogenicity islands in *Salmonella* (Marcus *et al.*, 2000). SPI 1 is approximately 40 kb in size, is located at 63 centisomes on the *S. typhimurium* chromosome, and has a G + C content significantly lower that that of the chromosome (42% compared to 52%). There are at least 35 genes encoded within SPI 1 including genes coding for a type III secretion system, effector, chaperone and regulatory proteins (Fig. 1.2) (Collazo and Galan, 1997).

The virulence phenotype associated with SPI 1 is invasion of epithelial cells. *S. typhimurium* mutants deficient in SPI 1 are attenuated for virulence in the mouse when infected via the oral route but not by intraperitoneal injection (Galan and Curtiss, 1989). This indicates that SPI 1 is required to penetrate the epithelium but not required for later stages of systemic infection. The ability of *S. typhimurium* to

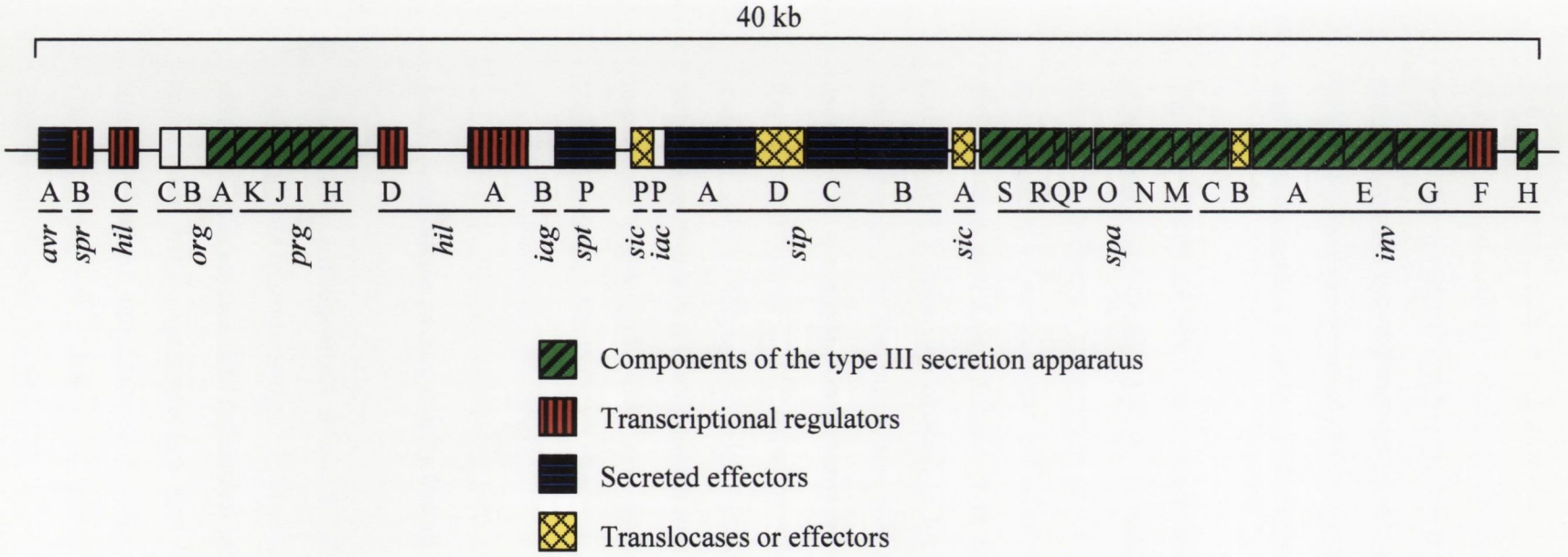


Fig 1.2. Genetic organisation of *Salmonella* pathogenicity island 1 (SPI 1).

SPI 1 genes encoding a type III secretion ststem, transcriptional regulators, translocated effectors and translocase/effectors are indicated. The length of each gene is approximately to scale.

induce its own uptake by non-phagocytic cells is dependent on the activity of the SPI 1-encoded type III secretion system (Watson *et al.*, 1995). Following attachment to the epithelium, the SPI 1 TTSS injects effector proteins into the host cell cytoplasm. These effectors are encoded both inside and outside of SPI 1 (Knodler *et al.*, 2002). Delivery of the SPI 1 effectors enables *S. typhimurium* to traverse the intestinal epithelium by inducing cytoskeletal rearrangements, which result in membrane ruffling and uptake of the bacteria into a vacuole (Francis *et al.*, 1993). Further secretion of SPI 1 effectors results in inflammation, fluid secretion, and cytokine release (Galan, 1996; Watson *et al.*, 1998).

The regulation of SPI 1 gene expression is complex and involves many factors (Eichelberg and Galan, 1999). Environmental and physiological conditions are known to influence expression of SPI 1 genes with oxygen levels, osmolarity, growth phase of bacteria and pH all exerting an effect on gene activity (Bajaj *et al.*, 1996; Lucas and Lee, 2000). It is thought that environmental conditions that activate SPI 1 gene expression are indicative of those found in the lumen of the small intestine, the location where SPI 1 activity is required. On a molecular level several SPI 1 encoded transcriptional activators are required for SPI 1 activity. Expression of these is influenced by a number of global regulatory proteins (Lucas and Lee, 2000). The nucleoid-associated proteins Fis, H-NS and HU are involved in SPI 1 gene regulation (Schechter *et al.*, 2003; Wilson *et al.*, 2001) as are the global regulators CsrA and SirA, and two-component signal transduction systems OmpR/EnvZ and PhoP/PhoQ (Bajaj *et al.*, 1996; Johnston *et al.*, 1996; Lawhon *et al.*, 2003; Lucas and Lee, 2000).

1.2.5 *Salmonella* pathogenicity island 2

Salmonella pathogenicity island 2 (SPI 2) is located at 31 centisomes on the *S. typhimurium* chromosome. It encodes a type III secretion system along with effectors, chaperones, and regulatory proteins. SPI 2 is associated with survival and replication inside macrophages and SPI 2 mutants are strongly attenuated for virulence when administered by the oral, intraperitoneal or intravenous route (Hensel *et al.*, 1995; Shea *et al.*, 1996). An extensive review of SPI 2 is provided later in this chapter (section 1.3).

1.2.6 *Salmonella* pathogenicity island 3

Salmonella pathogenicity island 3 (SPI 3) consists of a 17 kb insertion at the *selC*tRNA locus located at 82 centisomes on the *S. typhimurium* chromosome. SPI 3 contains at least ten open reading frames (ORFs) two of which, *mgtCB*, are required for survival inside macrophage and virulence in the mouse (Blanc-Potard and Groisman, 1997; Blanc-Potard *et al.*, 1999). The *mgtCB* genes encode a transport system that allows the uptake of magnesium by *S. typhimurium* under low Mg^{2+}conditions and their expression is tightly regulated by the PhoP/PhoQ two-component signal transduction system (Blanc-Potard and Groisman, 1997). The functions of the remaining SPI 3 genes are unknown, however they are not required for virulence.

1.2.7 *Salmonella* pathogenicity island 4

Very little is known about *Salmonella* pathogenicity island 4 (SPI 4). It consists of a 25 kb insertion at 92 centisomes on the *S. typhimurium* chromosome. Like many other pathogenicity islands it has a lower G + C content than the chromosome and is flanked by putative tRNA-like genes (Wong *et al.*, 1998). SPI 4 encodes 18 putative open reading frames (ORFs) some of which, based on sequence analysis, may encode a type I protein secretion system (Wong *et al.*, 1998). The function of SPI 4 is unknown, however one SPI 4 locus, designated ims98, has been shown to be required for intramacrophage survival (Baumler *et al.*, 1994).

1.2.8 *Salmonella* pathogenicity island 5

Salmonella pathogenicity island 5 (SPI 5) is located at 25 centisomes on the *S. typhimurium* chromosome and contains five open reading frames (Hong and Miller, 1998). The island is inserted at a tRNA gene and has a G + C content lower that that of the chromosome. SPI 5 appears to be involved in infection of the intestinal epithelium and not in systemic disease (Wood *et al.*, 1998). Experiments suggest that SPI 5 encoded proteins are bacterial effectors, secreted through the SPI 1 type III secretion system, that are responsible for inducing fluid secretion from host epithelial cells (Galyov *et al.*, 1997).

1.2.9 *Salmonella* plasmid virulence genes

All pathogenic serovars of *Salmonella* that cause systemic infection (including *S. typhimurium*) harbour high molecular weight virulence plasmids (Gulig, 1990). These virulence plasmids are essential for the bacteria to cause systemic disease. In the case of *S. typhimurium*, the virulence plasmid contributes to systemic infection in the mouse by increasing the replication rate of the bacteria inside host cells (Gulig and Doyle, 1993). The *S. typhimurium* virulence plasmid is approximately 90 kb, although the size of the virulence plasmid varies from 50 to 110 kb depending on the serovar (Chu *et al.*, 1999). It has been shown that an 8 kb region of the *Salmonella* virulence plasmid, conserved in all serovars, is sufficient to restore virulence to plasmid-cured strains (Gulig *et al.*, 1993). Encoded in this segment are five genes designated *spvRABCD* (for *Salmonella* plasmid virulence). SpvR is transcribed from its own promoter (P_{spvR}) while the *spvABCD* genes are transcribed as an operon from the *spvA* promoter (P_{spvA}) (Fig. 1.3). SpvR is a member of the LysR family of transcription regulators and is an activator of the P_{spvR} and P_{spvA} promoters. SpvB is a mono(ADP-ribosyl)transferase that ADP-ribosylates host cell actin, destabilising the cytoskeleton (Lesnick *et al.*, 2001; Otto *et al.*, 2000; Tezcan-Merdol *et al.*, 2001). The functions of SpvA, SpvC and SpvD are unknown but recently it was shown that *spvC* along with *spvB* were sufficient to restore partial virulence to plasmid-cured strains of *S. typhimurium* (Matsui *et al.*, 2001). This indicates that SpvC and SpvB are important virulence plasmid-encoded effector proteins.

In addition to SpvR, many other factors have been implicated in the regulation of *spv* gene expression. Like other virulence genes, environmental conditions play a part. Expression of the *spv* genes is induced during stationary phase and following uptake of the bacteria by macrophages (Marshall *et al.*, 2000). The induction observed *in vitro*, as the cells enter stationary phase, most likely occurs due to a dependence on RpoS, the stationary phase sigma factor (Chen *et al.*, 1995). RpoS expression is also induced following *S. typhimurium* entry into epithelial cells and macrophages (Chen *et al.*, 1996). Other known regulators of the *spv* system include H-NS, IHF, Lrp and CRP. H-NS and CRP both negatively regulate the *spv* genes and while it is thought that CRP exerts its effect on *spv* by influencing *rpoS* expression, H-NS repression of *spv* is independent of RpoS (O'Byrne and Dorman,

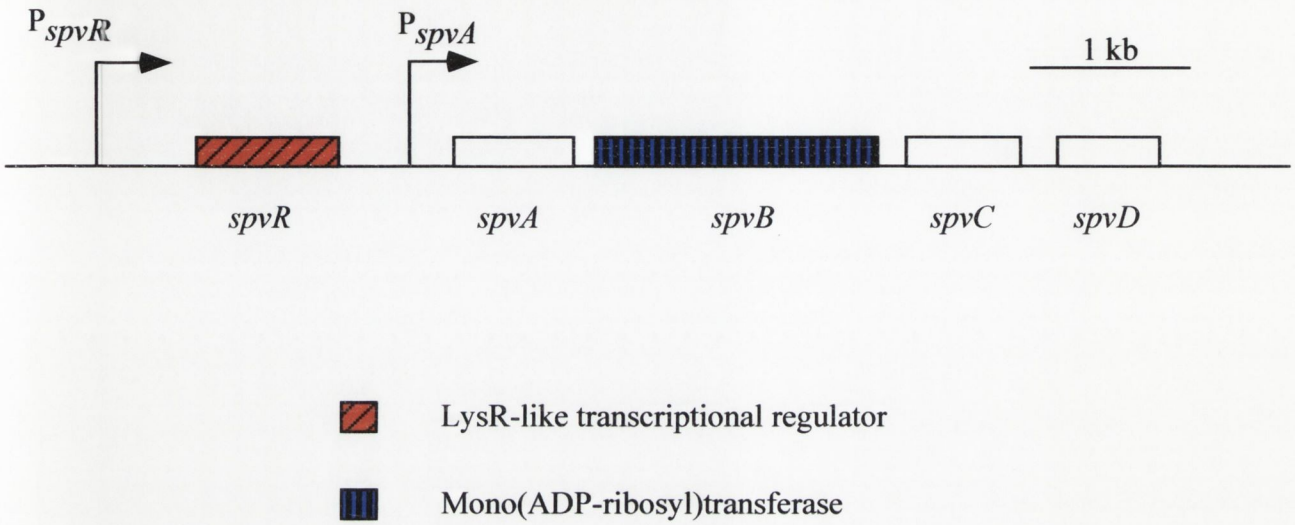


Fig 1.3. Genetic organisation of the *Salmonella* plasmid virulence (*spv*) genes. The *spvR* gene is transcribed from the P_{spvR} promoter while the *spvA*, *spvB*, *spvC* and *spvD* genes are transcribed on an operon from the P_{spvA} promoter. Functions of the SpvR and SpvB proteins are indicated.

1994a, b; Robbe-Saule *et al.*, 1997). Lrp is also a repressor of the *spv* genes, however only exerts an effect at the *spvA* promoter, while IHF is a positive regulator of *spvR* (Marshall *et al.*, 1999). The role of IHF in *spvR* gene expression is not fully understood, however, results indicate that it may be activating expression in a supercoiling-dependent manner (Marshall *et al.*, 1999).

1.2.10 Integrated bacteriophages

Horizontal gene transfer is widely regarded as the predominant force behind evolution of pathogenicity in bacteria (Ochman *et al.*, 2000). Pathogenicity islands are thought to have been acquired by horizontal transfer and recently it has emerged that lysogenic bacteriophages play an important role in disseminating *S. typhimurium* virulence determinants (Figueroa-Bossi *et al.*, 1997; Figueroa-Bossi and Bossi, 1999). Studies in *S. typhimurium* have identified several integrated bacteriophages that encode virulence genes (Figueroa-Bossi *et al.*, 2001). Among the best studied of these bacteriophages are the Gifsy and Fels phages. Studies have identified virulence genes encoded on the Gifsy-1 (Stanley *et al.*, 2000), Gifsy-2 (Ho and Slauch, 2001; Ho *et al.*, 2002), and Fels-1 (Figueroa-Bossi *et al.*, 2001) bacteriophages. These phages are fully functional and can be excised and used to infect other strains of *Salmonella* (Figueroa-Bossi *et al.*, 2001). The functions of these phage-encoded proteins are not fully understood but in most cases they are thought to encode effector proteins secreted through the SPI 1 or SPI 2 type III secretion systems. The repertoire of bacteriophages among closely related strains of *Salmonella* varies significantly with some bacteriophages unique to certain strains of the same serovar (Figueroa-Bossi *et al.*, 2001). It is thought that these bacteriophages disseminate virulence genes, are a mechanism for "fine tuning" virulence, and perhaps play a part in adapting strains of *Salmonella* to cause infection in specific hosts.

1.3 *Salmonella* pathogenicity island 2 (SPI 2)

The first indication that *S. typhimurium* contained a second type III secretion system came about during a study to identify bacterial virulence genes using signature-tagged mutagenesis (Hensel *et al.*, 1995). Four genes were identified that were required for virulence in mice and showed homology to genes encoding proteins of type III secretion systems. Analysis of the chromosomal location of the transposon insertions led to the identification of a 40 kb virulence gene cluster at 30 minutes on the *S. typhimurium* chromosome (Shea *et al.*, 1996). Simultaneously, another group identified the same pathogenicity island as being a chromosomal region unique to *S. typhimurium* that is required for virulence in mice (Ochman *et al.*, 1996).

The 40 kb SPI 2 insertion occurs at the $tRNA^{val}$ gene and is divided into 2 segments, each of which resulted from a distinct horizontal transfer event (Hensel *et al.*, 1997a; Hensel *et al.*, 1999). The smaller region is 15 kb in size and has a different codon frequency pattern and G + C content than the larger 25 kb region. In addition, the 15 kb region is not required for systemic infection and intracellular accumulation, and is present in *Salmonella enterica* and *Salmonella bongori* species (Hensel *et al.*, 1999). Encoded on the 15 kb region are five *ttr* genes involved in tetrathionate reduction, and seven open reading frames (ORF's) of unknown function. It has been suggested that this arm of SPI 2 was acquired first due to its presence in the phylogenetically older species *S. bongori.*

The larger, 25 kb section of SPI 2 is required for virulence and is only present in *Salmonella enterica* species (Hensel *et al.*, 1999). Within this region there are genes coding for a type III secretion system as well as regulatory, effector and chaperone genes (Cirillo *et al.*, 1998; Hensel *et al.*, 1998; Ochman *et al.*, 1996; Shea *et al.*, 1996). SPI 2 genes are designated *ssa* (for type III secretion system apparatus), *ssr* (for secretion system regulators), *ssc* (for secretion system chaperones) and *sse* (for secretion system effectors) (Hensel *et al.*, 1997b). This nomenclature system was based on sequence homologies to known type III secretion system apparatus, effector and chaperone proteins. Subsequent analyses of certain SPI 2 proteins have revealed that they were incorrectly named. For example, the SseA protein is a chaperone not an effector while the SsaB protein is an effector and not part of the

type III secretion system needle complex (Ruiz-Albert *et al.*, 2003; Uchiya *et al.*, 1999). Alternative names have been given for some of these (e.g. SpiC for SsaB) but for consistency throughout this work the *ssr*, *ssa*, *sse*, and *ssc* names have been used. The genetic organization of SPI 2 places functionally related genes together into four clusters called the regulatory, structural I, effector/chaperone, and structural II regions (Fig 1.4) (Cirillo *et al.*, 1998). It has been suggested that the transcriptional organization of the genes into operons follows this pattern of clustering with the *ssrAB* genes in the regulatory region on one operon, and the structural I, effector/chaperone and structural II genes all transcribed on distinct operons (Cirillo *et al.*, 1998). There have been some conflicting reports as to the organization of the transcription units in the structural II region. One report placed the location of the promoter downstream of the *ssaJ* gene and upstream of the *ssaK* gene. Using RT-PCR they were unable to link these genes on the same mRNA strand using primers in *ssaJ* and *ssaK* and therefore concluded that they are transcribed on separate transcripts (Hensel *et al.*, 1997b). Another report placed the structural II promoter further upstream, between the *sseG* and *ssaH* genes (Cirillo *et al.*, 1998). This report however failed to take into account the *ssaG* gene, which lies in this region. This omission makes it very difficult to identify the location of the promoter. The results suggest that perhaps more than one promoter is involved in transcription of the structural II region.

Shortly after the identification of SPI 2, work began to determine its role in *Salmonella* infection. Type III secretion systems form needle-like complexes that secrete or inject bacterial effector proteins into the host cell cytosol (Hueck, 1998). The apparatus spans the bacterial inner and outer membranes and translocon proteins form a pore in the host cell membrane creating a channel through which the effector proteins gain entry to the host cell (Frankel *et al.*, 1998). SPI 2 is the second such system encoded by *S. typhimurium* with the SPI 1 system involved in invasion of gut epithelial cells.

An investigation to identify bacterial genes preferentially expressed inside host cells identified the *ssaH* gene as being highly expressed inside macrophages (Valdivia and Falkow, 1997). This work led to an analysis that demonstrated that SPI 2 genes encoding structural, regulatory, effector and chaperone proteins are preferentially

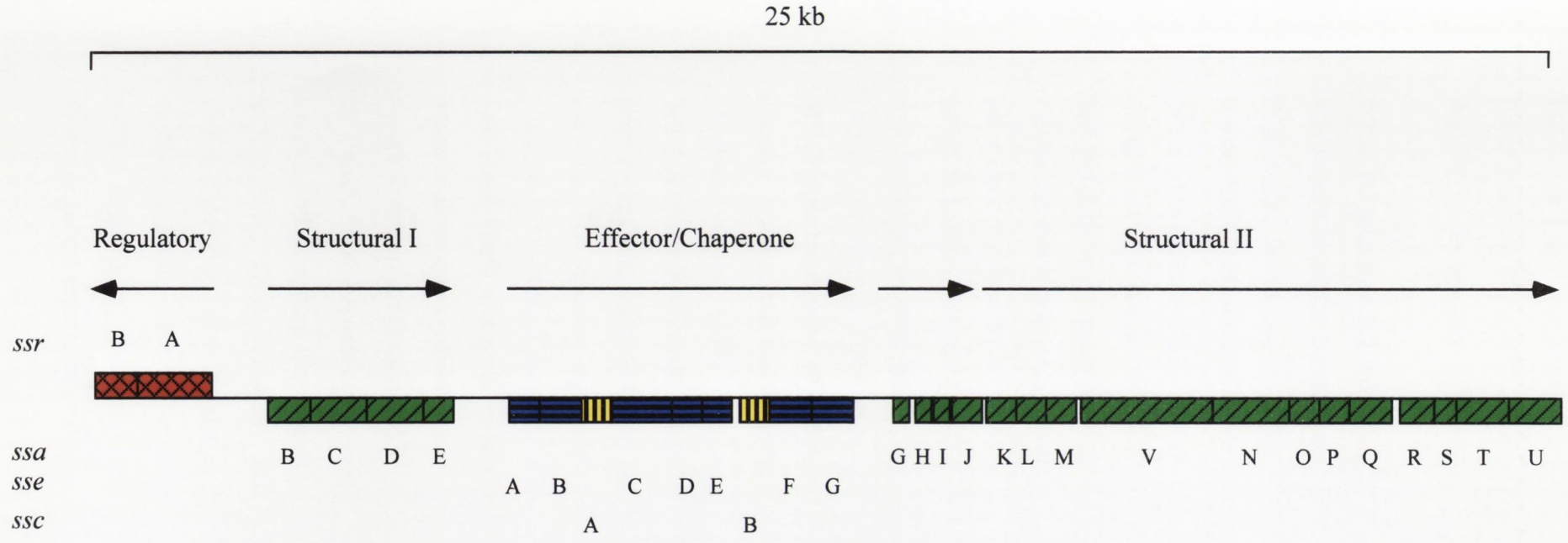


Fig 1.4. Genetic structure and organisation of *Salmonella* pathogenicity island 2 (SPI 2).

SPI2 genes encoding components of the type III secretion system (hatched boxes), regulatory proteins (shaded boxes), effector proteins (open boxes), and chaperones (filled boxes) are indicated. Arrows indicate the proposed transcriptional units.

expressed in the intracellular environment of the macrophage (Cirillo *et al.*, 1998). This work also demonstrated for the first time that the SsrA/SsrB system is required for expression of the SPI 2 genes and that mutations in SPI 2 genes results in an inability of the bacteria to replicate inside macrophages.

While the structure and function of SPI 2 was beginning to be understood, very little was known about the regulation of the genes within SPI 2. It was known that SPI 2 is activated inside macrophages and that the two-component signal transduction system SsrA/SsrB is required for this activation, but the specific signals that activate SPI 2 were unknown (Cirillo *et al.*, 1998). Conflicting reports have been published about what signals activate SPI 2 gene expression. Some groups have reported that low pH activates expression of SPI 2 genes (Cirillo *et al.*, 1998; Lee *et al.*, 2000). Others say that it does not (Deiwick *et al.*, 1999; Hensel, 2000). Low levels of Mg^{2+} have been reported by some to be required for SPI 2 expression (Deiwick *et al.*, 1999) but others disagree (Miao *et al.*, 2002). In the case of pH, it has been shown that bacterial survival inside macrophages decreases when acidification of the vacuole is inhibited (Rathman *et al.*, 1996). This means that low pH either activates SPI 2 gene expression or acts as a trigger for secretion of SPI 2 effectors. The effector protein SseB has been shown to be secreted only under conditions of low pH (Beuzon *et al.*, 1999). This result, along with the ambiguous reports concerning SPI 2 gene expression and low pH, suggests that acidic conditions do not influence gene expression, rather that they act as a trigger for the bacteria to release effector proteins. The situation regarding the role of Mg^{2+} in SPI 2 gene expression is less clear, as is the role of the PhoP/PhoQ two-component signal transduction system. The PhoP/PhoQ system is responsible for the regulation of at least 40 virulence genes in *Salmonella* (Miller *et al.*, 1989; Miller and Mekalanos, 1990). PhoQ is the sensor kinase of the system and PhoP the response regulator. When phosphorylated by PhoQ, PhoP becomes activated, inducing expression of PhoP-activated genes (*pag*s), and inhibiting the expression of PhoP-repressed genes (*prg*s) (Behlau and Miller, 1993; Groisman *et al.*, 1989; Miller *et al.*, 1989; Miller and Mekalanos, 1990). PhoP/PhoQ involvement in SPI 2 gene expression is a contentious issue. A number of studies have reported that SPI 2 gene expression decreases in a *phoP* mutant background (Deiwick *et al.*, 1999; Lee *et al.*, 2000; Worley *et al.*, 2000), however recent reports have disagreed with this and the prevailing attitude at the

moment is that SPI 2 expression is not regulated by PhoP/PhoQ (Beuzon *et al.*, 2001; Miao *et al.*, 2002).

Besides SsrA/SsrB, the only other known regulator of SPI 2 gene expression is the OmpR/EnvZ two-component signal transduction system (Feng *et al.*, 2003; Lee *et al.*, 2000). EnvZ, the sensor protein, detects changes in osmolarity, which it passes on to OmpR by phosphorelay (Forst and Roberts, 1994). OmpR, the response regulator, binds to promoter DNA and activates or represses gene expression. The OmpR/EnvZ system had previously been shown to be important for *S. typhimurium* virulence in mice (Dorman *et al.*, 1989), and now its role was extended to the regulation of SPI 2. OmpR was shown to bind to the *ssrA* promoter and be required for *ssrAB* transcription (Lee *et al.*, 2000). Recently another study identified five phospho-OmpR binding sites within the *ssrA* promoter and identified a promoter upstream of *ssrB* that is also OmpR/EnvZ-regulated (Feng *et al.*, 2003). It has been proposed that this promoter transcribes the *ssrB* gene, suggesting that transcription of the *ssrA* and *ssrB* genes is not linked.

Through the use of a variety of SPI 2 knock-out mutants, as well as strains carrying mutations in certain effector genes, SPI 2 has been shown to play a part in various aspects of *Salmonella* intramacrophage survival, including inhibition of lysosomal fusion with *Salmonella*-containing vacuoles (SCV) (Uchiya *et al.*, 1999), maintaining SCV membrane integrity (Beuzon *et al.*, 2000; Ruiz-Albert *et al.*, 2002), avoiding NADPH oxidase-dependent killing (Vazquez-Torres *et al.*, 2000), SPI 1 independent macrophage killing (van der Velden *et al.*, 2000) and actin cytoskeleton rearrangements (Meresse *et al.*, 2001). The number of diverse activities implies that several different effector proteins are involved and in certain cases these have been identified. In some of the above studies SPI 2 null mutants were used meaning that the effector proteins responsible are unknown (van der Velden *et al.*, 2000; Vazquez-Torres *et al.*, 2000). Effector proteins have been identified both within SPI 2 and also located elsewhere on the chromosome.

The SPI 2 encoded proteins SseB, SseC and SseD are secreted through the type III secretion system but are not in fact effector proteins. They are the SPI 2 components that form the translocon pore in the host cell membrane. They are not

required for secretion of effectors but are necessary for their translocation into the host cytosol (Klein and Jones, 2001; Nikolaus *et al.*, 2001).

The SseF and SseG proteins are translocated SPI 2 effectors (Hansen-Wester *et al.*, 2002). Their role inside the host cell is not fully understood, however, they have been shown to play a part in the formation of *Salmonella*-induced filaments (Sifs) (Kuhle and Hensel, 2002). Sifs are filamentous tubules, rich in lysosomal glycoproteins, that form around and extend from the SCV (Garcia-del Portillo *et al.*, 1993).

Another effector protein encoded within SPI 2 is SsaB (also called SpiC). SsaB is a translocated effector protein (not part of the type III secretion apparatus) whose functions are varied and disputed by different groups. SsaB was the first SPI 2 effector identified and studies with *ssaB* mutants showed that the protein was required to prevent fusion of the SCV with endosomes and lysosomes (Uchiya *et al.*, 1999). Additional work on SsaB has raised questions about this finding and on the role of SsaB as an effector protein. It has recently been shown that SsaB is required for secretion of the translocon proteins SseB, SseC and SseD (Freeman *et al.*, 2002; Yu *et al.*, 2002), as well as the translocation of SPI 2 effectors into infected macrophages (Freeman *et al.*, 2002). These results suggest that an *ssaB* mutant behaves much the same as a SPI 2 null mutant. This implies that the loss of SsaB in an *ssaB* mutant prevents effector translocation, which results in the *ssaB* phenotype observed by Uchiya *et al.* (1999). The SsaB protein *per se* does not prevent fusion of lysosomes with the SCV.

As well as SPI 2 encoded effectors, several non-SPI 2 encoded effector proteins are secreted through the SPI 2 type III secretion system (Waterman and Holden, 2003). Among these are SifA, SifB, SspH1, SspH2, SlrP, SseI and SseJ (Miao *et al.*, 1999; Miao and Miller, 2000). Most of these are regulated by the SsrA/SsrB system and contain a conserved signal sequence that targets them to the type III secretion system (Miao and Miller, 2000). The *sspH1*, *sspH2* and *sseI* genes are all located within lysogenic bacteriophages (Figueroa-Bossi *et al.*, 2001; Miao and Miller, 2000). These prophages are fully functional and most likely represent a mechanism of dissemination for effector protein genes throughout the *Salmonellae* species

(Figueroa-Bossi and Bossi, 1999; Figueroa-Bossi *et al.*, 2001). Several other genes have been identified that are regulated by the SsrA/SsrB system (Worley *et al.*, 2000). The functions of these genes are unknown and it has yet to be demonstrated that they are SPI 2 effectors. Interestingly, most of these SsrA/SsrB-regulated genes are located in uncharacterised regions of the chromosome that contain numerous bacteriophage-like genes. This supports the idea that integrating prophages are carriers of important *Salmonella* virulence determinants (Figueroa-Bossi *et al.*, 2001).

1.4 Nucleoid-associated proteins, supercoiling and Fis

1.4.1 Nucleoid-associated proteins

The *S. typhimurium* genome consists of a 4.8 million base pair, circular DNA molecule (McClelland *et al.*, 2001). This molecule, if linearized, would be over 1.5 mm long. The average *Salmonella* cell is a 1 μm X 0.5 μm cylinder, of which the nucleoid occupies approximately half (Azam *et al.*, 1999). The bacteria must condense the DNA to fit into this volume but also maintain it in a conformation that allows routine DNA transactions, such as transcription, to occur. It does this using a group of proteins that, due to functional similarity alone, have been named bacterial histone-like, nucleoid-associated proteins (hereafter called nucleoid-associated proteins) (Azam and Ishihama, 1999). The four major nucleoid-associated proteins in *S. typhimurium* are Fis, (factor for inversion stimulation), H-NS, (histone-like nucleoid structuring protein), IHF (integration host factor) and HU (heat-unstable nucleoid protein). These proteins contribute to the condensation of the genome by binding to the DNA and introducing bends or kinks in it. This helps compact the DNA allowing it to fit inside the cell and maintains it in a conformation that allows DNA transactions such as transcription to occur.

Nucleoid-associated proteins have the ability to bind to non-specific DNA sequences, however some, such as Fis and IHF, have been shown to bind preferentially to specific sequences of DNA (Finkel and Johnson, 1992; Goosen and van de Putte, 1995). The consensus IHF binding sequence is WATCAANNNNTTR while the Fis consensus is a more degenerate KNNYRNNWNNYRNNM (where W=A/T, R=A/G, Y=C/T, K=G/T, M=A/C, N=A/T/G/C). HU and H-NS do not bind to specific sequences of DNA, however they are thought to bind preferentially to specific DNA structures. H-NS has a high affinity for intrinsically curved DNA while HU is thought to bind preferentially to recombination and repair intermediates such as gapped or kinked DNA (Azam and Ishihama, 1999).

In addition to having a role in condensing the bacterial genome, the ability of nucleoid-associated proteins to bind to and influence the structure of DNA means that they are involved in a variety of other biological processes in the cell including

inversion, recombination and replication (Finkel and Johnson, 1992; O'Gara and Dorman, 2000; Schneider *et al.*, 2001).

Another critical biological process that nucleoid-associated proteins are involved in is transcription. All of the major nucleoid-associated proteins have been shown to be involved in the regulation of expression of a wide variety of genes (Dorman and Deighan, 2003; Schechter *et al.*, 2003; Williams and Rimsky, 1997). The mechanisms with which nucleoid-associated proteins influence gene expression are wide-ranging and varied. They can bind to the promoter DNA and compete with RNA polymerase or other regulatory proteins for binding, they can interact directly with RNA polymerase, they can alter DNA structure at a promoter influencing RNA polymerase binding, or, as is the case in many genes regulated by nucleoid-associated proteins, they can affect transcription by unknown mechanisms (Dorman and Deighan, 2003).

An important feature and characteristic of nucleoid-associated proteins is their expression profile *in vitro*. In *E. coli*, the major nucleoid-associated proteins have very distinct expression profiles when growing in batch culture (Azam and Ishihama, 1999). Fis levels are highest during early exponential phase growth and then decline as the cells divide. In contrast, IHF levels are highest in stationary phase while H-NS levels remain relatively constant throughout. The situation with HU is different again due to its ability to form both hetro- and homo-dimers of the two subunits HupA and HupB. Expression of each varies during growth so that at various stages A-A homodimers, B-B homodimers and A-B hererodimers (the activities and functions of which vary) can exist within the cell, with overall HU levels declining in stationary phase (Balandina *et al.*, 2001). Thus the protein composition of the bacterial nucleoid varies according to growth phase, which has important implications for the functions of nucleoid-associated proteins in regulating gene expression.

1.4.2 Supercoiling

Underwinding of the DNA double helix introduces negative supercoils, another important contributor to the compaction of DNA within the cell. The presence of

negative supercoils imparts free energy to the DNA helix, which is required for the DNA to unwind for processes such as transcription (Drlica, 1992). In fact, the transcription process itself alters local supercoiling levels, with positive supercoils introduced in front of RNA polymerase and negative supercoils introduced behind it (Liu and Wang, 1987).

Levels of supercoiling within the cell are controlled by the opposing actions of topoisomerase enzymes (Drlica, 1990). DNA gyrase, a heterodimer consisting of the products of the *gyrA* and *gyrB* genes, introduces negative supercoils into the DNA. Expression of the *gyrA* and *gyrB* genes is activated by relaxation of the DNA (Drlica, 1992). DNA topoisomerase I, encoded by the *topA* gene, removes negative supercoils and relaxes the DNA. Highly negatively supercoiled DNA induces expression of the *topA* gene (Tse-Dinh, 1985). The ability to increase supercoiling levels by gyrase in response to relaxed DNA, coupled to the ability to remove supercoils by topoisomerase I in response to highly negatively supercoiled DNA, represents a homeostatic feed-back loop that maintains the DNA at the optimal superhelical density.

Certain environmental conditions can induce changes in the superhelical density of the DNA. Following nutrient up-shift there is a rapid increase in supercoiling levels, followed by a gradual relaxation (Balke and Gralla, 1987). Conversely, entry into macrophages results in widespread relaxation of the DNA (Marshall *et al.*, 2000). As previously mentioned transcription of certain promoters is strongly influenced by the superhelical density of the DNA. Relaxed DNA activates the *gyrA* and *gyrB* genes, while highly supercoiled DNA activates *topA* gene expression (Drlica, 1992; Tse-Dinh, 1985). Other genes are known to be affected by supercoiling levels including the *fis* gene as well as several stable RNA genes (Schneider *et al.*, 1997, 2000). This connection between transcription and DNA superhelical density means that in response to a new environment, changes in supercoiling levels can act as a trigger to activate expression of genes that are required for growth or survival in that environment. In this way critical genes can be rapidly activated in response to environmental stimuli.

1.4.3 Fis

Fis is the factor for inversion stimulation and was originally identified as a protein that stimulated inversion of the *hin* invertible DNA element in *S. typhimurium* (Heichman and Johnson, 1990). Fis is a 98-amino acid, 11.2 kDa protein that binds to DNA as a homodimer. It binds to the consensus sequence KNNYRNNWNNYRNNM (where W=A/T, R=A/G, Y=C/T, K=G/T, M=A/C, N=A/T/G/C) and has a preference for binding sites located within regions of curved DNA. Upon binding Fis introduces a bend of between 40°–90° in the DNA. The DNA binding and bending abilities of Fis are achieved through a C-terminal helix-turn-helix (HTH) domain (Osuna *et al.*, 1991). In this HTH the two helices are separated by 25 Å instead of the usual 32-34 Å. This distance is too short to allow insertion into adjacent major grooves of the DNA therefore binding models show Fis bound to bent DNA in order to bring the major grooves closer together (Kostrewa *et al.*, 1991). The N-terminal portion of Fis is associated with its ability to stimulate Hin-mediated site-specific DNA inversion (Koch *et al.*, 1991; Osuna *et al.*, 1991). In the *hin* system, Fis binds to two sites and organises the DNA in such a way as to facilitate the formation of the invertasome, a nucleoprotein complex that includes Fis and the recombinase enzyme, located at the recombinase sites (Heichman and Johnson, 1990). Mutations in the N-terminal region of Fis disrupt formation of the invertasome indicating that this region is directly involved in inversion (Koch *et al.*, 1991; Osuna *et al.*, 1991).

Fis performs a similar function at other genetic switches (e.g. *cin* and *gin*), however its role extends beyond DNA inversion to incorporate a whole host of cellular functions (Finkel and Johnson, 1992). Fis is involved in bacteriophage λ site-specific recombination (Thompson *et al.*, 1987), DNA replication (Filutowicz *et al.*, 1992; Gille *et al.*, 1991) and transcriptional regulation of a wide variety of genes. Genes whose expression is influenced by Fis include those coding for stable RNA species (tRNA and rRNA) (Auner *et al.*, 2003), determinants of cellular supercoiling levels (*gyrA* and *gyrB*) (Keane and Dorman, 2003), nucleoid-associated proteins (*hns* and the *fis* gene itself) (Falconi *et al.*, 1993; Ninnemann *et al.*, 1992) and virulence genes (Goldberg *et al.*, 2001; Wilson *et al.*, 2001). Fis has the ability to act both as an activator and repressor of gene expression and does so via several

different mechanisms (Dorman and Deighan, 2003; Xu and Johnson, 1995). At certain promoters Fis activates expression by relieving H-NS mediated repression (Falconi *et al.*, 1993; Falconi *et al.*, 2001), while at others it represses by binding within the promoter. The best-studied examples of Fis regulated genes are the stable RNA genes. Here, multiple Fis binding sites are located in an upstream activating sequence (UAS), located between 60 to 150 bp upstream of the transcription start site. Binding of Fis to the primary site (usually located at position –71) results in activation of transcription due to direct interactions with RNA polymerase (Appleman *et al.*, 1998). In addition to direct interaction with RNA polymerase it has been shown that Fis regulation of stable RNA promoters is also supercoiling dependent (Schneider *et al.*, 1997). It is thought that Fis maintains the upstream DNA in a structure or conformation that stimulates transcription.

Most of the biological processes that Fis is involved in are sensitive to changes in DNA supercoiling (Finkel and Johnson, 1992; Travers *et al.*, 2001). The Fis protein itself plays an important role in controlling supercoiling levels within the cell. It does this by two mechanisms, firstly by binding to the DNA and constraining local supercoils, and secondly due to its involvement in the regulation of genes coding for DNA topoisomerases. Fis represses the gyrase genes, *gyrA* and *gyrB*, and activates the topoisomerase I gene *topA* (Keane and Dorman, 2003; Weinstein-Fischer *et al.*, 2000). The overall effect of this is to reduce the level of negative supercoiling within the cell or relax the DNA. Transcription of the *fis* gene is itself influenced by supercoiling, transcription levels being optimal under conditions of highly negatively supercoiled DNA (Schneider *et al.*, 2000). As previously mentioned environmental conditions can influence supercoiling levels within the cell. Following nutrient up-shift there is a rapid increase in supercoiling followed by a gradual decrease (Balke and Gralla, 1987). This peak in supercoiling levels coincides with a burst in *fis* expression, which then helps restore normal supercoiling levels to the cell (Schneider *et al.*, 1997; Schneider *et al.*, 1999). The burst in *fis* expression also coincides with activation of the stable RNA genes, which are in addition activated by negative supercoiling. In this way cellular supercoiling levels, Fis protein levels and transcription of stable RNA's are all interconnected and tightly regulated.

Recently it has emerged that Fis plays an important role in regulating expression of virulence genes in many different strains of enteric pathogens. Virulence genes in pathogenic strains of *E. coli* (Goldberg *et al.*, 2001; Sheikh *et al.*, 2001) and *Shigella* (Falconi *et al.*, 2001), as well as SPI 1 from *S. typhimurium* (Wilson *et al.*, 2001), have all been shown to be regulated by Fis. In this study the role of Fis is extended to include the regulation of SPI 2 and certain phage-encoded virulence genes.

Chapter 2

Materials and methods

2.1 Chemicals and growth media

2.1.1 Chemicals, reagents and radionucleotides

The supplier for each chemical or reagent used in this study is indicated in parenthesis after the product. DNA restriction and modifying enzymes were obtained from New England Biolabs or Roche Molecular Biochemicals. Radionucleotides were supplied by Amersham Pharmacia Biotech. Custom automated sequencing was performed by MWG Biotech. In addition, several molecular biology kits were used during this study. The basic principle of each kit is briefly described in the appropriate sections below, without giving an exhaustive protocol.

2.1.2 Growth media

Ingredients for preparing growth media were obtained from Difco or Oxoid. All media were sterilized by autoclaving at 120°C for 20 min prior to use, or storage at room temperature. Additional solutions not suitable for autoclaving, e.g. antibiotic solutions, were sterilized by filtration through sterile 0.2 μm Acrodisc Filters (PALL). All quantities listed below are for the preparation of 1 litre of medium in distilled, deionised water (ddH_2O). Media were supplemented with the appropriate antibiotics as required.

<u>LB broth and LB agar plates:</u>

LB agar plates were used throughout this study for reviving bacterial strains from frozen stocks, general culturing of strains, and selection of transformants and transductants. Bacterial strains were routinely grown in Luria-Bertani (LB) broth unless otherwise stated.

LB broth: 10 g Oxoid tryptone, 5 g yeast extract, 5 g NaCl.

LB agar: 10 g Oxoid tryptone, 5 g yeast extract, 5 g NaCl, 15 g agar.

Minimal medium 5.8:

Minimal medium 5.8 (MM 5.8) is a low pH, minimal medium previously described by Kox *et al.* (2000). It is used to simulate conditions inside a macrophage and has been shown to activate transcription of SPI 2.

MM 5.8: 1 g Casamino acids, 12.285 g bis tris, 11.2 ml glycerol, 50 ml 1 M tris-HCl pH 5.8.

After autoclaving 10 µl of 1 M $MgCl_2$ was added.

SOC medium:

SOC medium was used following transformation or electroporation of *S. typhimurium* or *E. coli* strains to increase the efficiency of transformation.

SOC medium: 20 g tryptone, 5 g yeast extract, 0.5 g NaCl.

After autoclaving 0.95 g $MgCl_2$, 1.2 g $MgSO_4$ and 1.8 g glucose was added.

Green agar:

Green agar plates were routinely used following bacteriophage P22-mediated generalized transduction to obtain isolates of *S. typhimurium* free of phages. On these plates phage-free colonies appear light green whereas pseudo-lysogens appear dark green. The dark green colour results from a lowered pH caused by bacterial lysis.

Green agar: 8 g tryptone, 1 g yeast extract, 5 g NaCl, 15 g agar.

After autoclaving 21 ml 40% glucose, 25 ml 2.5% (w/v) Alizarin yellow (freshly prepared) and 3.3 ml of 2% (w/v) Aniline blue were added.

2.1.3 Antibiotics, X-Gal and MUG

All stock antibiotic solutions were stored in aliquots at –20°C and those prepared in water were sterilized by filtration through 0.2 µm Acrodisc Filters (PALL). Carbenicillin, kanamycin and streptomycin were prepared as 50 mg/ml stock solutions in ddH_2O and used in media at a concentration of 50 µg/ml. Tetracycline

was prepared as a 12.5 mg/ml stock solution in 100% ethanol and used at a final concentration of 12.5 μg/ml. Chloramphenicol was prepared as a 25 mg/ml stock solution in 100% ethanol, and used at a final concentration of 25 μg/ml. Novobiocin was prepared at a stock concentration of 50 mg/ml in ddH_2O and used at the concentrations indicated.

X-Gal (5-bromo-4-chloro-3-indoyl-β-D-galactoside), a chromogenic substrate for β-galactosidase, was prepared as a 20 mg/ml stock solution in *N, N*-dimethyl formamide and stored in the dark at –20°C. X-Gal was used in agar plates at a final concentration of 20 μg/ml.

MUG (4-methylumbelliferyl-β-D-galactopyranoside) was prepared at a concentration of 4 mg/ml in DMSO (dimethylsulfoxide) and stored in the dark at –20°C.

2.2 Bacterial strains and culture conditions

2.2.1 Bacterial strains

All bacterial strains used in this study were derivatives of *Salmonella enterica* serovar *typhimurium* strains SL1344 and LT2, and *Escherichia coli* K12 and are listed in Table 2.1. Bacterial strains were maintained as permanent stocks in 15% (v/v) glycerol in LB broth and stored at –70°C.

2.2.2 Bacterial culture conditions

Bacterial cultures were routinely grown aerobically in liquid medium at 37°C with shaking, except where otherwise stated. Cultures were grown by inoculating single colonies into 3 ml of LB broth in sterile test-tubes and incubating at the required temperature overnight. Alternatively, or when larger volumes were required, cultures were grown by inoculating fresh media at a dilution of 1:100 from 3 ml overnight cultures.

Table 2.1. *S. typhimurium* and *E. coli* strains used in this study.

Strain	Relevant Genotype	Reference/ Source
Salmonella typhimurium		
LT2	Wild type	This lab
SL1344	Virulent wild type	Hoiseth and Stocker, (1981)
SL1344 *fis*	SL1344 *fis*::*cat*	Keane and Dorman, (2003)
SL1344 *ihf*	SL1344 *ihfB*::*cat*	Marshall *et al.*, (1999)
SL1344 *hns*	SL1344 *hns*::*kan*	J. Hinton
SL1344 *stpA*	SL1344 *stpA*::*kan*	J. Hinton
TH2285	LT2 *fis*::*cat*	K. T. Hughes
Escherichia coli		
XL-1	Routine cloning strain	Stratagene

When anaerobic and microaerophilic conditions were required (Chapter 5), cultures were incubated in 2.5 l gas jars along with the appropriate AnaeroGen Gas generating kit (Oxoid).

2.3 Eukaryotic cell line and growth conditions

2.3.1 Eukaryotic cell line

The murine, macrophage-like cell line J774A.1 was used throughout this study. J774A.1 cells were obtained from the American Type Culture Collection (ATCC) and permanent stocks were maintained in 5% (v/v) DMSO in TCM and stored in liquid nitrogen.

2.3.2 Eukaryotic cell growth conditions

J774A.1 macrophage-like cells were grown in Tissue Culture Medium (TCM), comprising Dulbecco's Modified Eagles Medium (DMEM; Sigma) supplemented with 10% (v/v) heat inactivated fetal bovine serum (Sigma). When required 100 U penicillin and 100 μg/ml streptomycin (Sigma) were added to the growth medium. Cells were routinely grown in 75 cm^2 tissue culture flasks at 37°C in a 5% CO_2 incubator.

2.4 Plasmids, bacteriophages and oligonucleotides

2.4.1 Plasmids

The plasmids used in this study are listed in Table 2.2 together with relevant details and source. Any necessary details of plasmid construction will be described in the appropriate results chapters.

Table 2.2. Plasmids used throughout this study.

Plasmid	Relevant characteristics	Source/Reference
pQF50	*lacZ* promoterless trap vector. Amp^R	Farinha and Kropinski, (1990)
pQFssrA	645 base pair *ssrA* promoter sequence upstream of promoterless *lacZ* in pQF50	This study
pQFssaB	690 base pair *ssaB* promoter sequence upstream of promoterless *lacZ* in pQF50	This study
pQFsseA	530 base pair *sseA* promoter sequence upstream of promoterless *lacZ* in pQF50	This study
pQFssaG	580 base pair *ssaG* promoter sequence upstream of promoterless *lacZ* in pQF50	This study
pQFssaH	250 base pair *ssaH* promoter sequence upstream of promoterless *lacZ* in pQF50	This study
pQFssaGH	810 base pair *ssaGH* sequence upstream of promoterless *lacZ* in pQF50	This study
pQFssaK	360 base pair *ssaK* promoter sequence upstream of promoterless *lacZ* in pQF50	This study
pQF[R:100]	327 base pair *spvR* promoter sequence upstream of promoterless *lacZ* in pQF50	Marshall *et al.*, (1999)
pQF[R:101]	pQF[R:100] with disrupted IHF binding site	This study
pQF[R:103]	pQF[R:100] with consensus IHF binding site	This study

Amp^R =Ampicillin resistant.

2.4.2 Bacteriophage

The bacteriophage used in this study was bacteriophage P22HT105/1 *int*-201, lysates of which were routinely stored at 4°C in the dark in 5 ml volumes over chloroform.

2.4.3 Oligonucleotides

The sequences and nomenclature of all oligonucleotides used in this study are listed in Table 2.3. Oligonucleotides were purchased from MWG-Biotech, Germany.

2.5 Transduction with bacteriophage P22

Bacteriophage P22 specifically recognises and binds to the O-antigen on the outer membrane of *Salmonella typhimurium*. After binding, double stranded linear DNA is injected into the host. The DNA circularises and is replicated first by O replication and then by rolling circle replication which generates long concatemers of double-stranded P22 DNA. These concatemers are resolved by cleavage by a phage-encoded nuclease, which cuts the DNA at specific sequences, 44 kb apart, called Pac sites. This DNA is packaged into new phage particles, which are released from the host by lysis after 50-100 of these particles have been produced.

In this study the P22 derivative used for generalized transduction in *Salmonella typhimurium* was P22 HT105/1 *int*-201. The high transducing frequency of this phage results from its nuclease having a lower specificity for the Pac sequence. This results in a high proportion of the phage heads carrying chromosomal DNA. Approximately 50% of the P22 HT (high transducing) phage heads carry random transducing fragments of host DNA. The *int* mutation prevents the formation of stable lysogens.

Table 2.3. Oligonucleotide primers used throughout this study.

Primer Name	Sequence[a]
ssrA_F[b]	5'-ATA CGG ATC CGA ATT CGT CGA CGG CAA GAC AAG GCT TAG GTA AGC-3'
ssrA_R[c]	5'-ATT AGG TAC CGG ATC CGC CTG ATT ACT AAA GAT GTT TGC-3'
ssaK_F[b]	5'-CGC GGA TCC CTT CTC CGA GCT CAG TTG CC-3'
ssaK_R[b]	5'-CGC GGA TCC CGA TTA AGA ATG GCG GTA CCG ATC-3'
ssaB_F[b]	5'-CGC GGA TCC CGT TCA GCT TCT TCA AAC-3'
ssaB_R[c]	5'-CGG GGT ACC CCT GAT ATC CTG AAT TAA TGG-3'
sseA_F[d]	5'-CAT GCC ATG GCC CGG TTA GAA GAT TTG CTG C-3'
sseA_R[c]	5'-CGG GGT ACC CGA TAT TCA CTA AAC GCA GCC-3'
ssaG_F[b]	5'-CGC GGA TCC GGA TTG GCC TTG CTA TTG C-3'
ssaG_R[c]	5'-CGG GGT ACC GGG TTG AGC AAA TCA TTA CC-3'
ssaH_F[b]	5'-CGC GGA TCC GGT AAT GAT TTG CTC AAC CC-3'
ssaH_R[c]	5'-CGG GGT ACC CGT TAG CGC TGG TAA CAT CG-3'
spvR11	5'-CCA AGC TTC AGT ACT GAT CTT GCG ATA CTG-3'
spvR14	5'-CCC AAG CTT CAG GTC ACC GCC ATC CTG TTT TTG C-3'
gyrA_F	5'-CGC GGA TCC GCC GCT TTC CAG TGC GTG CAG-3'
gyrA_R	5'-CGG GGT ACC CTA ACC GCT ATC CCT CTA G-3'
ssaK_F2	5'-GCT ATT TTT GCC TGA GAC G-3'
ssaK_R2	5'-CCA CTG TAG AAG CAA TTG C-3'
IHF_koF	5'-C CAG CTA TAC ATC ATA ACA GGA ATA TTA AAT CCA CTC AG-3'
IHF_koR	5'-CT GAG TGG ATT TAA TAT TCC TGT TAT GAT GTA TAG CTG G-3'
IHF_conF1	5'-C CAG CTA TAC ATC ATA ACA TAT CAA TTA AAT CCA CTC AG-3'
IHF_conR1	5'- CT GAG TGG ATT TAA TTG ATA TGT TAT GAT GTA TAG CTG G -3'
IHF_conF2	5'- C CAG CTA TAC ATC ATA ACA TAT CAA TTA AAT CCA CTC AG -3'
IHF_conR2	5'- CT GAG TGG ATT TAA TTG ATA TGT TAT GAT GTA TAG CTG G -3'

a. Restriction enzyme cleavage sites are underlined.

b. Primer tailed with restriction site for *Bam*HI.

c. Primer tailed with restriction site for *Kpn*I.

d. Primer tailed with restriction site for *Nco*I

2.5.1 Preparation of a P22 lysate

P22 lysates were routinely prepared as follows. The donor strain was grown overnight in 2 ml of LB broth at 37°C, with antibiotic selection as appropriate. This culture was used to inoculate 5 ml of fresh broth at a 1:200 dilution. This culture was incubated at 37°C with shaking until the OD_{600nm} reached approximately 0.15. At this point 10 µl of P22 phage stock (titre of approximately 10^{10} pfu/ml) was added and incubation continued for a further 4 hours. At this point 500 µl of chloroform was added, the culture mixed by vortexing and stored for 1 hour at 4°C. Cellular debris was removed by centrifugation in a bench-top centrifuge at 3,800 g for 20 min. The supernatant, containing the lysate was transferred to a clean tube and stored over chloroform.

2.5.2 P22 transduction

Genetic markers were mobilised using the general transducing properties of P22 lysates as follows. The recipient strain was grown overnight at 37°C in 2 ml of LB broth. 100 µl of the recipient culture was removed to a sterile 1.5 ml tube containing 100 µl of the donor P22 lysate. The mixture was incubated at 30°C without shaking for 30 min. 800 µl of LB broth was then added and the culture was incubated at 37°C with shaking for a further 30 min. The transduced cells were then harvested by centrifugation in a microfuge for 4 min at 20,800 g. 900 µl of the supernatant was then removed and the pellet resuspended in the remaining broth. Transduced cells were selected for by plating on agar plates with the appropriate antibiotic. The plates were incubated at 37°C overnight. True lysogens were then distinguished from pseudo-lysogens by 3 repeated single colony purifications on Green agar plates. On these plates true lysogens appear light green while pseudo-lysogens appear dark green. At this point transductants were deemed suitable for further experimentation.

2.6 Transformation with plasmid DNA

Plasmid DNA was transformed by two different methods. Either recipient cells were made competent by treatment with calcium chloride coupled with a heat-shock uptake of plasmid DNA, or transformation by electroporation, a high-voltage electroshock treatment. Greater transformation efficiencies can be achieved with electroporation than can be achieved with the $CaCl_2$ method. The $CaCl_2$ method is more cost effective and was routinely used for the transformation of plasmid DNA or ligation mixes. Electroporation was only sometimes used for transformation of precipitated ligation mixes where greater efficiency was required.

2.6.1 Transformation via calcium chloride method

An overnight culture of the strain to be made competent for transformation was used to inoculate 250 ml of LB broth and was grown to an OD_{600nm} of approximately 0.4. The cells were incubated on ice for 30 min and then pelleted by centrifugation at 5,800 g for 10 min, and the bacterial pellet resuspended in 100 ml of ice-cold $CaCl_2$ solution (60 mM $CaCl_2$, 15% glycerol, 10 mM PIPES). The cells were spun again for 10 min at 5,800 g, the supernatant removed and resuspended in 100 ml of ice-cold $CaCl_2$ solution. After incubation on ice for 30 min cells were again harvested as described above, and resuspended in 10 ml ice-cold $CaCl_2$ solution. At this stage cells were distributed into 500 µl aliquots and used directly, or stored at –70°C.

DNA (0.1–1 µg) in a volume not exceeding 10 µl, was added to 100 µl of competent cells and left on ice for 10 min, thus allowing the DNA to contact the bacterial surface. The tubes were then placed in a 42°C water bath for 2 min. This heat-shock treatment allows uptake of the plasmid DNA through the $CaCl_2$ induced competent bacterial membrane by an unknown mechanism (Mandel and Higa, 1970). 1 ml of SOC broth was added to the culture, which was then incubated at 37°C for 1 hour to allow phenotypic expression of the plasmid-borne antibiotic resistant marker. Subsequently, 10 µl and 100 µl samples of the transformation mix were plated onto appropriate selection plates. Cells to which no DNA had been added were treated in the same way and thus served as a control for contamination.

Following overnight incubation at 37°C, single colony transformants were purified on fresh selective agar plates.

2.6.2 Transformation via electroporation method

Typically 250 µl of an overnight culture of the strain to be made electrocompetent for transformation was used to inoculate 250 ml of LB broth and grown to an OD_{600nm} of 0.6. Cells were incubated on ice for 30 min and then pelleted by centrifugation at 5,800 g for 10 min and the bacterial pellet resuspended in 100 ml of sterile, ice-cold ddH_2O. The cells were again pelleted by centrifugation at 5,800 g for 10 min and resuspended in 100 ml cold ddH_2O. Following another centrifugation step, cells were resuspended in 500 µl of ice-cold 10% (v/v) glycerol. 120 µl aliquots were used directly or stored at –70°C.

The DNA to be electroporated (50–200 ng in 5 µl sterile water) was added to a 40 µl aliquot of electrocompetent cells and incubated on ice for 1 min. The mixture was then transferred to a pre-chilled electroporation cuvette (EquiBio, 0.2 cm gap width). The cuvette was placed in the Gene Pulser chamber (Bio-Rad) and an electroshock delivered. To the cuvette 1 ml of pre-warmed SOC broth was added and the contents transferred to a sterile tube and incubated at 37°C with aeration for 1 hour. This incubation allows phenotypic expression of the antibiotic resistance marker. Subsequently 100 µl of the transformation mix was plated onto appropriate selection plates. Cells to which no DNA had been added were treated in the same way and thus served as a control for contamination. Following overnight incubation at 37°C, transformants were single-colony purified.

2.7 Assays based on spectrophotometry

2.7.1 Monitoring bacterial growth

The growth of bacterial cultures was monitored by measuring the optical density of the culture at a wavelength of 600nm (OD_{600nm}). For routine measurement of OD_{600nm}, including estimation of cell quantity for β-galactosidase assays (section 2.7.2), 0.2–1 ml of the culture was transferred into a plastic disposable cuvette (Greiner), and brought to a final volume of 1 ml with LB broth. The OD_{600nm} value was measured in a spectrophotometer against a cuvette containing only LB broth as a blank. This value was linear in the range 0.1–0.8 and was multiplied by the dilution factor if necessary.

2.7.2 Assay of β-galactosidase activity

Transcriptional levels were routinely quantified by the β-galactosidase assay in which the *lac* genes are placed under the regulatory control of the promoter of a target gene. The transcription levels of the promoter are reflected in levels of the stable β-galactosidase enzyme, the product of the *lacZ* gene (Miller, 1992). In bacterial cells β-galactosidase cleaves the β-galactoside linkage of lactose, resulting in the formation of galactose and glucose. These then enter the glycolytic pathway. Several synthetic substrates such as ONPG (*o*-nitrophenyl-β-D-galactopyranoside), X-Gal, and MUG contain the β-galactoside linkage and thus can be hydrolysed by β-galactosidase. In the case of ONPG, which is colourless, cleavage results in the production of galactose, and *o*-nitrophenol, which is intensely yellow. Therefore, assaying the concentration of *o*-nitrophenol reports on the cellular level of β-galactosidase, which is determined by the activity of the target promoter.

The β-galactosidase assay used in this study has been previously described by Miller (1992). The bacterial culture to be assayed (50 µl) was transferred in duplicate into tubes containing 950 µl Z-buffer, 50 µl $CHCl_3$ and 25 µl of 0.1% SDS. Tubes were vortexed briefly to enhance permeabilization, and then incubated for 10 min at 28°C before addition of 200 µl of ONPG (4 mg/ml in ddH_2O). Incubation was continued

until a straw yellow colour was obtained (typically corresponding to an OD_{420nm} reading of 0.1-0.6). The reaction was stopped with the addition of 500 µl of 1 M Na_2CO_3, and the tubes were centrifuged at 20,800 g for 10 min to pellet cellular debris. 1 ml of the supernatant was transferred into a plastic cuvette and the OD_{420nm} determined. The amount of β-galactosidase activity was expressed in Miller units and calculated as follows:

$$\text{Activity (Miller units)} = \frac{1000 \times OD_{420nm}}{t \times V \times OD_{600nm}}$$

t = Reaction time in min (from addition of ONPG to addition of Na_2CO_3)
V = Volume of cells added in ml.

Each assay was performed in duplicate, and the mean values were determined from at least 3 independent experiments.

Z buffer: 60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 50 mM β-mercaptoethanol, 10mM KCl, 1 mM $MgSO_4$

2.7.3 Determination of nucleic acid concentration

The concentration of DNA and RNA samples was determined spectrophotometrically by measuring the absorbance at 260nm. Samples were diluted (typically 1:500) in ddH_20 before reading the OD_{260nm}. The concentration of nucleic acid was then determined according to the following formulae:

OD_{260nm} value of 1 corresponds to - 50 µg/ml of double-stranded DNA
40 µg/ml of single-stranded DNA or RNA

The purity of DNA or RNA was assessed by measuring the A_{280nm}. For pure DNA, uncontaminated by proteins or residual phenol the ratio of A_{260nm} to A_{280nm} is 1.8 while for RNA uncontaminated by proteins or residual phenol, the ratio of A_{260nm} to A_{280nm} is 2 (Sambrook *et al.*, 1989).

2.7.4 Determination of protein concentration by the Bradford assay

Protein concentration was determined using a Bio-Rad Protein Assay, which is based on the method of Bradford (1976), and measures the differential colour change (shift in absorbance from 465 to 595 nm) of Coomassie Brilliant Blue G-250 when protein binding occurs. The concentration of protein was determined by measuring several serial dilutions. The resulting OD_{595nm} measurements were compared to a standard curve determined by measuring several known concentrations of lysozyme.

2.8 Purification of RNA, plasmid DNA and chromosomal DNA

2.8.1 Small-scale isolation of plasmid DNA

The Wizard Plus *SV* Miniprep Kit (Promega) was routinely used to extract plasmid DNA from 5 ml cultures according to the guidelines provided. The procedure is based on a modified alkaline lysis method where bacteria are lysed and proteins denatured (SDS) in the presence of protease inhibitors. RNA is then degraded (RNase), and chromosomal and plasmid DNA denatured (NaOH). The lysis mixture is then neutralized with salts, causing protein and chromosomal DNA precipitation. Plasmid DNA rapidly re-anneals and debris is pelleted by centrifugation. The supernatant containing plasmid DNA is washed and desalted through a mini-column, and eluted in 100 µl ddH_2O.

2.8.2 Large-scale preparation of high purity plasmid DNA

The QIAGEN midi-plasmid purification kit was used to extract plasmid DNA from 100 ml overnight cultures of *E. coli*, according to the guidelines provided. Purification is based on a modified alkaline lysis procedure similar to that described in section 2.8.1, followed by binding of plasmid DNA to a column-based anion-exchange resin under low salt and pH conditions. A medium salt wash removes RNA, proteins, and other impurities, and the plasmid DNA is eluted with a high-salt

buffer. The DNA is then precipitated with isopropanol, desalted, and resuspended in 150 µl ddH_2O.

2.8.3 Purification of chromosomal DNA

Purification of chromosomal DNA for use in PCR was performed using the AGTC Bacterial Genomic DNA Purification Kit (Edge Biosystems). A 5 ml sample of an overnight culture was used for DNA extraction according to the guidelines provided. Briefly, the procedure involves conversion of bacteria to sphaeroplasts (spherical cells from which most of the cell wall has been removed) by incubation in a Tris-buffered solution containing lysozyme (cleaves peptidoglycan), sucrose (osmotic stress), and EDTA (chelates divalent metal ions, which are necessary cofactors for protease and DNase activity). Efficient lysis is then achieved by heating to 65°C in the presence of SDS (protein denaturant), NaCl (osmotic shock) supplemented with RNase. Latex beads are added that bind and clump denatured proteins and cellular debris. The mixture is centrifuged (debris pellets) and chromosomal DNA is extracted from the supernatant by precipitation with isopropanol. Chromosomal DNA is desalted, dried and uniformly resuspended in a final volume of 100 µl ddH_2O.

2.8.4 Isolation of RNA

RNA is a chemically unstable molecule and prone to digestion by ubiquitous RNases which require no cofactors to function, and can maintain activity even after autoclaving or boiling (Sambrook *et al.*, 1989). For these reasons certain precautions were used when isolating or handling RNA. These included wearing gloves, and using separate designated tips, tubes and electrophoresis tanks. All solutions were prepared with DEPC-treated ddH_2O, which inactivates RNases by covalent modification (1 ml DEPC/L ddH_2O, mixed overnight then autoclaved) or RNAse free water (Sigma). In addition, where possible, all steps in the isolation of bacterial RNA were performed quickly, and on ice.

Two methods of RNA purification were used in this study. RNA for use in primer extension analysis was prepared using the hot phenol method (below), while RNA for use in microarray analysis was prepared using the Promega SV Total RNA isolation kit (see section 2.19.1).

Total RNA was extracted from 7 ml samples of exponential phase cultures (OD_{600nm} of 0.6) by the hot-phenol method similar to that described previously (Kreig, 1996). Cells were harvested by centrifugation and resuspended in 0.5 ml of boiling lysis buffer (2% SDS, 10 mM EDTA, 50 mM sodium acetate (pH 5.3)). Hot phenol (0.5 ml, pH 5.3) was added, and the samples vortexed vigorously before incubation at 90°C for 5 min. After a 10 min incubation on ice, the aqueous layer was extracted twice with 1:1 phenol:chloroform and once with chloroform, and the RNA precipitated with ethanol at –70°C. Following resuspension, RNA concentration and purity was assessed by A_{260nm} and A_{280nm} measurements as described in section 2.7.3.

2.9 Manipulation of DNA *in vitro*

2.9.1 Restriction endonuclease cleavage of DNA

Typically 0.5–2.0 μg of plasmid DNA or purified PCR product were cut with 10 U of restriction enzyme in a 50 μl volume containing the reaction buffer supplied with the enzyme. For double digests involving simultaneous cleavage of DNA by two endonucleases, a suitable buffer was chosen in which both enzymes had >75% activity. Alternatively, double digests were performed sequentially in suitable buffers with ethanol precipitation of the DNA between digestions. Reactions were incubated at 37°C for 1–2 hours unless otherwise recommended.

2.9.2 Purification of linear DNA

Linear DNA fragments (PCR products or cleaved DNA) were purified for cloning, or for the preparation of labelled probes, using the Wizard PCR Prep DNA

purification system (Promega). The linear DNA fragments were purified directly, or from an agarose gel slice. For this, the DNA was electrophoresed through a 1 x TAE agarose gel containing 1 μg/ml of ethidium bromide. The desired DNA fragment was cut out using a surgical blade and purified following the guidelines supplied. Briefly, the procedure entails mixing DNA (from PCR reactions or a gel slice) in a buffer that provides the ions and environment where DNA is selectively bound with high affinity to a silica-based resin. The resin is then trapped in a mini-column and macromolecules, primers, salts and other impurities removed by syringing through 80% isopropanol. The resin is dried and DNA is eluted in ddH_2O.

2.9.3 Ligation of DNA molecules

T4 DNA ligase catalyses the ATP-dependent formation of phosphodiester bonds between adjacent 5'-phosphoryl and 3'-hydroxyl ends in double stranded DNA (Weiss *et al.*, 1968). Bacteriophage T4 DNA ligase supplied with the Rapid DNA ligation Kit (Roche Molecular Biochemicals) was routinely used to clone digested insert DNA into appropriately digested vectors according to the manufacturer's directions. Reactions were performed by incubating an estimated molar ratio of purified vector:insert DNA of 1:5 in a 20 μl volume with 1 μl of T4 DNA ligase. (DNA quantity was estimated by electrophoresing through a 1% TAE agarose gel and comparing band intensity to known standards). The mixture was incubated at room temperature for 20 min and typically a 10 μl sample was directly transformed into appropriate calcium chloride-competent cells.

2.9.4 Phenol extraction and ethanol precipitation of DNA/RNA

Contaminating proteinaceous debris was removed from DNA/RNA solutions by phenol/chloroform extractions, and then concentrated by ethanol precipitation as detailed in Ausubel *et al.* (1990). For nucleic acid extractions, an equal volume of phenol/chloroform (50% (v/v) sodium acetate buffered phenol (pH 5.2), 50% (v/v) chloroform) was mixed with the DNA or RNA sample (adjusted to a minimum volume of 400 μl if necessary), vortexed, and centrifuged for 4 min at 20,800 g. The aqueous (top) layer was carefully removed and an equal volume of chloroform

was added to remove traces of phenol. The contents were mixed and then separated by centrifuging at 20,800 g for 4 min. The DNA or RNA was concentrated by ethanol precipitation. 0.1 volumes of 3.5 M sodium acetate (pH 5.2) and 2 volumes of 100% ice-cold ethanol were added to the nucleic acid suspension. The contents were mixed, and incubated at –20°C for 15 min, before centrifugation at 20,800 g for 10 min. The pellet was washed in 500 µl of 70% ethanol, air-dried and resuspended in a suitable volume of ddH_2O.

2.10 Polymerase Chain Reaction

The polymerase chain reaction (PCR) was used for the amplification of DNA for preparation/confirmation of fragments during cloning strategies, and for generating probes for electrophoretic mobility shift assays. The PCR method is based on the ability of a thermostable DNA polymerase to amplify DNA, primed from oligonucleotides annealed to denatured single-stranded templates (Saiki *et al.*, 1988). The procedure involves thermal denaturation of the DNA template, allowing two specific oligonucleotides to hybridize to complementary sequences on opposite strands of the DNA, flanking the sequence to be amplified. The annealed primers are orientated with their 3' ends facing each other, such that DNA polymerase in the presence of dNTPs and Mg^{2+} will extend across the region of the original DNA template between the primers. Each synthesized strand is complementary to one of the primers, and can serve as template in further cycles of annealing and extension. The denaturation, annealing and extension steps are repeated for 25–35 cycles resulting in exponential amplification of the DNA region of interest.

2.10.1 Amplification of DNA

Two different thermostable polymerases were used in this study. *Taq* DNA polymerase (Roche Molecular Biochemicals) is a highly processive 5' to 3'-DNA polymerase purified in recombinant form, free of endo- or exo-nucleases (Sambrook and Russell, 2001). *Taq* polymerase lacks a 3'–5' exonuclease activity (proof-reading) and was routinely used for PCR when it was unimportant if the product

contained mutations, for example when checking plasmids for cloned inserts. *Pfx* polymerase (Gibco BRL) is also a highly processive 5' to 3'-DNA polymerase, free of endo- or exo-nucleases. *Pfx* harbours a 3' to 5' proof-reading exonuclease activity resulting in a reduced error rate of nucleotide misincorporation. *Pfx* polymerase was used for the amplification of probes for electrophoretic mobility shift assays, and for amplification of DNA fragments for cloning purposes.

PCR reactions were carried out by mixing 5 µl 10 x *Pfx* buffer, 0.2 mM of each dNTP, 100 pmol of each oligonucleotide, 1 U of *Pfx* polymerase, 1–3 mM $MgSO_4$, 10–100 ng template DNA and ddH_2O to a final volume of 50 µl in a 500 µl thin-walled PCR tube (Stratagene). PCR reactions using *Taq* DNA polymerase were performed as above, with the exception that 5 µl 10 x *Taq* buffer (includes 1.5 mM $MgCl_2$) and 1 U *Taq* polymerase were used. Reactions were set-up on ice and immediately placed into the Peltier Thermal Cycler. One tube contained no template DNA and served as a negative control. Routinely the reaction cycles were as follows:

1. 94°C, 2 min [denaturation]
2. oligo annealing temperature, 30 sec
3. 72°C, 1 min [extension]
4. 94°C, 30 sec [denaturation]
5. steps 2-4 above repeated, 30 cycles
6. 72°C, 10 min [final extension and renaturation]

The annealing temperature was typically set at 1°C below the theoretical melting temperature (Tm) of the oligonucleotides being used. The Tm was calculated using the formula Tm = 2 x (A+T) + 4 x (G+C) -2, where A, T, G and C refer to the base composition of the oligonucleotide (Sambrook and Russell, 2001). Extension time depended on expected length of PCR product (~1 min per kilobase).

DNA sequences were amplified from purified chromosomal or plasmid DNA. PCR amplification was also carried out using template DNA from a cell lysate. Here, a

single colony was scraped from the agar plate with a plastic tip and resuspended in 20 µl ddH_2O, boiled for 3 min and 2 µl used for each PCR reaction.

2.11 Gel electrophoresis

2.11.1 Agarose gel electrophoresis

Analysis of DNA and RNA samples was performed on 1% (w/v) agarose gels. Agarose gels were prepared as follows: 1g agarose was added to 100 ml TAE (40 mM Tris, 1 mM EDTA, 0.114% (v/v) glacial acetic acid) and heated to 100°C. Ethidium bromide was added, giving a final concentration in the gel of 1 µg/ml. Samples were prepared by adding 1µl of 10 X loading buffer (50 mM Tris pH 7.6, 60% (v/v) glycerol, 0.25% (w/v) bromophenol blue) to 9 µl sample and were electrophoresed through the gel at 80 V in TAE buffer for the required time. Separated samples were visualized under UV light.

2.11.2 SDS-PAGE

Proteins were separated by discontinuous polyacrylamide gel electrophoresis as described in Sambrook *et al.* (1989). The discontinuous buffer system uses buffers of different pH and composition in the stacking and separating gels. Consequently protein migration through the large pores in the stacking gel (5% acrylamide) is fast and the proteins become concentrated into a narrow band. However, migration through the narrow pores of separating gel (8%-16% acrylamide) is according to size. Both gels were prepared with 0.1% SDS. Since most proteins bind SDS in a constant weight ratio, this leads to similar charge densities for denatured proteins, and allows proteins to migrate according to size, not charge.

Separating gels (8%, 12% or 16%) were prepared according to Sambrook *et al.* (1989). The separating gel was then overlaid with 200 µl ethanol (excludes oxygen) and allowed to polymerize for 30 min. The 5% stacking gel was made by the mixing of 0.833 ml Protogel (National Diagnostics), 1.25 ml 0.5 M Tris-HCl (pH

6.8), 50 µl 10% SDS and 2.87 ml of ddH_2O. The gel was electrophoresed in 1 x Tris-glycine running buffer (25 mM Tris-HCl, 250 mM glycine (pH 8.3), 0.1% (w/v) SDS). Prior to loading, protein samples were denatured at 90°C for 10 min, centrifuged at 20,800 g for 1 min. Electrophoresis was performed at 150 V for 60–90 min.

2.11.3 Staining of proteins

Gels were washed in ddH_2O prior to overnight staining with Coomassie Brilliant Blue R-250 solution (0.25% (w/v) Coomassie Brilliant Blue R-250, 45% (v/v) methanol, 10% (v/v) acetic acid). Gels were then destained in coomassie destain solution (45% (v/v) methanol, 10% (v/v) acetic acid). When destained, protein bands appear blue against a clear background.

2.12 Fis antiserum

Immunising a New Zealand White rabbit with His-tagged purified Fis (a gift from O. Keane) generated antibodies specifically reactive against Fis. The initial injection was with 300 µg of Fis solubilized in Freund's complete adjuvant. Two further boosts were administered at two-weekly intervals each with 300 µg of protein in Freund's incomplete adjuvant. The generation of anti-Fis antibodies was monitored by Western immunoblotting, and after a primary test bleed when the titre was revealed to exceed 1:5000 the rabbit was exsanguinated. The resulting serum was absorbed against an *S. typhimurium fis*$^-$ lysate. The lysate was prepared by harvesting 100 ml of culture at OD_{600} = 1. This pellet was resuspended in 1 ml of sonication buffer (10% sucrose, 50-mM Tris-HCl (pH 7.5), 100 mM NaCl, 1 mM EDTA, 5 mM dithiothreitol) and lysis achieved by 3 cycles of freeze-thaw, sonication, and finally boiling for 10 min. Of this lysate 500 µl was absorbed overnight at 4°C against an equal volume of serum. Insoluble material was removed by centrifugation at 20,800 g for 30 min. The supernatant was retained and stored in aliquots at –20°C and used at an appropriate dilution in Western immunoblot analysis.

2.13 Western immunoblot analysis

Western immunoblottng is a sensitive technique whereby proteins (antigens) are (i) solubilized with SDS and β-mercaptoethanol (section 2.13.1) and separated by SDS-PAGE (section 2.11.2), (ii) irreversibly transferred to nitrocellulose membrane (section 2.13.2), (iii) the membrane is incubated with primary antibody and the antigen-antibody complexes detected with a secondary antibody and revealed by a chemiluminescent assay (section 2.13.3).

2.13.1 Preparation of total cellular extracts

Crude protein extracts from *in vitro* grown bacteria for Western immunoblot analysis were prepared as described below. The OD_{600nm} of each culture was measured. A volume of cells, which corresponded to 2 ml of culture per 1 OD_{600nm} unit, was taken for each culture. Bacteria were pelleted and washed in PBS. The pellets were then resuspended in 100 µl sonication buffer and 100 µl 2 x SDS loading buffer (150 mM Tris-HCl (pH 6.8), 1.2% (w/v) SDS, 30% (v/v) glycerol, 15% (v/v) β-mercaptoethanol), and boiled for 10 min. Typically 10 µl of this crude extract was used for immuno-detection in Western immunoblot analyses.

2.13.2 Transfer of proteins to nitrocellulose membrane

Following SDS-PAGE (section 2.11.2), gels were electroblotted to 0.2 µm PROTAN nitrocellulose membrane (Schleicher and Schuell) using a Mini Trans-blot electrophoretic transfer cell (Bio-Rad) filled with transfer buffer (25 mM Tris, 192 mM glycine, 0.02% (w/v) SDS, 20% (v/v) methanol), at 80 V for 2 hours at 4°C. Protein equal loading and consistent transfer to the nitrocellulose membrane was confirmed by staining the membrane with Ponceau S solution (2% Ponceau S (Sigma), 3% trichloroacetic acid) for 5 min followed by extensive washing with ddH_2O.

2.13.3 Detection of bound antigens

Nitrocellulose membranes were blocked for 1 hour in blocking buffer (5% (w/v) nonfat dry milk, in phosphate-buffered saline (PBS)). Antisera were diluted appropriately in blocking buffer (1:1000 for the Fis antisera) and incubated with the membrane overnight at 4°C. The membrane was washed 3 x 10 min with PBS+1% milk and incubated in blocking buffer containing HRP-linked anti-rabbit IgG for 1 hour. The blot was washed as before, and in the presence of a suitable chemiluminescent substrate (Pierce SuperSignal), the horseradish-peroxidase-mediated enzymic reaction results in a luminescent signal that visualizes the antigen-antibody complex, which can by detected by autoradiography (section 2.14). Typical exposures were from 5 min to 1 hour.

2.14 Autoradiography

In this study autoradiography was used to visualize and quantitate on X-ray film; (i) radio-emissions from molecules that incorporated [α-^{35}S]dATP or [γ-^{32}P]dATP (sequencing reactions, primer extension products and gel mobility shift assays); (ii) non-radioactive chemiluminescent emissions derived from horseradish peroxidase cleavage of chromogenic reagents (Western blots). In each case X-OMAT UV film (KODAK) was used. When photon emissions (chemiluminescent or radiation) strike a silver halide crystal (X-Ray film is coated with silver halides suspended in gelatin), the crystal adsorbs energy and releases an electron. This electron is attracted to a positively charged silver ion forming an atom of metallic silver. After an appropriate time the film was placed into a tray containing diluted Kodak LX-24 X-Ray developer for 3 min, a chemical solution which amplifies the signal by reducing exposed silver halide crystals to metallic silver. The film was washed briefly in water and then fixed in Kodak Industrex liquid fixer for a further 3 min. The fixer serves to convert any silver halide that was not reduced into soluble silver thiosulphate. Developed films were rinsed in a large volume of water. For quantitative analysis of signal intensity several exposures of varying times were taken.

2.15 DNA mobility shift assay

The association of purified Fis protein with the SPI 2 promoter regions was analysed by gel mobility shift assay. DNA probes were amplified by PCR with *Pfx* polymerase (Gibco-BRL), using *S. typhimurium* SL1344 chromosomal DNA as template. Amplified probes were purified as detailed in section 2.9.2, and labelled as described in section 2.16.1. Approximately 5 ng of probe was incubated with increasing concentrations of purified His-tagged Fis (O. Keane) from 0–60 ng for 15 min at room temperature in a 20 µl reaction containing 20 mM Tris-HCl (pH 7.5), 80 mM NaCl, 1 mM EDTA, 100 ng BSA, 25 µg/ml poly-[d(I-C).(dI-dC)], 10% glycerol and 1 mM DTT. Protein-DNA complexes were resolved by electrophoresis through a 7% polyacrylamide gel, for 5 hours at room temperature. The gel was dried and examined by autoradiography.

2.16 Primer extension

Primer extension analysis was used to map the *S. typhimurium ssrA* transcription start site. The principle of primer extension is that a ^{32}P-labelled primer hybridizes with its complementary RNA transcript. The addition of reverse transcriptase and dNTPs directs the synthesis of a DNA strand that is complementary to the RNA template. Extension stops when the 5' end of the mRNA template is encountered resulting in a single-stranded cDNA molecule of defined origin, whose length is determined by the transcription start site of the RNA molecule. The start site is then identified by electrophoresis of the synthesized cDNA alongside a DNA sequencing reaction that was generated using the same primer.

2.16.1 5'-end labelling of DNA using γ^{32}P-ATP

Primers were labelled with [γ-^{32}P]dATP using Bacteriophage T4 polynucleotide kinase (T4 PNK; New England BioLabs), which catalyses the transfer and exchange of a phosphate group from the γ-position of ATP to the 5'-hydroxyl terminus of the primer. 10 pmoles of primer (5 pmol/µl) was mixed with 50 µCi of [γ^{32}P]dATP (10 mCi/ml, 5,000 Ci/mmol), 1 µl 10 x kinase buffer (supplied with enzyme) and 15 U

T4 PNK in a 1.5 ml screw-cap Eppendorf tube. The reaction was incubated for 30 min at 37°C. The labelled oligonucleotide was then precipitated with 3 µl sodium acetate (3.5 M pH5.2) and 90 µl 100% ethanol at –20°C for 10 min. Following centrifugation, the pellet was washed with 70% ethanol, dried, and resuspended in 20 µl DEPC-treated ddH_2O (approximately 1 pmol/µl).

2.16.2 Primer-RNA annealing and cDNA synthesis

The AMV Reverse Transcriptase Primer Extension System (Promega) was used as per the manufacturer's instructions and is outlined below. Total cellular RNA was isolated from mid-exponential phase cultures as described in section 2.8.4. Approximately 10 µg of each RNA sample was mixed with 2 pmol of ^{32}P-labelled oligonucleotide and 5 µl of 2 X Primer extension buffer. RNA was denatured by incubating at 58°C for 20 min. The template and primer were annealed by cooling to 20°C for 10 min. Synthesis of cDNA was performed with the addition of the following to the template-primer reactions: 1.6 µl of ddH_2O, 5 µl of 2 X primer extension buffer, 1.4 µl of 40 mM sodium pyrophosphate and 1 U of AMV reverse transcriptase. Reactions were incubated at 42°C for 30 min. 20 µl of loading dye was added to the reaction mix and the samples heated to 90°C for 10 min. 20 µl of each extension product was analysed by denaturing PAGE alongside the DNA sequencing reactions.

2.17 DNA sequencing

The nucleotide sequences of DNA fragments of interest were determined using the dideoxy chain termination method developed by Sanger *et al.* (1977). The chain termination method utilizes DNA polymerase to synthesize a complementary copy of a single-stranded DNA template, primed from a specific annealed oligonucleotide. When the polymerase selects a deoxynucleotide analogue, dideoxynucleotide (ddNTP), for incorporation by base-pair matching to the template DNA, chain elongation is terminated. Chain growth depends on the formation of a phosphodiester bond between the 3'-OH group at the end of the growing primer and

the 5'-phosphate group of the incoming deoxynucleotide (dNTP). ddNTPs are efficiently recognized by DNA polymerase, however they lack a 3'-OH, and consequently prevent further chain elongation. This method of sequencing is designed such that in doing 4 separate reactions, a ddNTP (G, A, C or T) is introduced at every position of the complementary DNA corresponding to the template. Incorporation of [α-^{35}S] dATP in the reaction mixtures allows newly synthesized DNA to be labelled. Reactions are then electrophoresed side-by-side on a high-resolution denaturing polyacrylamide gel allowing the sequence to be read following autoradiography.

Plasmid denaturation, primer annealing, labelling and terminations reactions were carried out using the T7 Sequencing Kit (Pharmacia Biotech) according to the guidelines supplied.

2.17.1 Denaturing polyacrylamide gel electrophoresis

The products from sequencing and primer extension reactions were separated by electrophoresis on 7.5 M urea, 6% polyacrylamide gels in 1 x TBE buffer. The gels were prepared by mixing together 24 ml SequaGel concentrate, 10 ml SequaGel Buffer and 66 ml SequaGel Diluent (National Diagnostic). To this 300 µl of 10% (w/v) ammonium persulphate and 50 µl of *N*, *N*, *N'*, *N'*-tetramethylethylene diamine (TEMED) were also added. TEMED serves to enhance the formation of free radicals from ammonium persulphate. These free radicals in turn catalyse the polymerization of acrylamide, and formation of bisacrylamide crossbridges between polyacrylamide chains. The polymerized gel was pre-run in 1 x TBE at 70 W until the temperature of the gel had reached at least 50°C. Heating to this temperature and the presence of urea in the gel help maintain the sequencing reactions and primer extension products in a denatured state. Prior to loading, the 5 µl samples were heated to 90°C for 5 min. The gel was then run at 70 W for 90–120 min, dried under vacuum and exposed to X-ray film.

2.18 Infection of macrophage cell line

J774A.1 macrophages were infected with *Salmonella* bacteria and at various time points post-infection intracellular bacteria were harvested, enumerated and used subsequently for either gene expression assays or for Western immunoblots.

Typically, cells were seeded 72 hours prior to infection in 12 well plates, at a density of 2.5 x 10^5 cells per well in TCM. Overnight broth cultures of the bacterial strains for infection were diluted 1:50 into LB broth and grown with shaking at 37°C until the OD_{600nm} reached 1.0. Bacterial cells were harvested and resuspended in antibiotic free TCM. The TCM was removed from the macrophage monolayers and they were washed with antibiotic free TCM. TCM containing *S. typhimurium* was added to the monolayers at a multiplicity of infection of 100. Infected monolayers were incubated at 37°C in 5% CO_2 for 45 min to allow phagocytosis of the *Salmonella* to occur. Infected monolayers were washed twice with TCM and incubated in fresh media containing 100 μg/ml gentamicin to kill any remaining extracellular bacteria. This was denoted time zero (T=0). 30 minutes post-infection the first time point was harvested by washing the monolayers twice with antibiotic-free TCM and then lysing them with 0.5% Triton X-100 in PBS. The TCM containing 100 μg/ml gentamicin was removed from the remaining monolayers and replaced with TCM containing 20 μg/ml gentamicin. The monolayers were incubated in this medium for the remainder of the experiment. At 1-hour intervals post-infection the monolayers were harvested by washing twice with antibiotic-free TCM and then lysing them with 0.5% Triton X-100 in PBS. To determine the numbers of intracellular bacteria recovered, serial dilutions of the lysates were made and plated on agar plates with appropriate selection. The remainder of the lysates were spun down to remove PBS and stored at -20°C until assayed.

2.18.1 β-galactosidase assays

β-galactosidase activity from intracellular bacteria was assayed using the MUG (4-methylumbelliferyl-β-D-galactopyranoside) assay (Miller, 1992). Samples were mixed with 0.5 ml AB (100 mM NaCl, 60 mM K_2HPO_4, 40 mM KH_2PO_4) and

incubated at 37°C for 15 min. 50 µl of 4 mg/ml 4-methylumbelliferyl-β-D-galactopyranoside (MUG) was added and the reaction incubated in the dark at room temperature for 60 min. 0.5 ml of 0.4 M Na_2CO_3 was then added to stop the reaction. Serial dilutions of the samples were then made in ABN (50% AB + 50% 0.4 M Na_2CO_3) and placed in 96-well plates. The quantity of 4-methylumbelliferone (4-MU) produced was determined by reading luminesence with an excitation wavelength of 365 nm and emission wavelength of 455 nm in an LS 50B luminesencent spectrophotometer (Perkin-Elmer).

2.18.2 Western immunoblot analysis of bacteria recovered from intracellular environment

Western immunoblots were performed on bacteria recovered from the intracellular environment (Chapter 4). Certain modifications of the Western immunoblot technique (section 2.13) were employed to optimize detection. *In vivo* samples were resuspended in sonication buffer to give equal concentrations of bacteria based on viable counts. 30 µl of each sample, which corresponded to between 5 x 10^6 and 1 x 10^7 bacteria, was loaded on a 16% polyacrylamide gel. Following SDS-PAGE, electrotransfer to nitrocellulose was performed at 20 V for 16 hours at 4°C. Blocking of the membrane and all further washes took place in 10% (w/v) nonfat dry milk, in phosphate-buffered saline (PBS). Primary detection with Fis antisera at a 1:100 dilution was overnight at 4°C. Secondary detection with the HRP-linked anti-rabbit IgG took place for 3 hours.

2.19 Microarray analysis

Microarray analysis was carried out to determine the Fis regulon during growth in Minimal Medium 5.8 (MM5.8). A PCR product *S. typhimurium* genome array was used courtesy of J. Hinton. The procedure involved is based on that outlined in Eriksson *et al.* (2003) and involves isolation of RNA, reverse transcription to produce cDNA, labeling of cDNA with Cy5 dye, hybridization of the cDNA (and Cy-3 labeled genomic DNA) to the array, and finally detection in a GenePix

scanner. Results were normalized and imported into the GeneSpring software program for analysis. Arrays were hybridized in quadruplicate.

2.19.1 RNA isolation

RNA for use in microarray analysis was isolated from a volume of culture corresponding to 2 OD_{600} units (i.e. 2 ml of OD_{600} = 1 or 4 ml of OD_{600} = 0.5). The appropriate volume of culture was transferred to a tube containing 0.2 volumes of phenol/ethanol mix (95% (v/v) ethanol, 5% (v/v) phenol). Tubes were left on ice for 30 min. The phenol/ethanol helps to stabilize RNA and prevent degradation. After 30 mins samples were pelleted by centrifugation at 20,800 g. RNA was isolated from these pellets using the Promega SV Total RNA isolation kit according to the manufacturer's instructions. After elution the RNA was quantified, precipitated and resuspended at a concentration of 3 μg/μl in RNAse-free water (Sigma).

2.19.2 cDNA synthesis

5 μg of RNA was incubated with 5 μg of random hexamer primers. To facilitate annealing of the primers to the RNA the mixture was heated to 70°C for 10 min and then cooled on ice after which the following were added: 2.5 μl 10 X Reverse Transcriptase buffer, 1.6 μl 25 mM dNTP's, 0.3 μl 1 M DTT, 200 U Stratascript Reverse Transcriptase. Samples were incubated at 37°C overnight.

Following overnight incubation, 1.5 μl of 0.5 M EDTA and 15 μl of 0.1 M NaOH were added to each tube and samples heated to 70°C for 15 min. 15 μl 0.1 M HCl was added to neutralize the samples and the resulting cDNA purified using the Wizard PCR Prep DNA purification system (section 2.9.2).

2.19.3 Cy-dye labeling of DNA

The method of microarray analysis employed in this study uses genomic DNA as an internal positive control and reference. Genomic DNA is labeled green with Cy3-dCTP while cDNA derived from RNA is labeled red with Cy5-dATP. Both Cy-

labeled products are included in the array hybridizations. The binding of the genomic DNA (green) to each corresponding spot on the array acts as a base line or reference to which binding of the cDNA (red) is compared. The BioPrime DNA Labelling Kit (Gibco/BRL) was used for the labeling of both genomic DNA and cDNA and is outlined below.

2 μg of genomic DNA (to be labeled with Cy3) or the entire volume of eluted cDNA (to be labeled with Cy5) was ethanol precipitated and resuspended in 22 μl RNAse free water (Sigma). To this 20 μl of 2.5 X Random primer/reaction buffer mix from Gibco Kit was added and the samples heated to 100°C for 5 min then placed on ice for a further 5 min. At this point the genomic DNA was vortexed vigorously to aid fragmentation. To each sample the following was then added: 5 μl of 10 X dNTP mix (1.2mM each dATP, dGTP, dTTP; 0.6 mM dCTP), 3 μl of Cy5-dCTP for cDNA or Cy3-dCTP for genomic (1mM stock, Amersham) and 1 μl of Klenow from the kit. Samples were then incubated overnight at 37°C. Reactions were stopped by the addition of 5 μl of 0.5 M EDTA, purified using the Wizard PCR Prep DNA purification system and eluted in 100 μl nuclease-free H_2O.

2.19.4 DCE blocking of microarray slides

DNA microarrays are printed on Corning CMT-GAPS coated glass slides. Each slide contains 2 X arrays. Each array consists of 16 blocks of printed PCR products. The outline of each array was marked with a diamond pencil before blocking. Microarray slides were blocked with 1,2-dichloroethane (DCE) as follows.

Slides were incubated at 80°C for 2 hours to immobilize the DNA. Blocking solution was made up containing 1.5 g succinic anhydride dissolved in 300 ml anhydrous 1,2-dichloroethane, to which 3.75 ml N-methylimidazole was added. Slides were incubated, with gentle agitation, in blocking solution for 1 hour. After blocking, slides were washed for 3 min in 300 ml fresh 1,2-dichloroethane and then transferred to boiling water for 2 min to denature the DNA. Once denatured, slides were placed in ethanol for 1 min and then centrifuged at 290 g to dry.

2.19.5 Hybridizations

Hybridization mix was set up as follows. 50 µl of eluted genomic DNA was added to the 100 µl of eluted cDNA, ethanol-precipitated and resuspended in 9.38 µl ddH_2O. To this the following were added: 1.5 µl of 50 X Denhardt's solution, 2.25 µl of 20 X SSC, 1.125 µl of yeast tRNA, 0.375 µl of 1M HEPES pH7 and 0.37 µl of 10% (w/v) SDS. Samples were incubated at 100°C for 2 min then cooled to 20°C for 5 min. To set up the hybridizations, array slides were placed into the hybridization chambers (Die-Tech). The hybridization solution (15 µl) was applied towards one edge of the array. The edge of a 22 mm X 22 mm glass cover-slip was placed in the hybridization solution and the cover-slip gently lowered to cover the array taking care to exclude air bubbles and ensuring even distribution of the solution across the array. 3 X 10 µl drops of 3 X SSC were placed on the glass slide adjacent to the array to maintain humidity inside the hybridization chamber. Hybridization chamber lids were fastened securely and the chambers placed at 63°C overnight. The following day hybridization chambers were opened and the arrays washed as follows. Arrays were washed twice in wash solution (2 X SSC, 0.1% (w/v) SDS) at 63°C for 5 min with gentle agitation to remove cover-slips. Slides were then washed twice in 1 X SSC at 20 °C for 5 min, and twice in 0.2 X SSC at 20°C for 5 min. Slides were dried by centrifugation at 290 g for 5 min.

2.19.6 Scanning of microarray slides and data handling

After hybridization, microarray slides were scanned using a GenePix 4000 A scanner (Axon Instruments). Fluorescent spot intensities and local background data were quantified using the GenePix 3.0 software package. Data were then passed through quality control procedures outlined in Eriksson *et al.* (2003). Data that passed the quality controls were saved in .gpr file format which were then converted to .txt text files. The .txt files were imported into Microsoft Excel and using a custom designed macro program (S. Luccini), the cDNA data were normalized against the genomic DNA data. The resulting Excel file was converted into a .txt text document which was imported into the microarray analysis programme GeneSpring 6.0 (Silicon Genetics). All array analysis was carried out using

GeneSpring 6.0 and graphs of relevant data produced within the programme (see chapter 6).

2.20 Site-directed mutagenesis

Site-directed mutagenesis of the *spvR* IHF binding site (Chapter 3) was carried out using the QuikChange site directed mutagenesis kit (Stratagene) according to the manufacturer's instructions. Two oligonucleotide primers (each complementary to opposite strands of the dsDNA template) containing the desired mutation are used to generate a mutant plasmid containing staggered nicks by temperature cycling using the *PfuTurbo* enzyme. The original template DNA is digested using *DpnI*, which leaves the non-methylated mutant DNA intact. The nicked vector DNA containing the desired mutation is then transformed into XL-1 supercompetent cells and selected for with appropriate antibiotics. All mutations were confirmed by DNA sequencing (MWG-Biotech).

Chapter 3

The role of nucleoid-associated proteins in *Salmonella typhimurium* virulence gene regulation

3.1 Introduction

3.1.1 Nucleoid-associated proteins and virulence gene regulation

The genomic DNA of *S. typhimurium* is associated with several DNA binding, nucleoid-associated proteins (Azam and Ishihama, 1999). The exact composition and relative abundance of these proteins varies widely depending on many factors including growth phase and environmental conditions (Azam *et al.*, 1999). Bacterial nucleoid-associated proteins serve the same function as eukaryotic histones, condensing the DNA by binding to it, introducing bends or coils, and packaging it in such a way that it fits inside the cell. The major DNA binding proteins in *S. typhimurium* include Fis, H-NS, IHF and HU. In addition to their structural role, many nucleoid-associated proteins act as transcriptional regulators (Dorman and Deighan, 2003). Previous studies have shown that nucleoid-associated proteins are often involved in virulence gene regulation. Fis regulates virulence genes of several pathogenic *E. coli* species, including EPEC and EAEC (Goldberg *et al.*, 2001; Sheikh *et al.*, 2001), and along with HU and H-NS, regulates *Salmonella* pathogenicity island 1 (Schechter *et al.*, 2003; Wilson *et al.*, 2001). H-NS and IHF are involved in regulating *Shigella* virulence genes and the *spv* system in *S. typhimurium* (Marshall *et al.*, 1999; Porter and Dorman, 1997). There are various mechanisms by which nucleoid-associated proteins are involved in virulence gene regulation. They can influence local supercoiling levels, interact with RNA polymerase or compete for binding with transcriptional regulators, any of which can alter virulence gene expression (Dorman and Deighan, 2003).

3.1.2 *In vivo* expressed genes

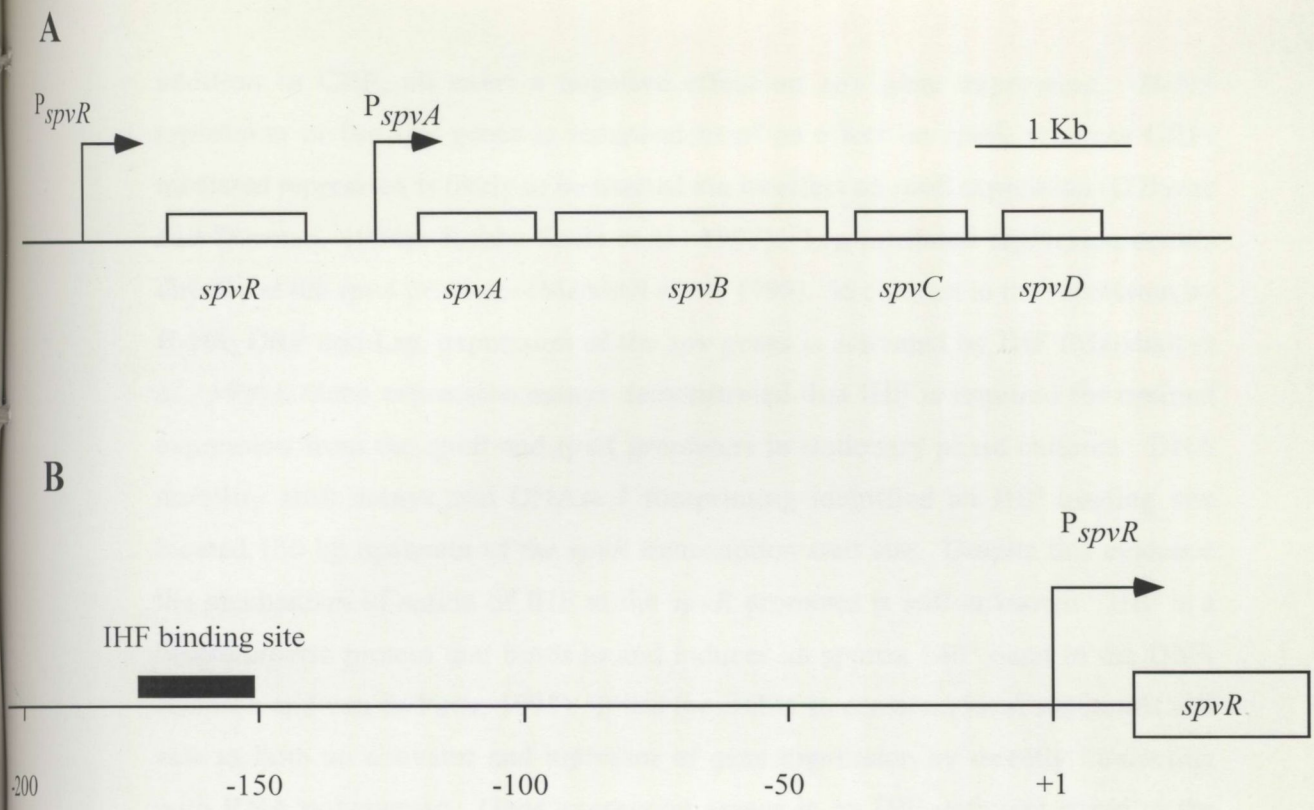
Experimental techniques such as differential fluorescence induction (DFI) and *in vivo* expression technology (IVET) have recently been used to identify *S. typhimurium* genes that are highly expressed and required for survival inside macrophages (Heithoff *et al.*, 1997; Valdivia and Falkow, 1997). The *Salmonella* plasmid virulence genes (*spv*) and *Salmonella* pathogenicity island 2 (SPI 2) are examples of such genes, their expression being strongly activated upon entry into macrophages (Cirillo *et al.*, 1998; Marshall *et al.*, 2000; Valdivia and Falkow,

1997). Expression of the *spv* system is known to be coordinated by several nucleoid-associated proteins (Marshall *et al.*, 1999; O'Byrne and Dorman, 1994a), however little is known about the regulation of SPI 2. The aim of this study was to investigate these *in vivo* activated genes to determine the role of nucleoid-associated proteins in their global regulation.

3.1.3 The *Salmonella* plasmid virulence genes (*spv*)

Many serovariants of *Salmonella enterica*, including Typhimurium, harbour large plasmids, which encode virulence determinants required for systemic infection (Gulig, 1990). Removal of these "virulence plasmids" abrogates bacterial growth *in vivo* and results in a decrease in virulence. The size of the virulence plasmid ranges from 50 to 110 kb in different strains, but an 8 kb region has been shown to be highly conserved among pathogenic species (Gulig *et al.*, 1993). Encoded on this region are the *Salmonella* plasmid virulence genes (*spv* genes). The *spv* operon consists of 5 genes termed *spvRABCD* (Fig. 3.1 A). SpvR is a LysR-like transcriptional regulatory protein that activates transcription from both its own promoter (*spvR* promoter) and the *spvA* promoter, which transcribes the *spvABCD* genes. The functions of SpvA, SpvC and SpvD are still unknown but the SpvB protein has been shown to be an ADP-ribosylase that ADP-ribosylates host cell actin and prevents actin polymerisation (Lesnick *et al.*, 2001; Otto *et al.*, 2000; Tezcan-Merdol *et al.*, 2001).

The regulation of the *spv* genes is well studied and in addition to SpvR, several other factors have been shown to be involved in *spv* gene expression. In particular, several nucleoid-associated proteins have been shown to be involved in both *spvR* and *spvA* expression. To date, the nucleoid-associated proteins H-NS (O'Byrne and Dorman, 1994a), IHF, and Lrp (Marshall *et al.*, 1999) have been shown to be involved in *spv* gene expression as well as CRP (cyclic AMP receptor protein) and the stationary phase sigma factor RpoS (O'Byrne and Dorman, 1994b; Robbe-Saule *et al.*, 1997). RpoS activates transcription of the *spv* genes and in this way links the expression of the *spv* genes to the physiological state of the cell, transcription being activated in stationary phase cultures, and as the bacteria enter macrophages (Chen *et al.*, 1995) (Chen *et al.*, 1996). The nucleoid-associated proteins H-NS and Lrp, in



C	**WATCAANNNNTTR**	consensus site
D	**GGTCAATTAAATC**	*spvR* IHF site
E	**GGAATATTAAATC**	disrupted *spvR* IHF site
F	**TATCAATTAATTG**	improved *spvR* IHF site

Fig. 3.1. The *Salmonella* plasmid virulence genes (*spv*) and the *spvR* IHF binding site.

A. Genetic structure and transcriptional organisation of the *spv* genes.

B. Locaton of the *spvR* IHF binding site 150bp upstream of the *spvR* transcription start site.

C. Sequence of the IHF consensus binding site. (W = A/T; R=A/G; N=A/T/C/G)

D. The *spvR* IHF binding site.

E. The *spvR* IHF binding site disrupted by removal of the critical CAA residues.

F. The "improved" *spvR* IHF binding site, mutated to match perfectly the consensus sequence.

addition to CRP, all exert a negative effect on *spv* gene expression. H-NS repression of the *spv* genes is independent of its effect on *rpoS*, whereas CRP-mediated repression is likely to be exerted via its effect on *rpoS* expression (O'Byrne and Dorman, 1994b; Robbe-Saule *et al.*, 1997). Lrp mediated repression occurs directly at the *spvA* promoter (Marshall *et al.*, 1999). In contrast to the repression by H-NS, CRP and Lrp, expression of the *spv* genes is activated by IHF (Marshall *et al.*, 1999). Gene expression assays demonstrated that IHF is required for optimal expression from the *spvR* and *spvA* promoters in stationary phase cultures. DNA mobility shift assays and DNAse I footprinting identified an IHF binding site located 150 bp upstream of the *spvR* transcription start site. Despite this evidence the mechanism of action of IHF at the *spvR* promoter is still unknown. IHF is a heterodimeric protein that binds to and induces an approx 140° bend in the DNA (Goosen and van de Putte, 1995). It has the ability to constrain local supercoils and acts as both an activator and repressor of gene expression by directly interacting with RNA polymerase. Gene expression assays in an IHF deficient strain in the presence of the DNA gyrase inhibitor novobiocin demonstrated an increase in *spv* gene expression as the DNA becomes more relaxed and IHF is not present (Marshall *et al.*, 1999). This result suggests a role for supercoiling in IHF mediated activation of the *spv* genes however it does not discount the possibility that IHF is interacting directly with RNA polymerase or competing with a repressor of the *spv* system. An investigation of the IHF binding site was carried out in an attempt to further elucidate the role of IHF in the regulation of the *spv* genes.

3.1.4 *Salmonella* pathogenicity island 2 (SPI 2)

The *Salmonella* chromosome contains groups of functionally related virulence genes termed pathogenicity islands (Groisman and Ochman, 1996). *Salmonella* pathogenicity island 1 (SPI 1) is the best characterised of these. It contains the genes associated with entry into epithelial cells as well as chaperone and regulatory proteins (Marcus *et al.*, 2000). *Salmonella* pathogenicity island 2 (SPI 2) has been discovered more recently and encodes genes associated with survival and replication inside macrophages (Hensel *et al.*, 1995; Ochman *et al.*, 1996). Like SPI 1, it codes for a type III secretion system, effector, chaperone and regulatory proteins (Cirillo *et al.*, 1998; Hensel *et al.*, 1997b; Shea *et al.*, 1996). Unlike SPI 1, little is known

about the genetic regulation of SPI 2. The SPI 2 encoded two-component signal transduction system, SsrA/B, activates transcription of the SPI 2 genes but at the commencement of this study no other regulatory proteins were known that activate or repress *ssrA* or any of the other SPI 2 promoters (Cirillo *et al.*, 1998). Subsequently the two-component signal transduction system OmpR/EnvZ has been shown to be involved in *ssrA* activation (Feng *et al.*, 2003; Lee *et al.*, 2000). A link between nucleoid-associated proteins and *Salmonella* virulence gene regulation is long established so an investigation was carried out to determine what role, if any, nucleoid-associated proteins play in SPI 2 gene regulation.

3.2 Results

3.2.1 Site-directed mutagenesis of the *spvR* IHF binding site

A previous study had demonstrated a role for IHF in *spv* gene expression (Marshall *et al.*, 1999). In an *ihf* background transcription from the *spvR* promoter was reduced by 50%. The *spvA* promoter, which is dependent on SpvR, also showed a 50% decrease in activity in the *ihf* background most likely due to the reduction in SpvR. In addition, mobility shift assay and DNAse I footprinting showed IHF binding to the *spvR* promoter DNA 150 bp upstream of the transcription start site. This IHF binding site closely matches the consensus IHF binding site (Fig. 3.1 B,C and D). These results indicate that IHF activates transcription from the *spvR* promoter, however the mechanism of action of IHF in activating transcription is not clear. β-galactosidase expression assays using novobiocin to relax the DNA demonstrated that supercoiling plays a role in IHF mediated, *spvR* activation (Marshall *et al.*, 1999). To further elucidate the role of IHF in *spvR* activation, site-directed mutagenesis was performed on the *spvR* IHF binding site. Site directed mutagenesis was carried out using the QuikChange™ site directed mutagenesis kit in plasmid pQF[R:100] (Marshall *et al.*, 1999). This plasmid contains a 327 bp *spvR* promoter fragment upstream of the promoterless *lacZ* gene in plasmid pQF50. The IHF binding site was mutated in two ways. Plasmid pQF[R:101] contains a disrupted IHF binding site created by substitution of the critical CAA triplet using primer pair IHF_koF and IHF_koR in the mutagenesis reaction. The resulting region no longer matches the consensus IHF binding sequence (Fig. 3.1 E). Conversely, plasmid pQF[R:103] (constructed following two rounds of site directed mutagenesis using primer pairs IHF_conF1, IHF_conR1 and IHF_conF2, IHF_conR2) contains an IHF binding site that has been altered to more closely match the consensus sequence for IHF binding (Fig. 3.1 F). It was hoped that through "improving" the binding site that the protein would have a higher affinity for the DNA and bind it more tightly. This could help in determining to what extent binding of IHF to the DNA and constraining supercoils, is involved in *spvR* regulation.

3.2.2 Effect of IHF binding site mutations on *spvR* promoter activity

Both mutations were carried out on a plasmid containing the *spvR* promoter upstream of a promoterless *lacZ* gene, therefore activity of the mutated and wild type promoters was assayed by β-galactosidase assay. The results of the β-galactosidase assays are shown in Fig 3.2. A two fold reduction in *spvR* expression is observed in plasmid pQF[R:101], where the IHF binding site has been disrupted. The mutation in plasmid pQF[R:103], designed to improve the binding site, had no effect on *spvR* expression. β-galactosidase activity from the *spvR* promoter in this construct is the same as in the wild type pQF[R:100]. These results indicate that removal of the CAA residues from the IHF binding site causes *spvR* transcription to drop to a level comparable to that observed from the wild-type promoter in an *ihf* mutant. This is consistent with a role for IHF in activating transcription from the *spvR* promoter by binding to the DNA upstream of the transcription start site. The finding that "improvement" of the binding site sequence had no effect on transcription indicates that while not a perfect match for the consensus IHF binding site, the *spvR* IHF binding site may already be optimised for IHF binding and activation of transcription.

3.2.3 Construction of SPI 2 reporter gene fusion

The *spv* genes are an example of *in vivo*-expressed genes that are activated for expression inside macrophages and required for full virulence. The virulence genes on *Salmonella* pathogenicity island 2 (SPI 2) are another example of these kind of *in vivo*-induced genes (Valdivia and Falkow, 1997). As nucleoid-associated proteins are known to play a critical role in regulating the *spv* system, an investigation was carried out to ascertain whether they are also involved in SPI 2 gene regulation. To this end, a SPI 2 promoter gene fusion was constructed. The SsrA/B two-component signal transduction system is required for expression of the SPI 2 genes (Cirillo *et al.*, 1998). SsrA and SsrB are transcribed on an operon from the *ssrA* promoter, whitch itself is positively autoregulated by SsrA/B. Consequently, expression from this promoter can be looked upon as a good indication of SPI 2 expression in general. A 645 bp fragment of DNA corresponding to the region

upstream of *ssrA* was amplified by PCR using oligonucleotides ssrA_F and ssrA_R (Table 2.3), and cloned into plasmid pQF50 to generate plasmid pQFssrA (Table 2.2, Fig 3.3). The cloned sequence contained the 5' region of the *ssrA* gene, the intergenic region between the divergently transcribed *ssrA* and *ssaB* genes, and part of the 5' sequence of *ssaB* (Fig. 3.3). The exact transcription start site for *ssrA* was unknown but was thought to lie in this region (Cirillo *et al.*, 1998). The amplified *ssrA* promoter fragment was flanked with *Bam*HI and *Kpn*I restriction sites at the 5' and 3' ends respectively. After digestion with *Bam*HI and *Kpn*I, the fragment was cloned into *Bam*HI/*Kpn*I cut pQF50. The order of these restriction sites in pQF50 ensured the promoter DNA was cloned into the vector in the correct orientation. Ligated plasmids were transformed into *E. coli* XL-1, and selected for on agar containing ampicillin. To confirm correct insertion of the fragment into the vector, plasmid DNA was isolated from recovered colonies and sequenced.

3.2.4 Identification of the *ssrA* transcription start site by primer extension

The region of *ssrA* promoter DNA amplified and cloned into pQF50 contained the intergenic region between the divergently transcribed *ssrA* and *ssaB* genes as well as some coding sequence from each. As these genes are divergently transcribed it was thought likely that the *ssrA* transcription start site would be located in this region although the exact location of the start site had not been reported at the time. To confirm that the *ssrA* transcription start site was located in this section of DNA and to identify the exact nucleotide where the *ssrA* transcript originates, primer extension analysis was carried out. RNA was isolated from *S. typhimurium* containing the pQFssrA plasmid. Using this as a template, a single stranded cDNA molecule was formed by extending a radiolabelled oligonucleotide using reverse transcriptase. This product was run on an acrylamide gel along with the products of a DNA sequencing reaction carried out using the same oligonucleotide. In this way the size of the primer extension product could be matched with the sequence and the transcription start site identified. In this case the transcription start site was identified as a T residue located 170 nucleotides upstream of the translation initiation codon (Fig. 3.4). This confirms that the DNA fragment cloned into pQFssrA contains the *ssrA* transcription start site as well as approximately 400 nucleotides of promoter sequence upstream of it.

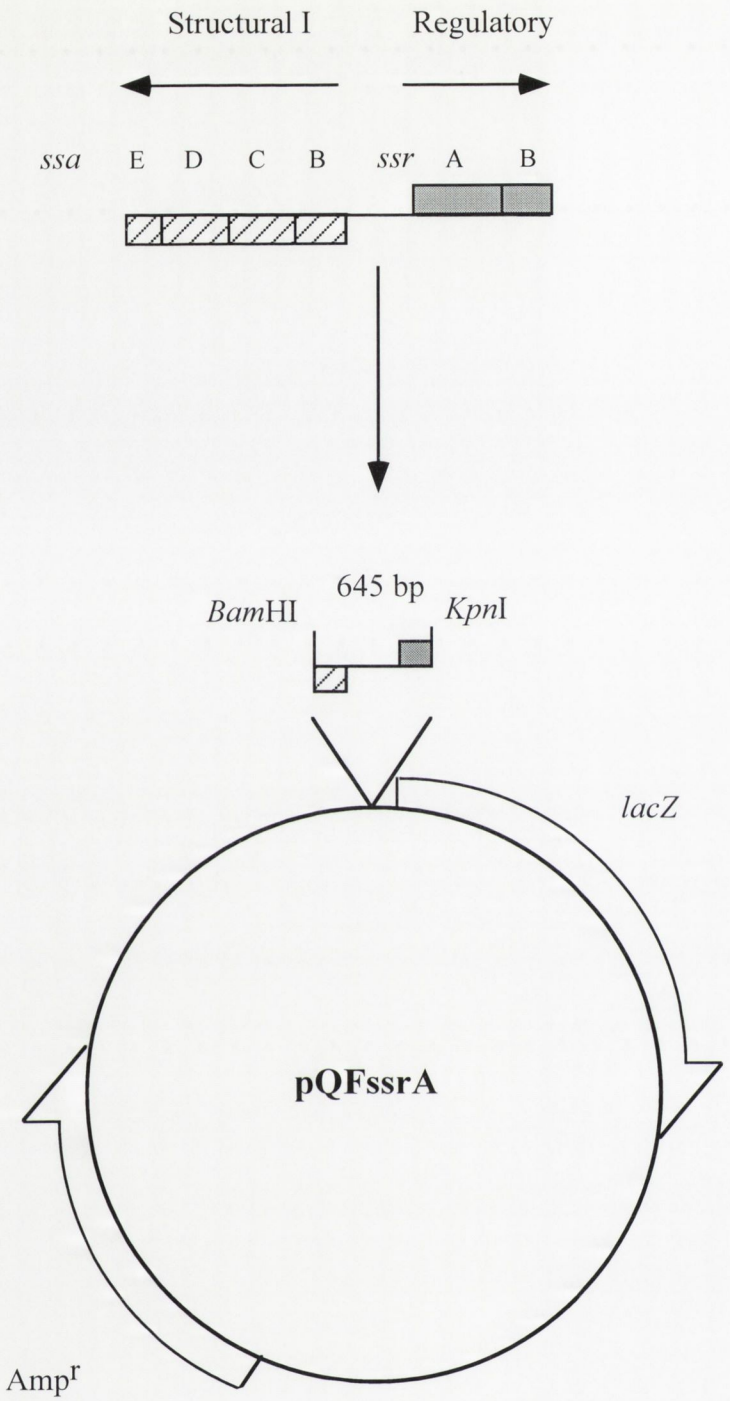


Fig. 3.3. Cloning of the *ssrA* promoter into plasmid pQF50.

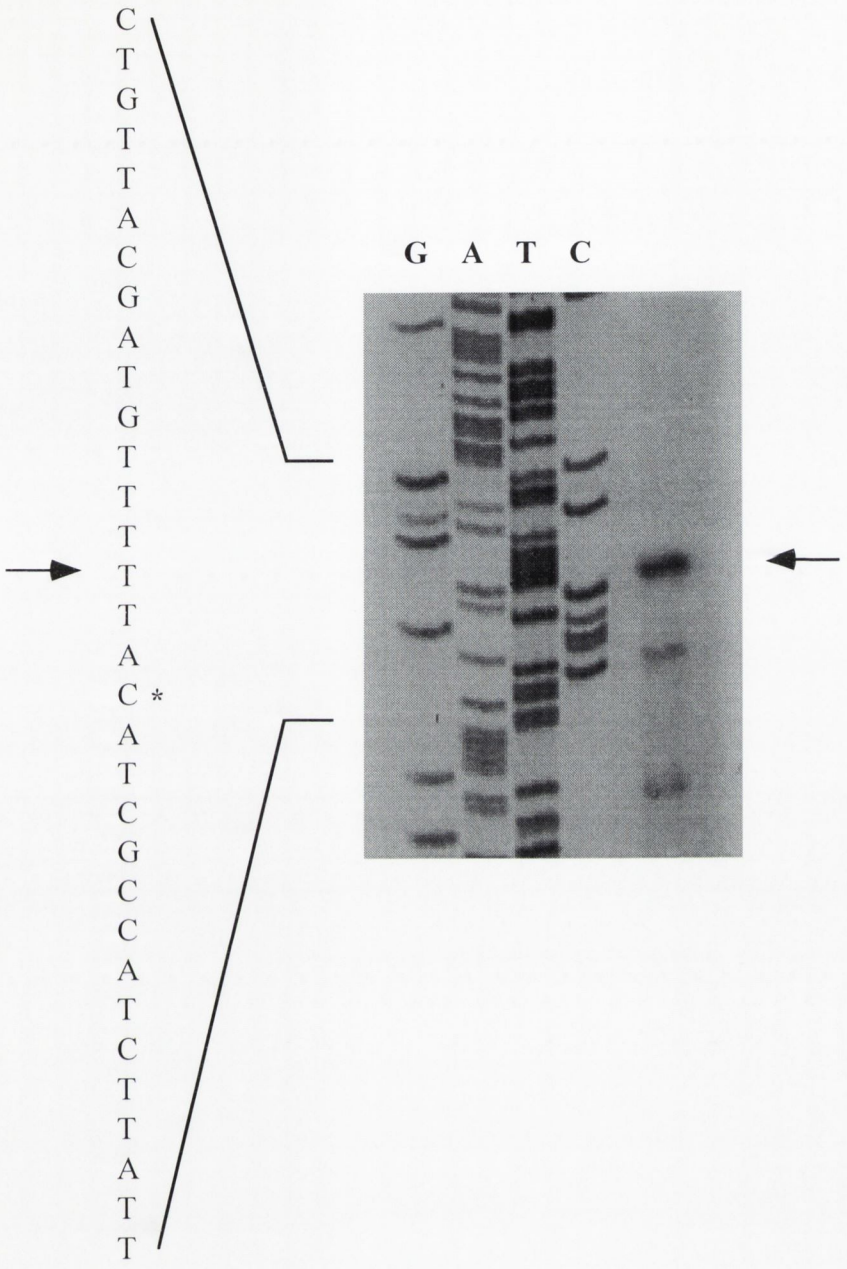
A 645 bp section of DNA, encorporating the 5' region of *ssrA*, the *ssrA-ssaB* intergenic region and part of the divergently transcribed *ssaB* gene, was amplified by PCR and ligated into the multiple cloning site of plasmid pQF50 upstream of the promoterless *lacZ* gene. The resulting plasmid was named pQFssrA.

Fig. 3.4. Primer extension analysis of *ssrA* transcription start site.

A. Primer extension. The four lanes corresponding to the four sequencing reactions are labelled G, A, T and C respectively. The primer extension product is run in the lane to the right of the sequencing reactions. The *ssrA* transcription start site is highlighted by an arrow. The transcription start site as reported by Feng *et al.* (2003) is marked with an asterisk (*).

B. *ssrA* promoter sequence. The *ssrA* coding DNA sequence is in CAPITALS and upstream promoter DNA is in lowercase letters. Transcription start site marked as in A above.

A



B

ctgttacgatgttttttacatcgccatcttattaaaaagtaattgtagtcatcg

actgggttatatatgaagaaatttatcttcctaatgataacaccatcgattaa

tcttctgatgaaactatatgtactgcgatagtgatcaagtgccaaagattttg

caacaggcaactggagggaagcatt ATGAATTTGCT

3.2.5 Effect of nucleoid-associated proteins on *ssrA* gene expression

The only known regulators of SPI 2 gene expression are the SsrA/B and OmpR/EnvZ two-component signal transduction systems (Lee *et al.*, 2000). Frequently, studies have highlighted the association between nucleoid-associated proteins and virulence gene regulation, the *spv* system in *S. typhimurium* being one example. Therefore an investigation was conducted to ascertain what role, if any, nucleoid-associated proteins play in *ssrA* and SPI 2 gene regulation. The pQFssrA plasmid, which harbours an *ssrA-lacZ* transcriptional reporter fusion, was used for this analysis. The plasmid was transformed into the wild type *S. typhimurium* strain SL1344 and isogenic strains harbouring mutations in various nucleoid-associated proteins. The mutants used were deficient in H-NS, IHF, Fis, or StpA. In addition to these strains an *S. typhimurium ompR* mutant was used as a positive control, as disruption of OmpR/EnvZ results in a decrease in *ssrA* transcription (Lee *et al.*, 2000). β-galactosidase assays were preformed on all strains following overnight growth in 3 ml of LB broth. Results (shown in Fig 3.5) confirm the role for OmpR/EnvZ in the regulation of SPI 2. A 6-fold decrease in β-galactosidase activity is observed in the *ompR* mutant. No effect on transcription is observed in either the *ihf* or *stpA* mutants. A 50% reduction in expression was observed in the *fis* mutant indicating a possible role for Fis in regulating this system. A 9-fold reduction in β-galactosidase activity was initially observed in the *hns* mutant strain. This result was extremely surprising. Normally H-NS acts as a repressor of transcription but here it appeared to be involved in activating transcription. However, previous studies have highlighted that for certain plasmids, plasmid copy number decreases in *hns* mutants (Deighan *et al.*, 2000). To investigate to what extent the reduced β-galactosidase levels in the *hns* mutant are as a result of plasmid copy number, plasmids were harvested from the wild type SL1344 and the SL1344 *hns* mutant. Following linearization with *Bam*H1, plasmids were analysed by agarose gel electrophoresis. Densitometry on the bands revealed a 10-fold reduction in plasmid copy number in the *hns* mutant (data not shown). Compensating for this reduction in copy number shows that β-galactosidase levels in the *hns* mutant are similar to those in the wild-type. No reduction in plasmid copy number was observed for any of the other mutant strains used in this study (data not shown).

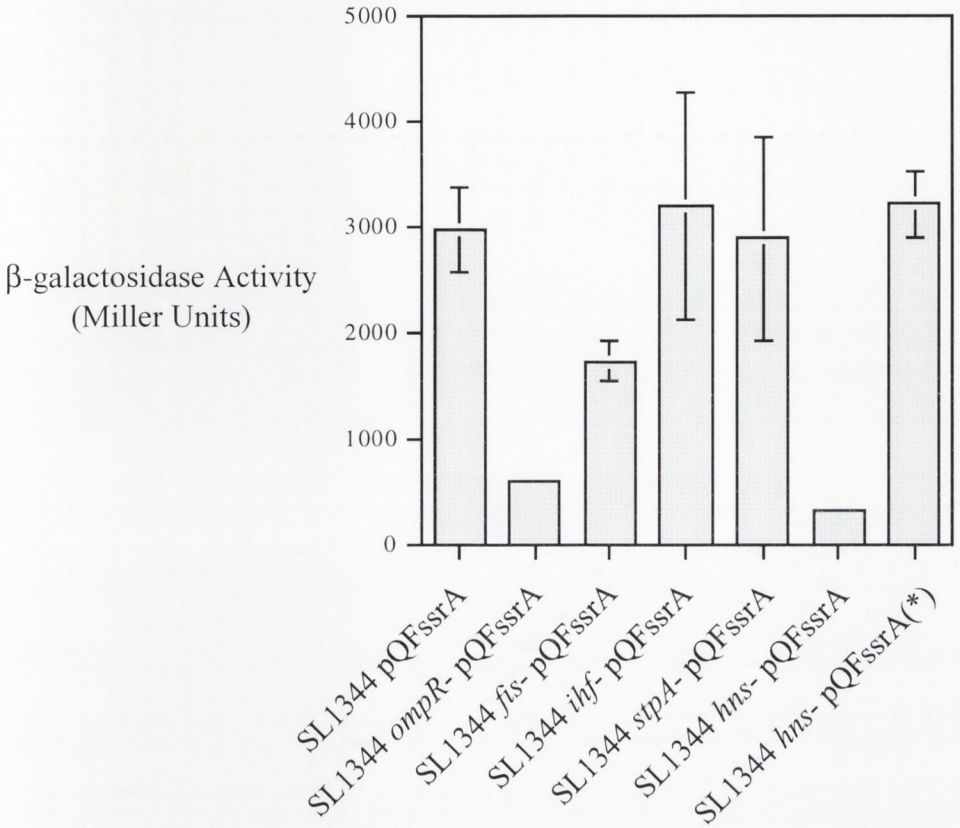


Fig. 3.5. Effect of nucleoid-associated proteins on *ssrA* transcription.

The plasmid pQFssrA, which contains an *ssrA-lacZ* fusion, was transformed into SL1344 and SL1344 strains with mutations in genes coding for the nucleoid associated proteins H-NS, IHF, Fis and StpA. An SL1344 *ompR* mutant was used as a control. β-galactosidase assays were then performed. A 5-fold reduction in *ssrA* transcription was observed in the *ompR* strain. Transcription levels in the *ihf* and *stpA* mutants were similar to the wild type. A 50% reduction in activity was observed in the *fis* mutant. An approx 9-fold reduction in activity was observed in the *hns* mutant. Further investigation revealed the copy number of the plasmid in the *hns* mutant had decreased 10 fold. Compensating for this results in wild type levels of *ssrA* expression (*). β-galactosidase assays using the template plasmid pQF50 produced 2 to 4 Miller units of activity (data not shown).

3.3 Discussion

A role for nucleoid-associated proteins in virulence gene regulation is long established in many different organisms and with many different sets of virulence genes (Dorman and Deighan, 2003). *S. typhimurium* is no exception with both SPI 1 and the *spv* genes previously shown to be regulated by one or more nucleoid-associated protein (Marshall *et al.*, 1999; O'Byrne and Dorman, 1994a; Schechter *et al.*, 2003). Regulation of virulence genes by nucleoid-associated proteins is one mechanism by which an organism can coordinate expression according to growth phase or environmental conditions (Robbe-Saule *et al.*, 1997). Many nucleoid-associated proteins display distinct expression profiles with levels changing depending on the physiology of the cell or certain environmental conditions (Azam *et al.*, 1999). As virulence gene expression can be a heavy metabolic load on the cell, it is paramount that they are only expressed under conditions favourable for infection. The onset of these conditions is often accompanied by a change in the physiology of the cell or environmental changes (Marshall *et al.*, 2000). This in turn can lead to an alteration in the levels of nucleoid-associated proteins which can then influence virulence gene expression (Schneider *et al.*, 1997). The aim of this study was to examine in more detail the role of nucleoid-associated proteins in *S. typhimurium* virulence gene regulation, in particular the role of IHF in *spv* gene expression, and to investigate what role, if any, nucleoid-associated proteins play in the regulation of the recently characterised SPI 2.

Previous studies had identified a role for IHF in *spvR* gene expression (Marshall *et al.*, 1999). An IHF binding site was located 150 bp upstream of the *spvR* transcription start site. β-galactosidase assays using a *spvR-lacZ* reporter fusion in both the wild type and *ihf* mutant background demonstrated a 2-fold reduction in expression in the *ihf* mutant. Further studies using novobiocin to relax the DNA indicated that the role of IHF in this system may be due to an effect on local supercoiling levels (Marshall *et al.*, 1999). To investigate this further site directed mutagenesis was carried out on the *spvR* IHF binding site in two ways (Fig. 3.1). Mutations were introduced that disrupted the CAA triplet, which is critical for IHF binding. The site was also mutated in such a way as to make it more closely match the consensus IHF binding site sequence. It was hoped that these mutations would

alter the affinity of IHF for the binding site. Where the site is disrupted IHF binding would be impaired or abolished and where the site is improved IHF would bind with a higher affinity. β-galactosidase assays using the *spvR-lacZ* reporter fusion demonstrated a 2-fold decrease in expression from the *spvR* promoter when using the disrupted site. This decrease is similar to that observed when using the unaltered promoter in an *ihf* mutant. This indicates that disruption of the IHF site results in a decrease in *spvR* expression due to a lack of upstream IHF binding. In contrast improving the IHF binding site had no effect on *spvR* expression. This result suggests that either the affinity of IHF for this binding site was not altered or that even with increased affinity tighter binding has no effect on promoter activity. The results indicate that binding of IHF upstream of the *spvR* promoter activates transcription and that the wild type IHF binding site may already be optimised for IHF binding.

Salmonella pathogenicity island 2 (SPI 2) has recently been identified as an important virulence determinant in *S. typhimurium* (Ochman *et al.*, 1996). Like the *spv* system, the SPI 2 genes are highly expressed inside macrophage and are required for virulence (Ochman *et al.*, 1996; Valdivia and Falkow, 1997). The only known regulators of the island are the two-component signal transduction systems OmpR/EnvZ and the SPI 2 encoded SsrA/B (Lee *et al.*, 2000). Nucleoid-associated proteins play an important role in *S. typhimurium* virulence gene regulation, e.g. *spv* and SPI 1, so a survey was carried out to determine if they are involved in regulation of SPI 2.

The SsrA/B two-component signal transduction system is required for expression of the SPI 2 genes (Cirillo *et al.*, 1998). The *ssrAB* genes are transcribed on an operon from the *ssrA* promoter. Activity from this promoter is a good indication of activity from SPI 2, therefore this promoter was cloned into a promoter trap vector, pQF50, upstream of the promoterless *lacZ*. To ensure the cloned fragment contained the *ssrA* transcription start site primer extension was carried out. The start site was identified as a T residue located at a position –170 bp from the translation start site. Recently, the *ssrA* transcription start site has been reported elsewhere by Feng *et al.* (2003). After primer extension analysis they report the transcription start site to be a C residue at position –167. While the two results place the start site in close

proximity to each other there is clearly some discrepancy. In Fig. 3.4 the primer extension product is a small concise band easily correlated to the T residue at –170 bp. In Feng *et al.* (2003) their primer extension product is diffuse and difficult to accurately attribute to one specific residue. The result clearly places the location of the transcription start site in this region of DNA and allows this construct to be used as a SPI 2 reporter fusion.

An investigation into the role of nucleoid-associated proteins in SPI 2 gene expression was carried out using the *ssrA-lacZ* reporter fusion. The plasmid containing the fusion, pQFssrA, was transformed into *S. typhimurium* strain SL1344 and isogenic strains carrying mutations in the following nucleoid-associated proteins, H-NS, IHF, Fis and StpA. As the *ssrA* promoter is known to be regulated by OmpR/EnvZ, an *ompR* mutant was included as a positive control. β-galactosidase assays showed no effect of the *ihf* or *stpA* mutations on *ssrA* expression. A 9-fold reduction in β-galactosidase activity was initially observed in the *hns* mutant but was later shown to be an artifact due to an effect of the *hns* mutant on plasmid copy number. Compensating the reduced β-galactosidase assay results for this decrease in copy number gives expression levels similar to those in the wild type bacteria. A 6-fold reduction in *ssrA* expression was observed in the *ompR* mutant, and a 2-fold reduction in expression was observed in the *fis* mutant. The role of OmpR in *ssrA* regulation was already known (Lee *et al.*, 2000), however Fis had not been previously implicated in SPI 2 gene regulation. This result indicated that in addition to its role in SPI 1 gene regulation, the Fis protein is involved in SPI 2 gene expression. It was decided to further investigate this potential role of Fis in SPI 2 gene regulation.

Chapter 4

The role of the nucleoid-associated protein Fis in *Salmonella* pathogenicity island 2 (SPI 2) gene regulation

4.1 Introduction

4.1.1 The Fis protein

Fis, the factor for inversion stimulation, is a 98-amino acid, 11.2-kDa nucleoid-associated protein. It was originally identified as a protein that was involved in inversion of the *hin* invertible DNA element in *S. typhimurium* (Heichman and Johnson, 1990). Since then Fis has been shown to have many different biological functions. In addition to its role in various genetic switches, Fis is involved in integration and excision of bacteriophages, DNA replication and also acts as a transcriptional regulator for many genes including those coding for stable RNA (rRNA and tRNA) (Finkel and Johnson, 1992; Wagner, 2000). Fis is also involved in modulating the superhelical density of the DNA within the cell (Travers *et al.*, 2001). It does this both directly, by binding to the DNA and influencing local supercoiling levels, and indirectly, by regulating the expression of the genes coding for DNA gyrase and DNA topoisomerase I.

Recently, a role has been identified for Fis in virulence gene regulation. Virulence genes in enteropathogenic *E. coli*, enteroaggregative *E. coli*, *Shigella flexneri* and *S. typhimurium*, have all been shown to be regulated by Fis (Falconi *et al.*, 2001; Goldberg *et al.*, 2001; Sheikh *et al.*, 2001; Wilson *et al.*, 2001). In this study, preliminary experiments indicated that Fis is also involved in *Salmonella* pathogenicity island 2 (SPI 2) gene regulation.

4.1.2 *Salmonella* pathogenicity island 2

Salmonella pathogenicity island 2 (SPI 2) was originally identified as a virulence locus in *S. typhimurium*, encoding a second type III secretion system, that is required for virulence in a mouse following intraperitoneal injection (Hensel *et al.*, 1995; Ochman *et al.*, 1996; Shea *et al.*, 1996). SPI 2 is located at 30 centisomes on the *S. typhimurium* chromosome and in addition to genes encoding a type III secretion system (*ssa* genes), it contains genes that code for effector (*sse*), chaperone (*ssc*) and regulatory proteins (*ssr*) (Fig 1.4). Functionally related genes in SPI 2 are clustered together into four groups, regulatory, structural I, effector/chaperone, and

structural II (Cirillo *et al.*, 1998). Reports outlining the organisation of the transcriptional units within SPI 2 are confusing and often contradictory. The regulatory genes *ssrAB* are transcribed on an operon from the *ssrA* promoter (this work and Feng *et al.* 2003). Recent reports suggest that in addition to being co-transcribed with *ssrA*, *ssrB* may have its own promoter (Feng *et al.*, 2003). Previous work on SPI 2 organization identified putative locations for three other promoters within SPI 2 (Cirillo *et al.*, 1998). Results indicated that promoters were located upstream of the *ssaB*, *sseA*, and *ssaH* genes, and that these promoters were responsible for transcription of the structural I, effector/chaperone and structural II regions respectively. Surprisingly, this study did not take into account the *ssaG* gene located upstream of *ssaH*. It was reported that the structural II promoter was located upstream of *ssaH* and downstream of *sseG*. The *ssaG* gene is located in this region but its presence was not acknowledged. In addition to this omission, this work disagreed with work carried out by Hensel *et al.* (1997b) which suggested that the structural II genes were transcribed from a promoter upstream of the *ssaK* gene and downstream of *ssaJ*. In this case the structural II genes upstream of *ssaK* would have to be transcribed from another promoter. Therefore, while it seems likely that the regulatory, structural I and effector/chaperone regions are transcribed from the *ssrA*, *ssaB* and *sseA* promoters, the organization of the transcriptional units in the structural II region is not fully understood.

4.2 Results

4.2.1 Cloning of SPI 2 promoters

Preliminary experiments using the *ssrA-lacZ* promoter fusion in gene expression assays indicated a potential role for Fis in SPI 2 regulation. To investigate this further, and to expand the investigation to encompass the entire pathogenicity island, several additional *lacZ* promoter fusions were constructed. Using the previous reports of Crillo *et al.* (1998) and Hensel *et al.* (1997) as a guide, regions of SPI 2 thought likely to contain promoters were amplified by PCR and ligated into plasmid pQF50 upstream of the promoterless *lacZ* gene. The regions included were those upstream of the following genes: *ssaB*, *sseA*, *ssaG*, *ssaH*, *ssaK* and a region containing the DNA upstream of *ssaG*, the entire *ssaG* gene, and the DNA upstream of *ssaH* (termed the *ssaGH* promoter fusion, see Fig 4.1). The DNA upstream of the *ssaB* and *sseA* genes was considered likely to contain the promoters for the structural I and effector/chaperone regions. The location of the structural II promoter or promoters was less clear. For this reason several constructs were made, containing different regions of DNA that potentially contained structural II promoters. These were the *ssaG*, *ssaH*, *ssaK* and *ssaGH* regions. Details of the primers used to amplify each promoter region are listed in Table 4.1. Cloning of these fragments into pQF50 generated the plasmids pQFssaB, pQFsseA, pQFssaG, pQFssaH, pQFssaK, and pQFssaGH (Fig 4.1). Using these plasmids, along with the pQFssrA plasmid, SPI 2 activity can be assayed in order to better understand the location of promoters within SPI 2 and to ascertain to what extent Fis is involved in SPI 2 gene regulation.

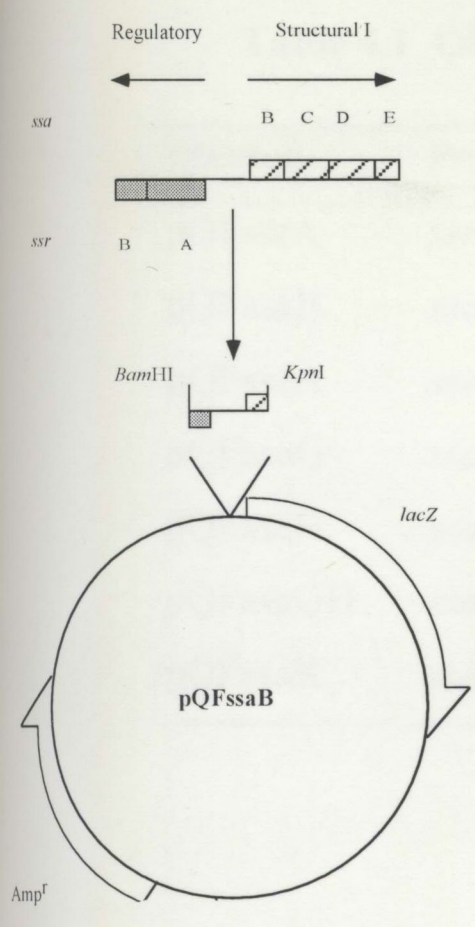
4.2.2 Analysis of expression levels from SPI 2 promoter fusions in LB broth

Each of the seven SPI 2 promoter fusion plasmids (Table 4.1) was transformed into *S. typhimurium* strain SL1344 and an SL1344 *fis* mutant. β-galactosidase assays were then performed on all strains to determine to what extent Fis is involved in regulation of each of the promoters within the pathogenicity island. It was also hoped that an insight could be gained into the location of the structural II promoter by analysis, in the wild-type strain, of the constructs containing the *ssaG*, *ssaH*,

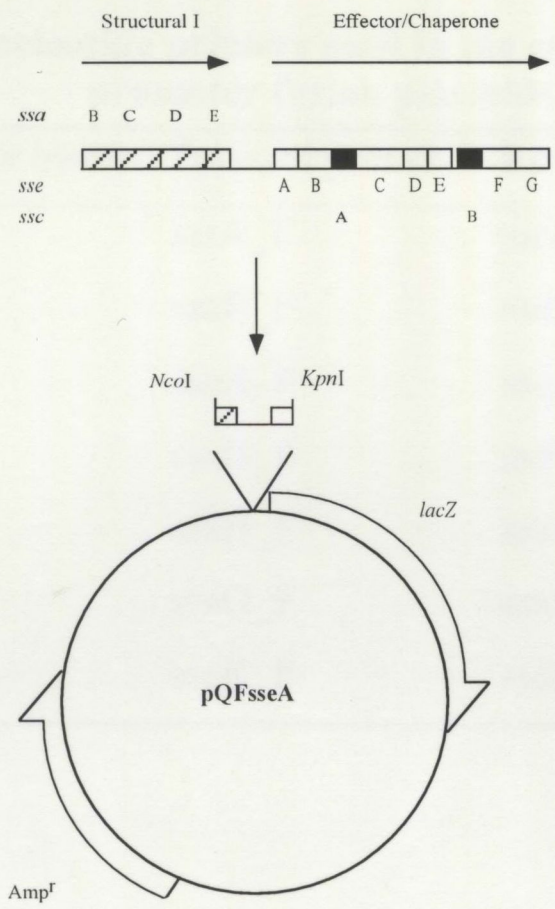
Fig. 4.1. Construction of SPI 2 promoter fusion plasmids.

Regions of DNA thought to contain SPI 2 promoters were amplified by PCR and ligated into plasmid pQF50 upstream of the promoterless *lacZ* gene. Six plasmids were constructed. For each plasmid, the area of DNA cloned is highlighted along with the restriction enzyme sites that were used for the cloning. All clonings were directional with the exception of the pQFssaK plasmid (F). In this case *Bam*HI was used for the cloning. Correct insertion of the promoter DNA into each construct was confirmed by DNA sequencing.

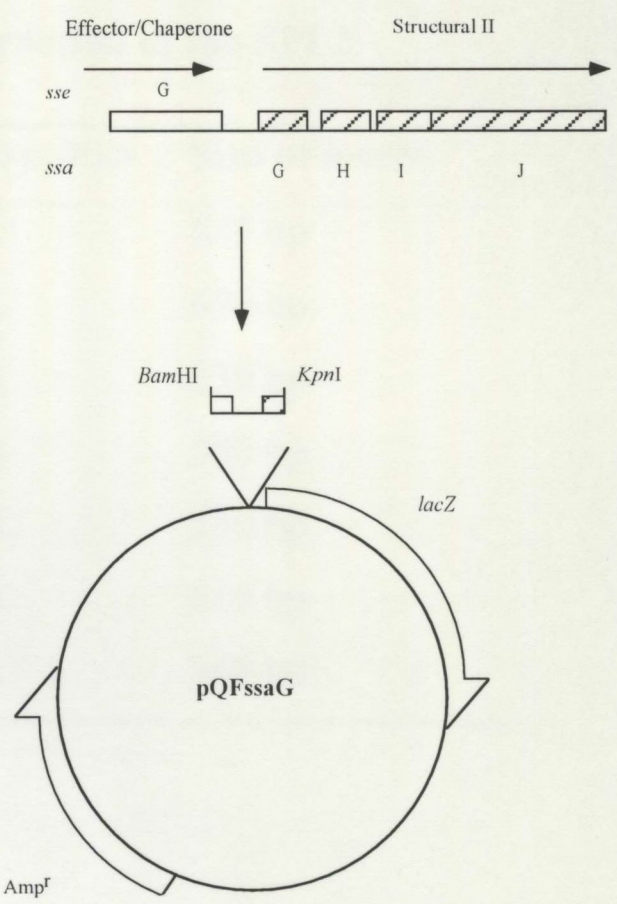
A


Regulatory
Structural I
ssa
B C D E
ssr
B A
BamHI
KpnI
lacZ
pQFssaB
Ampr

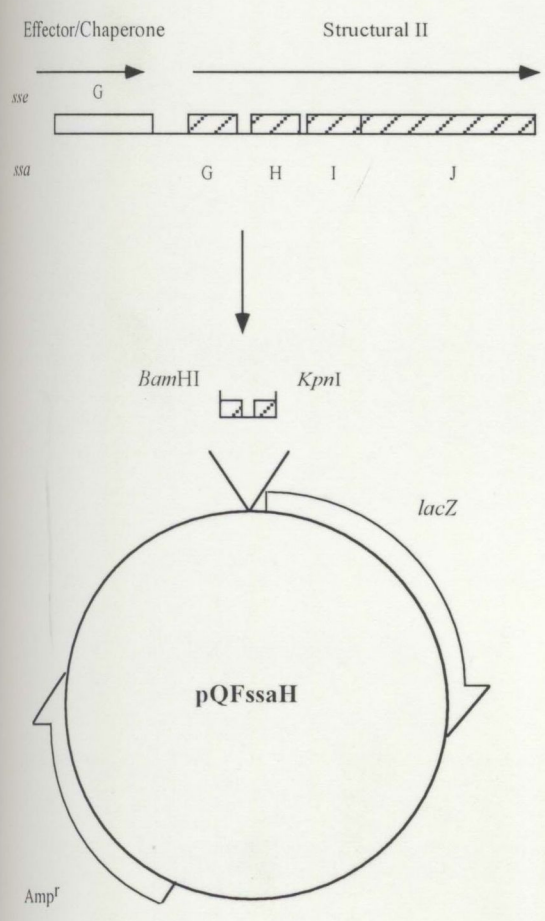
B


Structural I
Effector/Chaperone
ssa
B C D E
sse
A B C D E F G
ssc
A B
NcoI
KpnI
lacZ
pQFsseA
Ampr

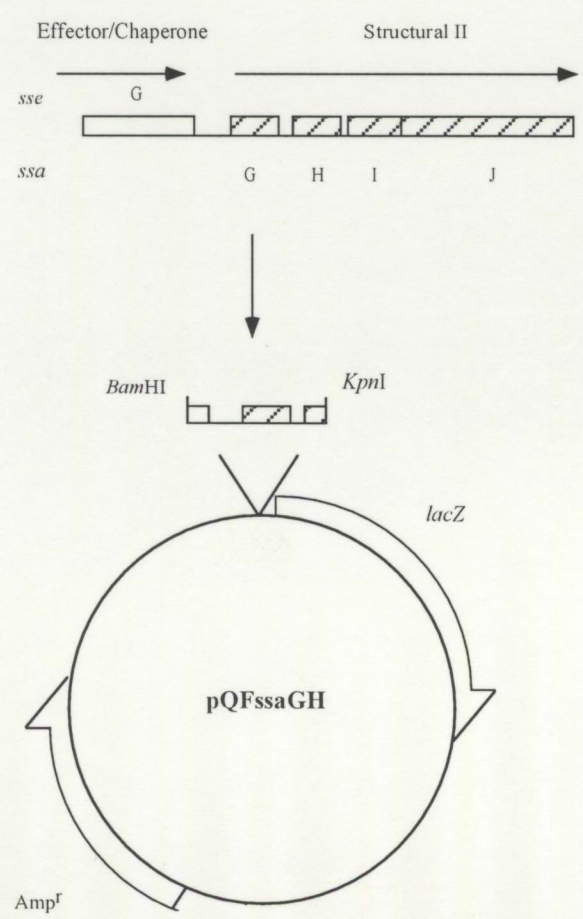
C


Effector/Chaperone
Structural II
sse
G
ssa
G H I J
BamHI
KpnI
lacZ
pQFssaG
Ampr

D


Effector/Chaperone
Structural II
sse
G
ssa
G H I J
BamHI
KpnI
lacZ
pQFssaH
Ampr

E


Effector/Chaperone
Structural II
sse
G
ssa
G H I J
BamHI
KpnI
lacZ
pQFssaGH
Ampr

F

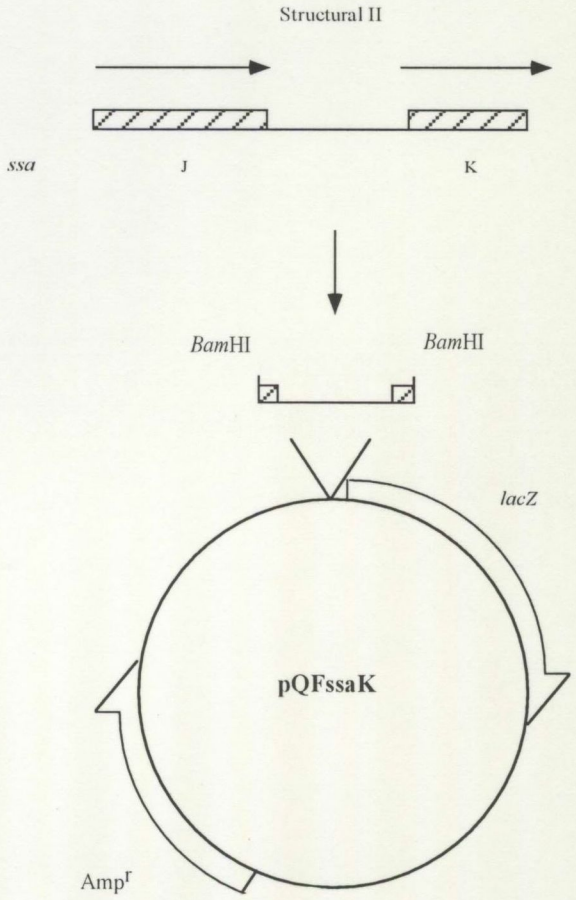

Structural II
ssa
J K
BamHI
BamHI
lacZ
pQFssaK
Ampr

Table 4.1 Oligonucleotide primers used in the construction of the SPI 2 promoter fusion plasmids.

Plasmid	Promoter insert	Forward primer	Reverse primer	Size of insert
pQFssrA	*ssrA*	ssrA_F	ssrA_R	645 bp
pQFssaB	*ssaB*	ssaB_F	ssaB_R	690 bp
pQFsseA	*sseA*	sseA_F	sseA_R	530 bp
pQFssaG	*ssaG*	ssaG_F	ssaG_R	580 bp
pQFssaH	*ssaH*	ssaH_F	ssaH_R	250 bp
pQFssaGH	*ssaGH*	ssaG_F	ssaH_R	810 bp
pQFssaK	*ssaK*	ssaK_F	ssaK_R	360 bp

ssaK and *ssaGH* regions. Results of β-galactosidase assays performed following overnight growth in 3 ml of LB broth, are shown in Fig 4.2. Decreased levels of β-galactosidase activity are observed from the *ssrA*, *ssaB*, *sseA*, *ssaG* and *ssaGH* promoter fusions in the *fis* mutant strains. No reduction in β-galactosidase levels was observed for the *ssaH* or *ssaK* promoter fusions. The fold reduction in expression in the *fis* mutants varies significantly between constructs. The smallest decrease was 2-fold for *ssrA-lacZ* and the largest, 73-fold for *ssaG-lacZ*. Analysis of β-galactosidase activity from the four constructs potentially containing the structural II promoter (*ssaG*, *ssaH*, *ssaGH*, and *ssaK*) in the wild type background, reveals high expression from both constructs containing the *ssaG* promoter region i.e. pQFssaG and pQFssaGH. Lower levels of expression were obtained from the *ssaH* and *ssaK* constructs, neither of which was affected in the *fis* mutant.

4.2.3 Analysis of expression levels from SPI 2 promoter fusions inside macrophages

SPI 2 is associated with survival and proliferation of the bacteria inside macrophages (Ochman *et al.*, 1996). Consequently, SPI 2 genes are strongly expressed *in vivo*, within macrophages (Cirillo *et al.*, 1998). In order to determine whether the observed effect of Fis on SPI 2 promoter function *in vitro* is pertinent in an *in vivo* setting, cultured J774A.1 macrophages were infected with *S. typhimurium* SL1344 and SL1344*fis* containing the SPI 2 promoter fusion plasmids. At various time-points post infection macrophages were lysed and the intracellular bacteria harvested. After enumeration of the recovered bacteria, β-galactosidase assays were performed, using MUG as a substrate, to determine levels of SPI 2 promoter activity during macrophage infection. β-galactosidase activity from the SPI 2 promoters in the wild type and *fis*$^-$ backgrounds, expressed in arbitrary units, could then be compared to ascertain to what degree Fis is involved in SPI 2 gene expression during macrophage infection. Results from the macrophage infections are shown in Fig 4.3. Infections were carried out using the pQFssrA, pQFssaB, pQFsseA, pQFssaG, pQFssaK and pQFssaH plasmids. Plasmid pQFssaGH was not included. In addition to the samples recovered from inside macrophages, β-galactosidase assays were performed on a sample of the inoculum used to infect the macrophage.

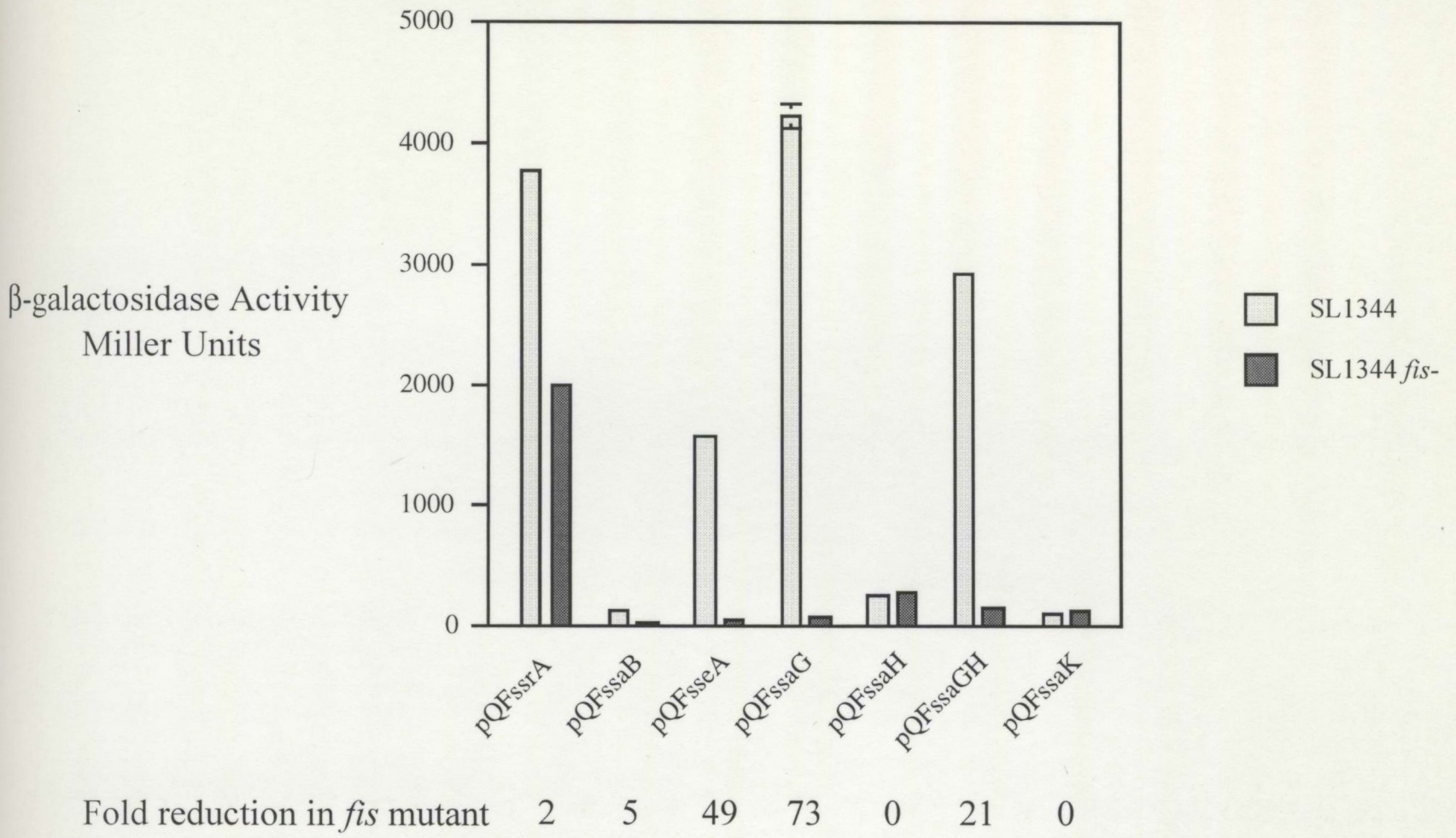
Fig. 4.2. Activity from SPI 2 promoter fusions in SL1344 and SL1344 *fis* growing in LB broth.

β-galactosidase assays were performed using the seven SPI2 promoter fusions in *S. typhimurium* strain SL1344 and an SL1344*fis* mutant.

A. Activity from SPI 2 promoter fusions in SL1344 □ and SL1344*fis* ■ . Reduced expression levels are observed in the *fis* mutants compared to the wild-type for constructs pQFssrA, pQFssaB, pQFsseA, pQFssaG and pQFssaGH. Fold reductions in *fis* mutant are indicated below each data set.

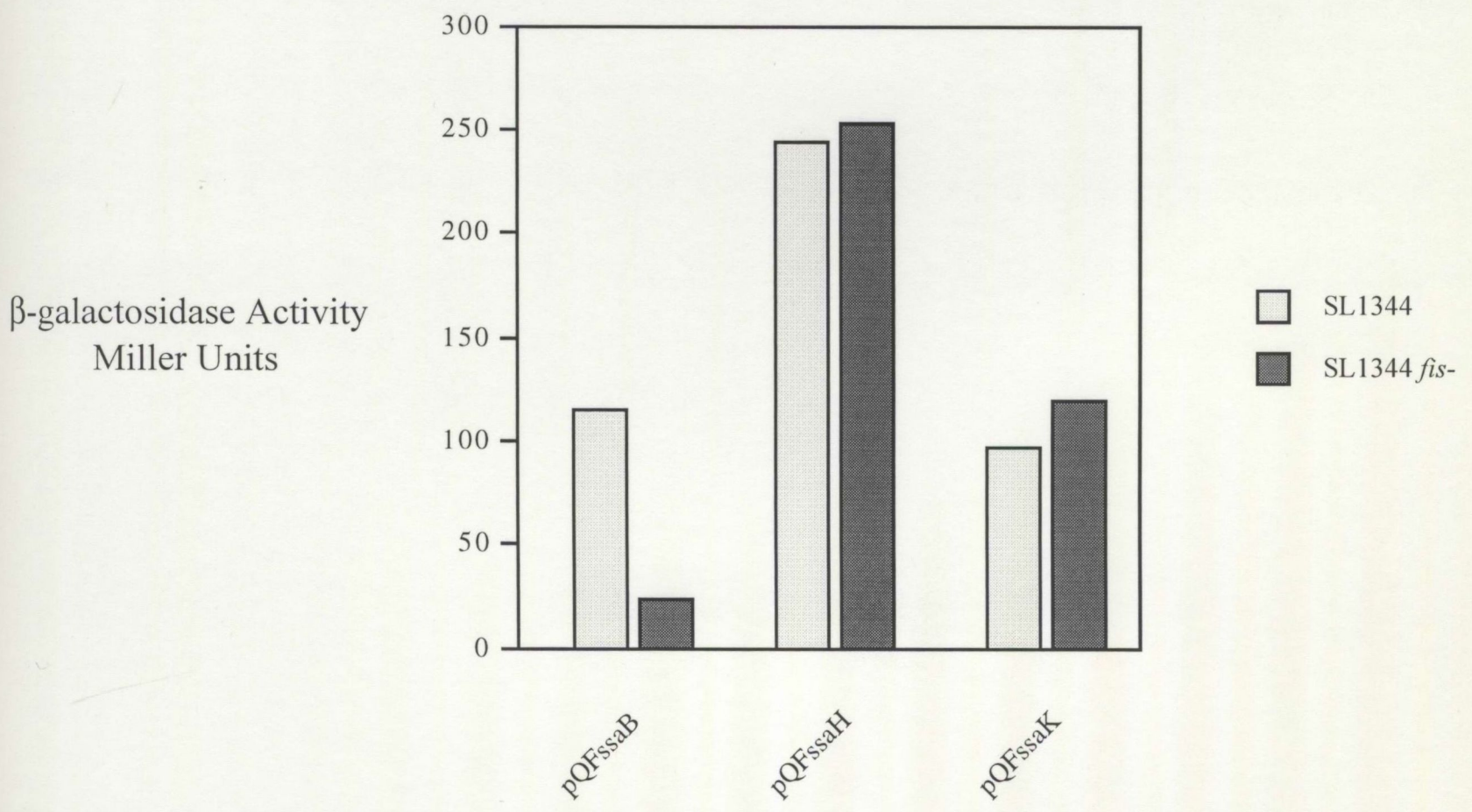
B. Activity from the pQFssaB, pQFssaH and pQFssaK constructs shown scaled up.

A



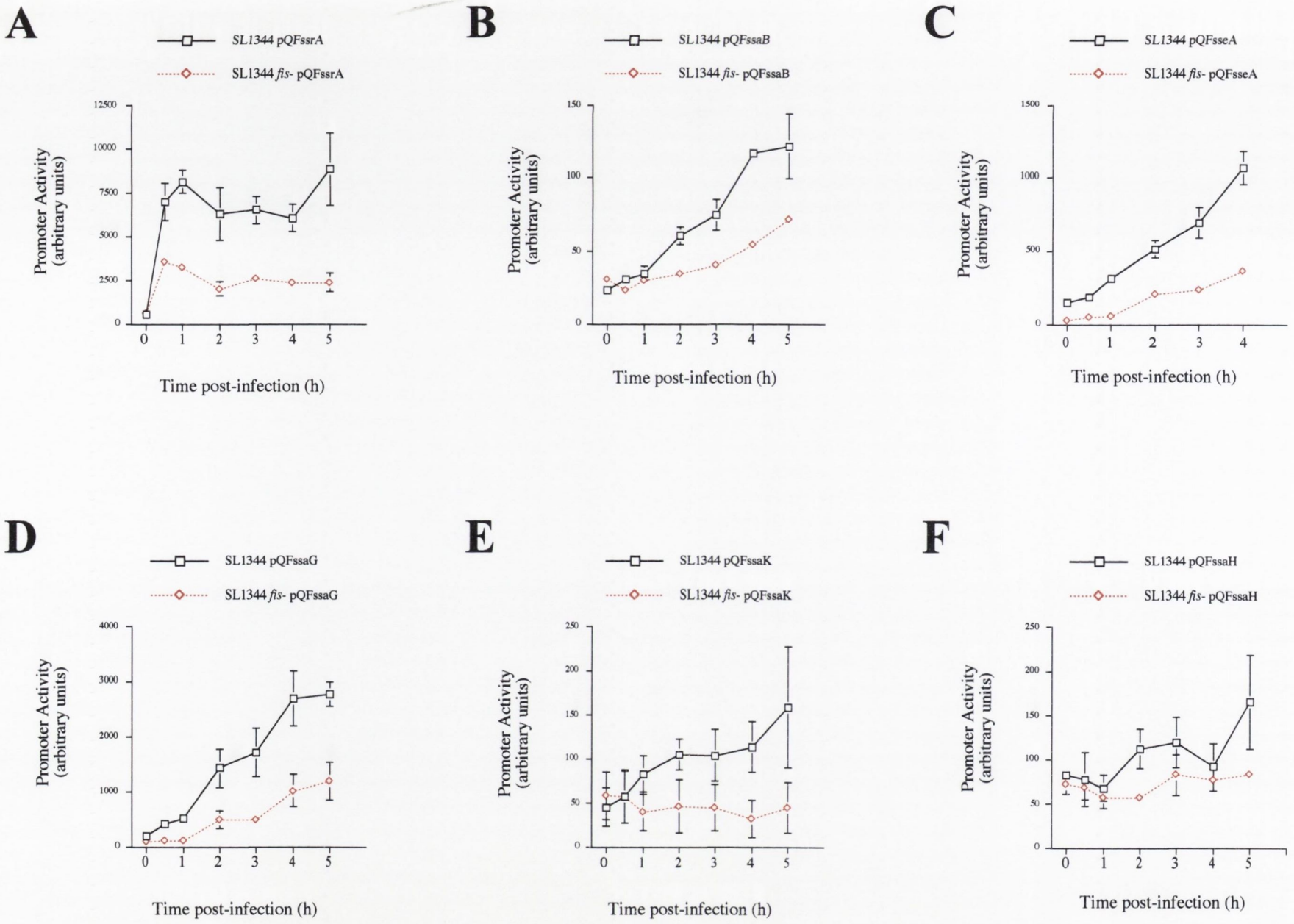
Fold reduction in *fis* mutant	2	5	49	73	0	21	0

B



Fold reduction in *fis* mutant	5	0	0

Fig 4.3. Activity from SPI 2 promoter fusions in SL1344 and SL1344 *fis,* inside macrophages. J774A.1 murine macrophage-like cells were infected with *S. typhimurium* strain SL1344 and an SL1344 *fis* mutant harbouring the SPI 2 promoter fusion plasmids pQFssrA (**A**), pQFssaB (**B**), pQFsseA (**C**), pQFssaG (**D**), pQFssaK (**E**) and pQFssaH (**F**). At various time-points post infection (30 min, 1 h, 2 h, 3 h, 4 h and 5 h) macrophages were lysed and the intracellular bacteria harvested, enumerated and used in gene expression assays. A sample of the inoculum used to infect the macrophages was also included (time-point 0). Results were graphed showing activity in the wild type strain versus activity in the *fis* mutant (**A** to **F**). A strong induction in expression is observed from all promoters in the wild type strain, with the exception of *ssaH.* In most cases induction is also observed in the *fis* mutant but to a lesser degree. The reduced inducton in the *fis* mutant results in a decrease in promoter activity in the *fis* mutants compared to the wild type at all time-points inside macrophages (with the exception of *ssaH*). Fold decrease varies but is within the range 2- to 5-fold at most time-points.


A
SL1344 pQFssrA
SL1344 fis- pQFssrA
Promoter Activity (arbitrary units)
Time post-infection (h)
B
SL1344 pQFssaB
SL1344 fis- pQFssaB
Promoter Activity (arbitrary units)
Time post-infection (h)
C
SL1344 pQFsseA
SL1344 fis- pQFsseA
Promoter Activity (arbitrary units)
Time post-infection (h)
D
SL1344 pQFssaG
SL1344 fis- pQFssaG
Promoter Activity (arbitrary units)
Time post-infection (h)
E
SL1344 pQFssaK
SL1344 fis- pQFssaK
Promoter Activity (arbitrary units)
Time post-infection (h)
F
SL1344 pQFssaH
SL1344 fis- pQFssaH
Promoter Activity (arbitrary units)
Time post-infection (h)

For the plasmids containing the *ssrA*, *ssaB*, *sseA*, *ssaG* and *ssaK* promoter regions, in the wild type SL1344, a strong induction of expression was observed as the bacteria enter the macrophage (Fig 4.3 A to E). Induction is also observed in the corresponding *fis* mutants but this induction is poor and always to a lesser degree than that observed in the wild type. The overall result of this poor induction in the *fis* mutants is that inside macrophages there is a 2- to 5-fold reduction in promoter activity in the *fis* mutants. For each promoter the exact fold decrease varies at different time-points but is within the range of 2- to 5-fold. The plasmid containing the *ssaH* promoter region displayed poor induction in both the wild type and *fis* mutant strains. It displayed little or no decrease in promoter activity in the *fis* mutant.

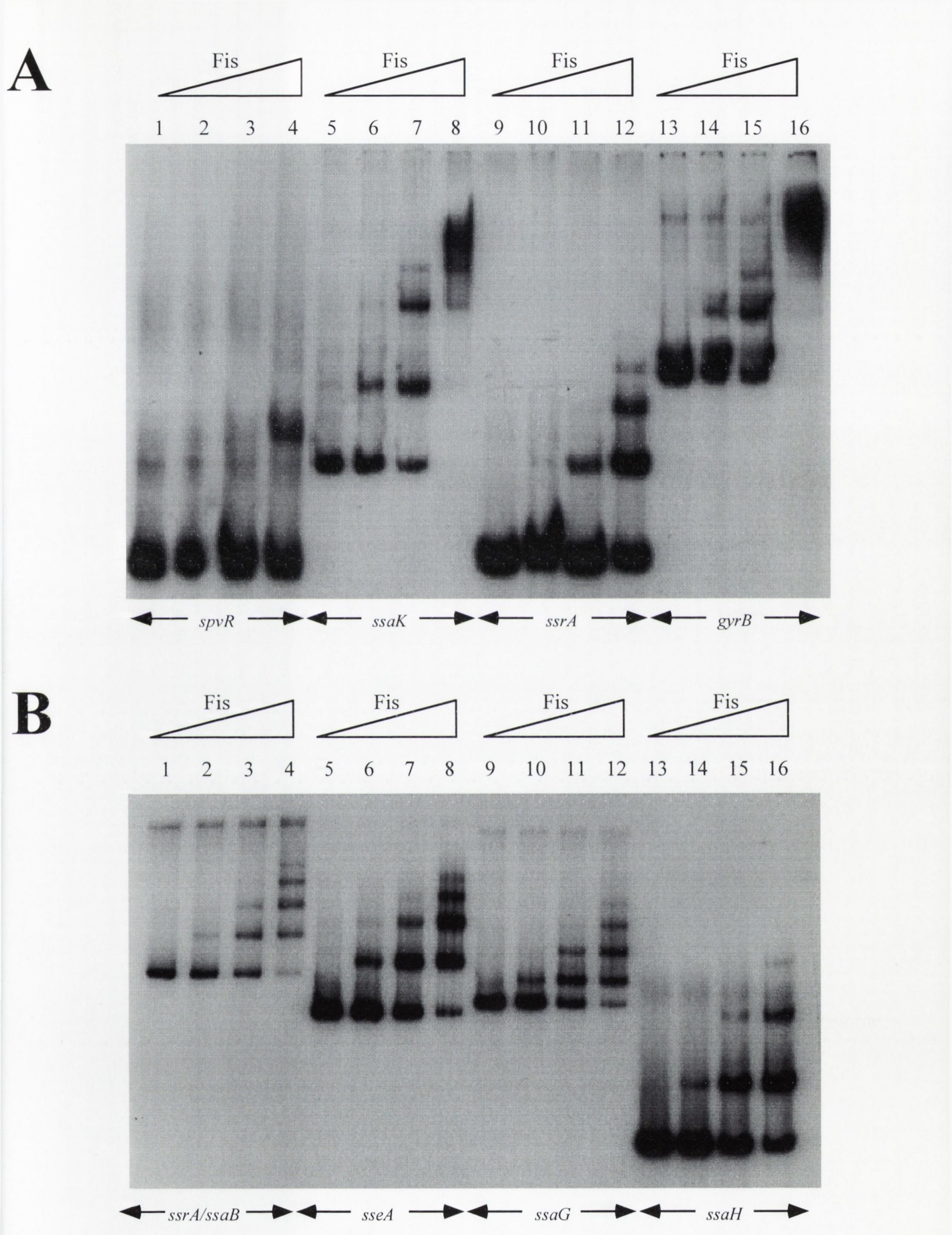
4.2.4 Interaction of Fis with SPI 2 promoters

Gene expression assays using the SPI 2 promoter fusion plasmids had indicated a role for Fis in the regulation of SPI 2 gene expression. Fis is a nucleoid-associated protein and a pleitropic regulator of gene expression. Therefore in order to establish if the observed effect of Fis on SPI 2 was as a direct result of Fis binding to the SPI 2 promoters and influencing promoter activity, DNA mobility shift assays were performed. Purified Fis protein containing an N-terminal six-histidine tag was provided as a gift by O. Keane. DNA fragments corresponding to the promoter regions of *ssrA*, *ssaB*, *sseA*, *ssaG*, *ssaK*, and *ssaH* were used. In addition, the *E. coli gyrB* and *S. typhimurium spvR* promoters were used as positive and negative controls respectively. DNA fragments were radiolabelled with [γ-^{32}P]dATP and incubated with increasing concentrations of Fis protein. Samples were then analysed on a non-denaturing polyacrylamide gel. Binding of the protein to the DNA forms a complex that runs more slowly through the gel resulting in a "shift" in the mobility of the DNA band visualised by autoradiography. Results of the DNA mobility shift assays using the Fis protein and SPI 2 promoters are shown in Fig 4.4. All of the SPI 2 promoter fragments used (*ssrA/ssaB, sseA, ssaG, ssaH, ssrA* and *ssaK*) bind the Fis protein. In addition, Fis binds to multiple sites at each promoter. For the *ssaK*, 190 bp *ssrA* (Fig 4.4 A), *sseA*, *ssaG*, and *ssaH* (Fig 4.4 B) promoters, three distinct complexes can be seen. This indicates that Fis is binding to three distinct binding sites at these promoters. For the *ssrA/ssaB* fragment, which spans

Fig 4.4. Interaction of Fis protein with SPI 2 promoter sequences by DNA mobility shift assay. DNA fragments corresponding to promoter regions of SPI 2 were amplified by PCR and radiolabelled with [γ-^{32}P]dATP. Radiolabelled fragments were incubated with 0 ng (lanes 1, 5, 9, 13), 4 ng (lanes 2, 6, 10, 14), 20 ng (lanes 3 ,7, 11, 15) or 60 ng (lanes 4 ,8, 12 ,16) purified Fis protein and separated on polyacrylamide gels. Mobility of the resulting bands was analysed by autoradiography. The *E. coli gyrB* and *S. typhimurium spvR* promoters were also used, as positive and negative controls respectively.

A. Results of DNA mobility shift assay using the *S. typhimurium spvR* (lanes 1-4) and *E. coli gyrB* (lanes 13-16) controls, along with a 360-base pair fragment of the *ssaK* promoter (lanes 5-8) and a 192-base pair fragment of the *ssrA* promoter (lanes 9-12). The *ssrA*, *ssaK* and *gyrA* promoter fragments each formed three distinct complexes indicating the presence of three Fis binding sites (lanes 5-16). For the *spvR* promoter (lanes 1-4) a shift was only observed at the highest concentrations of Fis (lane 4). In this case (and also in lanes 8 and 16) the high concentration of Fis resulted in non-specific association of the protein to the DNA .

B. Results of DNA mobility shift assay for the *ssrA/ssaB* (lanes 1-4), *sseA* (lanes 5-8), *ssaG* (lanes 9-12) and *ssaH* (lanes 13-16) promoter fragments. A 690-base pair DNA fragment spanning the *ssrA/ssaB* intergenic region, a 530-bp *sseA* promoter fragment, a 580-bp *ssaG* promoter fragment, and a 250-bp *ssaH* promoter fragment were used. For each, the formation of at least three distinct complexes can be seen.


A
Fis
Fis
Fis
Fis
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
spvR
ssaK
ssrA
gyrB
B
Fis
Fis
Fis
Fis
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
ssrA/ssaB
sseA
ssaG
ssaH

the entire *ssrA-ssaB* intergenic region (and encompasses the 190 bp *ssrA* fragment used in Fig 4.4 [A]) and contains two divergently transcribed promoters, at least five complexes are formed, again indicating that Fis binds to multiple sites at each promoter. Fis was also demonstrated to bind to the *E. coli gyrB* promoter at multiple sites but not to the *S. typhimurium spvR* promoter. In certain cases, non-specific association of the protein and the DNA was observed (Fig 4.4 A, lanes 4,8 and 16). This was likely to have arisen as a result of the previously described ability of high concentrations of Fis to form non-specific complexes with DNA (Schneider *et al.*, 1997). The results show that Fis has the potential to affect the SPI 2 promoters directly by binding to them.

4.2.5 Fis antiserum

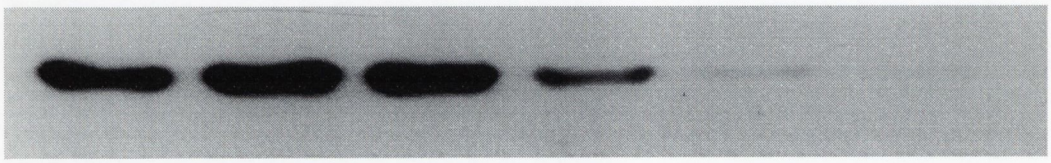
The Fis protein has a very characteristic expression profile *in vitro* in *E. coli* (Azam *et al.*, 1999). Induction of Fis occurs following nutrient up-shift and approximately 60,000 dimers of Fis are found within the cell during early exponential growth. After this Fis levels decrease so that by the onset of stationary phase, it is almost undetectable (Ball *et al.*, 1992; Ninnemann *et al.*, 1992). A previous investigation into the expression profile of nucleoid-associated proteins during macrophage infection reported an induction of the *hns* and *ihf* promoters inside macrophage (Marshall *et al.*, 2000). No report had been made in relation to Fis levels. To find out if the *S. typhimurium* Fis expression profile matches that of *E. coli* and to attempt to determine Fis levels during macrophage infection, antibodies were raised against the purified his-tagged Fis protein (O. Keane). Three injections, each containing 300 μg Fis protein in either Freund's complete or incomplete adjuvant, were administered to a New Zealand White rabbit at two-weekly intervals. Serum from a test bleed was assayed by Western immunoblot, and found to contain anti-Fis antibodies at a dilution of 1:5000. The rabbit was exsanguinated and serum stored at 4°C. Prior to use in Western blots, serum was adsorbed against a lysate of SL1344*fis*.

4.2.6 Analysis of Fis protein levels *in vitro*

To find out if the expression profile of Fis in *Salmonella* is similar to that of *E. coli*. Western immunoblots were performed. *S. typhimurium* SL1344 was grown in LB broth and at various time-points throughout the growth cycle the OD_{600nm} was measured and a volume of cells which corresponded to 2 ml of culture per 1 OD_{600nm} unit was taken and used to prepare crude protein extracts. Equal volumes of each extract was loaded on an SDS-PAGE gel and analysed by Western blot using the Fis-antiserum. Results demonstrated that Fis has a similar expression profile in *S. typhimurium* as in *E. coli* (Fig 4.5) (Azam *et al.*, 1999). Fis levels are highest in early exponential phase and steadily decline thereafter so that by stationary phase, it is barely detectible.

4.2.7 Analysis of Fis protein levels during macrophage infection

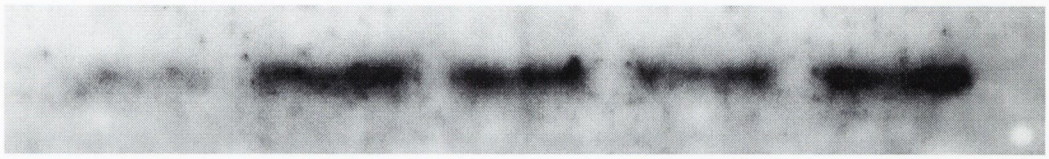
Results so far have indicated that Fis is required for full activation of the promoters in SPI 2. The expression profile of Fis in *S. typhimurium* demonstrates that it is abundant in early exponential phase after which levels drop, however, nothing is known about Fis levels during macrophage infection. In an attempt to establish Fis protein levels during macrophage infection, Western blots were performed on bacteria recovered from the intracellular environment. At various time-points post infection macrophages were lysed and intracellular bacteria recovered and enumerated by viable counts. The remaining bacteria were pelleted and stored at –20°C. Each bacterial pellet was resuspended in an appropriate volume of lysis buffer to give a lysate corresponding to between 1×10^5 and 3×10^5 bacteria per µl. Equal volumes of these lysates were loaded on SDS-PAGE gels and used for Western blot analysis. A sample of the inoculum used to infect the macrophages was also included. For consistency this sample was also enumerated by viable count and treated in the same way as the recovered *in vivo* samples. Western analysis of intracellular Fis levels was preformed at least 4 times and a representative blot is shown in Fig 4.6. Results indicate that Fis is induced following uptake of the bacteria into the macrophage and that once induced Fis levels remain high. Inside macrophage Fis protein levels remained relatively constant however occasional fluctuations were observed. This *in vivo* expression profile is in stark contrast to the



OD600	0.168	0.3	0.546	1.075	2.14	2.2

Fig. 4.5. Western immunoblot analysis of Fis protein levels *in vitro* during growth in LB broth.

Samples of *S. typhimurium* strain SL1344 growing in LB broth were harvested at various times during the growth cycle (OD_{600} values for each sample are given above). Equal quantities of each lysate were loaded on an SDS-PAGE gel and analysed by Western blot. Results demonstrated a similar expression profile to that of *E. coli.* Fis levels are highest during early exponential phase and decrease steadily thereafter.



inoculum (OD_{600} = 1) 30 mins 1h 2h 3h

Fig. 4.6. Western immunoblot analysis of Fis protein levels during macrophage infection. J774A.1 macrophages were infected with SL1344 bacteria and at various time-points post infection macrophages were lysed, bacteria recovered, enumerated, and used in Western blots analysis with the anti-Fis antiserum. A sample of the inoculum bacteria used (in which the OD_{600} = 1) was also included. Fis levels increased 4-fold following entry into macrophages and remained high thereafter.

expression pattern seen in both *S. typhimurium* and *E. coli* cells growing in LB broth, indicating a novel regulatory mechanism and role for Fis during macrophage infection.

4.2.8 Analysis of promoter DNA sequence

Using the results generated here, along with the information on the *ssrA* OmpR binding sites (Feng *et al.*, 2003; Lee *et al.*, 2000), a map of the intergenic region between *ssrA* and *ssaB* was constructed (Fig 4.7). The locations of the *ssrA* and *ssaB* translation initiation codons are indicated, along with the *ssrA* transcription start site (this work) and the location of the OmpR and phospho-OmpR binding sites (Feng *et al.*, 2003; Lee *et al.*, 2000). An analysis of the DNA in this region, using the "Mac Target Search" DNA analysis software, identified a potential location for the –10 and –35 regions of the *ssrA* promoter.

Using the "Mac Target Search" DNA analysis software, an analysis of the *ssrA-ssaB* DNA was carried out looking for potential Fis binding sites. The consensus Fis binding site is degenerate with very few absolutely conserved residues (Finkel and Johnson, 1992). As a result this consensus site is easily matched to any target DNA sequence making accurate determinations of Fis binding sites, based on alignments with this consensus site, very difficult. To aid in the search for Fis sites a search file was created based on 37 known Fis binding sites (Finkel and Johnson, 1992). Residues at each position in the binding site were given a weighting depending on the frequency with which they appear at that position in all 37 sites. Highly conserved residues were given heavy weighting while lowly conserved residues were lightly weighted. An analysis of the target DNA sequence using the Fis binding site program identifies potential matches based on total scores obtained for each residue in the 15 bp site. It was hoped that in this way regions of DNA showing homology with conserved residues of Fis binding could be accurately identified as potential Fis binding sites. Using this program with the *ssrA-ssaB* DNA region, several potential Fis binding sites were identified (Fig 4.7). None of the binding sites identified is a perfect match for the consensus Fis site, however as this consensus is so degenerate this was not surprising. While this sequence analysis gives a good indication as to the location of Fis binding sites, further biochemical

Fig. 4.7. Analysis of the *ssrA-ssaB* DNA sequence.

5' regions of the *ssrA* (blue) and *ssaB* (red) coding DNA sequences (CDS), and the *ssrA-ssaB* intergenic region (black) is shown. The *ssrA* transcription start site is indicated with an arrow along with the putative -10 and -35 regions. The OmpR binding site A1, identified by Lee *et al.* (2000) is shown (blue box) and the five phospho-OmpR binding sites B1 to B5, identified by Feng *et al.* (2003) are also shown (red boxes). Potential Fis binding sites identified by sequence analysis are indicated (green and underlined). The location of one potential Fis binding site places it between the -10 and -35 of the *ssrA* promoter.

ggatccgaattcgtcgacggcaagacaaggcttaggtaagctt

tccaggtcatttaagaacaaagaaatagaaaatgcttctgaga

ssaB CDS

aaatttctcctctggcaggatgcccatcaatagtcattatcca

ggatcggctattaccttcggccctgatatcctgaattaatgga

atgccttttaaaactgccagcatgaatccctcctcagacataa

atgggagtttctatcaaattcgctcacaaccacatccgtaaaa

agcctgattcacatttatttcgactatactcttcttgtacaata

tcaggatgctgtctacatatacccttgtcacaggcgattctatc

attcggattttccgataaattcacaattacattttcagcattga

-35

cataaaaacttacaatttgaaaaaattatttattaaataaactgt

A1 ssrA +1

-10

tacgatgttttttacatcgccatcttattaaaaagtaattgtagt

B1 B2

catcgactgggttatatatgaagaaatttatcttcctaatgata

B3

acaccatcgattaatcttctgatgaaactatatgtactgcgat

B4

agtgatcaagtgccaaagattttgcaacaggcaactggaggga

B5

agcattatgaatttgctcaatctcaagaatacgctgcaaacat

ssrA CDS

ctttagtaatcaggcggatccggtaccccc

evidence, e.g. DNAse I footprinting is required to identify the exact location(s) of Fis binding. One of the potential Fis binding sites identified here is located between the –10 and –35 regions of the *ssrA* promoter. Another is located approx 20 bp upstream of the –35. The proximity of these sites to the *ssrA* promoter indicates that they are good candidates for Fis to bind to this promoter and influence gene expression.

The "Mac Target Search" Fis binding site search file was also used to identify Fis binding sites at the *sseA*, *ssaG*, and *ssaK* promoters. Potential Fis binding sites were located at each promoter but in the absence of further biochemical evidence and identification of the transcription start sites of each of these promoters it is difficult to accurately locate binding sites in these areas.

4.3 Discussion

Nucleoid-associated proteins are increasingly being associated with regulation of virulence genes in pathogenic bacteria. A prime example of this is the Fis protein, which has been shown to be involved in the regulation of virulence genes in many different enteric pathogens, including *S. typhimurium* (Wilson *et al.*, 2001). Fis is required for full activation of the *hilA* and *invF* regulatory genes of SPI 1. In a *fis* mutant, expression of the SPI 1 type III secretion system genes is reduced and the mutant is attenuated for virulence in orally infected mice. Now, for the first time, a role for Fis in SPI 2 gene regulation has been shown. Specifically, Fis has been shown to be required for full expression of SPI 2 promoters both *in vitro* and during macrophage infection.

Following a preliminary result with an *ssrA-lacZ* promoter fusion that indicated a role for Fis in regulation of the *ssrA* promoter (chapter 3), a series of *lacZ* promoter fusion plasmids were made which contained regions of SPI 2 DNA thought likely to contain promoters. These included the *ssaB* (structural I) promoter and the *sseA* (effector/chaperone) promoter. The exact location of certain SPI 2 promoters is in doubt (i.e. the structural II promoter[s]) so several constructs were made to cover all potential scenarios (the *ssaG*, *ssaH*, *ssaK* and *ssaGH* promoters). These promoter fusions were then used in gene expression assays in the wild type and *fis* mutant bacteria. Results from cultures grown overnight in LB broth (Fig 4.2), demonstrated that Fis is required for full expression from the *ssaB*, *sseA*, *ssaG* and *ssaGH* promoters, in addition to the *ssrA* promoter. No effect of Fis was observed at the *ssaH* or *ssaK* promoters although both these promoters did demonstrate significant transcriptional activity. An analysis of the four promoters in the structural II region showed considerably higher expression from the *ssaG* and *ssaGH* promoters than from the *ssaH* or *ssaK* promoters. This result indicates that there is a strong promoter located upstream of *ssaG*, transcription of which continues through the *ssaG* gene and into *ssaH*. This result implies that the *ssaG* promoter transcribes a polycistronic transcript containing several of the structural II genes. It is unknown how long this transcript is as there does appear to be functionally active promoters located upstream of the *ssaK* and *ssaH* genes. Interestingly, neither of these promoters is responsive to Fis in LB broth. Previous work (Hensel *et al.*, 1997b),

claimed that *ssaJ* and *ssaK* were not co-transcribed. Taking this into account along with the promoter data shown here, it seems that there are at least two promoters responsible for the transcription of the structural II region, i.e. the *ssaG* and *ssaK* promoters. The *ssaH* promoter may still be significant under certain circumstances. Its failure to respond to Fis may mean that this promoter is only active under certain specific conditions to which other promoters in the island do not respond. An understanding of how each component of the type III secretion system contributes to its function will be important in determining this. This may also help explain the difference in strength between the *ssaG* and *ssaK* promoters. Greater quantities of certain components of the type III secretion system may be required for assembly so these may be transcribed from stronger or multiple promoters.

SPI 2 genes are associated with survival and proliferation of the bacteria inside macrophages and are highly expressed in the intracellular environment (Cirillo *et al.*, 1998). To determine if the observed *in vitro* effect of Fis on SPI 2 gene expression also applied during macrophage infection, gene expression assays were performed on bacteria containing the SPI 2–*lacZ* reporter fusions, following infection of cultured J774A.1 macrophages. Results (Fig 4.3) show that in the wild type bacteria, the *ssrA* promoter is rapidly activated following entry into the macrophage. Levels of expression from this promoter remain consistently high thereafter. The *ssaB*, *sseA*, *ssaG* and *ssaK* promoters demonstrate a more gradual induction over the five-hour time-course of this experiment. This result is consistent with the ascribed role for SsrA/B as a two-component signal transduction system that is required for transcription of the SPI 2 genes (Cirillo *et al.*, 1998). Rapid activation of the *ssrA* promoter results in high levels of SsrA and SsrB, which in response to signals from the macrophage activate transcription from the rest of the SPI 2 promoters. This accounts for the delayed induction seen in these promoters. The *ssaH* promoter responded poorly to the intracellular environment. Highest expression levels inside macrophages were just over 2-fold higher than the LB grown inoculum. This result indicates that this region does not contain a promoter that responds to the intracellular environment in the same way as the rest of the SPI 2 promoters.

β-galactosidase activity from the SPI 2 promoter fusions was also determined in *fis* mutant bacteria during growth inside macrophages. Whereas in the wild type bacteria a strong induction was observed from the *ssrA*, *ssaB*, *sseA, ssaG* and *ssaK* promoters, in the corresponding *fis* mutants a very weak induction was observed. At all time-points inside macrophages, levels of transcription were lower in the *fis* mutants than in the wild-type bacteria. The *ssaH* promoter was an exception displaying little or no decrease in transcription in the *fis* mutant. For promoters responsive to the presence of Fis, the fold decrease in expression varied but was usually within the range 2- to 5-fold. In certain cases this agreed well with the LB *in vitro* data (e.g. *ssrA*), however for some (e.g. *ssaG*) the fold difference is much smaller than that observed *in vitro*. There are several possibilities as to why this is the case. *In vitro* β-galactosidase assays were performed using stationary phase cultures grown in a nutrient-rich medium. Physiologically, these cells are very different from cells surviving inside a macrophage. Levels of global regulatory proteins e.g. nucleoid-associated proteins as well as supercoiling levels will be very different, which can have unpredictable consequences on gene expression profiles (Azam *et al.*, 1999; Marshall *et al.*, 2000). This makes it difficult to draw accurate comparisons between them. The effect of Fis is clear, and it appears that under conditions where SPI 2 is required, i.e. inside a macrophage, removal of Fis results in a 2- to 5-fold decrease in transcription.

One other factor must be taken into account when analysing SPI 2 promoter activity in the *fis* mutant bacteria. The SsrA/B two-component signal transduction system is required for activation of the SPI 2 genes (Cirillo *et al.*, 1998). In a *fis* mutant expression from the *ssrA* promoter decreases resulting in decreased production of SsrA and SsrB, which in turn will result in decreased transcription from the downstream SPI 2 promoters (i.e. *ssaB*, *sseA*, *ssaG* and *ssaK*). This means that the observed decrease in activity from these promoters may be as a result of an indirect effect and is passed on through SsrA/B. Alternatively, Fis may be affecting each promoter individually, meaning that the observed effect is a combination of both direct and indirect (through SsrA/B) effects. To investigate this further, DNA mobility shift assays were performed. Direct interaction of Fis with SPI 2 promoter DNA would indicate that Fis has a direct effect at that promoter. The results clearly

indicated that Fis does bind to the DNA at each of the SPI 2 promoters (Fig 4.4), including the *ssaH* promoter. This result suggests that Fis exerts a direct effect at each promoter and that the observed decrease in transcription in the downstream SPI 2 promoters is a result of both direct local effects of Fis and indirect effects, channelled through SsrA/B. Surprisingly, the *ssaH* promoter DNA also bound the Fis protein. Expression from this promoter was unaltered in a *fis* background both in LB and inside macrophages therefore it is difficult to explain why Fis would bind there. One possible explanation for this concerns the mechanism of action of Fis in this system. Fis acts as a conventional transcription activator but can also affect transcription levels due to its role in supercoiling (Travers *et al.*, 2001). Binding to the DNA and influencing local supercoiling levels is one mechanism of action of Fis in modulating gene expression. If this is the mechanism employed by Fis in regulation of SPI 2, binding of Fis throughout the island would be expected as in this way it could constrain local supercoils and exert a positive effect on gene expression. Evidence that supports this argument and shows SPI 2 promoters are sensitive to supercoiling levels will be presented in chapter 5.

Most nucleoid-associated proteins display characteristic expression profiles during *in vitro* batch culture, and Fis is no exception. By generating antibodies against purified Fis protein, and using them in Western immunoblot analyses it was shown that Fis has a similar expression profile in *S. typhimurium* as it has in *E. coli* (Azam *et al.*, 1999). Fis levels are highest immediately following sub-culture into fresh medium after which they decline. In addition to establishing Fis levels in *S. typhimurium* growing *in vitro*, for the first time, Fis levels were determined for bacteria in the intracellular environment. Western blots were performed on bacteria recovered from the intracellular environment and they showed that Fis is induced upon entry into the macrophage and that its levels remain high in the cell thereafter. This expression profile is in contrast to the expression profile of Fis *in vitro* indicating a novel mechanism of regulation and function for Fis during macrophage infection. The finding that Fis is required for activation of the SPI 2 genes combined with the novel expression pattern of Fis *in vivo,* and the evidence of Fis binding to the SPI 2 promoters clearly demonstrates that Fis regulates the genes on SPI 2. This raises the interesting possibility that the nucleoid-associated protein Fis

co-ordinately regulates expression of two different pathogenicity islands in *S. typhimurium*.

Chapter 5

Environmental and physiological factors affecting SPI 2 gene expression

5.1 Introduction

5.1.1 Two-component signal transduction systems

Two of the known regulators of *Salmonella* pathogenicity island 2 (SPI 2) are two-component signal transduction systems, namely the SsrA/SsrB and OmpR/EnvZ systems (Cirillo *et al.*, 1998; Lee *et al.*, 2000). Two-component signal transduction systems consist of a sensor kinase (usually membrane bound) and a cytoplasmic response regulator protein (Perraud *et al.*, 1999). In the membrane, the sensor kinase is activated by specific external signals, e.g. environmental conditions, and phosphorylates the response regulator. Following phosphorylation the response regulator binds to the DNA and exerts an effect on gene expression. OmpR/EnvZ is the best studied two-component signal transduction system with EnvZ being the membrane bound sensor kinase and OmpR the cytoplasmic response regulator (Forst and Roberts, 1994). The external stimulus sensed by EnvZ is osmolarity. Genes regulated by OmpR/EnvZ are therefore influenced by the osmolarity of the environment outside the cell. The predicted amino acid sequences for SsrA and SsrB indicate that they form another two-component signal transduction system homologous to OmpR/EnvZ (Ochman *et al.*, 1996; Shea *et al.*, 1996). SsrA shows homology to EnvZ and is therefore likely to be the membrane bound sensor while SsrB shows homology to OmpR and is likely the response regulator. Both SsrA and SsrB are encoded within SPI 2 and are required for activation of the SPI 2 genes (Cirillo *et al.*, 1998) as well as other effectors encoded outside of SPI 2 (Worley *et al.*, 2000). Very little is known about the mechanism by which SsrA/SsrB activates gene expression and the environmental signal sensed by SsrA is unknown.

5.1.2 Environmental conditions affect SPI 2 gene expression

SPI 2 genes are specifically induced upon bacterial entry into macrophages and are required for intracellular survival and proliferation (Ochman *et al.*, 1996). Activation of the SPI 2 genes involves the two-component signal transduction systems SsrA/SsrB and OmpR/EnvZ (Cirillo *et al.*, 1998; Lee *et al.*, 2000). Both these systems activate gene expression in response to environmental stimuli. In the case of OmpR/EnvZ the signal is osmolarity (Forst and Roberts, 1994). For

SsrA/SsrB the signal is unknown. Whatever the signal for SsrA/SsrB, it is clear that expression of the SPI 2 genes is influenced by certain environmental conditions (Deiwick *et al.*, 1999). Identifying environmental conditions that influence SPI 2 expression has become a contentious topic of investigation and has lead to some interesting and often contradictory reports. Deiwick *et al.* (1999) studied SPI 2 gene expression levels and SsaP and SscA protein levels under various conditions and in various media and concluded that Mg^{2+} deprivation and phosphate starvation are required for SPI 2 gene expression. Miao *et al.* (2002) refute this in their study saying that Mg^{2+} deprivation and phosphate starvation are not required for SPI 2 activation. Lee et al. (2000) conclude that low pH activates SPI 2 expression while both Deiwick *et al.* and Miao *et al.* disagree. SPI 2 genes are activated inside macrophages and are regulated by at least 2 two-component signal transduction systems. It is evident that SPI 2 genes are activated by certain macrophage-associated environmental signals but the precise nature of these signals remains unclear. Using the SPI 2 promoter fusions created in this study, an investigation was carried out to determine what macrophage-associated conditions influence SPI 2 gene expression.

5.1.3 Environmental and physiological conditions inside macrophages

As part of its infectious cycle, *S. typhimurium* has the ability to survive and replicate inside host cells, including epithelial cells and macrophages (Alpuche-Aranda *et al.*, 1994). Macrophage cells express numerous antimicrobial factors such as reactive oxygen species, reactive nitrogen species and antimicrobial peptides, yet in spite of these *Salmonella* still has the ability to proliferate inside these cells (Vazquez-Torres and Fang, 2001). Upon entry into the macrophage the bacteria rapidly form a modified phagolysosome termed the *Salmonella*-containing vacuole (SCV) (Alpuche-Aranda *et al.*, 1994). The bacteria reside within the SCV, which displays reduced antimicrobial activity and is less acidic than the classic phagolysosome. The reduced antimicrobial activity observed within the SCV is mediated by the bacteria, and in particular by the SPI 2 system (Fang and Vazquez-Torres, 2002). The type III secretion system coded for by SPI 2 forms a translocon that injects bacterial effector proteins into the host cell cytoplasm. These effector proteins

interfere with trafficking of the SCV in relation to the endocytotic pathway resulting in the reduced antimicrobial environment within the SCV, which facilitates bacterial survival and replication. As shown in chapter 4 there is a rapid induction of the SPI 2 promoters inside macrophages in response to the environmental signals encountered inside the early SCV.

In association with the environmental changes as the bacteria enter the macrophage, certain physiological changes also occur. A previous study has shown that as the bacteria enter macrophages there is a global relaxation of the DNA (Marshall *et al.*, 2000). This relaxation is accompanied by induction of the DNA-relaxation-activated gyrase genes (*gyrA* and *gyrB*) but no induction of the DNA-relaxation-repressed topoisomerase I gene (*topA*). Changes in topology of the DNA are known to influence gene expression (Schneider *et al.*, 2000) raising the possibility that macrophage-induced genes e.g. SPI 2, are affected by relaxation of the DNA.

The bacteria undergo a substantial change in environmental and physiological conditions as they enter macrophages and these conditions are responsible for the induction of genes important for survival inside the cell (Marshall *et al.*, 2000). To determine which signals are involved in SPI 2 activation an investigation was carried out looking at activity of the SPI 2 promoter fusions in various environmental conditions to determine how they reacted to the various stimuli.

5.2 Results

5.2.1 Temperature regulation of SPI 2 genes

Salmonella infection is spread through the fecal-oral route. As a result the bacteria must undergo periods outside the host before infecting a new one. Infection of a new host is accompanied by a shift in temperature to 37°C. For many pathogens (e.g. *Shigella flexneri*) a shift in temperature to 37°C is an environmental signal that activates expression of virulence genes (Dorman *et al.*, 1990). To determine if the SPI 2 genes are temperature-regulated, the SPI 2 promoter fusions were used for gene expression assays, in the wild type SL1344 strain following growth at 30°C and 37°C. Results of the β-galactosidase assays (shown in Fig 5.1) show that the SPI 2 promoters are preferentially expressed at 37°C. For each promoter the fold increase in expression at 37°C compared to 30°C varies with the smallest being 2.5-fold (*ssrA*) and the largest 12-fold (*sseA*). This is the first demonstration that SPI 2 genes are thermo-regulated.

5.2.2 Effect of supercoiling on SPI 2 promoter activity

Changes in DNA topology have previously been shown to influence bacterial gene expression (Schneider *et al.*, 2000). *S. typhimurium* DNA becomes relaxed following uptake of the bacteria by macrophages (Marshall *et al.*, 2000). SPI 2 genes are rapidly activated following uptake by the macrophage thus raising the possibility that supercoiling levels influence SPI 2 gene expression. Furthermore, the involvement of Fis is consistent with sensitivity to changes in DNA topology (Auner *et al.*, 2003; Keane and Dorman, 2003). To investigate this possibility gene expression assays were performed following growth of the bacteria in media containing increasing concentrations of novobiocin. Novobiocin is a DNA gyrase inhibitor and therefore the addition of sub-lethal concentrations to the media causes a relaxation of the DNA in a dose dependent manner (Marshall *et al.*, 1999). The *ssrA-lacZ* promoter fusion was used in SL1344 grown overnight in 3 ml LB broth. Concentrations of novobiocin used were 0, 12.5, 25, 50 and 75 μg/ml (Marshall *et al.*, 1999). Results of the β-galactosidase assays are shown in Fig. 5.2. An increase

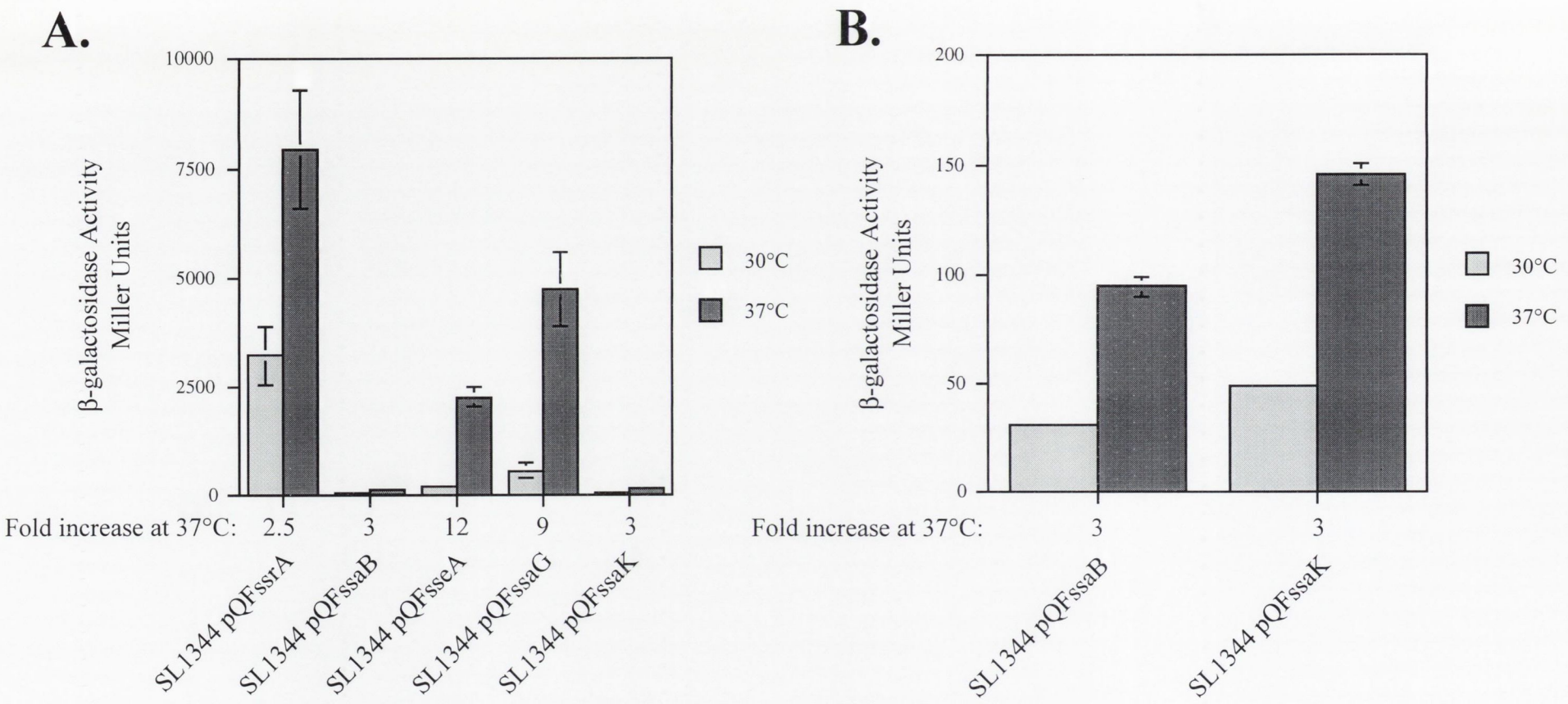


Fig 5.1. Thermoregulation of SPI 2 promoters.

A. β-galactosidase activity from the *ssrA*, *ssaB*, *sseA*, *ssaG* and *ssaK* promoter fusions in SL1344 following growth at 30°C and 37°C. Fold increase in expression at 37°C is indicated below each.

B. β-galactosidase activity from the *ssaB* and *ssaK* promoter fusions shown scaled up.

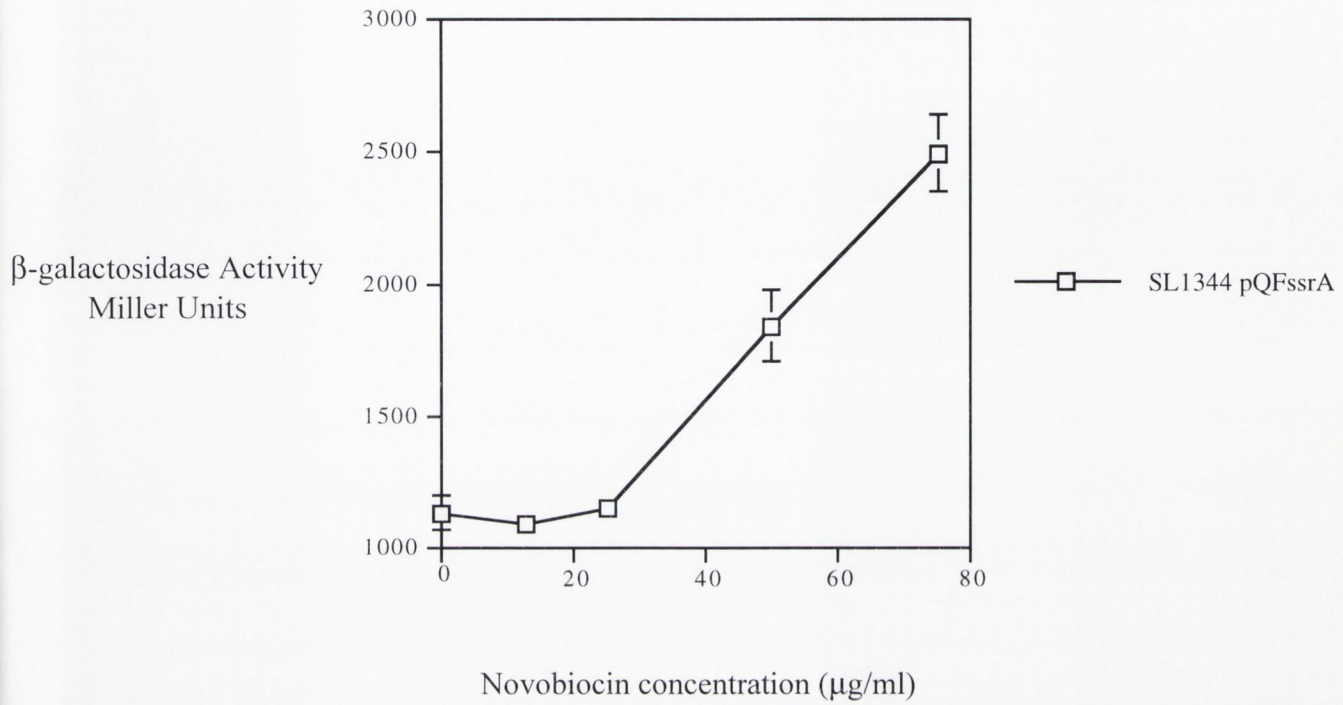


Fig 5.2. Effect of increasing novobiocin concentrations on *ssrA* promoter activity. SL1344 pQFssrA was grown overnight in LB containing increasing concentrations of the DNA gyrase inhibitor novobiocin. β-galactosidase assays were then performed to determine the effect of relaxation of the DNA on *ssrA* transcription. At novobiocin concentrations above 25 μg/ml an increase in transcription was observed from the *ssrA* promoter.

in *ssrA* transcription is observed when the novobiocin concentration is above 25 μg/ml. This result shows that the *ssrAB* operon is induced by relaxation of the DNA.

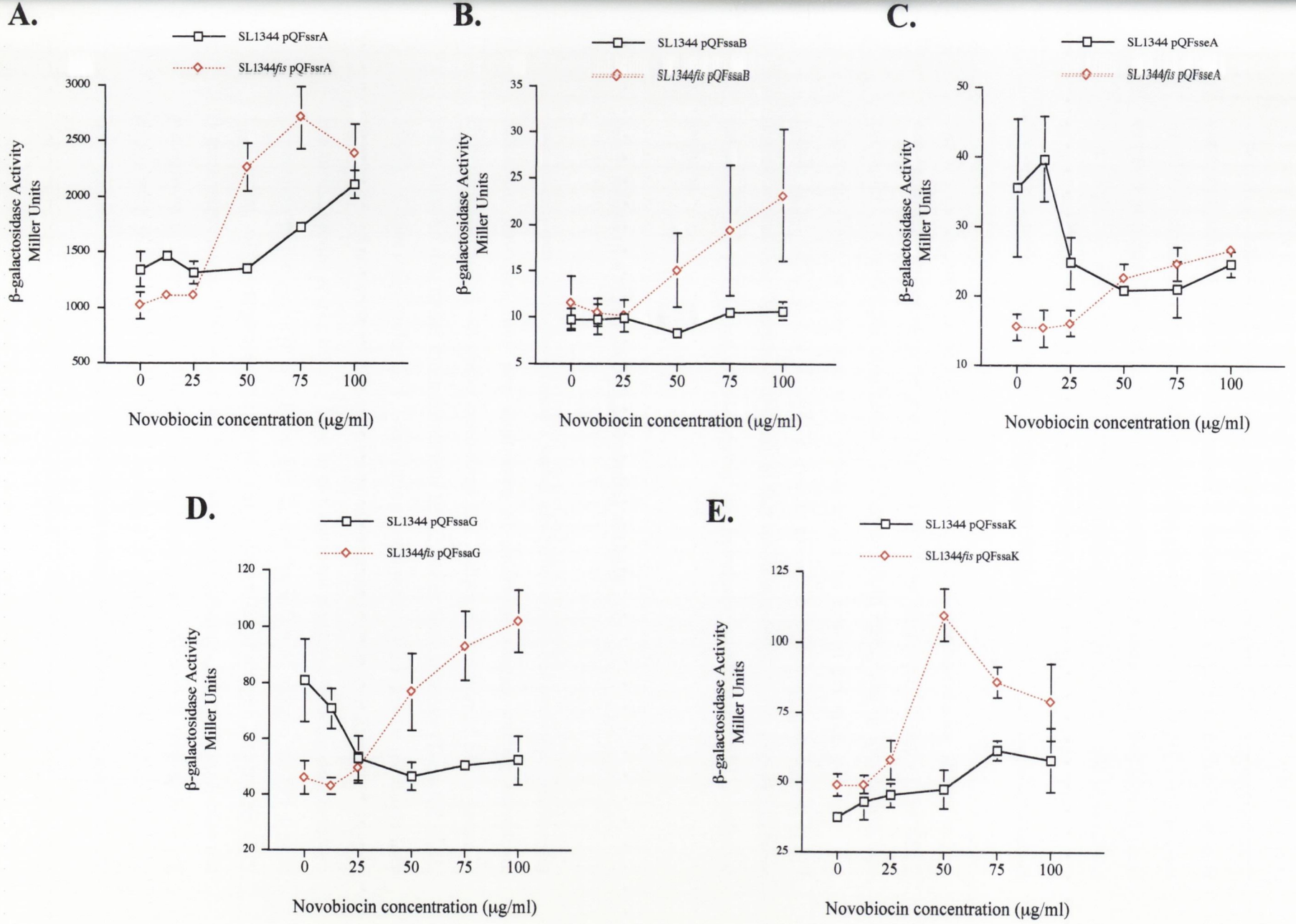
The finding that *ssrA* is induced by relaxation of the DNA raises some interesting possibilities. In chapter 4 Fis was shown to be involved in SPI 2 gene regulation by an unknown mechanism. Fis has previously been shown to regulate genes by binding to the promoter DNA and influencing local supercoiling levels (Auner *et al.*, 2003). The possibility therefore exists that the observed effect of supercoiling on *ssrA* transcription may be mediated by Fis. To examine this further expression assays were again performed using the SPI 2 promoter fusions in both the wild type SL1344 and SL1344*fis* backgrounds following growth in media containing increasing concentrations of novobiocin. Results of the β-galactosidase assays performed using the *ssrA*, *ssaB*, *sseA*, *ssaG* and *ssaK* promoter constructs are shown in Fig 5.3. In the wild type background an increase in transcription is again observed from the *ssrA* promoter as novobiocin levels increase, however no increase is observed from the rest of the SPI 2 promoters. A drop in transcription levels are seen from the *sseA* and *ssaG* promoters. In contrast to this an increase in transcription is observed from all promoters in the *fis* mutant background as novobiocin levels increase. In the case of *ssrA* where an increase is also observed in the wild type, the increase in transcription in the *fis* mutant begins at lower concentrations of novobiocin. These results indicate that *ssrAB* expression is activated by relaxation of the DNA, a condition known to accompany uptake of the bacteria by macrophages. In addition, results indicate that the previously identified involvement of Fis in SPI 2 regulation may be through an effect of Fis on DNA supercoiling.

5.2.3 Effect of pH on SPI 2 expression

Inhibition of phagosome acidification by the vacuolar ATPase inhibitor bafilomycin reduces the intracellular replication of *S. typhimurium* (Rathman *et al.*, 1996). This observation suggests that low pH either activates expression of SPI 2 genes or that it triggers the secretion of virulence determinants. Previous studies on this topic have

Fig 5.3. Effect of increasing novobiocin concentrations on SPI 2 promoter activity in wild type and *fis* mutant SL1344.

SL1344 and SL1344*fis* harbouring the pQFssrA (**A**), pQFssaB (**B**), pQFsseA (**C**), pQFssaG (**D**), and pQFssaK (**E**) plasmids were grown overnight in LB containing increasing sub-lethal concentrations of novobiocin. β-galactosidase assays were then performed. In the wild type background an increase in transcription was observed from the *ssrA* promoter as novobiocin concentration increased. No increase in expression was observed from the *ssaB* or *ssaK* promoters and a decrease was observed from the *sseA* and *ssaG* promoters. In the *fis* mutants an increase in transcription is observed for all promoters as novobiocin concentration increases.


A.
SL1344 pQFssrA
SL1344fis pQFssrA
β-galactosidase Activity
Miller Units
3000
2500
2000
1500
1000
500
0
25
50
75
100
Novobiocin concentration (μg/ml)
B.
SL1344 pQFssaB
SL1344fis pQFssaB
β-galactosidase Activity
Miller Units
35
30
25
20
15
10
5
0
25
50
75
100
Novobiocin concentration (μg/ml)
C.
SL1344 pQFsseA
SL1344fis pQFsseA
β-galactosidase Activity
Miller Units
50
40
30
20
10
0
25
50
75
100
Novobiocin concentration (μg/ml)
D.
SL1344 pQFssaG
SL1344fis pQFssaG
β-galactosidase Activity
Miller Units
120
100
80
60
40
20
0
25
50
75
100
Novobiocin concentration (μg/ml)
E.
SL1344 pQFssaK
SL1344fis pQFssaK
β-galactosidase Activity
Miller Units
125
100
75
50
25
0
25
50
75
100
Novobiocin concentration (μg/ml)

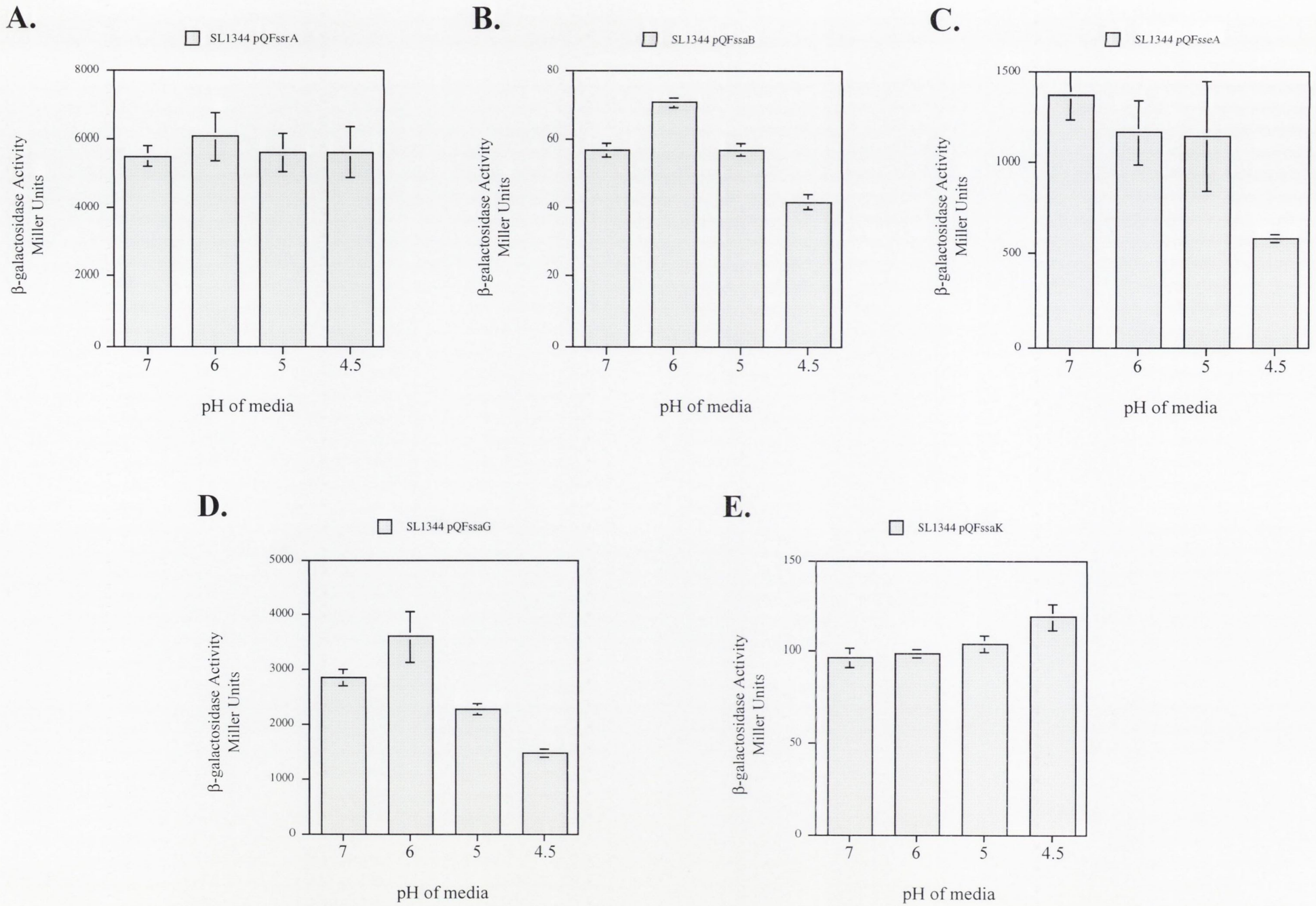
produced conflicting results. Lee *et al.* (2000) reported that medium with a pH of 4.5 was activating for SPI 2 gene expression while medium with a pH of 7 was repressing. Miao *et al.* (2002) found that expression of the SPI 2 dependent *sspH2* gene was induced at both pH 4.5 and pH 7. In addition, experiments by Beuzon *et al.* (1999) demonstrated that shifting bacteria grown in medium at pH 7 to medium at pH 5 or below induced secretion of the SseB protein indicating that low pH acts as a trigger for secretion of SPI 2 effector proteins. To determine if pH is involved in activation of SPI 2 gene expression, bacteria harbouring the SPI 2 promoter fusion plasmids were grown in media at pH 7, 6, 5, and 4.5 and β-galactosidase assays performed to determine the effect of reduced pH on promoter activity. Results for the *ssrA*, *ssaB*, *sseA*, *ssaG* and *ssaK* promoter fusions are shown in Fig. 5.4 (A to E). No increase in promoter activity was observed for any of the SPI 2 promoters in acidic pH conditions. In fact a 2-fold reduction in expression was observed from the *sseA* and *ssaG* promoters at the lowest pH used, pH 4.5. The results agree with Miao *et al.* (2002). Acidic pH is not required for activation of SPI 2 gene expression. Very low pH may in fact result in a reduction in expression from certain SPI 2 promoters, most notably the promoter transcribing SPI 2 effector proteins.

5.2.4 Effect of oxygen on SPI 2 promoter activity

Salmonella pathogenicity islands 1 and 2 are both critical for systemic *Salmonella* infection however they are expressed at different stages during infection. Experiments carried out under conditions that induce expression of SPI 1 genes *in vitro*, showed that under these conditions SPI 2 genes were inactive (Deiwick *et al.*, 1999). Conversely under SPI 2-activating conditions SPI 1 genes are silent (see chapter 6). Activation of SPI 1 genes *in vitro* occurs under conditions of oxygen limitation (Bajaj *et al.*, 1996). Due to the apparent converse relationship between expression of the genes in SPI 1 and SPI 2, and the effect of oxygen on SPI 1 gene expression, an investigation was carried out to determine if oxygen levels affect SPI 2 gene expression. The wild type SL1344 strain harbouring the pQFssrA plasmid was grown in a variety of different conditions designed to provide varying levels of oxgenation to the culture. Different volumes of culture, (3 ml, 25 ml or 200 ml), were grown in differently sized vessels (test tubes, 250 ml conical flasks, 250 ml

Fig 5.4. Effect of pH on SPI 2 gene expression.

S. typhimurium bacteria harbouring the SPI 2 promoter fusion plasmids were grown in LB broth at pH 7, 6, 5 and 4.5. β-galactosidase assays were then performed to determine the effect of reduced pH on promoter activity. No effect on transcription was observed from the *ssrA*, *ssaB* or *ssaK* promoters in media with reduced pH (**A**, **B** and **E**). The *sseA* and *ssaG* promoters showed a slight (2-fold) reduction in promoter activity at the lowest pH used (pH4.5) (**C** and **D**). The result shows that SPI 2 promoters are not activated by acidic pH.


A.
SL1344 pQFssrA
β-galactosidase Activity
Miller Units
8000
6000
4000
2000
0
7
6
5
4.5
pH of media
B.
SL1344 pQFssaB
β-galactosidase Activity
Miller Units
80
60
40
20
0
7
6
5
4.5
pH of media
C.
SL1344 pQFsseA
β-galactosidase Activity
Miller Units
1500
1000
500
0
7
6
5
4.5
pH of media
D.
SL1344 pQFssaG
β-galactosidase Activity
Miller Units
5000
4000
3000
2000
1000
0
7
6
5
4.5
pH of media
E.
SL1344 pQFssaK
β-galactosidase Activity
Miller Units
150
100
50
0
7
6
5
4.5
pH of media

baffled conical flasks or 2 l conical flasks), with or without shaking to provide the bacteria with a range of oxygenation levels. β-galactosidase assays were then performed to determine how the various aeration levels affected *ssrA* promoter activity. Conditions for each culture and results obtained from them are shown in Fig. 5.5. The OD_{600} of each culture was measured to determine if *ssrA-lacZ* β-galactosidase activity correlated with culture density, however, no pattern could be established (data not shown). Interestingly, the cultures in which aeration levels were highest, i.e. those where the volume of culture = 10% of the volume of the flask, (i.e. 25 ml in 250 ml flasks and 200 ml in 2 l flasks) showed lowest levels of expression of *ssrA-lacZ*. In addition, for these cultures no difference in expression levels was observed when grown with or without shaking. Cultures grown with moderate or low levels of aeration (3 ml test tube cultures and 200 ml in 250 ml flasks) showed increased levels of *ssrA-lacZ* expression with highest levels arising from 200 ml cultures grown in 250 ml flasks. This result suggests that reduced or low levels of aeration activate *ssrA* gene expression. However when activity from the *ssrA* promoter in low aeration cultures with and without shaking was examined a decrease in expression in the static cultures was observed. This result indicates that increased aeration levels in these cultures results in an increase in *ssrA* transcription and so contradicts the initial observation that increased aeration reduces *ssrA* gene expression.

The results above clearly indicate that oxygen levels are important in influencing *ssrA* gene expression, however due to the ambiguity of the results the exact role of oxygen and the aeration level that maximally induces *ssrA* expression is difficult to establish. The *ssrA* promoter is just one of the SPI 2 promoters and, as has been previously demonstrated, the SPI 2 promoters can react differently to various stimuli. To further investigate the effect of oxygen on SPI 2 gene expression, the *ssaB*, *sseA*, *ssaG*, and *ssaK* promoters were assayed, along with *ssrA*, for their response to oxygen. Experiments were carried out in test tubes to negate any effect of varying size and shape of culture container on promoter activity, and in addition to growing cultures with and without shaking aerobically, cultures were grown with and without shaking anareobically (<1% oxygen atmosphere) and under microaerophillic conditions (6% oxygen). β-galactosidase assays were then

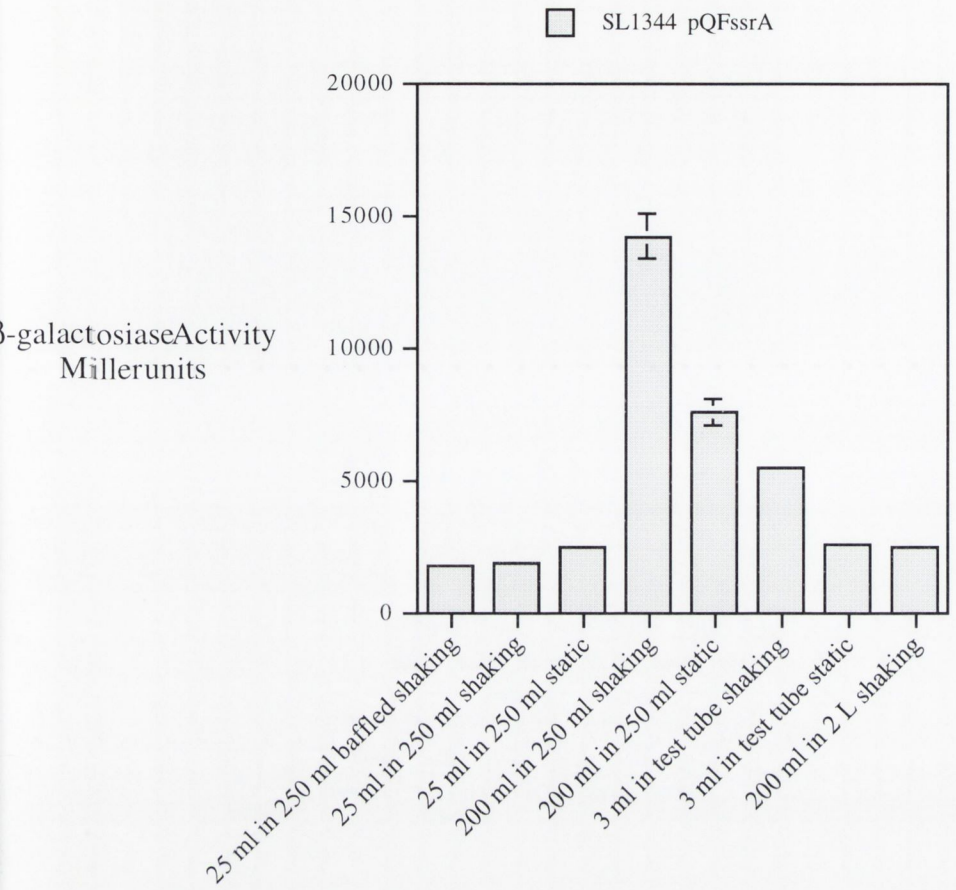


Fig 5.5. Effect of varying oxygen conditions on *ssrA* promoter activity.

LB cultures of *S. typhimurium* SL1344 containing the pQFssrA plasmid were grown under conditions of varying oxygenation and β-galactosidase assays performed. Volume of culture, type of culture container used and culture conditions i.e. shaking or static, are indicated below each datum point. Conditions where oxygenation levels were expected to be highest (i.e. those where volume of culture = 10% volume of flask {25 ml in 250 ml flasks and 200 ml in 2 l flasks}) showed lowest levels of *ssrA* experssion and no difference between baffled and non-baffled flasks or shaking and static cultures. Under conditions of moderate or low oxygenation (i.e. test tube cultures and 200 ml culture in 250 ml flasks) higher levels of expression were observed from the *ssrA* promoter and a decrease in expression was observed for cultures grown statically versus those grown with shaking.

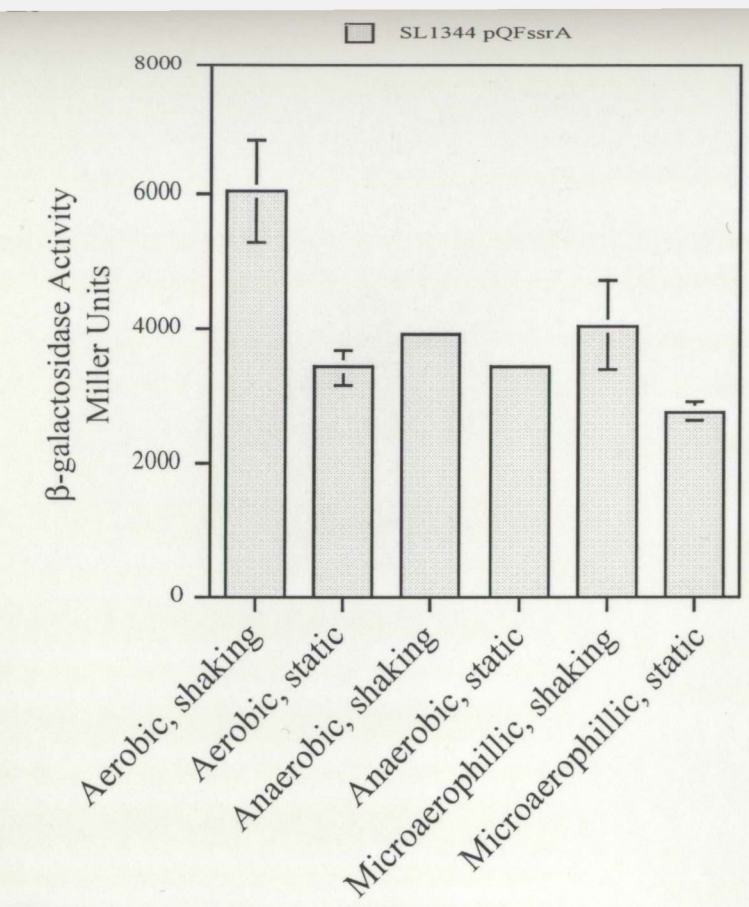
performed on all cultures to determine the effect of the various conditions and the effect of shaking on transcription and the results are shown in Fig. 5.6. Transcription levels from the *ssaB* and *ssaK* promoters did not change under any of the conditions assayed. There was no variation in transcription levels between static and shaking cultures or between aerobic, anaerobic or microaerophillic cultures. This result indicates that oxygen levels do not affect these promoters. The *ssrA*, *sseA* and *ssaG* promoters did respond to variations in oxygen levels. Cultures grown aerobically showed higher levels of transcription when grown with shaking than when grown statically. This result is because of higher aeration levels in the shaking cultures as demonstrated by the fact that cultures grown anaerobically showed no difference in transcription levels when grown with or without shaking. For each promoter a slight decrease in transcription was observed between shaking and static cultures grown microaerophillically. This result is consistant with the role for oxygen seen in the aerobic and anaerobic cultures. Interestingly, for the *sseA* and *ssaG* promoters transcription levels from cultures grown anaerobically were slightly higher than those from cultures grown aerobically without shaking. Aerobic static cultures clearly do not have high aeration levels however oxygen levels would be higher than those of anaerobic cultures. It is therefore surprising that the anaerobic cultures would display increased transcription levels. The results indicate that under moderate oxygen conditions increases in aeration activates transcription of the *ssrA*, *sseA* and *ssaG* promoters but not the *ssaB* and *ssaK* promoters. High levels of oxygen appear to repress the *ssrA* promoter.

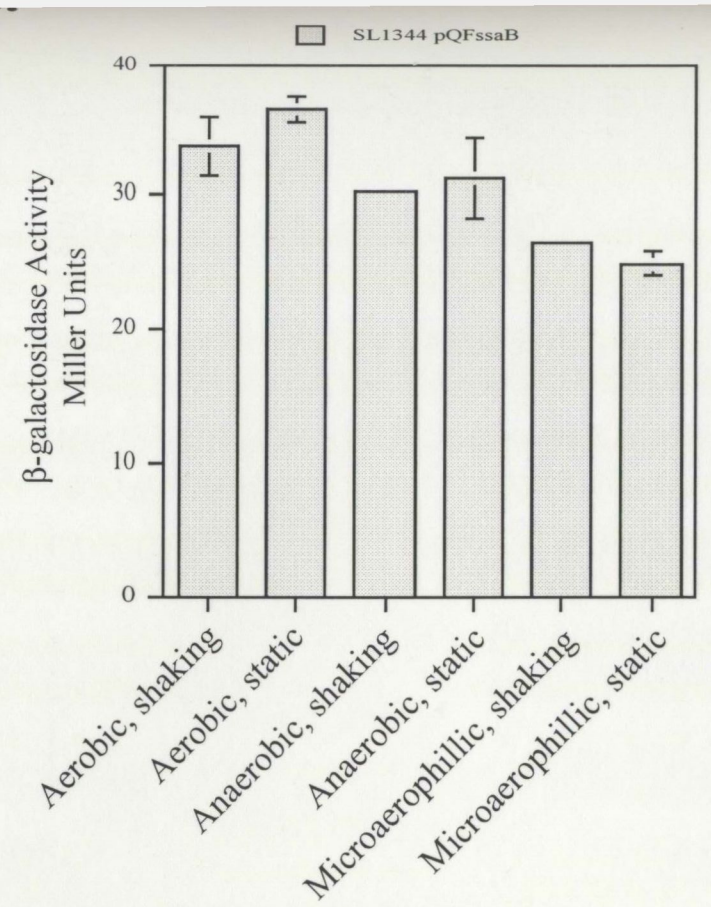
5.2.5 Influence of reactive oxygen species on SPI 2 gene expression

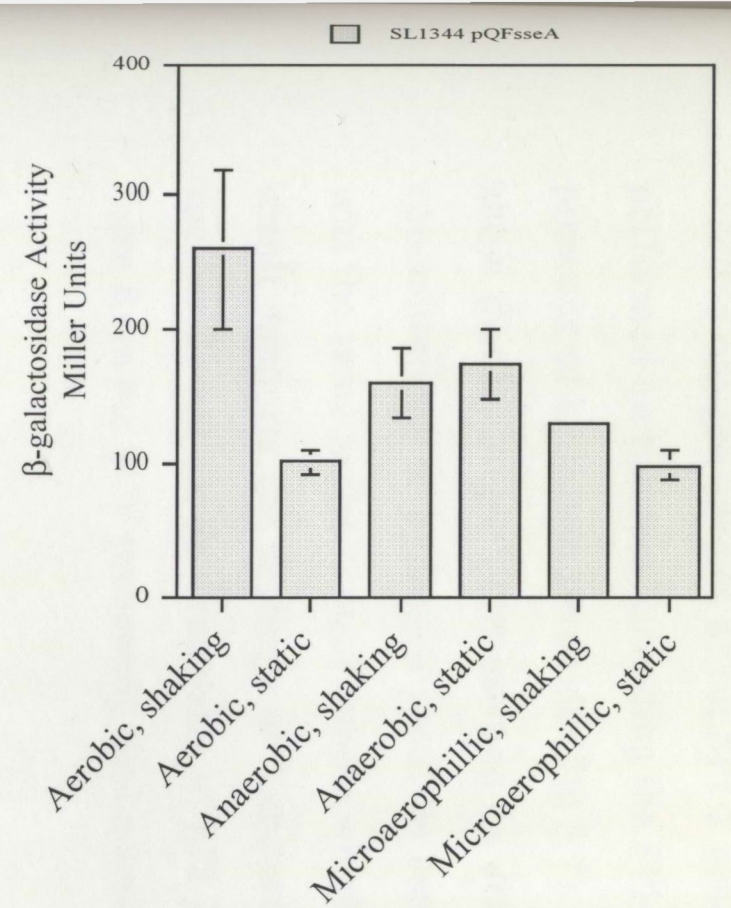
NADPH (nicotinamide adenine dinucleotide phosphate) oxidase plays a critical role in innate host defence against microbial infection. The NADPH oxidase catalyses the reduction of oxygen to superoxide, a precursor to several potent antimicrobial, reactive oxygen species, e.g hydrogen peroxide, hydroxyl radical, and peroxynitrate (Ischiropoulos *et al.*, 1992; Miller and Britigan, 1997). Release of the reactive oxygen species into the phagosome results in killing of the invading microbe. It has been suggested that one function of SPI 2 is to prevent the release of reactive oxygen species into the SCV thus allowing intracellular survival of the bacteria (Vazquez-Torres *et al.*, 2000). To test this hypothesis, and to determine whether

Fig. 5.6. Effect of aerobic, anaerobic and microaerophillic conditions on SPI 2 promoter activity.

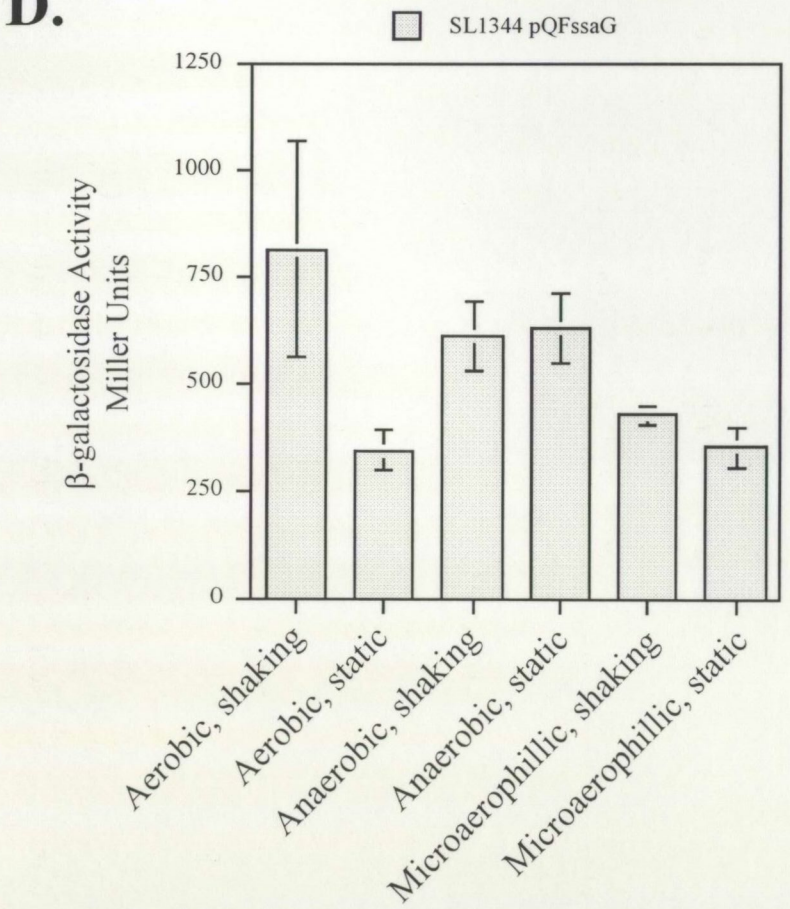
Cultures of *S. typhimurium* strain SL1344, harbouring the pQFssrA (**A**), pQFssaB (**B**), pQFsseA (**C**), pQFssaG (**D**), or pQFssaK (**E**) promoter fusion plasmids, were grown overnight aerobically, anaerobically and microaerophilically, both with and without shaking. β-galactosidase assays were then performed to determine how each promoter was affected by these conditions. The *ssaB* and *ssaK* promoters were not affected by oxygen levels (**B and E**). No change in β-galactosidase activity was observed between different aeration conditions or between static and shaking cultures. The *ssrA*, *sseA* and *ssaG* promoters were affected by oxygen levels (**A, C and D**). For shaking cultures, highest levels of promoter activity were observed under aerobic conditions. Cultures grown anaerobically and microaerophillically showed reduced levels of β-galactosidase activity. Cultures grown statically displayed a different profile. Anaerobic conditions resulted in highest levels of β-galactosidase activity with slightly reduced levels observed in cultures grown aerobically and microaerophillically. No difference in β-galactosidase activity was observed between shaking and static cultures grown anaerobically, however a decrease in activity was observed at the *ssrA*, *sseA* and *ssaG* promoters between static and shaking cultures when grown aerobically. This demonstrates that oxygen levels influence expression of these promoters.


SL1344 pQFssrA
β-galactosidase Activity
Miller Units
8000
6000
4000
2000
0
Aerobic, shaking
Aerobic, static
Anaerobic, shaking
Anaerobic, static
Microaerophillic, shaking
Microaerophillic, static

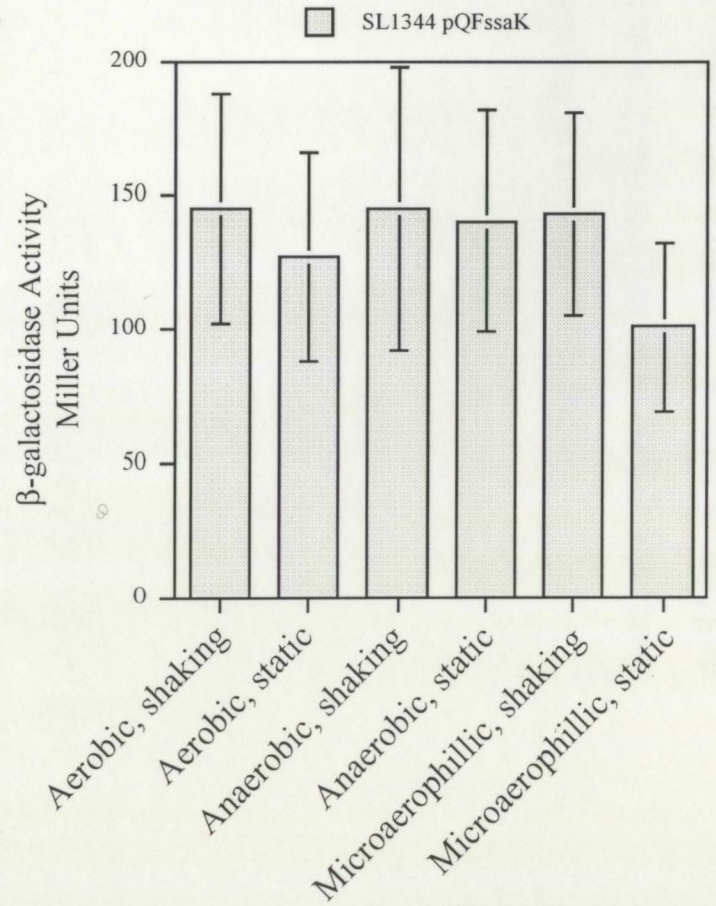

SL1344 pQFssaB
β-galactosidase Activity
Miller Units
40
30
20
10
0
Aerobic, shaking
Aerobic, static
Anaerobic, shaking
Anaerobic, static
Microaerophillic, shaking
Microaerophillic, static


SL1344 pQFsseA
β-galactosidase Activity
Miller Units
400
300
200
100
0
Aerobic, shaking
Aerobic, static
Anaerobic, shaking
Anaerobic, static
Microaerophillic, shaking
Microaerophillic, static

D.


SL1344 pQFssaG
β-galactosidase Activity
Miller Units
1250
1000
750
500
250
0
Aerobic, shaking
Aerobic, static
Anaerobic, shaking
Anaerobic, static
Microaerophillic, shaking
Microaerophillic, static

E.


SL1344 pQFssaK
β-galactosidase Activity
Miller Units
200
150
100
50
0
Aerobic, shaking
Aerobic, static
Anaerobic, shaking
Anaerobic, static
Microaerophillic, shaking
Microaerophillic, static

reactive oxygen species affect SPI 2 gene expression, β-galactosidase assays were performed on bacteria harvesting the SPI 2 promoter fusion plasmids, grown in the presence of hydrogen peroxide (Fig 5.7). The results of the β-galactosidase assays show that hydrogen peroxide had no effect on SPI 2 promoter activity. At both concentrations of hydrogen peroxide used (0.5 mM and 1 mM) promoter activity was the same as the control sample which did not contain hydrogen peroxide. This result supports the findings of Vazquez-Torres *et al.* (2000). Reactive oxygen species do not act as a regulator of SPI 2 gene expression because SPI 2 excludes them from the SCV preventing the bacteria coming in contact with them.

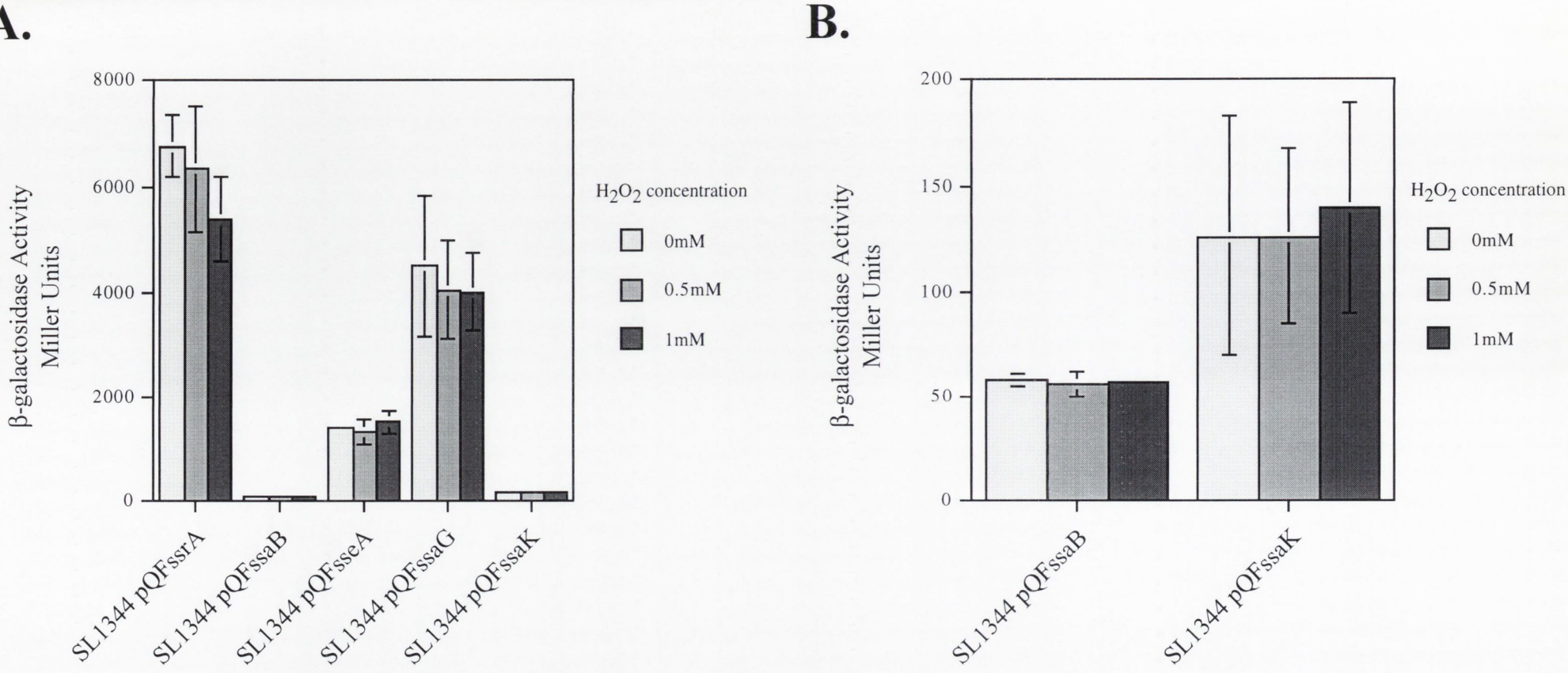


Fig 5.7. Effect of hydrogen peroxide on SPI 2 promoter activity.

A. β-galactosidase activity from the *ssrA*, *ssaB*, *sseA*, *ssaG* and *ssaK* promoter fusions in SL1344 following growth in LB containing 0, 0.5 or 1 mM H_2O_2. No effect on transcription is observed

B. β-galactosidase activity from the *ssaB* and *ssaK* promoter fusions shown scaled up.

5.3 Discussion

SPI 2 genes are preferentially expressed and are required for survival inside macrophages (Cirillo *et al.*, 1998). Environmental signals within the macrophage are critical for SPI 2 gene activation, however exactly what these signals are and their influence on SPI 2 remains unclear. Osmolarity is a known regulator of SPI 2 due to the involvement of the OmpR/EnvZ two-component system in SPI 2 gene regulation (Lee *et al.*, 2000). Other environmental signals proposed to effect SPI 2 expression include low Mg^{2+} and low pH, however conflicting reports have raised questions over the input of these conditions (Deiwick *et al.*, 1999; Miao *et al.*, 2002). In this study the effect of various environmental and physiological conditions on SPI 2 promoter activity was determined. Conditions assayed included temperature, pH, oxygen, DNA superhelical density and hydrogen peroxide.

Previous investigations into the effect of environmental conditions on SPI 2 have focused on one or two specific genes and interpreted their activity to represent activity of the island as a whole (Deiwick *et al.*, 1999; Miao *et al.*, 2002). In this investigation activity of promoters throughout the island were examined and their response to various environmental stimuli investigated. Results demonstrated that each SPI 2 promoter has the ability to react independently to a given environmental condition. The ability to respond differently to the same signal may reflect the organization of functionally related genes into operons within SPI 2. Activation of the secretion system apparatus genes (structural I and structural II regions) may be induced differentially to the effector and chaperone genes (effector/chaperone region), which may then be induced in response to a different signal. In this way temporal and sequential activation of the genes within the island can be achieved.

All SPI 2 promoters assayed displayed preferential expression at 37°C compared to 30°C. As SPI 2 expression is required for survival inside the host, induced expression at 37°C would be expected. While not surprising, this is nonetheless the first demonstration of temperature regulation of SPI 2 genes.

The effect of pH and hydrogen peroxide on SPI 2 gene expression is the topic of conflicting reports at present (Deiwick *et al.*, 1999; Lee *et al.*, 2000; Miao *et al.*,

2002; Vazquez-Torres *et al.*, 2000). In this study pH and hydrogen peroxide were shown not to affect transcription from the SPI 2 promoters. Acidification of the SCV has been shown to be required for survival of the bacteria inside macrophages. Results here agree with Miao *et al.* (2002) in suggesting that low pH does not activate SPI 2 transcription. This supports the findings of Beuzon *et al.* (1999) who report that low pH triggers release of SPI 2 effector proteins not their transcription. In the case of hydrogen peroxide, it has been suggested that *Salmonella* effector proteins prevent the release of reactive oxygen species into the SCV therefore the bacteria do not come in contact with them (Vazquez-Torres *et al.*, 2000). Hydrogen peroxide had no effect on SPI 2 promoter activity supporting this proposed mechanism.

As well as changes in environmental conditions as the bacteria enter the macrophage, changes in physiological conditions also occur. Bacterial DNA becomes relaxed upon entry into macrophages (Marshall *et al.*, 2000). Previous studies have shown that changes in the superhelical density of DNA can alter gene expression (Auner *et al.*, 2003). The Fis protein is also known to be involved in modulating DNA supercoiling levels and can influence transcription in a supercoiling dependent manner (Schneider *et al.*, 1997). In light of these results, and the recently discovered role for Fis in SPI 2 gene expression, an investigation was carried out to determine if supercoiling levels affect SPI 2 gene expression and if the role of Fis in SPI 2 gene regulation is as a result of its role in modulating supercoiling levels. Of the SPI 2 promoters, the *ssrA* promoter alone was found to be activated by relaxation of the DNA in the wild type SL1344 strain. In the SL1344 *fis* mutant all of the SPI 2 promoter were found to be activated by relaxed DNA. This result indicates that the role of Fis in SPI 2 regulation may be through an effect on supercoiling however the exact mechanism by which it is involved is unclear. Fis is required for activation of the SPI 2 genes *in vitro* and *in vivo*. SPI 2 genes are activated upon entry into macrophages, a condition where the DNA is known to become relaxed (Marshall *et al.*, 2000). In the absence of Fis the SPI 2 promoters are more strongly induced as the DNA becomes relaxed. These results appear contradictory, however the novobiocin experiments have certain limitations. Increasing novobiocin concentration causes relaxation of the DNA but it is unknown what concentration of novobiocin produces DNA with the same superhelical density

as that of bacteria entering macrophages. In addition novobiocin is an antibiotic drug and therefore has a deleterious effect on the bacterial cell. It is difficult to account for this in the β-galactosidase experiments. The results clearly indicate that the *ssrA* promoter is a supercoiling-sensitive promoter, activated by relaxation of the DNA. None of the other SPI 2 promoters responded in this way indicating an ability to respond differently to a particular stimulus. Fis, which is involved in SPI 2 regulation, may be influencing transcription in a supercoiling-dependent manner.

Salmonella pathogenicity island 1 (SPI 1), like SPI 2, is regulated by various environmental conditions (Bajaj *et al.*, 1996). Oxygen level is one condition that influences transcription of SPI 1 genes and results here show that it is also important in SPI 2 gene expression. The *ssrA* promoter was repressed by high aeration conditions but activated under moderate or low levels. Experiments with all SPI 2 promoters again demonstrated their ability to react differently to a given stimulus as the *ssaB* and *ssaK* promoters showed no effect of oxygen levels on their transcription while the *ssrA*, *sseA* and *ssaG* promoters all displayed an oxygen dependent expression profile. Results indicate that for these promoters oxygen is positively effecting transcription up until a certain point after which it becomes a negative influence. SPI 1 is activated by low levels of oxygen and, as with the Fis protein, SPI 2 appears to be influenced in the same way.

Chapter 6

Microarray analysis of the Fis regulon during growth in Minimal Medium 5.8 (MM5.8)

6.1 Introduction

6.1.1 DNA microarray analysis

Advances in genome sequencing and robotics have revolutionised molecular biology. Nowadays research papers report the sequence of entire genomes or genomic expression profiles whereas little over a decade ago the sequencing or expression profile of a single gene warranted an entire paper. One application arising from the genomics revolution has been the use of DNA microarrays in global gene expression profiling. DNA microarrays consist of either oligonuleotide or cDNA products corresponding to every gene in the genome, deposited onto glass slides in an ordered grid. Hybridization of fluorescently labelled cDNA, produced by reverse transcription of total genomic RNA harvested under certain conditions, to the microarray slide and subsequent analysis of fluorescent intensity gives an indication of the expression of every gene in the cell under a specific condition. Comparison of gene expression profiles under various conditions can reveal how gene expression in an organism responds to those particular conditions. Similarly, comparison of the genomic expression profile of a wild type strain with a strain carrying a mutation reveals how that particular mutation affects global transcription levels. The Fis protein is known to be involved in regulation of many different genes in *S. typhimurium* and so is known as a global regulator. This work shows that Fis is also involved in regulating the genes in SPI 2. In light of this, and as part of a study to determine the entire Fis regulon, DNA microarray analysis was carried out using the wild type SL1344 and the SL1344*fis* mutant. Initial microarray experiments in LB broth confirmed that Fis was required for full expression of the SPI 2 genes (Kelly *et al.*, submitted). SPI 2 genes are preferentially expressed and are required for survival inside macrophages, so to understand better the role of Fis in regulating SPI 2, microarray analysis of the Fis regulon was carried out following growth in the SPI 2 inducing medium, minimal medium 5.8 (MM 5.8). This is a low pH, minimal medium designed to simulate the intracellular environment of the macrophage (Deiwick *et al.*, 1999; Kox *et al.*, 2000). Recent results catagorising the transcriptional profile of *S. typhimurium* inside macrophage have shown that MM5.8 provides a poor representation of the intracellular environment (Eriksson *et al.*, 2003). For most genes, expression in MM5.8 and expression inside macrophage

does not correlate. The SPI 2 genes (and certain other *in vivo* expressed genes) are exceptions showing similar expression levels inside macrophage and in MM5.8. As such MM5.8 can be considered a SPI 2-inducing medium. It was hoped that analysis of the role of Fis during growth in this medium would help elucidate the role of Fis on SPI 2 and further our understanding of the role of Fis during infection.

6.2 Results

6.2.1 Growth of *S. typhimurium* in minimal medium 5.8 (MM5.8)

As MM5.8 is a stringent medium the ability of *S. typhimurium* to grow in it was assessed prior to use in microarray experiments. SL1344 and SL1344*fis* do not grow in MM5.8 when inoculated from colonies on agar plates so the following culture strategy was developed. LB cultures were used as an initial step and two rounds of sub-inoculation into MM5.8 were used to minimise transfer of nutrients from the LB into the MM5.8. Cultures were grown as follows. In test tubes, 3 ml of LB broth was inoculated from colonies growing on agar plates. These LB cultures were incubated at 37°C overnight with shaking. The following day 25 ml of MM5.8 in 250 ml conical flasks was inoculated with 250 µl of the overnight LB cultures (i.e. a sub-culture ratio of 1:100) and again grown overnight at 37°C with shaking. These overnight MM5.8 cultures were then use to inoculate fresh MM5.8 in either 500 ml or 250 ml conical flasks. The above procedure was rigorously adhered to during every use of MM5.8 due to the fact (as seen in the previous chapter) that small differences in growth conditions can alter gene expression resulting in difficulties in generating reproducible data. Growth curves for SL1344 and SL1344*fis* in MM5.8 are shown in Fig 6.1. Both strains have a doubling time of approx 61 min during exponential growth however the *fis* mutant displayed a slightly increased lag phase prior to exponential phase growth.

6.2.2 Fis protein levels during growth in MM5.8

Microarray analysis of the Fis regulon consists of a comparison of the genome expression profiles of the wild type and *fis* mutant *S. typhimurium* under a specific condition or conditions. In this case the conditions used are growth in MM5.8. To determine at what stage of growth to perform the microarray analysis the expression profile of Fis was determined for bacteria growing in MM5.8. As shown in chapter 4, Fig 4.5, the expression profile for Fis during growth in LB broth shows high levels of protein in early exponential phase growth after which levels decline. For bacteria inside macrophages a more constant expression profile is seen with similar levels of Fis at 30 min and 3 h post-infection (Fig 4.6). Fis protein levels during

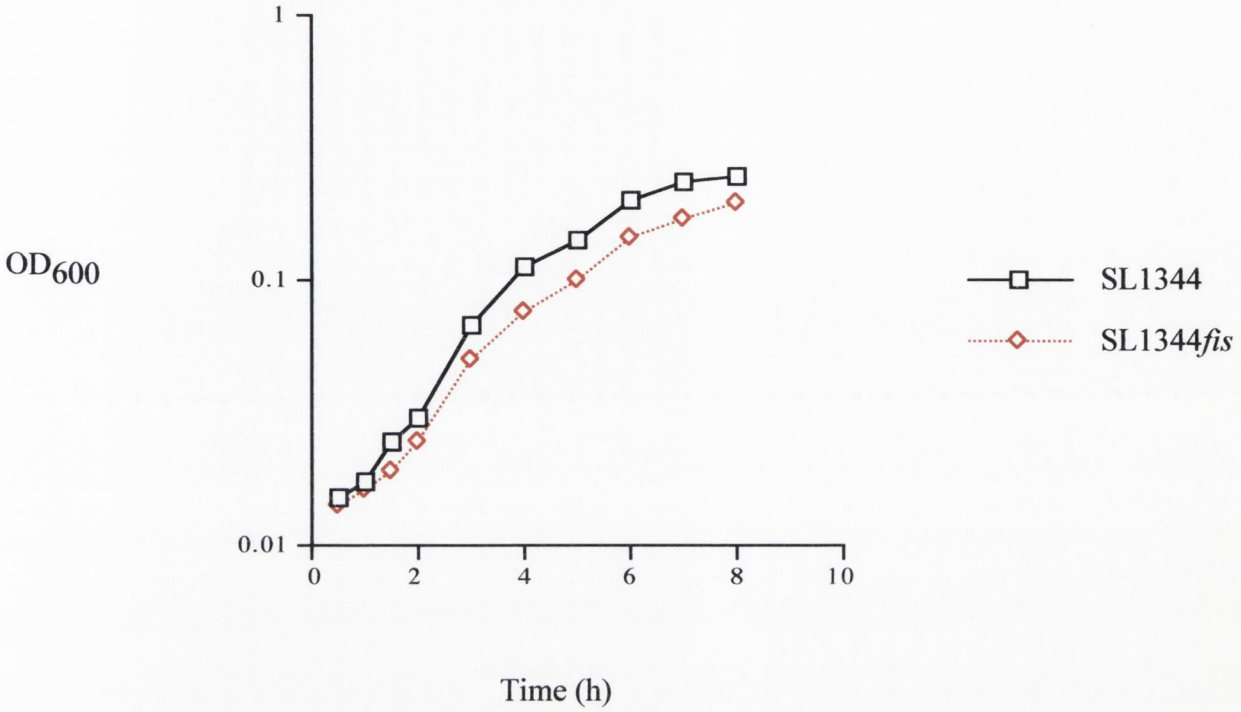


Fig 6.1. Growth of SL1344 and SL1344*fis* in MM5.8.

S. typhimurium SL1344 and SL1344*fis* were grown overnight in 3 ml LB cultures, subcultured into MM5.8, grown overnight, and then used to inoculate fresh MM5.8 cultures. Growth of the two strains was measured by OD_{600} at various time-points post inoculation and the log growth plotted against time. Both strains have similar doubling times (61 min) while the *fis* mutant displays a slightly increased lag phase prior to exponential growth. Experiment performed at least 3 times and representative data is shown.

growth in MM5.8 were determined by Western blotting using samples of SL1344 taken at regular time-points throughout the growth cycle in MM5.8. Results of the Western blot analysis are shown in Fig. 6.2. As in LB broth, an induction of Fis is observed in MM5.8 following nutrient upshift or sub-culture. In LB, Fis levels then decrease rapidly whereas in MM5.8 they remain high. Only when bacterial growth begins to slow down and the cells enter stationary phase do Fis levels drop. This sustained induction of Fis is reminiscent of the situation inside macrophages where Fis levels remain constant after an initial induction following uptake of the bacteria by the macrophage.

6.2.3 Microarray procedures and data handling

Based on the results obtained from the Western blot analysis of Fis levels in MM5.8, two time-points were chosen to isolate RNA from for the microarray experiment. The time-points chosen were 2.5 hours and 5 hours post sub-culture, which represented early and late exponential phase growth. The wild type and *fis* mutant bacteria were grown as previously outlined and at the corresponding time-point samples were harvested and RNA prepared as outlined in chapter 2. This RNA, along with genomic DNA, was labelled using the Cy3 and Cy5 dyes and then used in microarray hybridizations as detailed in chapter 2. The *S. typhimurium* microarrays used in this study were PCR product arrays provided by J. Hinton in the Institute for Food Research (IFR) in Norwich, England. All microarray work was carried out in Norwich and was funded by a Marie Curie fellowship grant. For each time-point four arrays were hybridized and the data presented are the average of all four arrays. Procedures for slide blocking, microarray hybridization, data handling and analysis are detailed in chapter 2. Once data had passed statistical analysis they were imported into the GeneSpring 6.0 software program where all further analysis was carried out. From within the GeneSpring 6.0 program a Microsoft Excel file containing the results for all genes that passed statistical filtering was created. This Microsoft Excel file is included on CD-ROM at the back of this manuscript. Genes are ranked according to their expression ratio. The expression ratio is a numerical value representing the fold decrease in expression in the *fis* mutant compared to the wild type. A cut off point of 2-fold was imposed on all microarray data. Genes with

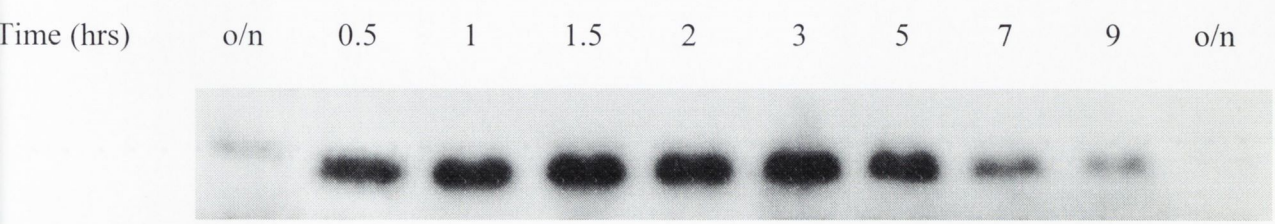


Fig. 6.2. Fis protein levels during growth in MM5.8.

S. typhimurium SL1344 was grown in MM5.8 as described and samples were taken at various time-points and used for Western immunoblot analysis with the anti-Fis antibody. Time post sub-culture for each sample is indicated above. Induction of Fis occurs following nutrient upshift and Fis levels remain high during exponential phase growth. Fis levels decline after 5 hours which corresponds with a slow down in bacterial growth and entry into stationary phase.

an expression ratio above 2 were deemed to be activated by Fis and those with a ratio below 0.5 repressed by Fis.

6.2.4 *S. typhimurium* gene expression profile in MM5.8

MM5.8 medium was used here as it has previously been shown to activate expression of SPI 2 genes (Deiwick *et al.*, 1999). Microarray analysis of the Fis regulon in this medium will indicate in more detail the role of Fis in regulating SPI 2 and other *in vivo* expressed genes, under activating conditions similar to those found inside macrophages. To verify that this medium is SPI 2-inducing, and to determine the effects of growth in this medium on bacterial gene expression, the microarray results from the wild type SL1344 grown in MM5.8 were compared to microarray results from SL1344 grown in LB broth (Kelly *et al.*, submitted). Comparisons were made between samples taken during early (2.5 h in MM5.8 and 1 h in LB) and late (5 h in MM5.8 and 4 h in LB) growth. At the early time-point, expression of 1,976 genes was affected by a factor of 2 or greater in MM5.8 compared to LB. Of these, 1,032 genes displayed increased expression while 944 displayed decreased expression in MM5.8. At the later time-point, 2,105 genes showed changes in expression of 2-fold or more with 1,243 being up in MM5.8 and 862 down. A comparison of those genes affected at both time-points showed that in total, expression of 2,812 genes was altered during growth in MM5.8 (compared to LB), with 1,268 genes affected at both time-points. A list of all genes and their expression levels in MM5.8 compared to LB is given in a Microsoft Excel document titled "Supplementary material 1.xls". There are 4 sheets in this document. Sheet 1 contains the results for all genes listed alphabetically. Sheets 2 and 3 contain the genes whose expression was altered 2-fold in the early and late time-points listed with those most up-regulated at the top and descending. Finally, sheet 4 contains the 2,812 genes whose expression was altered 2-fold or more in MM5.8.

An analysis of the activity of the SPI 2 genes in MM5.8 confirmed that the majority of SPI 2 genes are induced in this medium. Fig. 6.3B shows a graphical representation of the induction of 27 of 29 SPI 2 genes in MM5.8. The *sseA* and *sseB* genes were the only SPI 2 genes not induced in MM5.8. Also included are graphs of the global gene expression profile (Fig 6.3A), and SPI 1 activity in MM5.8

Fig. 6.3. Analysis of *S. typhimurium* wild type gene expression profile in MM5.8 compared to LB.

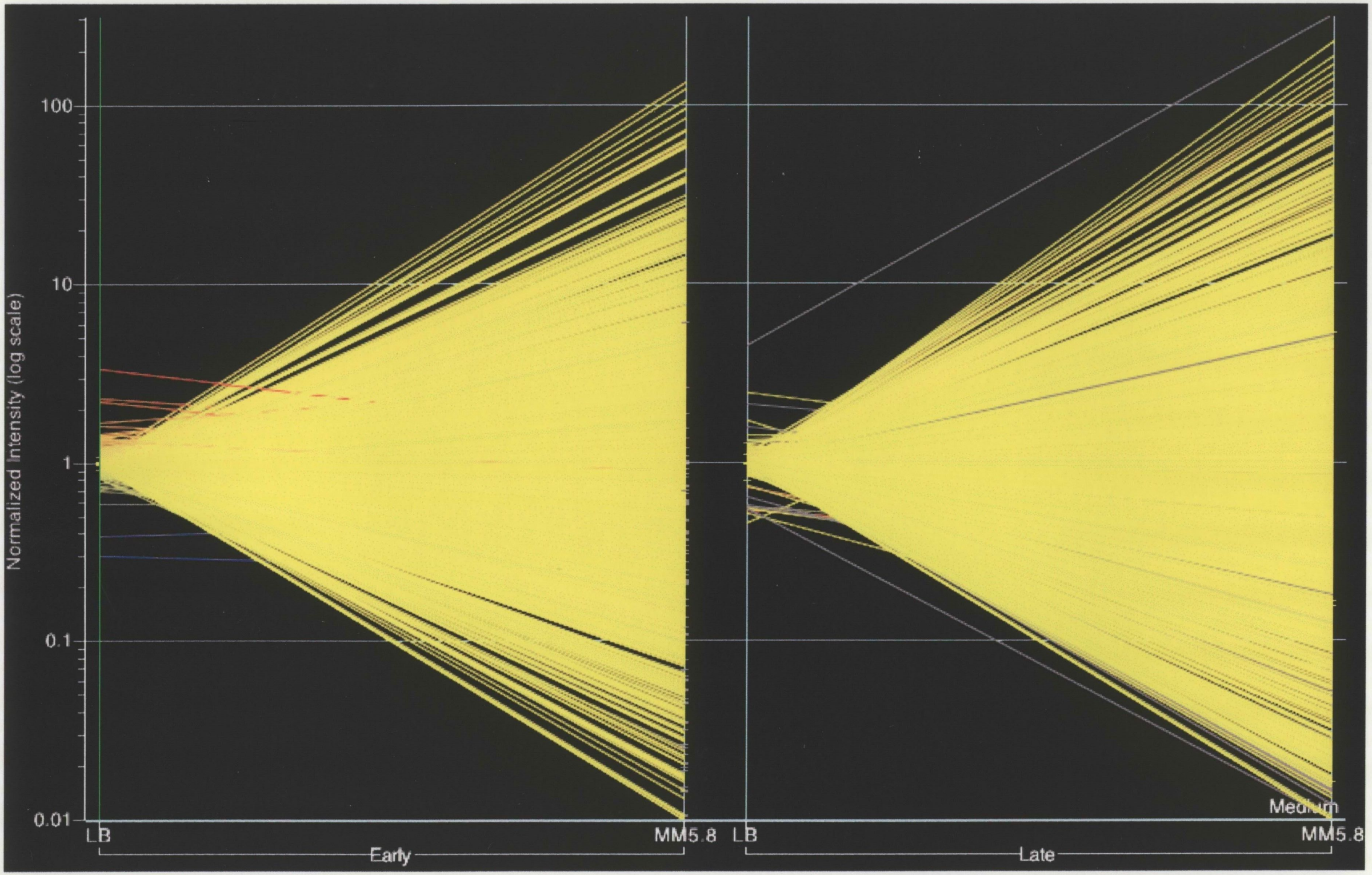
Graphical representation of fold difference in gene expression in MM5.8 compared to LB broth at both early (left) and late (right) time-points. An increase from left to right on the graphs indicates up-regulation in MM5.8 compared to LB, while a decrease indicates down-regulation in MM5.8. Gene expression values are normalized against the LB data and fold differences in expression are indicated on the Y axis.

A. *S. typhimurium* expression profile in MM5.8 at the early and late time-points. 4,231 genes are represented of which 2,812 genes were up or down-regulated 2-fold or more (see "Supplementary material 1" excel file).

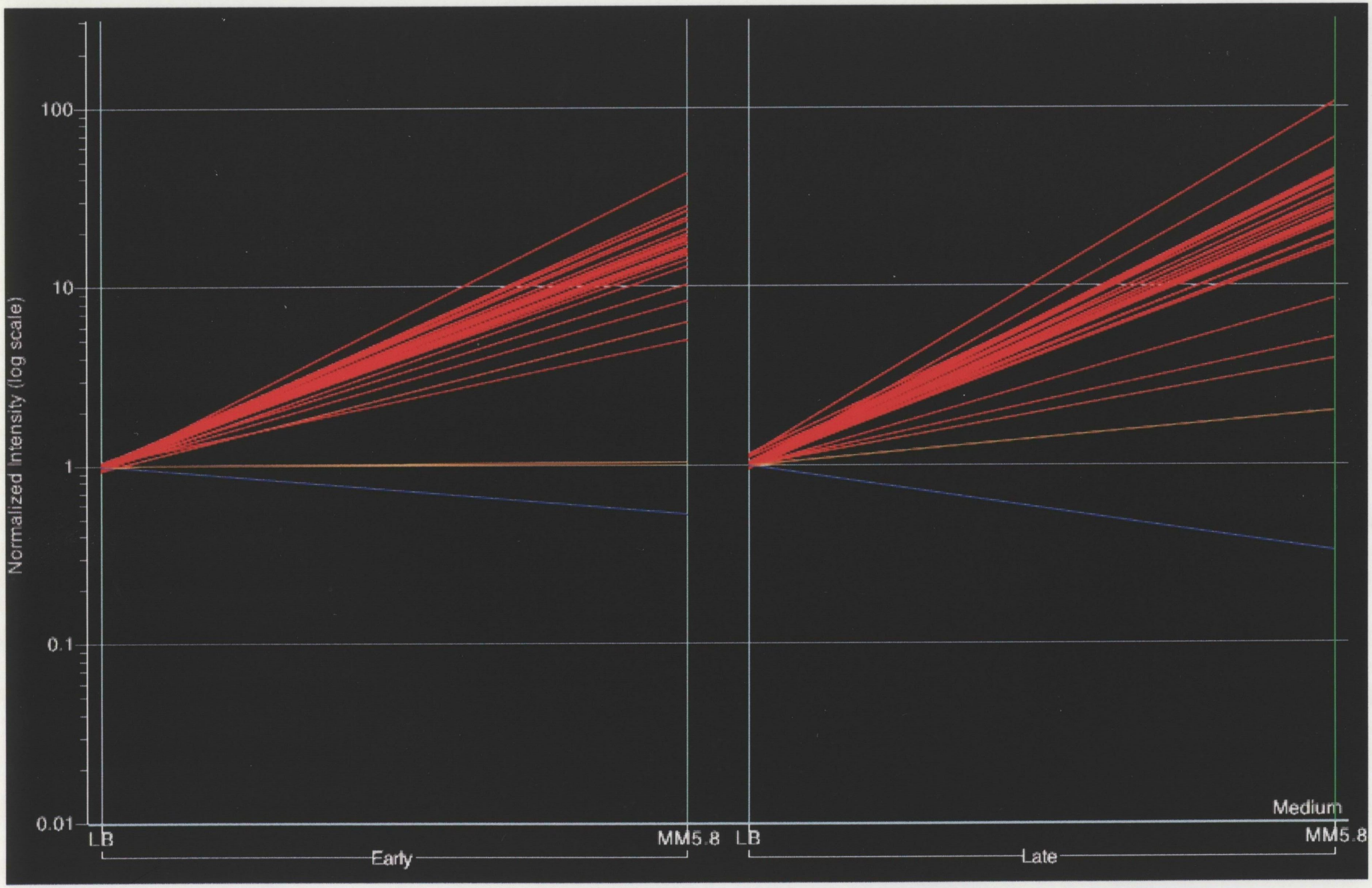
B. SPI 2 expression profile in MM5.8. 29 SPI 2 genes are represented and for 27 of these an induction in expression in MM5.8 is observed.

C. SPI 1 expression in MM5.8. 30 SPI 1 genes are represented and show reduced expression in MM5.8.

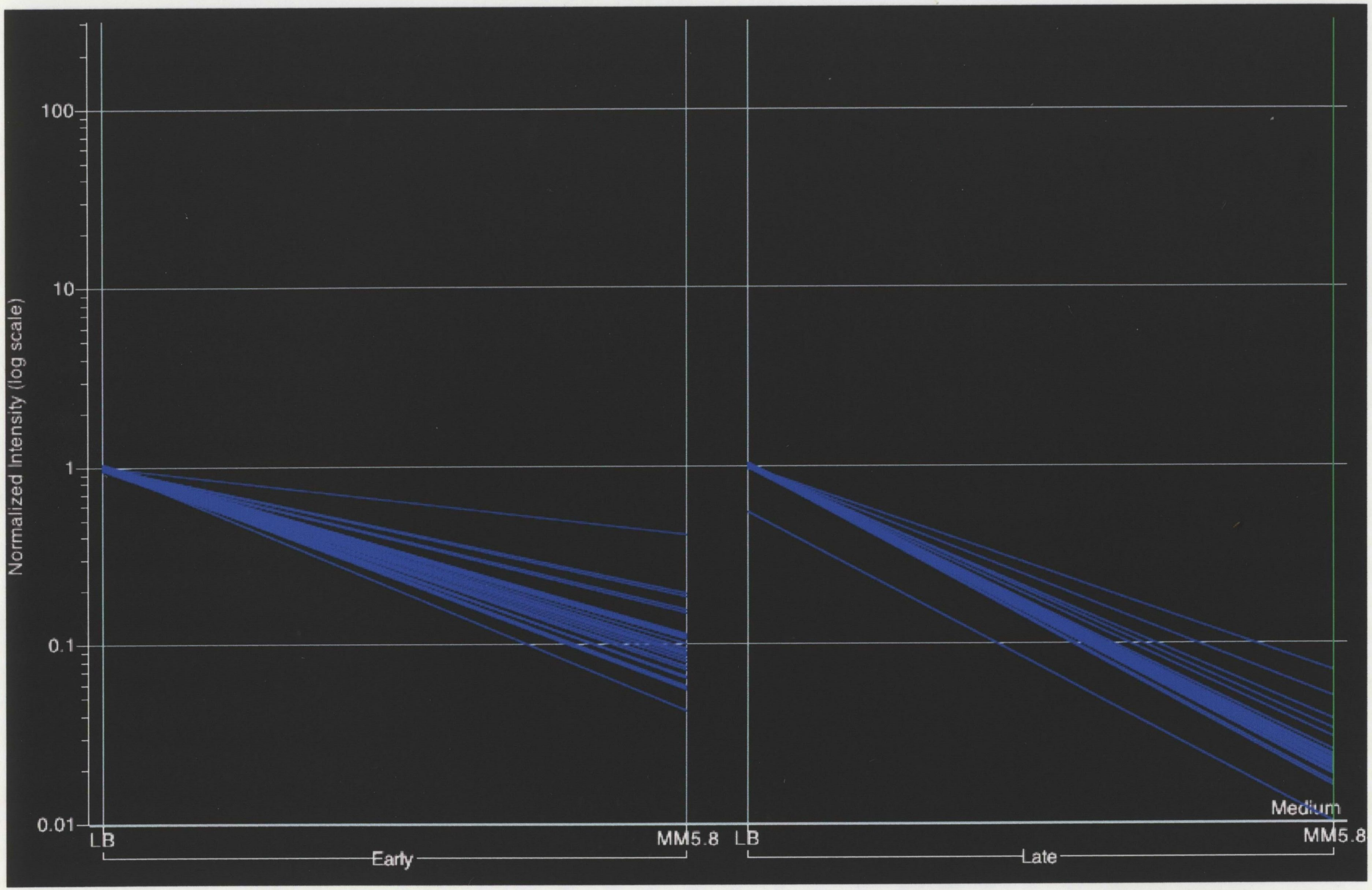
A


100
10
1
0.1
0.01
Normalized Intensity (log scale)
LB
MM5.8
Early
LB
Medium
MM5.8
Late

B


100
10
1
0.1
0.01
Normalized Intensity (log scale)
LB
MM5.8
LB
MM5.8
Medium
Early
Late

C


100
10
1
0.1
0.01
Normalized Intensity (log scale)
LB
MM5.8
Early
LB
Medium
MM5.8
Late

(Fig 6.3C). In contrast to SPI 2, SPI 1 genes are down-regulated in MM5.8. The results here confirm that the majority of SPI 2 genes are activated in MM5.8.

6.2.5 The Fis regulon in MM5.8

Following the analysis of wild type *S. typhimurium* gene expression in LB and MM5.8, the gene expression profiles of the wild type SL1344 and SL1344 *fis* mutant, in MM5.8 were compared. Again results were generated for the early (2.5 h) and late (5 h) time-points. A cut off value of 2-fold was again employed. Results were generated for 4,654 genes and are shown in Microsoft Excel file "Supplementary material 2.xls". In sheet 1, results are shown alphabetically with each gene's expression ratio at 2.5 h and 5 h along with a brief gene description. The expression ratio is the wild type expression value divided by the expression value in the *fis* mutant and is therefore a measure of the fold change in activity in the *fis* mutant. Analysis of the results for the 2.5 h time-point showed that 52 genes had expression levels that were altered by 2-fold or more in the *fis* mutant. Exactly half of these (26) displayed increased expression in the *fis* mutant and half showed decreased expression in the *fis* mutant. At the 5 h time-point 136 genes showed greater than 2-fold changes with 45 showing increases in expression in the *fis* mutant and 91 decreases in expression. Combining these results generated a list of 170 genes whose expression was up or down 2-fold in the *fis* mutant, and of these 18 were affected at both time-points. Sheets 2 and 3 of "Supplementary material 2.xls" list the genes that were affected at the 2.5 h and 5 h time-points while sheet 4 contains a full list of the 170 affected genes and the 18 genes affected at both time-points.

82 of the 170 genes found to be affected by Fis in MM5.8 have unknown or only suggested functions. The genes found to be most affected by Fis were virulence genes, including genes from SPI 1, 2 and 5, and bacteriophage genes. The results obtained for some of these are detailed below.

Results with the SPI 2 promoter fusion plasmids have shown that Fis is required for the activation of SPI 2 genes in LB broth and inside macrophages. As a result, the microarray results showing the activity of the SPI 2 genes in MM5.8 in the wild type

Table 6.1. Effect of Fis on SPI 2 genes during growth in MM5.8

Gene	Description	Wild type/*fis* expression ratio		2-fold in *fis*[-a]	1.7-fold in *fis*[-b]
		2.5 h	5 h		
ssrA	Secretion system regulator	1.29	1.85		+
ssrB	Secretion system regulator	1.26	2.79	+	+
ssaB	Secretion system apparatus	1.41	1.57		
ssaC	Secretion system apparatus	1.32	2.18	+	+
ssaD	Secretion system apparatus	ND[c]	ND[c]		
ssaE	Secretion system apparatus	1.74	1.57		+
sseA	Secretion system effector	0.95	0.98		
sseB	Secretion system effector	1.12	1.01		
sscA	Secretion system chaperone	1.77	1.70		+
sseC	Secretion system effector	1.92	2.56	+	+
sseD	Secretion system effector	2.03	1.85	+	+
sseE	Secretion system effector	1.79	2.43	+	+
sscB	Secretion system chaperone	1.80	1.48		+
sseF	Secretion system effector	1.88	1.32		+
sseG	Secretion system effector	1.77	1.13		+
ssaG	Secretion system apparatus	1.56	1.58		
ssaH	Secretion system apparatus	1.42	3.47	+	+
ssaI	Secretion system apparatus	1.41	2.03	+	+
ssaJ	Secretion system apparatus	1.56	1.95		+
ssaK	Secretion system apparatus	1.60	2.55	+	+
ssaL	Secretion system apparatus	1.92	1.84		+
ssaM	Secretion system apparatus	1.77	1.80		+
ssaV	Secretion system apparatus	1.84	2.07	+	+
ssaN	Secretion system apparatus	2.20	2.93	+	+
ssaO	Secretion system apparatus	2.89	3.12	+	+
ssaP	Secretion system apparatus	2.74	2.70	+	+

ssaQ	Secretion system apparatus	ND[c]	ND[c]		
ssaR	Secretion system apparatus	1.38	3.11	+	+
ssaS	Secretion system apparatus	1.28	2.97	+	+
ssaT	Secretion system apparatus	1.41	2.58	+	+
ssaU	Secretion system apparatus	1.70	1.74		+

a. Gene expression altered 2-fold or more in *fis* mutant.

b. Gene expression altered 1.7-fold or more in *fis* mutant.

c. No Data.

and *fis* mutant were of particular interest. The results obtained for 29 of the 31 SPI 2 genes are shown in Table 6.1. No data were obtained for the *ssaD* or *ssaQ* genes. 15 of the 29 genes had an expression ratio of 2 or more indicating that Fis is required for their activation in MM5.8. This only accounts for approximately half of the genes in SPI 2. Upon closer examination of the 14 genes that had an expression ratio below 2 it was revealed that in most cases their expression ratio was just below the 2-fold cut off point. Reduction of the cut off to 1.7-fold results in 25 of the 29 SPI 2 genes being included. While 2-fold is the standard cut off point in these types of experiment, due to the relatively low expression ratios of the genes most highly regulated by Fis in this medium (5.7-fold at 2.5 h and 20.9-fold at 5 h), reduction of the cut off point may be warranted. In support of this argument, the *fis* gene itself shows an expression ratio of 1.9 at 5 h. This is below the 2-fold cut off even though Fis is known to be negatively autoregulatory (Ninnemann *et al.*, 1992). The SPI 2 microarray results support the findings in chapter 4. Fis is involved in activation of SPI 2 gene expression. Results here also suggest that certain SPI 2 genes may be more dependent on Fis for activation than others as some of the secretion system apparatus (*ssa*) genes in the structural II region showed the highest expression ratios of all the SPI 2 genes.

While SPI 2-encoded virulence genes featured prominently in the list of genes most affected by Fis in MM5.8, other virulence genes were also shown to be regulated by Fis in this medium. Table 6.2 contains a list of 15 virulence genes or virulence associated genes that also passed the 2-fold cut off. Not surprisingly, 3 SPI 2 regulated genes, were found to be regulated by Fis. *srfJ, sseJ* and *sspH2* are not located within SPI 2 but are activated by the SsrA/B system. They all had expression ratios above 2 indicating that, like SPI 2, they are activated by Fis. The SPI 1 chaperone *sicP*, and a gene of unknown function, STM0972, that is homologous to the SPI 1 secreted protein SopD, were also shown to require Fis for activation. Three genes from SPI 5, *pipA*, *pipB* and *pipD*, were shown to require Fis for activation. A gene of unknown function STM2780, that shows sequence homology to *pipB*, was also shown to require Fis for activation. All of the virulence genes that are regulated by Fis were found to be activated by Fis with the exception of the *spvA* and *spvB* virulence plasmid encoded genes. Both these genes showed a 2-fold repression by Fis in MM5.8. This result is surprising in light of the fact that

Table 6.2. Virulence determinants affected by Fis during growth in MM5.8

Gene	Description	Wild type/*fis* expression ratio	
		2.5 h	5 h
hha	Hemolysin expression-modulating protein (involved in environmental regulation of virulence factors)	0.94	2.33
pipA	SPI 5 pathogenicity island-encoded protein	2.14	1.98
pipB	SPI 5 pathogenicity island-encoded protein	2.33	3.14
pipD	SPI 5 pathogenicity island-encoded protein	1.58	2.58
pqaA	PhoPQ-regulated protein	2.05	3.10
sicP	SPI 1 chaperone	1.05	2.38
sifA	Lysosomal glycoprotein (lgp)-containing structures; replication in macrophages	1.91	2.26
spvA	*Salmonella* virulence plasmid encoded gene	0.46	0.73
spvB	*Salmonella* virulence plasmid encoded gene	0.61	0.44
srfJ	Activated by transcription factor SsrB	2.29	1.82
sseJ	*Salmonella* translocated effector: regulated by SPI-2	1.83	3.39
sspH2	Leucine-rich repeat protein, induced by SsrA/B	2.07	1.55
STM0972	Homologous to SPI 1 secreted protein SopD	1.46	2.65
STM2780	Homologue of SPI 5-encoded *pipB*	1.47	2.93
ybjX	Homologue of *Shigella* virulence gene *virK*	1.07	2.09

Fis has never been shown to affect the *spv* system in LB or in macrophages. Also there is no evidence that Fis can bind to the *spvR* promoter (chapter 4) as would be expected if Fis acted as a repressor there.

S. typhimurium harbours several fully functional bacteriophages that are capable of excising from the chromosome under specific conditions (Figueroa-Bossi *et al.*, 1997). These phages have been implicated in the horizontal transfer of genes, in particular virulence genes, between different strains and different species of bacteria (Figueroa-Bossi *et al.*, 2001). Examples of these phages in *S. typhimurium* are the Gifsy and Fels prophages. The *S. typhimurium* chromosome harbours at least 2 Gifsy, Gifsy-1 and Gifsy-2, and 2 Fels, Fels-1 and Fels-2, prophages. The Gifsy prophages have been shown to be important for *S. typhimurium* virulence in mice and are known to encode several virulence genes (Figueroa-Bossi *et al.*, 2001). Strains that are cured of the Gifsy-2 prophage are significantly attenuated in a mouse model of infection. Microarray analysis of the Fis regulon showed that many of the genes on Gifsy-1, Gifsy-2, Fels-1, and Fels-2 are regulated by Fis. Table 6.3 contains a list of all Gifsy and Fels encoded genes that passed the 2-fold cut off. Of the 24 listed, 13 are Gifsy-1, 7 are Gifsy-2, 2 are Fels-1, and 2 are Fels-2. Interestingly, in most cases Fis is acting as a repressor i.e. the expression ratio is below 0.5. For 3 Gifsy-1 and 3 Gifsy-2 genes Fis is an activator of expression, the expression ratio being over 2. This is the first indication that Fis in involved in the regulation of prophage genes and another putative connection between Fis and regulation of virulence genes.

Interestingly, the genes that were most affected by Fis in MM5.8 are a cluster of phage-like genes from STM2233 to STM2239 (with the exception of STM2238 as no data was collected for it). Results obtained for these genes are included in Table 6.3. The expression ratios for these genes are significantly higher than any other genes on the chromosome and indicate that Fis is crucial for the expression of these genes in MM5.8. Comparison of the expression of these genes in MM5.8 with their expression in LB shows that they are induced in MM5.8 (Fig. 6.4). The functions of these genes are unknown however some are homologous to phage proteins indicating the possibility that this is another lysogenic phage whose expression in regulated by Fis.

Fig 6.4. Induction of *S. typhimurium* Fis-regulated, putative bacteriophage sequences in MM5.8. *S. typhimurium* coding sequences STM2233, STM2234, STM2235, STM2236, STM2237 and STM2239 show homology to phage genes and are strongly dependent on Fis for activation. Comparison of their expression in MM5.8 with that in LB shows that these genes are strongly induced in MM5.8 indicating a potential *in vivo* role during infection. Induction is shown for early (graph on left) and late (graph on right) time-points with LB data on the left and MM5.8 data on the right of each graph.

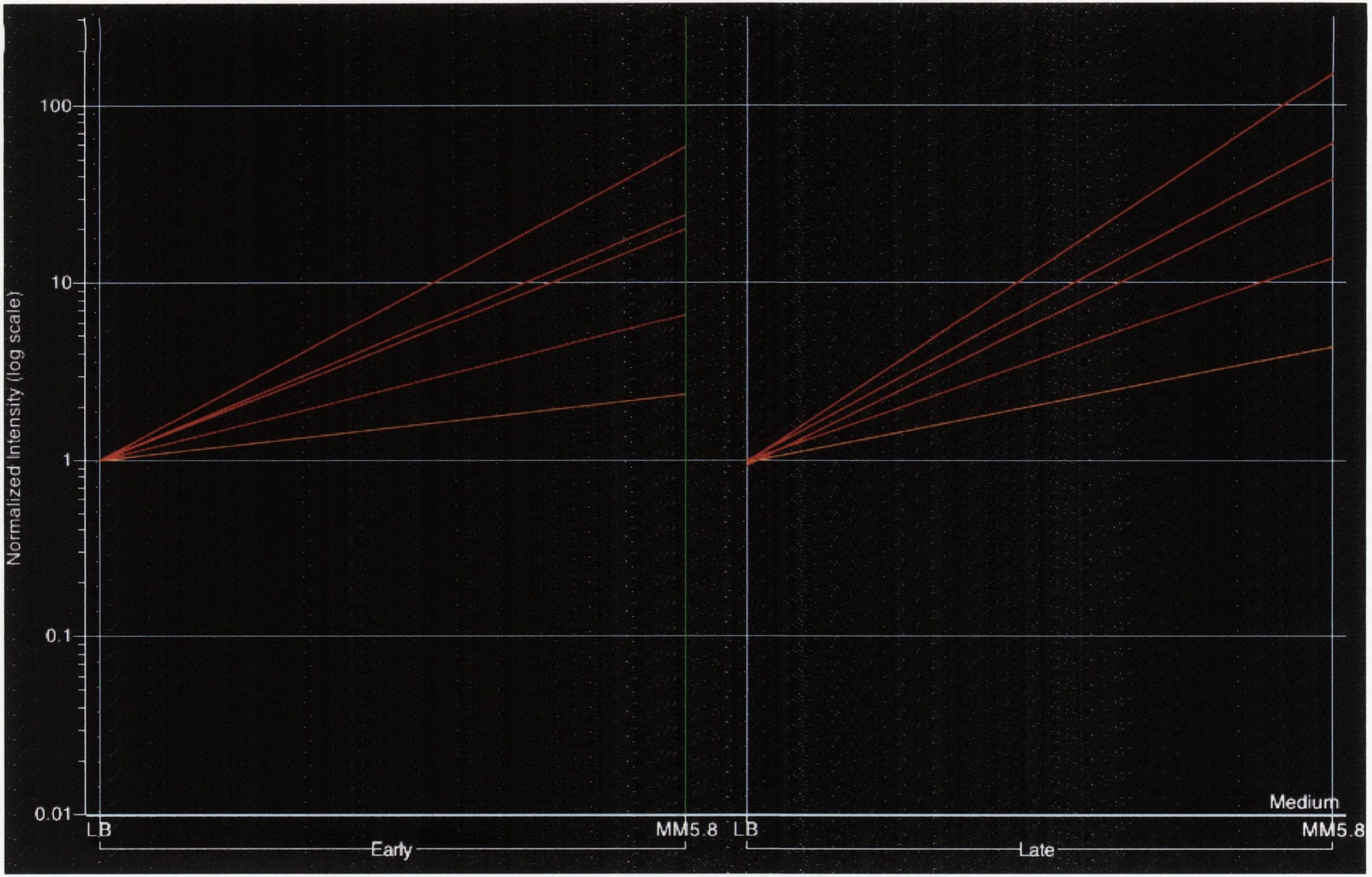

Normalized Intensity (log scale)
100
10
1
0.1
0.01
LB
MM5.8
Early
LB
Medium
MM5.8
Late

Table 6.3. Bacteriophage elements affected by Fis during growth in MM5.8

Designation	Description	Wild type/*fis* expression ratio	
		2.5 h	5 h
	Gifsy-1 prophage		
STM2584 (*gogB*)	Gifsy-1 prophage: leucine-rich repeat protein	1.24	2.12
STM2585	Gifsy-1 prophage: similar to transposase	1.21	2.13
STM2589	Gifsy-1 prophage: similar to host specificity protein-J in phage lambda	0.48	1.25
STM2601	Gifsy-1 prophage: similar to minor capsid protein FII of phage N15	0.47	0.99
STM2603	Gifsy-1 prophage: similar to head protein gp7 of phage 21	0.39	1.18
STM2604	Gifsy-1 prophage: similar to head protein gpshp of phage 21	0.35	0.90
STM2605	Gifsy-1 prophage: similar to head-tail preconnector gp5 of phage 21	0.38	0.87
STM2607	Gifsy-1 prophage: similar to head to tail joining protein	0.44	1.09
STM2609	Gifsy-1 prophage: similar to DNA packaging protein Nu1 of phage 21	0.39	1.01
STM2610	Gifsy-1 prophage gene	0.37	0.84
STM2611	Gifsy-1 prophage gene	0.39	0.83
STM2614	Gifsy-1 prophage gene	0.71	2.15
STM2620	Gifsy-1 prophage gene	0.46	1.01
	Gifsy-2 prophage		
STM1011	Gifsy-2 prophage gene	0.40	0.52
STM1013	Gifsy-2 prophage; probable regulatory protein	0.29	0.64
STM1019	Gifsy-2 prophage gene	0.31	0.58
STM1020	Gifsy-2 prophage gene	0.38	0.92
STM1026	Gifsy-2 prophage gene	0.75	2.24

STM1052	Gifsy-2 prophage gene	2.83	1.82
STM1053	Gifsy-2 prophage gene	2.19	1.45
	Fels-1 prophage		
STM0893	Fels-1 prophage; putative integrase	1.12	0.50
STM0925	Fels-1 prophage; putative host specificity protein	0.60	0.40
	Fels-2 prophage		
STM2698	Fels-2 prophage: similar to gpE in phage P2	1.04	0.44
STM2722	Fels-2 prophage: similar to gpP, ATP charging, in phage P2	0.11	0.44
	Putative phage		
STM2233	Putative cytoplasmic protein	4.20	15.07
STM2234	Putative tail fiber assembly protein	5.51	20.96
STM2235	Putative phage protein	4.11	9.65
STM2236	Putative phage protein	5.77	12.57
STM2237	Putative inner membrane protein	5.58	14.37
STM2238	Putative phage protein	ND[a]	ND[a]
STM2239	Putative phage protein; homology to antiterminator protein Q of phage P5	2.08	1.23

a. No Data.

6.3 Discussion

DNA microarrays are now the method of choice for genomic expression profiling. In this study a PCR product microarray was used to determine the *S. typhimurium* Fis regulon during growth in medium MM5.8, a stringent medium designed to simulate the signals and environmental conditions encountered inside the macrophage and activate SPI 2 gene expression (Deiwick *et al.*, 1999). This analysis is part of an ongoing investigation into Fis that has already determined the Fis regulon in LB broth (Kelly *et al.*, submitted) and will eventually explore the Fis regulon inside macrophages. The medium MM5.8 was chosen as activation of *in vivo*-expressed genes can be achieved in it and until recently microarray analysis using bacteria recovered from the intracellular environment was beyond the limitations of this type of analysis (Deiwick *et al.*, 1999). Recent advances in RNA purification methods have made *in vivo* microarray analyses a possibility and this type of experiment is now planned to determine exactly the Fis regulon inside macrophage (Eriksson *et al.*, 2003).

An analysis of Fis protein levels during growth in MM5.8 revealed a surprising correlation with Fis levels inside macrophage. In LB broth, following nutrient upshift, there is a rapid increase in Fis levels followed by a prompt decrease (Fig 4.5). Inside macrophage Fis is maintained at a constant level (Fig 4.6). In MM5.8 induction of Fis is seen following nutrient upshift and Fis levels remain at a constantly high level until bacterial growth begins to slow and the bacteria enter stationary phase (Fig. 6.2). In this way the Fis expression pattern in MM5.8 resembles more that of intracellular bacteria than LB grown bacteria. This result suggests that the functions of Fis during growth in MM5.8 are similar to its functions inside macrophage.

In chapter 4, using promoter fusion plasmids, Fis was shown to regulate expression of SPI 2 genes in LB broth and inside macrophages. For that reason, the microarray results concerning the SPI 2 genes were of particular interest. Using the microarray results to compare the activity of the SPI 2 genes in MM5.8 and LB (Kelly *et al.*, submitted), confirmed that the majority of them are activated in MM5.8 (Fig. 6.3). This result confirms that SPI 2 and SPI 1 are reciprocally expressed (Fig. 6.3).

Next, SPI 2 activity in the wild type and *fis* mutant strains growing in MM5.8 was compared. 15 SPI 2 genes were expressed more than 2-fold less in the absence of Fis, again confirming that Fis regulates SPI 2. Most SPI 2 genes that did not show a greater than 2-fold reduction in expression in the *fis* mutant were just under the 2-fold cut-off. 25 of the 29 SPI 2 genes show a reduction in expression of 1.7-fold or greater in the *fis* mutant. Examination of the expression ratio of the SPI 2 genes in the wild type and *fis* mutant indicates that certain genes in the structural II region display a higher dependency on Fis for activation than others within SPI 2. If SPI 2 genes are differentially expressed at different times then this may reflect the time points chosen or it may mean that certain SPI 2 genes are more dependent on Fis for activation than others. Taken together the results confirm a role for Fis in activating transcription of the SPI 2 genes.

Many (83) of the genes found by microarray analysis to be regulated by Fis in MM5.8 were of unknown function. Apart from SPI 2 genes, several other virulence genes were found to require Fis for expression. Genes associated with SPI 1 and SPI 5 were identified along with several SPI 2 regulated genes located outside SPI 2. Strangely, two plasmid virulence genes *spvA* and *spvB* were identified as being repressed by Fis. Fis is not implicated in regulating the *spv* system but these genes are under the control of several other proteins including H-NS, IHF, CRP and Lrp (Marshall *et al.*, 1999; O'Byrne and Dorman, 1994a, b). The observed 2-fold increase in transcription in the *spv* genes may be due to an indirect effect of Fis on one or more of these proteins. Alternatively the effect observed on the *spv* genes could be as a result of Fis-mediated changes in supercoiling levels in MM5.8.

30 of the 170 genes that passed the 2-fold cut off point are bacteriophage or bacteriophage-like genes. Both Gifsy prophages (Gifsy-1 and Gifsy-2) and both Fels prophages (Fels-1 and Fels-2) contain genes that were affected by Fis. The Gifsy prophages carry important virulence determinants as they contain several genes required for virulence in the mouse (Figueroa-Bossi *et al.*, 2001). Loss of Gifsy-2 results in attenuation of the bacteria. An involvement of Fis in regulation of these prophage genes is another link between Fis and virulence gene regulation.

The genes found to be most dependent on Fis for activation are a cluster of genes of unknown function designated STM2233 to STM2237. These genes, many of which code for putative phage proteins, are located in a region of the chromosome that contains several phage-like genes as well as several SPI 2 associated genes. STM2231 and STM2244 are homologous to macrophage survival gene *msgA* and the SPI 2 and Fis regulated *sspH2* is also located in this region. Interestingly, immediately upstream of this cluster of genes is a proline tRNA gene. Pathogenicity islands are known to insert at tRNA sites and bacteriophage have previously been implicated in horizontal gene transfer. All this evidence suggests that in addition to the identified virulence genes at this location, this cluster of genes may contain other important virulence determinants. This cluster of genes may represent another pathogenicity island or lysogenic prophage containing important virulence genes.

Results from the microarray analysis have confirmed a role for Fis in the regulation of *S. typhimurium* virulence genes. Genes on SPI's 1 and 2 were confirmed as being Fis regulated. In addition to this Fis was shown to regulate many other virulence factors including the Gifsy prophage. These results show that the Fis protein may play a bigger role than previously thought in *S. typhimurium* infection.

Chapter 7

General Discussion

Bacterial nucleoid-associated proteins serve many diverse functions within the cell. In many cases they bind to the DNA and act in a structural capacity, condensing the genome or arranging the DNA for recombination, insertion and excision (Azam and Ishihama, 1999; Finkel and Johnson, 1992; O'Gara and Dorman, 2000). Another important cellular process that nucleoid-associated proteins are frequently involved in is transcription. All of the major nucleoid-associated proteins (i.e. H-NS, IHF, HU and Fis) have been shown to be involved in the regulation of a wide variety of genes (Dorman and Deighan, 2003). The mechanisms employed by nucleoid-associated proteins in gene regulation are numerous and varied. Examples include direct interaction with RNA polymerase, competition for binding at promoter regions, manipulation of DNA to facilitate recruitment of RNA polymerase, and modulation of promoter DNA superhelical density to facilitate transcription initiation (Dorman and Deighan, 2003).

Expression of the major nucleoid-associated proteins varies significantly during batch culture, with each displaying a characteristic expression profile (Azam *et al.*, 1999). Environmental conditions such as nutrient availability or entry into macrophages can also greatly influence the expression of these proteins (Marshall *et al.*, 2000) (Ninnemann *et al.*, 1992). Therefore, having genes under the control of nucleoid-associated proteins is one mechanism of coordinating their expression with the physiology or environmental conditions of the cell.

Expression of virulence genes can be a heavy metabolic burden on the cell. Therefore it is important that they are only expressed at the correct moment during infection i.e. when the benefit of their expression outweighs the burden. The transition from the external environment to the inside of the host represents a substantial change in environmental conditions for the bacteria. This environmental transition is accompanied by changes in the levels of nucleoid-associated proteins. Many enteric pathogens have exploited this by having expression of virulence genes under the control of nucleoid-associated proteins. Virulence genes in *Salmonella*, *Shigella* and the *E. coli* pathogens EPEC and EAEC are all regulated by nucleoid-associated proteins (Beloin and Dorman, 2003; Goldberg *et al.*, 2001; Sheikh *et al.*, 2001; Wilson *et al.*, 2001).

In this study the role of nucleoid-associated proteins in *Salmonella* virulence gene regulation was investigated. The nucleoid-associated protein Fis was identified as a positive regulator of the recently characterised *Salmonella* pathogenicity island 2 (SPI 2). In addition, work was carried out on the *Salmonella* plasmid virulence (*spv*) genes to further elucidate the role of IHF in their regulation (Marshall *et al.*, 1999).

Binding of IHF 150 bp upstream of the *spvR* transcription start site is required for full expression from the *spvR* promoter (Marshall *et al.*, 1999). The mechanism by which IHF mediates this activation is not known. However, results using novobiocin (to relax the DNA) have indicated that supercoiling is involved. Site-directed mutagenesis was carried out on the *spvR* IHF binding site. One mutation disrupted the critical CAA triplet within the binding site while a second mutation was a closer match to the consensus IHF binding site. It was hoped that the affinity of IHF for each of these sites would be altered and give an indication as to how this affects *spvR* transcription. Results demonstrated that disrupting the IHF site reduces transcription and that "improving" the binding site does not affect transcription levels. This may indicate that improving the site does not induce tighter binding of IHF or that tighter binding has no affect on promoter activity.

The main focus of this work was the role of Fis in SPI 2 gene expression. Using a *lacZ* transcriptional fusion to the SPI 2 *ssrA* promoter, an investigation was carried out to identify potential transcription regulators. From a screen of mutants deficient in various nucleoid-associated proteins, Fis emerged as a potential regulator of SPI 2 gene expression. To investigate this effect further, five additional SPI 2 promoter fusions were made. The regions of SPI 2 cloned were those previously reported to contain promoters including areas where the location of the promoter is in dispute (i.e. the structural II promoter) (Cirillo *et al.*, 1998; Hensel *et al.*, 1997b). Results *in vitro*, in LB broth, and *in vivo*, inside macrophages, demonstrated that Fis is required for full activation of the SPI 2 promoters. The fold decrease in promoter activity in the absence of Fis varied considerably in LB but inside macrophages was consistently within a 3- to 5-fold range. Activation of the SPI 2 promoters inside macrophages occurred at different rates. The *ssrA* promoter, which transcribes the SsrA/B system, was rapidly activated following entry into macrophages. The

remaining SPI 2 promoters demonstrated a more gradual induction, perhaps reflecting their dependence on SsrA/B for activation.

It was hoped that by analysis of promoter activity an insight could be gained into the location of the SPI 2 structural II promoter. Previous reports have disagreed about the location of this promoter (Cirillo *et al.*, 1998; Hensel *et al.*, 1997a). Three potential locations for this promoter were cloned, i.e. the regions upstream of *ssaG*, *ssaH* and *ssaK*. The region upstream of *ssaG* generated considerably higher levels of transcription than either *ssaH* or *ssaK*. This result implies that a strong promoter is located upstream of *ssaG*. When a *lacZ* fusion containing the *ssaGH* region (i.e. the *ssaG* promoter, *ssaG* gene and *ssaH* promoter) was used, transcription levels generated were only slightly lower that those from the *ssaG* construct. This implies that the *ssaG* promoter transcribes at least the *ssaG* and *ssaH* genes, however how much longer this transcript is remains unknown. While activity from the *ssaH* and *ssaK* promoters is low, activity is nonetheless observed. In LB-grown bacteria Fis had no effect on either of these promoters while in macrophages an effect was observed at the *ssaK* promoter but not at *ssaH*. Are the regions upstream of *ssaH* and *ssaK* functionally relevant promoters? Taking into account that the *ssaH* "promoter" does not respond to Fis and that the *ssaG* promoter produces a transcript that includes *ssaH*, it is unlikely that this region contains a biologically relevant promoter. The activity observed in the *ssaH-lacZ* construct may result from a redundant promoter left over following construction of the pathogenicity island. In the case of the *ssaK* promoter, Hensel *et al.* (1997) reported that *ssaJ* and *ssaK* were not located on the same transcript. Inside macrophages the *ssaK* promoter showed reduced activity in the *fis* mutant. Taking these into account it appears likely that the *ssaK* upstream region does contain a promoter; hence there are at least two promoters responsible for transcription of the structural II region. Further biochemical investigation such as primer extension and Northern blotting are required to conclusively prove the location(s) and number of promoters in this region.

Experiments *in vitro* and *in vivo* demonstrated that in the absence of Fis, expression from the SPI 2 promoters decreases. As previously mentioned expression of the SPI 2 genes is also dependent on SsrA/B (Cirillo *et al.*, 1998). Therefore any reduction

in *ssrA* promoter activity would also result in a decrease in activity from the rest of the SPI 2 promoters. This raises the possibility that the decrease in expression observed at the SPI 2 promoters in a *fis* mutant is a result of decreased expression from the *ssrA* promoter. In this scenario Fis only directly affects transcription from the *ssrA* promoter and the effects observed at the other SPI 2 promoters are indirect (Fig 7.1 A). Alternatively Fis could be exerting a direct effect at each promoter and the decrease in expression observed in a *fis* mutant results from a combination of both direct (Fis at the promoter) and indirect (Fis effect on SsrA/B) effects (Fig 7.1 B). To determine if Fis is regulating each promoter directly DNA mobility shift assays were performed. Fis was shown to bind to all of the SPI 2 promoters. This suggests that in addition to an indirect effect through SsrA/B, Fis has the potential to directly influence transcription at each promoter. Further experimental evidence, such as *in vitro* transcription, is required as indisputable proof that Fis affects transcription directly at these promoters.

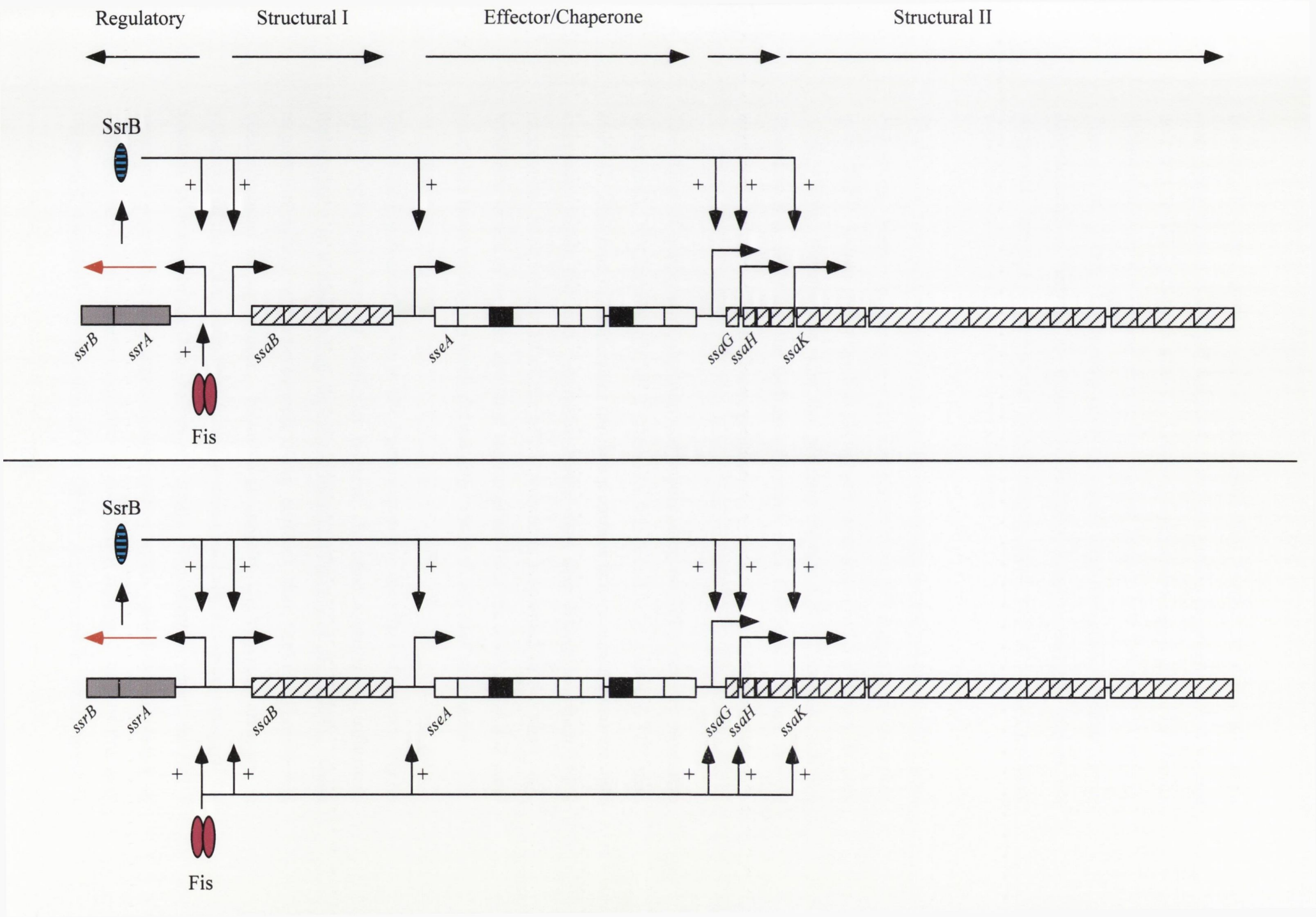
Gene expression data and DNA mobility shift analysis clearly indicated a role for Fis in SPI 2 gene expression, however no information was available concerning Fis expression levels inside macrophages. To investigate this, *S. typhimurium* were recovered from the intracellular environment and used in Western immunoblot analyses. This is the first demonstration of *S. typhimurium* nucleoid-associated protein levels during infection of a cultured cell line. Results show that Fis is induced following uptake of the bacteria by macrophages and that its levels remain constant in the cell thereafter. This expression profile indicates a novel regulation mechanism and function of Fis inside macrophages.

Results here have shown that Fis is a regulator of SPI 2 gene expression. The only other known regulators of SPI 2 are the two-component signal transduction systems SsrA/B and OmpR/EnvZ (Cirillo *et al.*, 1998; Lee *et al.*, 2000). Two-component systems regulate gene expression in response to environmental stimuli. OmpR/EnvZ responds to osmolarity but the environmental signal SsrA/B responds to is unknown. As the bacteria enter macrophages they undergo environmental and physiological changes. These changes are responsible for activation of the SPI 2 genes. To determine what specific environmental conditions activate expression of the SPI 2 promoters, expression analysis was preformed using the SPI 2-*lacZ* promoter

Fig. 7.1. Direct and indirect effects of Fis at SPI 2 promoters.

A. Fis activates expression of the *ssrA* promoter. This results in production of SsrA and SsrB which in response to environmental signals activate transcription from the downstream SPI 2 promoters (*ssaB*, *sseA*, *ssaG*, *ssaH* and *ssaK*). In the absence of Fis, reduced expression from the downstream promoters results from the decrease in SsrA/SsrB production.

B. Fis has a direct role in activating transcription from all of the SPI 2 promoters. In the absence of Fis, reduced expression from the downstream promoters results from (i) the absence of Fis at the promoter, and (ii) the reduction in SsrA/SsrB levels.


Regulatory
Structural I
Effector/Chaperone
Structural II
SsrB
ssrB
ssrA
ssaB
sseA
ssaG
ssaH
ssaK
Fis
SsrB
ssrB
ssrA
ssaB
sseA
ssaG
ssaH
ssaK
Fis

fusions. Various environmental conditions that would be expected inside macrophages, such as low pH and reactive oxygen species (hydrogen peroxide), had no effect on SPI 2 promoter activity. Previous reports have stated that SPI 2 effector proteins prevent the release of reactive oxygen species into the SCV (Vazquez-Torres *et al.*, 2000). The bacteria never encounter these molecules therefore are not programmed to respond to them. In addition, reactive oxygen species are immensely harmful to the bacteria. An extremely rapid reaction to their presence would be required if the bacteria were to survive.

There have been several conflicting reports concerning the role of pH in SPI 2 gene expression. Some reports suggest that low pH activates SPI 2 gene expression (Lee *et al.*, 2000). Others report that low pH triggers secretion of SPI 2 effectors (Beuzon *et al.*, 1999). Results here show that pH does not affect SPI 2 promoter activity. Therefore it is likely that the findings of Beuzon *et al.* (1999) are correct, low pH triggers the release of SPI 2 effector proteins.

The effect of temperature, aeration and supercoiling on SPI 2 activity were also determined. Not surprisingly SPI 2 expression was found to be greater at 37°C than at 30°C. This type of temperature regulation ensures that virulence genes are only expressed inside the host. Aeration levels were also shown to affect SPI 2 expression, however not all of the SPI 2 promoters responded in the same way. Moderate levels of oxygen activated transcription from the *ssrA*, *sseA* and *ssaG* promoters however oxygen levels did not affect the *ssaB* and *ssaK* promoters.

Due to the involvement of Fis in SPI 2 gene expression the effect of supercoiling on SPI 2 expression was of particular interest. In other systems Fis influences transcription in a supercoiling-dependent manner (Travers *et al.*, 2001). Gene expression assays were performed using cultures that contained sub-lethal concentrations of novobiocin. Novobiocin inhibits DNA gyrase resulting in relaxation of the DNA, a condition known to occur naturally following uptake of the bacteria by macrophages (Marshall *et al.*, 2000). The ways in which the SPI 2 promoters responded to changes in supercoiling again varied demonstrating an ability to respond differently to specific environmental signals. The *ssrA* promoter responded positively to relaxation of the DNA. An increase in transcription is

observed when the DNA becomes relaxed. None of the other SPI 2 promoters were activated by relaxation of the DNA in the wild type strain. Interestingly, in the *fis* mutant, all of the SPI 2 promoters were activated by relaxation of the DNA. This result suggests that the role of Fis in SPI 2 gene expression may be through an effect on supercoiling, however it does highlight certain inconsistencies. Fis is required for SPI 2 activation as the bacteria enter macrophages, a condition that is known to be accompanied by relaxation of the DNA (Marshall *et al.*, 2000). Results here indicate that in the absence of Fis SPI 2 promoters are strongly induced by relaxation of the DNA but that when Fis is present only *ssrA* is activated by relaxed DNA. These experiments, while indicating a role for supercoiling in SPI 2 regulation, have certain limitations. It is unknown what concentration of novobiocin results in DNA that is relaxed to the same extent as when the bacteria enter macrophages. In addition, the different ways in which novobiocin affects the wild type and *fis* mutant bacteria is difficult to determine and account for. With Fis present the bacteria are better able to cope with changes in supercoiling. The effects of novobiocin on the *fis* mutant may be more severe than on the wild type strain. Evidence that supports a link between Fis, supercoiling and SPI 2 gene regulation came from the DNA mobility shift assays. Fis was found to bind throughout the island including at the *ssaH* promoter. Gene expression assays with this promoter showed that Fis has no effect on its expression. It is surprising therefore that the protein should bind there. It has been proposed that in certain systems Fis binds to the DNA and maintains it in a conformation that is favourable for transcription (Travers *et al.*, 2001). If a similar mechanism were to be employed by Fis in the regulation of SPI 2, binding of the protein throughout the island would be expected. This could account for the presence of Fis at the *ssaH* promoter.

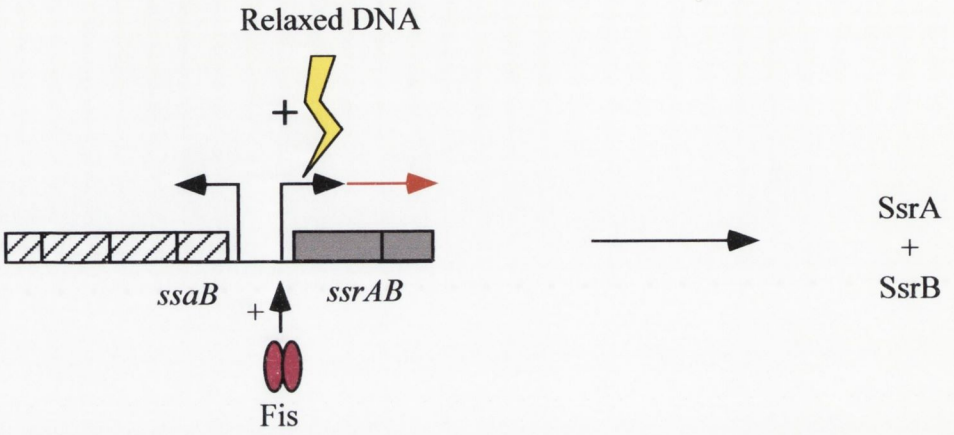
The exact mechanism with which supercoiling and Fis influence SPI 2 gene expression is difficult to establish. As is the case with other environmental factors certain promoters appear capable of responding differently than others. This may reflect an ability of SPI 2 to sequentially activate expression of genes in response to different environmental conditions during macrophage infection. Initial signals, e.g. relaxed DNA, activate expression from the *ssrA* promoter resulting in the production of SsrA and SsrB (Fig. 7.2 A). Once produced SsrA/B in conjunction with other environmental signals, e.g. moderate oxygen levels, activate transcription of certain

Fig. 7.2. Sequential activation of SPI 2 promoters by environmental conditions.

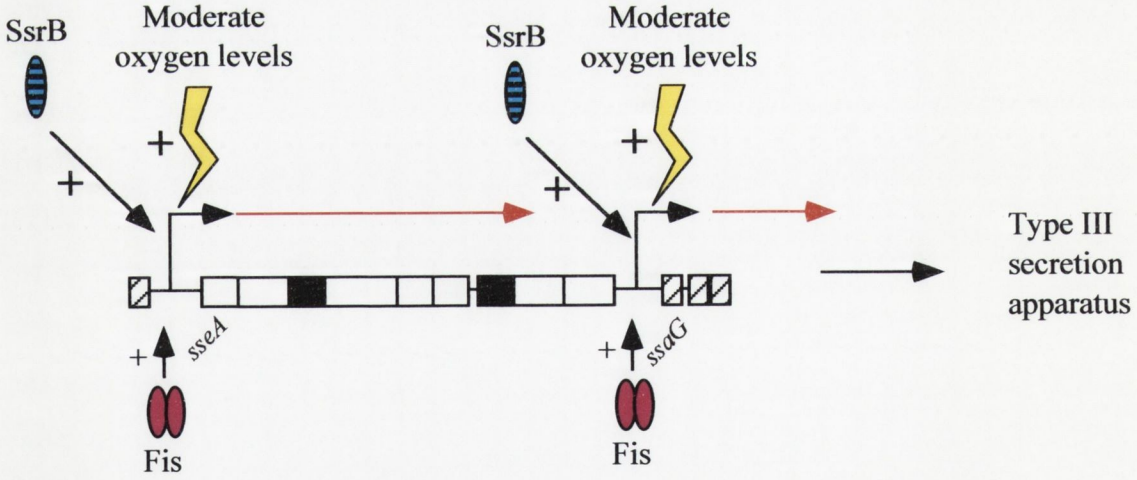
A. Rapid relaxation of the DNA following entry of the bacteria into macrophages activates transcription from the *ssrA* promoter. This results in the production of SsrA and SsrB which form a two-component signal transduction system required for activation of the downstream SPI 2 promoters.

B. Moderate oxygen levels, along with SsrB and Fis, activate expression from the *sseA* and *ssaG* promoters. This results in the production of certain components of the type III secretion machinery.

A.


Relaxed DNA
+
ssaB
ssrAB
+
Fis
SsrA
+
SsrB

B.


SsrB
Moderate
oxygen levels
+
+
SsrB
Moderate
oxygen levels
+
+
+
sseA
Fis
+
ssaG
Fis
Type III
secretion
apparatus

type III secretion system components (Fig 7.2 B). Following this, expression of the chaperone and effector proteins and their subsequent secretion into the host cell facilitates bacterial survival inside the macrophage. A mechanism such as this would account for the induction kinetics of the SPI 2 genes inside macrophages. The *ssrA* promoter displayed a rapid induction while the downstream SPI 2 promoters demonstrated a more gradual, delayed induction.

Further conformation of the role of Fis in SPI 2 gene regulation came following DNA microarray analysis of the Fis regulon during growth in miminal medium 5.8 (MM5.8). MM5.8 is a medium that was shown here and elsewhere to activate transcription of SPI 2 genes (Deiwick *et al.*, 1999). It is a low pH, stringent medium, which contains low levels of magnesium. Results from the microarray analysis showed that transcription of 15 SPI 2 genes were found to have decreased by a factor of 2 or more in the *fis* mutant. The fold decrease in expression in the *fis* mutant, of these 15 genes ranged from 2- to 3.47-fold (Table 6.1). These fold differences are similar to those observed when using the SPI 2-*lacZ* promoter fusions inside macrophages i.e. a 2- to 5-fold range (Fig 4.3). Interestingly, the fold decreases in SPI 2 gene expression observed in the *fis* mutant during growth in MM5.8 and inside macrophages are considerably lower than those observed for bacteria growing in LB broth. In LB the fold differences range from 2- to 73-fold (Fig 4.2). Although surprising, these results were confirmed by microarray analysis of the Fis regulon during growth in LB (A. Kelly, personal communication). During this analysis the fold decreases in expression of SPI 2 genes in the *fis* mutant were shown to be in the same range as those observed by promoter fusion in this study. The reason for this difference is unknown. Perhaps the fact that SPI 2 is not as highly expressed in LB as it is in MM5.8 or macrophages and the different roles of Fis during growth in LB, MM5.8 and macrophages contributes to this difference.

Other virulence genes found to be regulated by Fis included genes associated with SPI 1 and SPI 5. Interestingly, a large number of bacteriophage and bacteriophage-like genes were also found to be regulated by Fis. Several genes in the Gifsy and Fels prophages were Fis regulated. These fully-functional integrated phages have recently been shown to encode transmissible virulence factors that can be secreted via the SPI 1 and/or SPI 2 type III secretion systems (Figueroa-Bossi *et al.*, 2001).

It is interesting to note that Fis, which regulates the SPI 1 and SPI 2 systems, is also involved in the regulation of these phage-encoded virulence determinants. Many horizontally acquired genes insert at tRNA sites, which are amongst the most highly Fis regulated genes in the cell. Fis is also required for the insertion and excision of many bacteriophages. Perhaps the presence of Fis at these insertion sites combined with the lack of sequence specificity in the Fis binding site has aided its recruitment as a regulator of horizontally-acquired genes.

The microarray analysis also identified a cluster of genes, highly dependent on Fis for expression, that show homology to bacteriophage proteins. This cluster is found close to a tRNA gene, in a region of the chromosome that also contains macrophage survival genes and SPI 2 regulated genes. All of these factors indicate that this region may contain another integrated bacteriophage that carries virulence determinants.

In conclusion, this work has identified a role for the nucleoid-associated protein Fis in regulating expression of *Salmonella* pathogenicity island 2 genes. Fis is required for activation of the SPI 2 promoters as the bacteria enter macrophages and its own expression was induced intracellularly and maintained at a constant level. Several environmental factors were also identified as being important in SPI 2 regulation. Temperature, oxygen and supercoiling levels all influence SPI 2 promoter activity with the role of supercoiling and Fis possibly connected. Microarray analysis confirmed the role of Fis in SPI 2 activation and also identified roles for Fis in regulating expression of the Gifsy and Fels prophages as well as a previously unidentified cluster of bacteriophage-like genes. The results show that Fis plays a bigger role in infection and virulence gene regulation than previously thought. In addition to regulating SPI 1, Fis has now been shown to regulate SPI 2 as well as several effector proteins encoded outside these pathogenicity islands. This work confirms the role of Fis as a global regulatory protein and furthers our knowledge of its role in virulence gene regulation.

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