Gene Regulation and the Fis Nucleoid-Associated Protein in *Salmonella enterica* serovar Typhimurium.

by

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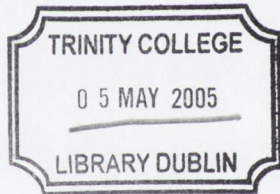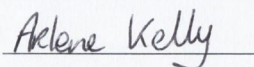
DECLARATIONS

I, Arlene Kelly, 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., Goldberg, M.D., Carroll, R.K., Danino, V., Hinton, J.C., and Dorman, C.J. 2004. A global role for Fis in the transcriptional control of metabolism and type III secretion in *Salmonella enterica* serovar Typhimurium. Microbiology. **150**:2037-2053.

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Summary

The Fis protein was found to influence the superhelical density of DNA and its ability to do this differed between *Salmonella enterica* serovar Typhimurium (*S. typhimurium*) and *Escherichia coli* (*E. coli*). Induction of the Fis protein to high levels in *S. typhimurium* resulted in the concomitant relaxation of the DNA in good correlation with Fis as a transcriptional repressor of the *gyr* genes that encode DNA gyrase.

The regulon of genes whose expression is influenced by Fis in *S. typhimurium* was defined by DNA microarray analysis. The data indicate that Fis activates the transcription of virulence genes located in the pathogenicity islands SPI-1, SPI-2, SPI-3, SPI-4 and SPI-5. Genes involved in the motility and chemotaxis of *S. typhimurium* require Fis for full expression. Among the most strongly Fis-repressed genes are those involved in metabolism and transport, many of which are concerned with survival in the mammalian gut. Many global regulators of gene expression, including members of the family of nucleoid-associated proteins, are also Fis-regulated.

Fis regulation of motility is due to a direct effect as the Fis protein interacts with the upstream regions of representative flagellar genes. *S. typhimurium fis* mutants display reduced motility compared to the wild-type, independent of flagellar phase variation and resulting partly from a decrease in flagellin protein. Fis is required for the full expression of SPI-2 genes. It binds at three sites within the *ssrA* promoter region of the *ssrAB* two-component regulator of SPI-2. The protein levels of the response regulator SsrB are severely reduced in the absence of Fis. Limiting oxygen induces SPI-2 gene expression, an environmental condition that results in specific SPI-2 genes becoming more dependent on Fis for full induction.

This study reveals for the first time that Fis is a regulator of the three *S. typhimurium* type III secretion protein export machineries encoded by SPI-1, SPI-2 and the flagellar regulon. The data from this study also indicate a key role for Fis in

coordinating the expression of both housekeeping and virulence factors to assist *S. typhimurium* during life in the gut lumen and during invasion and systemic infection of host cells.

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John you have taught me the biggest lesson of all, how to love. For all your patience, understanding and support I thank you.

For my 'Little Nana'

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I go at what I am about

as if there was nothing else

in the world for the time being.

Charles Kingsley

Chapter 1

Introduction

1.1 General introduction

1.1.1 *Salmonella*

Salmonella is a Gram-negative facultative intracellular pathogen. The genus *Salmonella* is divided into two species, *Salmonella enterica* and *Salmonella bongori*, which are subdivided into subspecies or serovars. *Salmonella enterica* serovars infect a wide range of hosts including poultry, cattle, pigs and humans while *S. bongori* serovars are usually only isolated from cold blooded animals (Ohl and Miller, 2001). Certain serovars infect a wide variety of hosts while others are specific to only a few hosts. For example *Salmonella enterica* serovar Typhimurium can infect mice, cattle and humans but *Salmonella enterica* serovar Typhi is exclusively a human pathogen (Ohl and Miller, 2001). *Salmonella* infections are usually acquired from contaminated food or water and are commonly associated with ingestion of improperly cooked poultry products, particularly eggs. *Salmonella* is a leading cause of foodborne diseases worldwide. Salmonellosis (the disease caused by *Salmonella*) has several clinical manifestations ranging from gastroenteritis to typhoid fever and bacteraemia. Serotypes associated with gastroenteritis orchestrate an intestinal inflammatory and secretory response whereas serovars concerned with causing typhoid fever establish a systemic infection through their ability to survive and proliferate in mononuclear phagocytes (Ohl and Miller, 2001). The nature of the disease caused depends on the infecting *Salmonella* serovar and the recipient host (Darwin and Miller, 1999a). *Salmonella* enterica serovar Typhimurium causes a self-limiting gastroenteritis characterized by diarrhoea in healthy humans and bacteraemia is a rare complication of the very young and immunocompromised (Jones and Falkow, 1996; Wallis and Galyov, 2000). In mice *Salmonella* enterica serovar Typhimurium causes a systemic disease, which serves as an animal model to study typhoid fever (Kingsley and Baumler, 2000), which is a systemic infection in humans infected with *Salmonella* enterica serovar Typhi. Typhoid fever is still a serious problem in many tropical areas of the world (www.who.int/vaccines/en/typhoid.shtml). While *Salmonella* enterica serovar

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Typhimurium is frequently associated with a foodborne disease in humans it is also a major cause of calf morbidity and mortality (Gibson, 1961; Smith *et al.*, 1994; Zhang *et al.*, 2002). *Salmonella* enterica serovar Typhimurium also manifests as a gastroenteritis in calves with similar clinical and pathological features to the human gastroenteritis caused by the same serovar (Frost *et al.*, 1997; Tsolis *et al.*, 1999a; Zhang *et al.*, 2002). Therefore, the calf is a model of human gastroenteritis caused by *Salmonella* enterica serovar Typhimurium.

1.1.2 *Salmonella* infection

Following ingestion *Salmonella* must survive the harsh acidic environment of the stomach before entering the small intestine (Fig. 1.1). Several acid survival systems allow the bacteria to endure low pH *in vitro* and it is proposed that these same systems are employed to help the bacteria adapt to the acidic environment of the stomach (Lucas and Lee, 2000; Slauch *et al.*, 1997). Next *Salmonella* colonizes the small intestine and attaches to the intestinal lining using fimbriae, filamentous appendages on the cell surface for adherence (Fig. 1.1) (Clegg *et al.*, 1996; Darwin and Miller, 1999a; Finlay and Brumell, 2000; Lucas and Lee, 2000). *Salmonella* penetrates the intestinal barrier by targeting and invading the specialized M cells of the follicle-associated epithelium of Peyer's patches (Jones *et al.*, 1994) and absorptive enterocytes (Fig. 1.1), (Frost *et al.*, 1997). *Salmonella* promotes its own uptake into these non-phagocytic cells by a process known as bacterial mediated endocytosis (Francis *et al.*, 1992). *Salmonella* uses a type III secretion system to translocate deleterious effector proteins to the host cell cytosol that mediate its uptake (Galan, 1996). The effector proteins induce major cytoskeletal rearrangement of the host cell in the vicinity of the adhering bacteria resulting in denuding of the microvilli and ruffling of the cell membrane (Fig. 1.1). The membrane ruffles surround and engulf the invading bacteria into large vesicles. Serovars of *Salmonella* that cause gastroenteritis can infect adjacent epithelial cells and secrete effector proteins into the host cell cytosol (Galyov *et al.*, 1997). This elicits

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Fig. 1.1. Hypothetical model of the *Salmonella* infection. *Salmonella* is typically acquired through the consumption of contaminated food or water. Following ingestion it must survive a number of stressful environments during the course of infection. It endures the harsh acidic pH of the stomach before adhering to and invading the cells that line the epithelium of the small intestine. The SPI-1 type III secretion system is required by *Salmonella* to invade the intestinal barrier. It secretes effector proteins into the epithelial cell, via the type III secretion system, which induce cytoskeletal rearrangements, denuding of the microvilli, ruffling of the membrane and subsequent bacterial uptake. Once inside the host cell *Salmonella* can infect adjacent epithelial cells. Secretion of effector proteins also promotes secretion of cytokines, inflammation and fluid secretion which result in gastroenteritis. *Salmonella* exits at the basolateral surface of the epithelial cells and is engulfed by the phagocytic macrophages. The intracellular bacteria reside in a *Salmonella* containing vacuole (SCV) in the macrophage where they have the ability to survive and replicate. SPI-2 mediates survival and proliferation in macrophages. *Salmonella* evades the host's immune responses from within the macrophage and is transported in the macrophage via the lymphatics and bloodstream to the liver and spleen to cause a systemic infection. Upon reaching the liver and spleen the *Salmonella* virulence plasmid supports the continued survival and replication of *Salmonella*. *Salmonella* cells that "hide" in the gall bladder cause a chronic infection.

Salmonella in contaminated food or water

— *Salmonella*

Acidic pH of the stomach

Secretion of cytokines

Inflammation

Fluid secretion

Diarrhoea

Gut lumen

SPI-1

Membrane ruffling

Denuding of microvilli

Columnar epithelial cell

Small intestine

M cell

Basolateral surface of epithelial cells

SPI-2

Macrophage

Lymphatics and bloodstream

Virulence plasmid

Liver, spleen and gall bladder
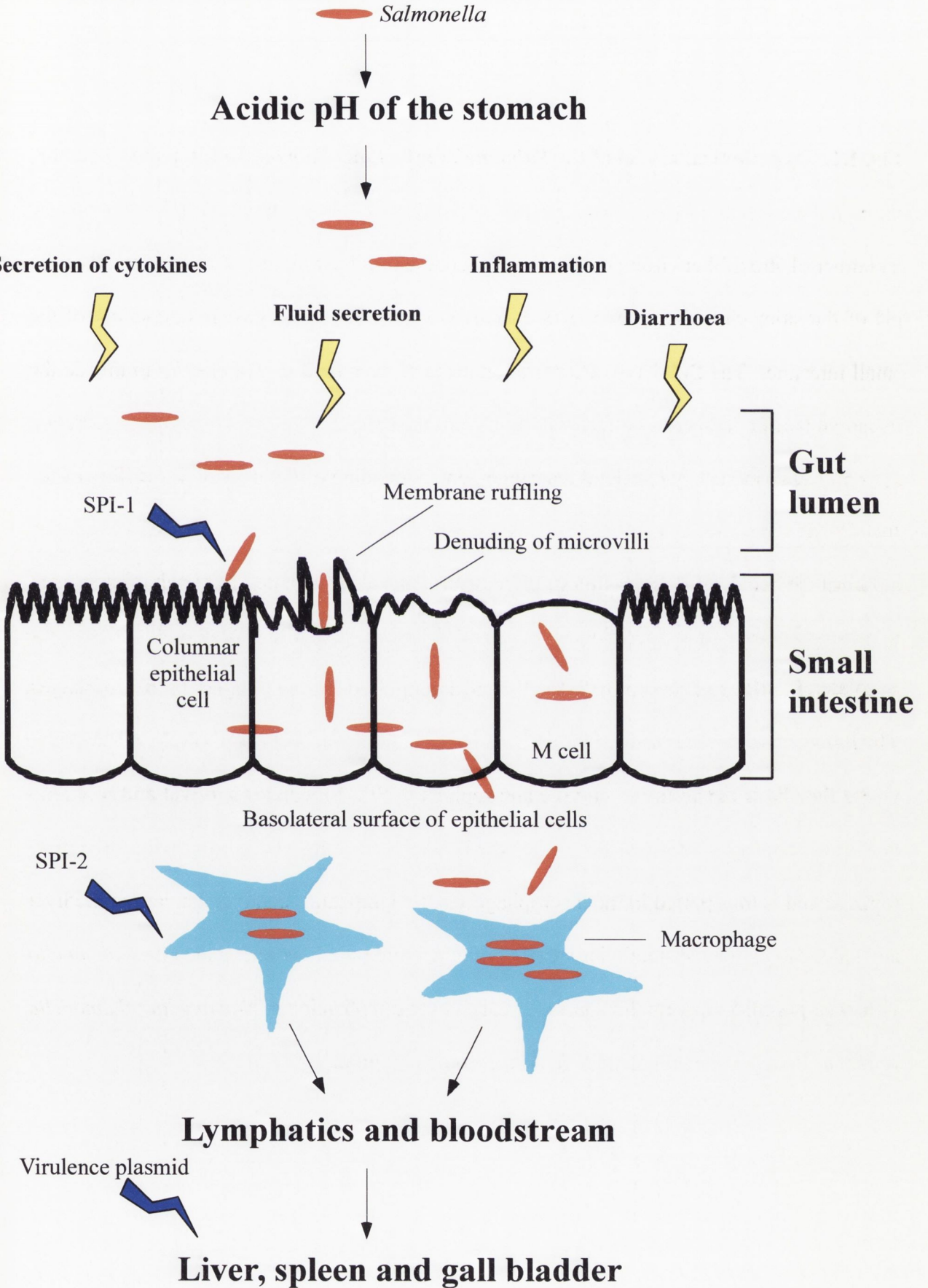
cytokine release, inflammation and fluid secretion, which result in the gastroenteritis disease (Fig. 1.1).

It is serovars of *Salmonella* that cause a systemic infection that are transcytosed to the basolateral surface of the intestinal epithelia where they exit and reach the Peyer's patches. At the Peyer's patches *Salmonella* encounters and is engulfed by phagocytic submucosal macrophages (Fig. 1.1). Within the macrophage *Salmonella* resides in a *Salmonella*-containing vacuole (SCV) where it survives and replicates and can importantly evade the host's immune system (Alpuche-Aranda *et al.*, 1994). While evading the host's immune system *Salmonella* can spread to the mesenteric lymph nodes, liver and spleen via the lymphatics and bloodstream. A second type III secretion system is employed to secrete effector proteins that interfere with the antimicrobial functions of the macrophage and thus is required for *Salmonella* survival in macrophages (Cirillo *et al.*, 1998; Hensel, 2000; Vazquez-Torres *et al.*, 2000). Upon reaching the liver and spleen the *Salmonella* virulence plasmid is involved in the continued survival and proliferation of the bacteria in these tissues (Fig. 1.1) (Guiney *et al.*, 1995).

Salmonella enterica serovar Typhimurium (hereinafter called *S. typhimurium*) is the *Salmonella* serotype used throughout this study. *S. typhimurium* causes infection of mammalian hosts by coordinating the expression of a number of key virulence factors. These factors include those encoded by genes located in five pathogenicity islands and the *Salmonella* large virulence plasmid (Fig.1.2) (Marcus *et al.*, 2000). The pathogenicity islands are often integrated at tRNA loci and consist of DNA sequences that differ from the rest of the *Salmonella* genome in the G+C content and by different codon usage. This property reflects the acquisition of pathogenicity islands by horizontal gene transfer (Blanc-Potard *et al.*, 1999). *Salmonella* pathogenicity island-1 (SPI-1) encodes the ability to invade the epithelial cells of the small intestine (Galyov *et al.*, 1997; Hardt *et al.*, 1998; Mills *et al.*, 1995; Watson *et al.*, 1995; Wood *et al.*, 1996) and *Salmonella* pathogenicity island-2 (SPI-2) encodes the ability to survive and replicate inside macrophages (Cirillo *et al.*, 1998; Hensel *et al.*, 1998; Hensel, 2000;

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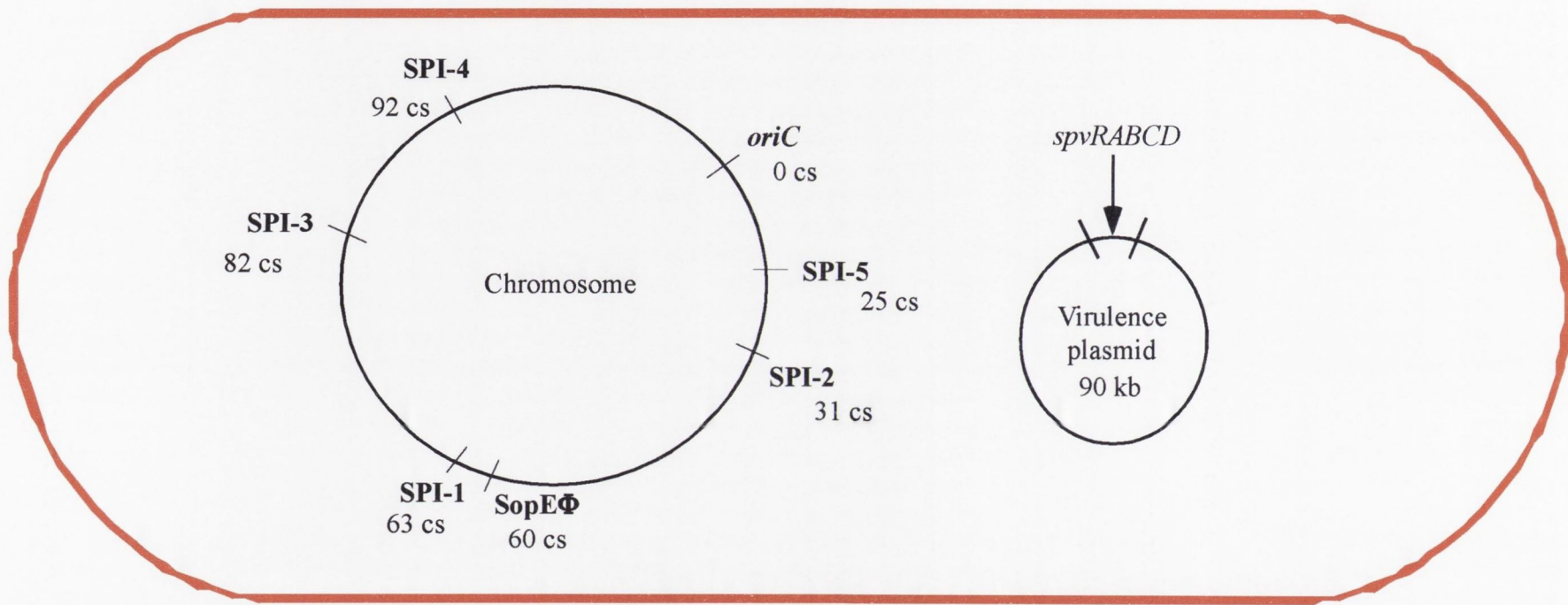
Fig. 1.2. Schematic representation of the *S. typhimurium* chromosome and the large virulence plasmid. The five *S. typhimurium* pathogenicity islands are labelled on the chromosome. The 90 kb large virulence plasmid including the highly conserved 8 kb region encoding the *spvRABCD* genes is indicated. The SopEΦ at 60 cs and the origin of replication *oriC* are also indicated.

Ochman *et al.*, 1996; Waterman and Holden, 2003) and cause systemic infection in mice (Hensel *et al.*, 1995; Shea *et al.*, 1996). Along with regulatory, chaperone and effector proteins these two large virulence loci encode type III secretion systems.

1.1.3 Type III secretion systems

Type III protein secretion systems are specialised organelles essential for the virulence of many bacterial pathogens including *Salmonella* (Cornelis and Van Gijsegem, 2000; Galan and Collmer, 1999; Galan, 2001; Hueck, 1998). Type III secretion systems are dedicated protein export machineries for the delivery of virulence proteins into the host cell cytoplasm (Fig. 1.3). The type III secretory system begins by forming a channel through the bacterial inner and outer membranes. Translocon proteins are delivered through this channel to the cell surface where they remain associated and function to form a pore in the host cell membrane. Therefore, the completed type III secretion apparatus is a needle-like complex that injects proteins from the bacterial cytosol to the host cell cytosol through a continuous pathway or hollow tunnel spanning the bacterial membrane envelope and the host cell membrane (Fig.1.3) (Cornelis and Van Gijsegem, 2000; Galan and Collmer, 1999; Galan, 2001; Hueck, 1998). Effector proteins are secreted into the host cell cytosol where they modulate or interfere with cellular functions. Information in the first ~120 amino acids of the effector proteins targets these proteins to the type III secretion apparatus and this domain is not cleaved upon secretion (Sory *et al.*, 1995). This domain is also the binding site for chaperones, which are not secreted but are released upon secretion of the effector protein (Page and Parsot, 2002). The chaperone protein prevents incorrect folding and premature degradation of the effector protein.

In addition a third type III secretion pathway exists in *S. typhimurium* and is used to facilitate the deployment and assembly of flagella on the cell surface for motility (Section 1.1.12 and Fig. 1.10), (Chilcott and Hughes, 2000; Macnab, 2003).

Fig. 1.3. Hypothetical model of the *S. typhimurium* SPI-1 type III secretion system. Type III secretion systems are multicomponent organelles specialised in the delivery of effector proteins from the bacterial cytosol to the host cell cytosol. The type III secretion apparatus is composed of a needle-like complex that spans the bacterial envelope, links to and also spans the host cell membrane. Therefore one continuous channel is formed between the bacterial cytosol and the host cell cytosol. The bacterial effector proteins targeted to the type III secretion apparatus are released from their chaperone proteins and secreted via the type III secretion pathway into the eukaryotic host cell, where they have deleterious effects on host cell physiology. SPI-1 encodes the structural components of a type III secretion machinery (e.g., InvA, InvC, InvG, PrgH, PrgI, PrgK, and SpaO), the proteins involved in the translocation of effector molecules into the host cell cytosol (e.g., SipB, SipC and SipD) and the effector proteins that modulate host cell functions (e.g., SopE1, SopB, SipA, SptP and AvrA). Although the majority of effector proteins are encoded on SPI-I some are not. SopB is encoded within SPI-5 and SopE1 is encoded within a cryptic bacteriophage SopEΦ located at centisome 60 on the *S.typhimurium* chromosome and both these effector proteins are targeted for secretion to the SPI-1 type III secretory apparatus. OrgB and InvC energize export of effector proteins through the type III secretory apparatus. The translocation of effector proteins via the SPI-1 encoded type III secretory system allow *S. typhimurium* to invade non-phagocytic cells, such as M cells of the small intestinal epithelium.

SPI-1 type III secretion system

M cell

Translocators
SipD   SipB   SipC

PrgI

Bacterial outer membrane

InvG

PrgH

Bacterial periplasm

PrgK

Bacterial inner membrane

SpaO

OrgB

InvC
(ATPase)

Inner membrane components:
SpaP   SpaQ   InvA   SpaS   SpaR

Effector protein

Chaperone protein

Effector proteins:

SipA   SptP   SopE1   AvrA   SopB

(SopEΦ)   (SPI-5)

Chaperone proteins:
SicP   SpaM   SpaT

SPI-1

63 cs

- ■ (blue) Components of the type III secretion apparatus
- ■ (pale yellow) Transcriptional regulators
- ■ (red) Secreted effectors
- ■ (black) Translocases or effectors
- □ (white) Function unknown

avr spr hil   org   prg   hil   iag spt sic iac   sip   sic   spa   inv

A B C C B A K J I H D   A B P P P A D C B A S R Q P O N M C B A E G F H

40 kb

fhlA   mutS
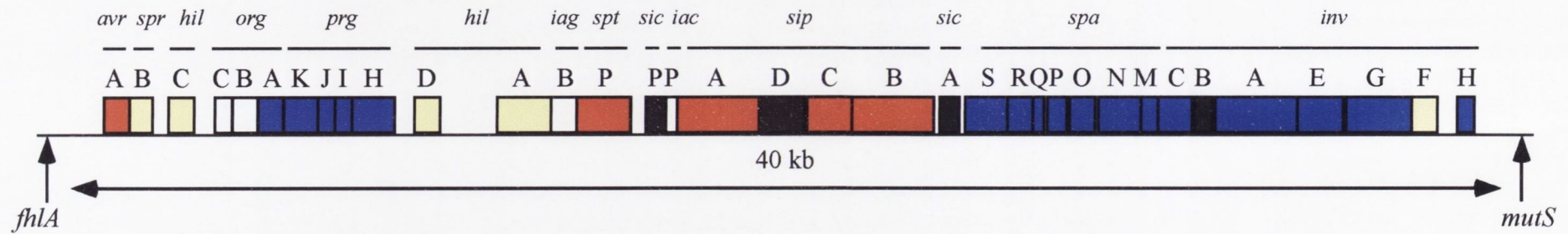
Fig 1.4. Genetic organisation of *Salmonella* pathogenicity island-1 (SPI-1). SPI-1 is approximately 40 kb in size and is located at 63 centisomes on the *S. typhimurium* chromosome. SPI-1 is flanked by the genes *fhlA* and *mutS*. SPI-1 genes encoding a type III secretion system, transcriptional regulators, secreted effectors and translocase/effectors are indicated. The length of each gene is approximately to scale.

AraC/XylS-like proteins that positively regulate the expression of the regulatory gene *hilA* by alleviating repression and/or directly activating transcription at the *hilA* regulatory region (Bajaj *et al.*, 1995; Boddicker *et al.*, 2003; Eichelberg and Galan, 1999; Olekhnovich and Kadner, 2002; Schechter *et al.*, 1999; Schechter and Lee, 2001). The *hilA* gene encodes the HilA OmpR/ToxR-like protein that activates the transcription of *invF*, which encodes the InvF protein, an AraC/XylS-like protein (Eichelberg and Galan, 1999). InvF binds to the promoter region of the SPI-1 structural gene operon to activate transcription, as does HilA (Darwin and Miller, 1999b; Lostroh *et al.*, 2000; Lostroh and Lee, 2001). HilC and HilD can also activate some SPI-1 genes independent of their positive regulation of *hilA* (Akbar *et al.*, 2003).

Regulatory inputs that influence the expression of the SPI-1 genes come from an array of proteins encoded outside of SPI-1 including EnvZ/OmpR, FliZ, HilE, PhoB, PhoP/PhoQ, SirA/BarA, CsrA, RtsA, and from the environmental stimuli oxygen and osmolarity (Bajaj *et al.*, 1996; Ellermeier and Slauch, 2003; Goodier and Ahmer, 2001; Lawhon *et al.*, 2003; Lucas and Lee, 2000; Lucas *et al.*, 2000). The nucleoid-associated proteins Fis, H-NS and HU are also involved in the regulation of SPI-1 gene expression (Schechter *et al.*, 2003; Wilson *et al.*, 2001). A role for Fis in controlling the expression of the SPI-1 genes was demonstrated, where Fis is required for the full expression of the *hilA* and *invF* regulatory genes (Wilson *et al.*, 2001). The virulence of a *fis* mutant is attenuated 100-fold when administered orally to mice and *S. typhimurium* strains deficient in the Fis protein also have a 50-fold reduction in their ability to invade Hep-2 cells. The H-NS protein is known largely as a repressor of transcription (Dorman, 2004) and it has a negative influence here too as it acts in opposition to Fis, repressing SPI-1 gene expression (Schechter *et al.*, 2003).

It has been demonstrated that the proteins CsrA (encoded by the carbon storage regulatory gene), RtsA an AraC/XylS-like protein and RtsB a DNA binding protein containing a helix-turn-helix motif, FliZ a member of the flagellar regulon and the two-component regulatory system SirA/BarA coordinate the expression of SPI-1 genes with

flagellar genes (Ellermeier and Slauch, 2003; Goodier and Ahmer, 2001; Lawhon *et al.*, 2003; Lucas *et al.*, 2000).

The main factor inducing the expression of SPI-1 genes is the change in pH from acidic to mildly alkaline (Daefler, 1999). These conditions reflect the physiology of *in vivo* infections where *S. typhimurium,* after oral ingestion, passes through the acidic environment of the stomach to the mildly alkaline milieu of the small intestine where expression of SPI-1 genes is required to invade the intestinal barrier (Fig. 1.1). Low aeration and high osmolarity enhance the *S. typhimurium* invasive phenotype (Lee and Falkow, 1990) and are also conditions that increase the level of negative supercoiling in the bacterial DNA (Dorman *et al.*, 1988; Higgins *et al.*, 1988). In good correlation with this the SPI-1 gene promoters were demonstrated to be sensitive to alterations in the superhelicity of the DNA (Galan and Curtiss, 1990), indicating SPI-1 genes are members of the regulon of genes influenced by DNA topology (Dorman, 1991; Hatfield and Benham, 2002; Steck *et al.*, 1993). The fact that alterations in the DNA topology influence the expression of SPI-1 genes is consistent with the fact that these genes are regulated by Fis, a nucleoid-associated protein that can modulate the topology of DNA (Schneider *et al.*, 1997; Schneider *et al.*, 1999).

1.1.5 *Salmonella* pathogenicity island-2 (SPI-2)

SPI-2 is 40 kb in size and located at 31 centisomes (cs) on the *S. typhimurium* chromosome (Fig. 1.5) and has a lower G+C content than that of the remainder of the *S. typhimurium* genome suggesting it was acquired by horizontal gene transfer (Ochman *et al.*, 1996; Shea *et al.*, 1996). This large virulence locus is inserted adjacent to the tRNA^{ValV} gene (Hensel *et al.*, 1997a). *S. typhimurium* requires SPI-2 for intracellular replication in host cells such as the macrophage (Cirillo *et al.*, 1998; Hensel *et al.*, 1998; Hensel, 2000; Ochman *et al.*, 1996) and for systemic infection (Fig. 1.1) (Hensel *et al.*, 1995; Shea *et al.*, 1996). SPI-2 mutants have severely attenuated virulence compared to

SPI-2

31.0 cs

30.5 cs
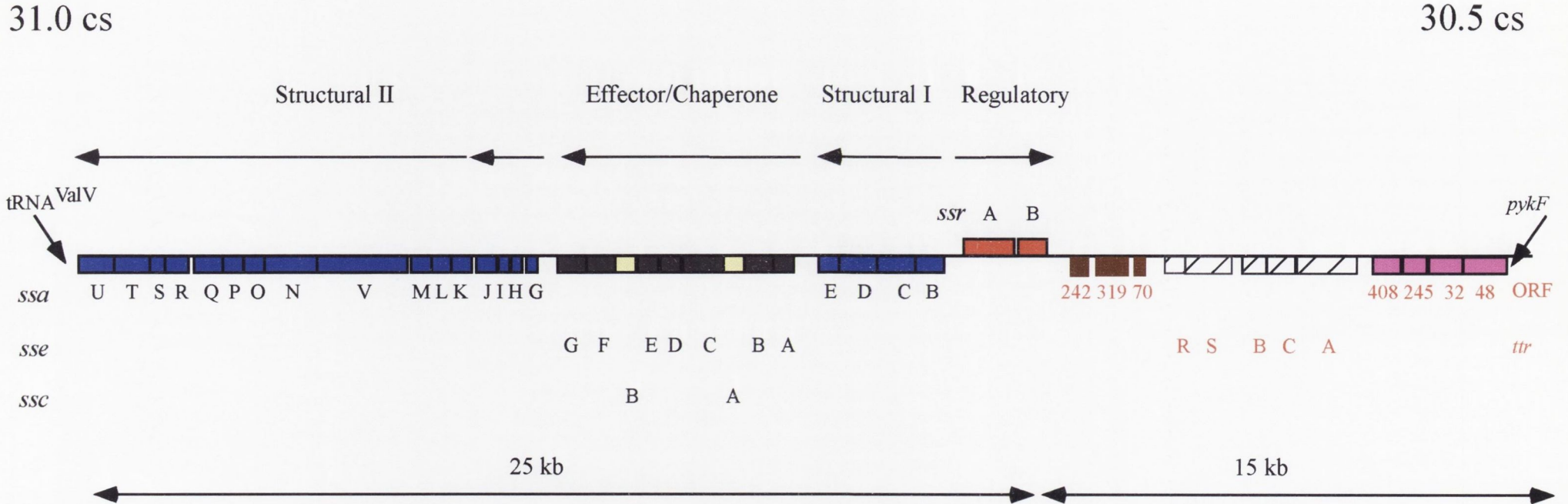
Fig. 1.5. Genetic structure and organisation of *Salmonella* pathogenicity island-2 (SPI-2). The 25 kb virulence region of SPI-2 includes SPI-2 genes encoding the type III secretion apparatus (*ssa*, blue boxes), regulatory proteins (*ssr*, red boxes), effector proteins (*sse*, grey boxes) and chaperones (*ssc*, yellow boxes). Horizontal arrows indicate the proposed transcriptional units. The 15 kb region of SPI-2 encoding genes of unknown function (brown and pink boxes) and the genes encoding the tetrathionate reductase (hatched boxes) are indicated.

the wild-type regardless of whether they are infected by the oral, intraperitoneal or intravenous routes (Hensel *et al.*, 1995; Ochman *et al.*, 1996; Shea *et al.*, 1996). In contrast SPI-1 mutants are attenuated for virulence when administered orally to mice but are fully virulent when administered intraperitoneally (Shea *et al.*, 1996). SPI-1 is an older genetic acquisition and confers the ability to invade the intestinal barrier and invoke the inflammatory response. Following the discovery of SPI-1 a signature-tagged mutagenesis experiment revealed *S. typhimurium* mutants attenuated for virulence when administered intraperitoneally to mice (Hensel *et al.*, 1995; Shea *et al.*, 1996). These mutant genes were related to genes that encode type III secretion systems (Shea *et al.*, 1996). Subsequent mapping of theses genes revealed they were clustered on a pathogenicity island distinct to SPI-1, which given the name SPI-2 (Shea *et al.*, 1996). Also, SPI-2 genes were discovered independently when a region of *S. typhimurium* not present in *E. coli* was sequenced (Ochman *et al.*, 1996).

SPI-2 is divided into two genetic elements, a 25 kb region and a 15 kb region acquired independently by horizontal gene transfer (Fig. 1.5), (Hensel *et al.*, 1999b). The 15 kb region of SPI-2 is present in *S. enterica* species and *S. bongori* species but the 25 kb region is only present in *S. enterica* species (Hensel *et al.*, 1999b). Given that *S. bongori* is the phylogenetically older species of salmonellae, the 15 kb region is considered a more ancient insertion than the 25 kb region (Hensel *et al.*, 1999b). It is proposed that the tRNA^{ValV} gene of a common ancestor of the *Salmonella* species served as a targeting region for the insertion of the 15 kb portion of SPI-2 and subsequently for the insertion of the 25 kb portion of SPI-2 (Hensel *et al.*, 1999b).

The 25 kb region is required for the virulence functions of SPI-2 and is flanked by the tRNA^{ValV} gene at 31 cs and *ssrB* (Fig. 1.5), (Hensel *et al.*, 1999b). The smaller 15 kb region of SPI-2 is flanked by ORF 242 and *pykF* at 30.5 cs and is not required for virulence (Fig. 1.5), (Hensel *et al.*, 1999b). The 15 kb region harbours a cluster of *ttr* genes involved in anaerobic tetrathionate reduction and also harbours seven open reading frames (ORFs) of unknown function (Fig. 1.5), (Hensel *et al.*, 1999a; Hensel *et al.*, 1999b).

Within the 25 kb region of SPI-2 are the genes encoding a type III secretion system as well as regulatory, chaperone and effector proteins (Fig. 1.3), (Cirillo *et al.*, 1998; Hensel *et al.*, 1998; Ochman *et al.*, 1996; Shea *et al.*, 1996). Here, 31 genes are organized into four operons termed regulatory, structural I, structural II and effector/chaperone (Fig. 1.5), (Cirillo *et al.*, 1998; Hensel *et al.*, 1998; Shea *et al.*, 1996). Nomenclature has been proposed for SPI-2 virulence genes according to their general function based on sequence homologies to known type III secretion systems (Hensel *et al.*, 1997b). Genes encoding components of the type III secretion apparatus are designated *ssa* (<u>s</u>ecretion <u>s</u>ystem <u>a</u>pparatus), genes encoding type III substrate proteins and their specific chaperones are designated *sse* (<u>s</u>ecretion <u>s</u>ystem <u>e</u>ffector) and *ssc* (<u>s</u>ecretion <u>s</u>ystem <u>c</u>haperone), respectively and genes encoding regulatory proteins are termed *ssr* (<u>s</u>ecretion <u>s</u>ystem <u>r</u>egulator). Subsequent analyses of SPI-2 genes revealed that some have been incorrectly named. SseA was initially described as a putative effector protein (Hensel *et al.*, 1998) and was revealed in a later investigation to be a chaperone of the translocon components SseB and SseD (Ruiz-Albert *et al.*, 2003; Zurawski and Stein, 2003). SsaB was also incorrectly described as a member of the type III secretion apparatus and was renamed SpiC. SpiC inhibits phagosome-lysosome fusion and interferes with intracellular trafficking (Uchiya *et al.*, 1999).

There have been conflicting reports about the location of the transcriptional start sites within the structural II operon (Fig. 1.5). One report organizes the genes *ssaK, L. M, V, N, O, P, Q, R, S, T, U* into a single operon and describes the *ssaJ* gene directly upstream of *ssaK* as being the terminal gene of another operon (Hensel *et al.*, 1997b). Another report found a promoter upstream of *ssaH* and proposed it to drive the transcription of all the genes downstream (Cirillo *et al.*, 1998). However this study placed the promoter upstream of *ssaH* and downstream of *sseG* and did not consider the *ssaG* gene, which lies between *sseG* and *ssaH*. Despite the conflicting reports it is clear that more than one promoter is involved in the transcription of the structural II region of SPI-2.

SPI-2 gene expression is not dependent on an intact secretion apparatus unlike the regulation of the flagellar regulon of *S. typhimurium* where flagellar gene expression is linked to assembly (Cirillo *et al.*, 1998). SPI-2 genes encoding structural, regulatory,

effector and chaperone proteins are preferentially expressed in the intracellular environment of the macrophage (Cirillo *et al.*, 1998; Valdivia and Falkow, 1997) and SPI-2 is required for replication inside macrophages (Cirillo *et al.*, 1998; Hensel *et al.*, 1998). The SsrA/SsrB two-component signal transduction system encoded on SPI-2 by the *ssrA* and *ssrB* regulatory genes is required for the activation of the SPI-2 type III secretion apparatus, chaperones and effector proteins and in addition also activates a regulon outside of SPI-2, including at least five effectors proteins that are secreted by the SPI-2 type III secretion apparatus (*sspH2*, *sseI*, *sseJ*, *sifA* and *sifB*) (Beuzon *et al.*, 2000; Miao and Miller, 2000; Worley *et al.*, 2000).

Conflicting reports have been published about what environmental signals are responsible for the activation of SPI-2 gene expression. SPI-2 gene expression was greatly diminished in macrophages where vacuolar acidification was blocked (Cirillo *et al.*, 1998), in good correlation with the finding that low pH activated the expression of SPI-2 genes (Lee *et al.*, 2000) and that inhibition of vacuolar acidification decreased the survival of *S. typhimurium* in macrophages (Rathman *et al.*, 1996). Reports that are in disagreement with this find that acidic pH does not induce SPI-2 gene expression (Deiwick *et al.*, 1999; Hensel, 2000; Miao et al., 2002). However, the secretion of the SseB effector protein was rapidly induced upon a shift in the pH of the bacterial medium to an acidic pH (Beuzon *et al.*, 1999). Therefore acidic pH may or may not induce SPI-2 gene expression but it appears to be a trigger for the secretion of SPI-2 effector proteins. In a study that found acidic pH did not affect transcription of the SsrA/SsrB regulon, alkaline pH was reported to inhibit transcription of the SsrA/SsrB regulon (Miao *et al.*, 2002). The induction of SPI-2 gene expression by magnesium deprivation and phosphate starvation has been reported (Deiwick *et al.*, 1999) but since another group disagrees entirely (Miao *et al.*, 2002), the influence of these environmental cues on SPI-2 gene expression is unclear. The regulation of SPI-2 by PhoP/PhoQ is also subject to much controversy since there are reports of decreased SPI-2 gene expression in a *phoP* mutant background (Deiwick *et al.*, 1999; Lee *et al.*, 2000; Worley *et al.*, 2000) while more recent reports show SPI-2 gene expression is not regulated by PhoP/PhoQ (Beuzon *et al.*, 2001; Miao *et al.*, 2002).

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Besides SsrA/SsrB the only other known regulator of SPI-2 is another two component regulatory system OmpR/EnvZ. This is a two-component signal transduction system largely studied in *E. coli*, where it was discovered to respond to changes in osmolarity and to regulate the expression of the major outer membrane porins OmpF and OmpC (Alphen and Lugtenberg, 1977). *S. typhimurium* also has the OmpR/EnvZ two-component regulatory system and a *S. typhimurium ompR* mutant was found to have profoundly attenuated virulence (Dorman *et al.*, 1989). EnvZ is the sensor protein, which detects changes in osmolarity that are passed on to OmpR, the response regulator by phosphorelay (Forst and Roberts, 1994). OmpR was found to positively regulate the two-component regulatory system SsrA/SsrB by binding directly upstream of the *ssrA* promoter (Lee *et al.*, 2000). In a more recent study phosphorylated OmpR was shown to bind five sites, one upstream and four downstream of the *ssrA* transcriptional start site and the OmpR/EnvZ regulation of *ssrA* that was previously described was confirmed (Feng *et al.*, 2003). The discovery of a transcriptional start site upstream of *ssrB* suggested that transcription of *ssrA* and *ssrB* is not linked (Feng *et al.*, 2003). Three phosphorylated OmpR binding sites were revealed downstream of the *ssrB* promoter and *ssrB* was also shown to be OmpR/EnvZ regulated (Feng *et al.*, 2003). Therefore, OmpR, the response regulator of a two-component regulatory system, was found to activate separately the transcription of the sensor kinase (*ssrA*) and the response regulator (*ssrB*) of another two component regulatory system.

In a study to investigate the effect of the environmental signals, low Ca^{2+}, osmolarity and pH on SPI-2 gene expression it was elucidated that the increase in SPI-2 gene expression in response to these environmental cues is mediated mainly through SsrA/SsrB and any effect on SPI-2 gene expression mediated through OmpR/EnvZ requires a functional SsrA/SsrB (Garmendia *et al.*, 2003). This is in good correlation with the studies that described OmpR and phospho-OmpR binding sites at *ssrA* and *ssrB* regulatory regions (Feng *et al.*, 2003; Lee *et al.*, 2000).

The principal role of SPI-2 is to facilitate the replication of intracellular bacteria within membrane-bound *Salmonella* containing vacuoles (SCVs). Effectors within and outside

of SPI-2 encode the ability to avoid NADPH oxidase-dependent killing (Vazquez-Torres *et al.*, 2000), inhibit fusion between lysosomes and SCVs (Uchiya *et al.*, 1999), induce a delayed apoptosis-like host cell death (van der Velden *et al.*, 2000), control SCV membrane dynamics (Beuzon *et al.*, 2000) (Ruiz-Albert *et al.*, 2002), assemble a meshwork of F-actin around the SCV (Meresse *et al.*, 2001), accumulate cholesterol around the SCV (Catron *et al.*, 2002) and interfere with the localization of inducible nitric oxide synthase to the SCV (Chakravortty *et al.*, 2002). Such events that allow *S. typhimurium* to prevail and proliferate in intracellular compartments such as the macrophage were discovered by studying SPI-2 knockout mutants and a variety of mutations in effector genes. This also resulted in some cases in revealing the specific effector protein that is responsible for a particular function.

The first SPI-2 encoded effector protein to be identified and characterized was SpiC (originally named *ssaB*) (Uchiya *et al.*, 1999). Mutation of *spiC* resulted in a greater number of SCVs undergoing fusion with endosomes and lysosomes (Uchiya *et al.*, 1999). *spiC* mutants were also defective in their ability to survive in the macrophage and displayed reduced virulence compared to the wild-type (Uchiya *et al.*, 1999). The host cell protein TassC was proposed to be a target for SpiC and when TassC expression was abolished from host cells this promoted the survival of *spiC* mutants in macrophages (Lee *et al.*, 2002). Recent studies however have revealed that SpiC is necessary for the translocation of SPI-2 effectors in macrophages and necessary for the *in vitro* secretion of the SPI-2 translocon proteins SseB, SseC and SseD (Freeman *et al.*, 2002; Yu *et al.*, 2002). It is now thought that a *spiC* mutant does not reflect just the loss of SpiC protein but the loss of all SPI-2 effector proteins since a functional translocon is required for the transport of all effector proteins. A *spiC* mutant is also defective in its ability to produce Sifs (*Salmonella*-induced filaments) (Guy *et al.*, 2000). The production of these tubular extensions of the SCV, called Sifs, is dependent on the effector protein SifA (Stein *et al.*, 1996), and since a *spiC* mutant cannot deliver SifA it therefore cannot produce Sifs either. It is possible the phenotype observed by Uchiya *et al* (1999) is a result of the *spiC* mutant being defective in the translocation of all effector proteins. However, perhaps it is also possible that SpiC has a dual function, as

an effector, inhibiting the fusion of SCVs with endosomes and lysosomes and also functions to promote effector secretion.

SseB, SseC and SseD are SPI-2 encoded proteins that function as a translocon (Klein and Jones, 2001; Nikolaus *et al.*, 2001). Although these proteins are secreted they are not effectors as they assemble into a translocator complex on the bacterial surface, where they remain associated and function in the translocation of effector proteins from the cytosol of *S. typhimurium* to the host cell cytosol (Klein and Jones, 2001; Nikolaus *et al.*, 2001). Recently it was revealed that SseA (initially described as a putative effector protein) is a chaperone for SseB and SseD (Ruiz-Albert *et al.*, 2003).

Secretion of SseG and SseF through the SPI-2 type III secretion system has been reported (Hansen-Wester *et al.*, 2002). SseG and SseF are SPI-2 encoded effectors that are translocated to the SCV membrane, Sifs and other endosomal compartments (Kuhle and Hensel, 2002). Their role inside the host cell is not fully understood however, they have been shown to be involved in Sif production (Guy *et al.*, 2000; Kuhle and Hensel, 2002). Sifs are extensions of the SCV (Garcia-del Portillo *et al.*, 1993).

Several effectors translocated to the host cell cytosol via the SPI-2 type III secretion apparatus are located outside of SPI-2 (Waterman and Holden, 2003). SspH1 and SspH2 proteins are not encoded by SPI-2, contain leucine-rich repeats, are secreted by the SPI-2 type III secretion system and are important for virulence of *S. typhimurium* in calves (Miao *et al.*, 1999). Another SPI-2 effector protein not encoded on SPI-2 is SlrP, which also contains leucine-rich repeats and is important for virulence in mice (Tsolis *et al.*, 1999b). On the basis of similarity to SspH1, SspH2 and SlrP amino termini, four other effector proteins, SifA, SseI, SseJ and SifB were identified (Miao and Miller, 2000). These seven effectors have similar translocation signal domains that target them for translocation across the phagosome membrane by the SPI-2 type III secretory apparatus (Miao and Miller, 2000). SspH1 and SlrP are also translocated by the SPI-1 type III secretory system during *S. typhimurium* invasion (Miao and Miller, 2000). SspH1 and SlrP are also different to the other effectors as their expression is not

dependent on SsrA/SsrB (Miao and Miller, 2000). Despite the fact they are also translocated by the SPI-1 type III secretory system, they are not regulated by HilA either, which suggests that these two effectors are constitutively expressed (Miao and Miller, 2000). Constitutive expression of SspH1 and SlrP is perhaps the way the bacterial cell ensures a supply of these effector proteins under both SPI-1 and SPI-2 inducing environments. The genes encoding these seven effector proteins were all acquired by horizontal gene transfer at different times or from different sources (Miao and Miller, 2000). The genes *sseI*, *sspH1* and *sspH2* are located within lysogenic bacteriophages and this may represent a common mechanism for the dissemination of *S. typhimurium* effectors (Figueroa-Bossi and Bossi, 1999; Figueroa-Bossi *et al.*, 2001; Miao and Miller, 2000). Gifsy-2 and Gifsy-3 prophages are competent for lytic growth and lysogeny of *Salmonella* naive strains and harbour *sseI* and *sspH1*, respectively (Figueroa-Bossi *et al.*, 2001; Miao and Miller, 2000). It is proposed the 25 kb virulence portion of SPI-2 was acquired by one genetic horizontal transmission event, which conferred virulence and a selective advantage to the bacteria and so was retained in the population. Similarly it is proposed that the acquisition of the effector proteins located outside of SPI-2 would have promoted virulence and a selective advantage (Waterman and Holden, 2003). Other genes located outside of SPI-2 have been identified that are SsrA/SsrB regulated but their functions are unknown and it has yet to be determined if they are SPI-2 effectors (Worley *et al.*, 2000). However, these genes are located in regions that contain bacteriophage-like genes, which supports the idea that integrating prophages are a mechanism of supplying and spreading *Salmonella* virulence determinants (Figueroa-Bossi *et al.*, 2001; Worley *et al.*, 2000).

1.1.6 SPI-3, SPI-4 and SPI-5

SPI-3, SPI-4 and SPI-5 (Fig. 1.6) are the three remaining *Salmonella* pathogenicity islands, which are not as well characterized as SPI-1 and SPI-2 but like SPI-1 and SPI-2 they were also probably horizontally acquired. SPI-3 is 17 kb in size and is inserted

downstream of the *selC*tRNA gene at 82 centisomes on the *S. typhimurium* chromosome (Fig. 1.6), (Blanc-Potard and Groisman, 1997). Two of the SPI-3 open reading frames (ORFs) include the operon *mgtCB*. The *mgtCB* operon encodes a high affinity magnesium transporter required for growth under conditions of limiting Mg^{2+}. Transcription of the *mgtCB* operon is induced under conditions of low Mg^{2+} by the PhoP/PhoQ two-component signal transduction system (Blanc-Potard and Groisman, 1997; Marcus *et al.*, 2000). The *mgtC* gene is required for intramacrophage survival and for *S. typhimurium* virulence in mice (Blanc-Potard and Groisman, 1997; Blanc-Potard *et al.*, 1999). None of the other SPI-3 genes are PhoP-regulated nor are they required for survival in macrophage or for invasion of epithelial cells. The function of these remaining genes is unknown but since their distribution varies between serovars of *Salmonella* it is postulated they contribute to host specificity or chronic infection (Marcus *et al.*, 2000).

SPI-4 is a 25 kb insertion flanked by putative tRNA genes at 92 centisomes on the *S. typhimurium* chromosome (Fig. 1.6), (Wong *et al.*, 1998). There is very little known about SPI-4 however it is thought the open reading frames (ORFs) it harbours might constitute a single operon. 3 open reading frames (ORFs) based on sequence analysis are proposed to encode a type I protein secretion system that mediates toxin secretion, however there is no experimental evidence to support this hypothesis (Wong *et al.*, 1998). A transposon insertion in an open reading frame (ORF) termed ORF-L resulted in a defect in intramacrophage survival, indicating ORF-L or a downstream gene in SPI-4 is responsible for this phenotype (Baumler *et al.*, 1994).

SPI-5 is a small 7 kb island inserted at the *serT*tRNA gene at 25 centisomes on the *S. typhimurium* chromosome (Fig. 1.6), (Hong and Miller, 1998). This pathogenicity island is also detected in the serovars Dublin, Enteritidis, Choleraesuis, Galinarum and Pullorum. SPI-5 is involved in the infection of epithelial cells of the small intestine but not in systemic disease (Wood *et al.*, 1998). Experiments suggest that SPI-5 encodes effector proteins targeted to the SPI-1 type III secretion system, which is consistent with a role for SPI-5 in infection of the intestinal barrier (Galyov *et al.*, 1997). The secretion

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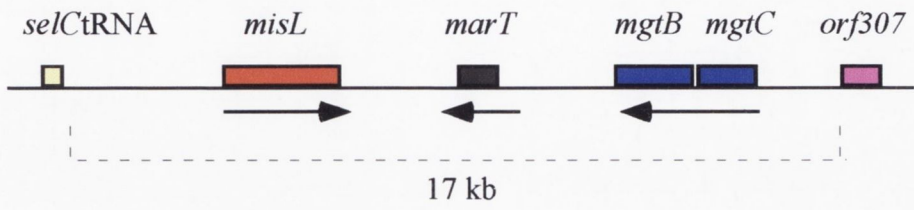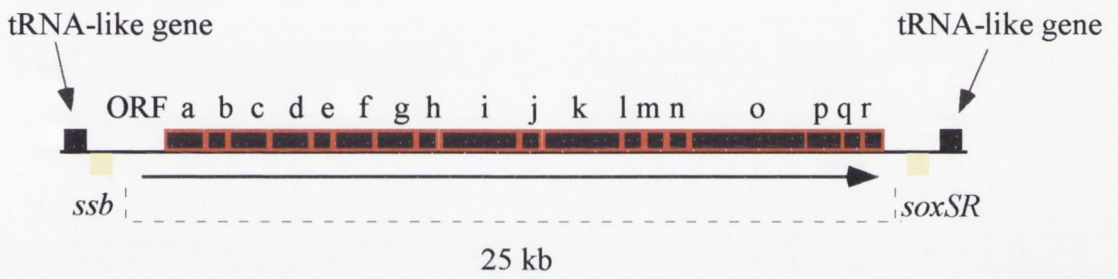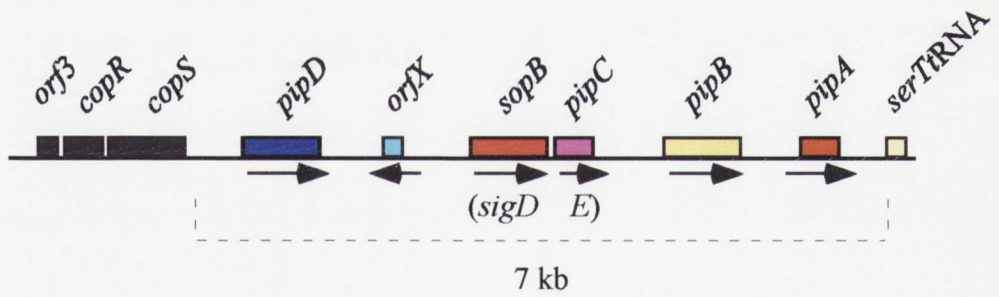
Fig. 1 6. Schematic representation of *Salmonella* pathogenicity island-3 (SPI-3), *Salmonella* pathogenicity island-4 (SPI-4) and *Salmonella* pathogenicity island-5 (SPI-5).

of these effector proteins is reported to stimulate inflammatory responses and fluid secretion from the host cells (Darwin and Miller, 1999a). For example, the PipC/SigE and SopB/SigD proteins encoded within SPI-5 are secreted by the SPI-1 secretion apparatus and are associated with the invasion phenotype (Hong and Miller, 1998). The invasive ability of *S. typhimurium* was reduced 10-fold when the SPI-5 encoded *pipC* gene that encodes the PipC/SigE protein was mutated (Hong and Miller, 1998).

1.1.7 The *S. typhimurium* virulence plasmid

All pathogenic non-typhoidal serovars of *Salmonella* (including *S. typhimurium*) harbour a large virulence plasmid required for the bacteria to cause systemic infection (Fig. 1.2), (Gulig, 1990). The size of the virulence plasmid varies between 50-100 kb depending on the serovar it is isolated from and it is estimated to be present at 2.75 copies per cell (Chu *et al.*, 1999; McClelland *et al.*, 2001). In the case of *S. typhimurium* the virulence plasmid is 90 kb in size and contributes to systemic infection in the mouse by increasing the growth rate of the bacteria inside host cells (Fig. 1.1), (Gulig and Doyle, 1993). Although the size of the plasmid may vary a region of 8 kb is highly conserved. This 8 kb region encodes five genes *spvRABCD* (designated <u>S</u>almonella <u>p</u>lasmid <u>v</u>irulence (*spv*) genes) and is sufficient to restore virulence to plasmid-cured strains (Fig. 1.7), (Gulig *et al.*, 1993).

The *spvR* gene is transcribed from its own promoter, P$_{spvR}$ and the *spvABCD* operon is transcribed from the *spvA* promoter, P$_{spvA}$ (Fig. 1.7). The *spvR* gene encodes a transcriptional activator of the LysR family, which activates the transcription from both P$_{spvR}$ and P$_{spvA}$ promoters. SpvR has been shown to bind two sites along the *spvA* promoter in a hierarchical fashion, with binding to a proximal site being dependent on binding to a more distal site (Sheehan and Dorman, 1998). SpvB is a mono(ADP-ribosyl)transferase that ADP ribosylates actin and inhibits the polymerization of G actin to F actin thereby destabilizing the cytoskeleton of the host cell (Lesnick *et al.*, 2001;
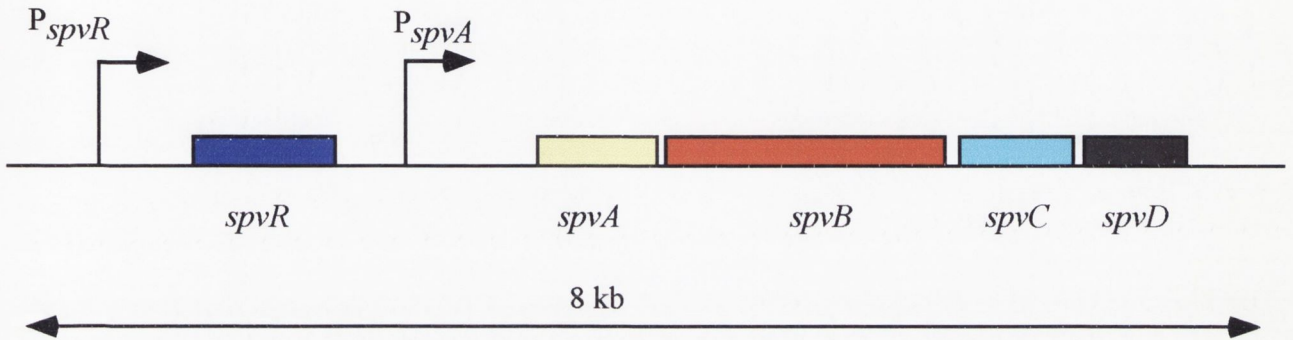
Fig 1.7. Genetic organisation of the *Salmonella* plasmid virulence (*spv*) genes.

Non-typhoidal *Salmonella* serovars harbour a large virulence plasmid. The size of the plasmid varies depending on the serovar. However within all plasmids is a highly conserved 8 kb five gene cluster designated *spvRABCD*. The *spvR* gene encodes a member of the LysR family of transcriptional activators and is transcribed from the P_{spvR} promoter. The *spvA*, *spvB*, *spvC* and *spvD* genes are transcribed as an operon from the P_{spvA} promoter. SpvB is a mono(ADP-ribosyl)transferase.

Otto *et al.*, 2000; Tezcan-Merdol *et al.*, 2001). Although the functions of SpvC and SpvD are unknown they are known to play a role in virulence as it was demonstrated that SpvC along with SpvB restore partial virulence to plasmid-cured strains of *S. typhimurium* (Matsui *et al.*, 2001).

The expression of the *spv* genes is induced in cultures during stationary phase of growth in LB broth, in mice and following phagocytosis of *S. typhimurium* by macrophages (Heithoff *et al.*, 1997; Marshall *et al.*, 2000; Rhen *et al.*, 1993). The stationary phase sigma factor RpoS is required for the full expression of the *spv* genes and it too is also upregulated in epithelial cells and macrophages (Chen *et al.*, 1995; Chen *et al.*, 1996). In addition to SpvR and RpoS the expression of the *spv* genes is also regulated by H-NS, IHF, Lrp and CRP. The nucleoid-associated protein H-NS and the cAMP receptor protein CRP have both been shown to repress the expression of the *spv* genes in stationary phase, where the CRP repression is mediated through RpoS and the H-NS repression is not (O'Byrne and Dorman, 1994a, b). The leucine-responsive regulatory protein Lrp negatively regulates the expression of the *spvABCD* structural operon by binding to the *spvA* promoter (Marshall *et al.*, 1999). The nucleoid-associated protein IHF has a negative influence on *spvR* gene expression and influences the response of the *spv* genes to alterations in the superhelicity of the DNA (Marshall *et al.*, 1999). Consistent with this, the *spv* promoters were shown to be supercoiling sensitive, where increasing the level of negative supercoiling downregulates the *spv* genes (O'Byrne and Dorman, 1994a). Some other loci on the plasmid outside of the highly conserved 8 kb region include a fimbrial operon, designated *pef,* a conjugal transfer gene *traT*, *rck* and *rsk* loci all of which may play a role in other stages of the infection process (Rotger and Casadesus, 1999).

1.1.8 The PhoP/PhoQ two-component regulatory system

Two-component signal transduction systems are an important mechanism used by *S. typhimurium* to sense the environment and respond appropriately. These systems are composed of a pair of proteins, one is a sensor kinase frequently associated with the cytoplasmic membrane and the other is a response regulator that can bind DNA. The sensor kinase is phosphorylated at a histidine residue within a conserved domain upon receipt of a specific environmental signal and it transmits the phosphate group to an aspartate residue in a conserved receiver domain in the response regulator. Typically phosphorylation of the response regulator activates it whereby it will bind specific sequences of DNA to modulate the transcription of specific sets of genes to respond to the original environmental stimulus that instigated this chain of events (Miller *et al.*, 1989; Perraud *et al.*, 1999). Over 40 genes in *S. typhimurium* are regulated by the two-component signal transduction system PhoP/PhoQ (Groisman *et al.*, 1989; Miller *et al.*, 1989). PhoQ is the sensor kinase sensing Mg^{2+}, Mn^{2+} and Ca^{2+} and PhoP is the response regulator in this two-component regulatory system (Lejona *et al.*, 2003). The PhoP/PhoQ system activates *pag* genes (<u>P</u>ho<u>P</u> <u>a</u>ctivated <u>g</u>enes) and represses *prg* genes (<u>P</u>ho<u>P</u> <u>r</u>epressed <u>g</u>enes). *phoP* or *phoQ* mutants are severely attenuated for virulence because they are incapable of intramacrophage survival (Fields *et al.*, 1986; Miller *et al.*, 1989). These mutants also display increased susceptibility to antimicrobial peptides, bile salts and pH (Bearson *et al.*, 1998; Fields *et al.*, 1989; Miller *et al.*, 1990; van Velkinburgh and Gunn, 1999). However, it is not certain if PhoP/PhoQ is an activator of the SPI-2 type III secretion system following *S. typhimurium* phagocytosis by macrophages and it is an issue of much debate (Beuzon *et al.*, 2001; Deiwick *et al.*, 1999; Miao *et al.*, 2002). It is certain that PhoP/PhoQ is induced inside macrophages and many *pag* genes switched on inside macrophages are required by *S. typhimurium* for intracellular survival (Blanc-Potard and Groisman, 1997). It is thought that Mg^{2+} is the environmental signal that PhoP/PhoQ responds to when *S. typhimurium* are within the macrophage and from within the macrophage PhoP/PhoQ is responsible for repressing SPI-1 gene expression (Bajaj *et al.*, 1996; Pegues *et al.*, 1995). It is also

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indicated that this two-component regulatory system negatively influences the expression of flagellar genes either directly or indirectly (Adams *et al.*, 2001). Interestingly the PhoP/PhoQ system is yet another example of how the SPI-1 and flagellar genes are coordinately expressed.

1.1.9 Nucleoid-associated proteins

The condensation of the eukaryotic chromosomes is different to that of the prokaryotic chromosome (Bendich and Drlica, 2000). Both are similar however in that they both employ proteins to package and compact the DNA. These proteins are termed nucleoid-associated proteins in prokaryotes and histone proteins in eukaryotes. The nucleoid-associated proteins are grouped together due to functional similarity alone (Azam and Ishihama, 1999). The *S. typhimurium* chromosome is a circular DNA molecule consisting of 4.8 million base pairs (McClelland *et al.*, 2001). Where linearized the chromosome measures approximately 1.5 mm in length while the *S. typhimurium* cell is a 1 μm X 0.5 μm cylinder of whose volume the nucleoid (region that contains the DNA genome and its associated proteins) occupies approximately half (Azam *et al.*, 1999). Compaction of the bacterial chromosome is not a job for one protein alone but instead it is the concerted effort of all the nucleoid-associated proteins where the absence of one nucleoid-associated protein is compensated for by the other members of the group (Trun and Marko, 1998). However more recently the individual mechanisms used by the nucleoid-associated proteins H-NS, IHF and Fis in modulating the conformation of the genome as a whole has been better established (Dame *et al.*, 2000; Holbrook *et al.*, 2001; Schneider *et al.*, 2001). Some of this compaction is due negative supercoiling of the bacterial DNA (Stuger *et al.*, 2002). The chromosome is carefully compacted into a conformation that still allows DNA transactions such as the transcription of genes to occur. The four major and best-characterized nucleoid-associated proteins in *S. typhimurium* are Fis (factor for inversion stimulation), H-NS (histone-like nucleoid

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structuring protein), IHF (integration host factor) and HU (heat-unstable nucleoid protein).

IHF and Fis bind regions of the DNA containing a consensus sequence. IHF displays great specificity in DNA binding whereas Fis binds to a degenerate consensus binding sequence (Dorman and Deighan, 2003; Finkel and Johnson, 1992; Goosen and van de Putte, 1995). The consensus IHF binding sequence is WATCAANNNNTTR while the Fis consensus is more degenerate KNNYRNNWNNYRNNM (W=A/T, R=A/G, Y=C/T, K=G/T, M=A/C, N=A/T/G/C). While HU and H-NS are described as binding non-specifically to the DNA, HU is attracted to DNA containing structural aberrations and H-NS displays a preference for binding sites of intrinsic curvature (Azam and Ishihama, 1999; Dorman and Deighan, 2003; Dorman, 2004). The relative abundance of these nucleoid-associated proteins varies greatly during the growth of *S. typhimurium* in batch culture (Azam *et al.*, 1999). Fis protein is optimally expressed at the end of lag phase but its levels decline rapidly thereafter. In contrast, IHF protein levels are maximal as cells enter stationary phase and the concentration of the H-NS protein is maintained generally constant throughout growth. The *hupA* and *hupB* genes encode the subunits of the HU protein and although its subunit composition varies with growth the overall concentration of HU decreases as cells enter stationary phase (Balandina *et al.*, 2001). Therefore different stages of growth represent the abundance of some nucleoid-associated proteins and the near absence of others.

As well as organizing the genetic material within the bacterial nucleoid the nucleoid-associated proteins are also involved in transcription (Dorman and Deighan, 2003). The nucleoid-associated proteins are often described as global regulators as they each influence the transcription of a wide range of genes. The mechanisms by which they contribute to transcription are varied. These proteins can modulate transcription by interacting directly with the RNA polymerase, competing with the RNA polymerase, altering the DNA topology at the promoter, and by interacting with other nucleoid-associated proteins or regulatory proteins at promoter regions in a cooperative or antagonistic fashion. One mechanism by which H-NS represses transcription involves

H-NS binding to more than one site of intrinsic curvature at the promoter region. H-NS binding at these phased sites results in a loop formation of the DNA bringing the H-NS binding sites together and trapping the RNA polymerase (Dorman and Deighan, 2003; Dorman, 2004; Schroder and Wagner, 2000). Fis binding to sites that overlap with H-NS binding sites can disrupt H-NS repression (Falconi et al., 1996; Falconi et al., 2001). Due to their ability to bind and alter the structure of the bacterial DNA, the nucleoid-associated proteins are involved in other DNA transactions such as recombination, replication and inversion (Dorman and Deighan, 2003).

1.1.10 Fis

Fis the factor for inversion stimulation was identified originally as a factor that stimulated DNA inversion at the *hin* invertible DNA element in *S. typhimurium* (Heichman and Johnson, 1990; Huber et al., 1985; Johnson and Simon, 1985; Kahmann et al., 1985). This genetic switch is part of the mechanism behind flagellar phase variation in *S. typhimurium* (Fig. 1.11), (Bonifield and Hughes, 2003; Haykinson et al., 1996; Macnab, 1996). Fis is a 98 amino acid, 11.2 kDa DNA binding protein that can bend the DNA between 40° and 90° (Hengen et al., 1997). Fis binding sites have a degenerate consensus sequence and are often located within regions of intrinsic curvature (Hengen et al., 1997). The proposed core consensus sequence is 5'-KNNYRNNWNNYRNNM-3' (Finkel and Johnson, 1992; Hubner and Arber, 1989). Fis binds the DNA as a homodimer and the crystal structure of the wild-type Fis protein revealed each monomer consists of four α helices, A-D, but disorder in the N-terminal amino acids prevented resolution of this domain (Kostrewa et al., 1991; Yuan et al., 1991). A K36E Fis mutant with an ordered N-terminal domain was also crystallized and two β-sheets in this region were revealed (Safo et al., 1997). The two N-terminal β-sheets are involved in activation of DNA inversion reactions catalysed by the Hin and Gin recombinases while the C-terminal α helices C and D harbouring the helix-turn-helix (HTH) domain are involved in DNA binding and bending (Koch et al., 1991;

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Kostrewa *et al.*, 1991; Osuna *et al.*, 1991; Safo *et al.*, 1997). The α helices of the monomers are tightly intertwined to form a globular dimer with two protruding HTH motifs (Kostrewa *et al.*, 1991; Yuan *et al.*, 1991) (Safo *et al.*, 1997). The HTH domains of the Fis dimer are composed of the two α helices C and D in each monomer and it is the D helices ('recognition' helices) of the dimer that are separated by 25 Å instead of the usual 32-34 Å (Finkel and Johnson, 1992; Kostrewa *et al.*, 1991; Yuan *et al.*, 1991). This distance is too short to allow insertion into adjacent major grooves of the DNA. Since Fis is known to bend DNA upon binding, models of DNA binding show Fis bound to bent DNA in order to bring the major grooves closer together (Finkel and Johnson, 1992; Kostrewa *et al.*, 1991; Yuan *et al.*, 1991).

The gene that encodes Fis is part of an operon with one open reading frame (ORF) of unknown function (*yhdG*) preceding the *fis* gene (Fig. 1.8) (Ball *et al.*, 1992; Ninnemann *et al.*, 1992). The *E. coli* Fis protein is expressed in a rapid and transient manner. When stationary phase *E. coli* cells are subcultured into rich medium, Fis protein levels increase from less than 100 to over 50,000 copies per cell, prior to the first cell division (Ball *et al.*, 1992; Nilsson *et al.*, 1992; Ninnemann *et al.*, 1992). As cells enter exponential growth nascent synthesis is largely shut off and Fis protein levels decrease as a function of cell division and become virtually undetectable during late exponential and stationary phases of growth (Ball *et al.*, 1992; Nilsson *et al.*, 1992; Ninnemann *et al.*, 1992). However, exponentially growing cells can experience another burst of Fis expression if they are shifted to a richer medium (Ball *et al.*, 1992). The sudden increase in Fis protein concentration is due to a rapid but transient increase in *fis* mRNA in response to nutrient upshift (Ball *et al.*, 1992). In addition, the *fis* mRNA expression pattern is not regulated at the level of *fis* mRNA decay (Pratt *et al.*, 1997). The magnitude of *fis* expression is related to the nutritional quality of the medium, so that higher Fis protein and mRNA levels are measured in nutritionally richer medium (Ball *et al.*, 1992; Nilsson *et al.*, 1992). Cells growing in minimal medium do not display sudden fluctuations in Fis concentrations (Nilsson *et al.*, 1992). Since Fis protein levels mirror the mRNA expression pattern it was always considered that Fis regulation occurs largely at the transcriptional level (Ball *et al.*, 1992). However it was
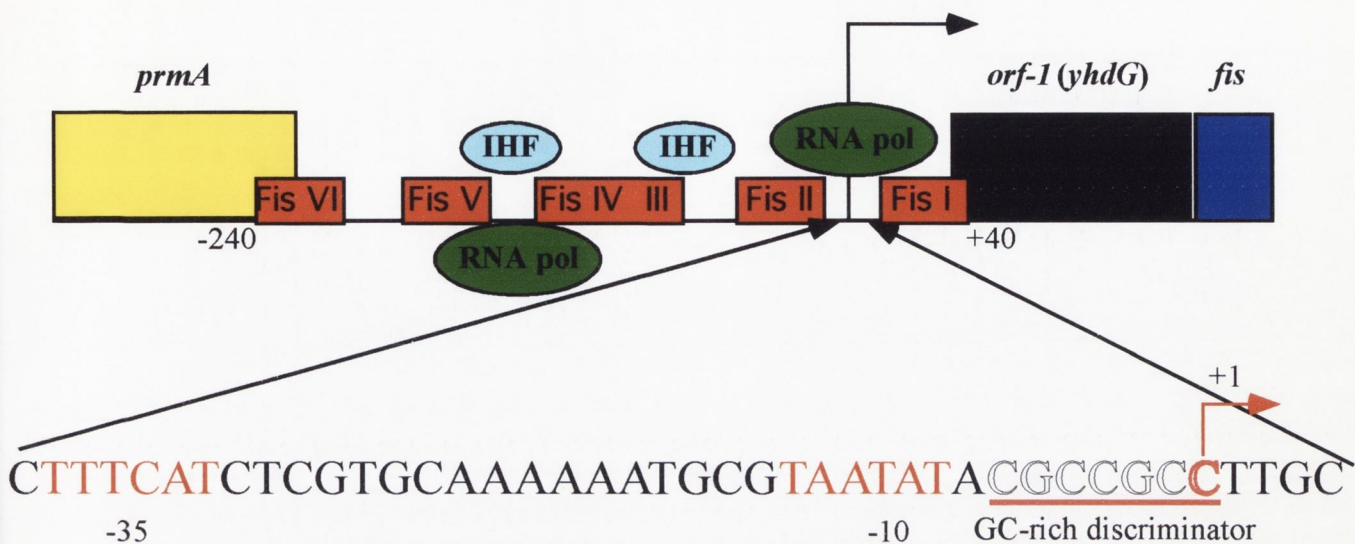
Fig. 1.8. The *fis* operon. The *fis* gene is located in an operon with one open reading frame of unknown function referred to as *yhdG* in *S. typhimurium*, preceding it. *fis* and the upstream ORF are cotranscribed. The *fis* promoter region contains six Fis binding sites that overlap two RNA polymerase binding sites, suggesting the mechanism of Fis repression may involve exclusion of RNA polymerase. IHF, a transcriptional activator of the *fis* promoter binds to two sites in the *fis* operon regulatory region. Fis and IHF exert antagonistic effects at the *fis* promoter and their binding sites overlap. The sequences TTTCAT and TAATAT are the -35 and -10 regions, respectively. The predominant transcription initiation nucleotide is +1C. A GC-rich motif is located downstream of the -10 region. The overall *fis* operon organisation in *S. typhimurium* is the same as that in *E. coli* and the Fis protein amino acid sequences are identical between the two species. The ORF upstream of *fis* is also well conserved. The *fis* promoter region is conserved between *E.coli* and *S. typhimurium* from -49 to +94, relative to the *E. coli* +1, but the sequence from -50 to -252 shows a 49% nucleotide divergence.

reported recently that *fis* is also regulated at the translational level by BipA, a translation factor with GTPase activity (Owens *et al.*, 2004). In the absence of BipA, *fis* transcript levels are abundant while Fis protein levels are severely reduced which indicates BipA is required to regulate the translation of Fis.

The Fis protein negatively regulates its own synthesis, where maximum *fis* mRNA levels are 10-fold higher in the absence of Fis (Ball *et al.*, 1992; Ninnemann *et al.*, 1992; Osuna *et al.*, 1995; Pratt *et al.*, 1997). The *fis* promoter region contains Fis binding sites that overlap RNA polymerase binding sites, suggesting the mechanism of Fis mediated repression may involve exclusion of RNA polymerase from the promoter (Fig. 1.8), (Ball *et al.*, 1992). In keeping with this model is the fact that only mutations in Fis that reduce DNA binding are defective in autoregulation (Ball *et al.*, 1992). The presence of a total of six Fis binding sites (I-VI) within the *fis* operon promoter region (Fig. 1.8) raises the possibility that Fis binding induces a highly wrapped nucleoprotein complex that contributes to repression (Ball *et al.*, 1992). However, when the *fis* promoter region is truncated to sequentially remove the Fis binding sites it is discovered that Fis site II centered at –42 plays the predominant role in autoregulation while sites I and III play only minor roles and sites IV, V and VI do not appear to contribute to autoregulation (Fig. 1.8), (Pratt *et al.*, 1997). Although Fis autoregulation controls the magnitude of *fis* mRNA levels the characteristic *fis* expression pattern can still be observed in the absence of Fis protein (Ball *et al.*, 1992; Osuna *et al.*, 1995).

In addition to growth phase regulation and autoregulation, the *fis* promoter is also strongly repressed by the stringent response (Ninnemann *et al.*, 1992). Stringent control is inhibition by signaling molecules pppGpp and ppGpp, which accumulate in the cell under conditions of metabolic and nutritional stresses such as amino acid starvation and carbon, nitrogen or phosphate limitation (Chatterji and Ojha, 2001). Synthesis of the signaling molecules is catalysed by the proteins RelA and SpoT. The growth phase regulation and stringent control of the *fis* promoter depend on the presence of a GC-rich discriminator motif downstream of the –10 region (Fig. 1.8), (Ninnemann *et al.*, 1992). A *fis* promoter containing mutations in the GC-rich motif shows significant activity in

late logarithmic phase and even in stationary phase, indicating that such a mutation abolishes growth phase regulation. Under conditions of amino acid starvation (stringent), the wild-type *fis* promoter is shut off and no detectable *fis* mRNA observed, while the mutant *fis* promoter lacking the GC-rich motif is no longer subject to stringent control and maintains *fis* mRNA production. However, a wild-type *fis* promoter maintains its expression profile in a *relAspoT* strain, indicating that growth phase regulation is not dependent on ppGpp levels (Ball *et al.*, 1992).

In an investigation to dissect further the *fis* promoter it was established that the sequences TTTCAT and TAATAT are the −35 and −10 regions respectively, optimally separated by 17 bp (Fig. 1.8), (Walker *et al.*, 1999). Mutating the GC-rich motif and the −35 region, but not the −10 region reduce the *fis* response to stringent control (Walker *et al.*, 1999). Changing the predominant transcription initiation nucleotide from +1C to the preferred A or G results in altered expression of the *fis* promoter, where high levels of *fis* mRNA are detected in late logarithmic and stationary phase (Walker *et al.*, 1999). It is implied that the acute growth phase regulation of the *fis* promoter relies on an inefficient transcription initiation process that is achieved with promoter sequences deviating from the −10 and −35 consensus sequences and, more importantly upon the availability of the least favoured transcription initiation nucleotide CTP.

Pratt *et al.* (1997) investigated what other processes affect *fis* expression and discovered that the nucleoid-associated protein, integration host factor (IHF) had a role to play. IHF stimulates transcription from the *fis* promoter by binding to a site centered at approximately −144 and also to a weak site from −79 to −49, relative to the transcriptional start site (Fig. 1.8). The *fis* promoter is stimulated 3-4 fold by IHF. While Fis and IHF exert antagonistic effects on the *fis* promoter, they are not necessary for growth phase-dependent regulation of *fis* expression (Osuna *et al.*, 1995; Pratt *et al.*, 1997). The *fis* promoter region from −36 to +7 lacks all the known Fis- and IHF-binding sites and it alone still retains the characteristic *fis* regulation profile (Fig. 1.8), (Pratt *et al.*, 1997). The *fis* gene is also regulated by the global regulator CRP, whose activity is dependent on cAMP levels (Nasser *et al.*, 2001). CRP positively regulates *fis*

gene expression in the absence of Fis but together Fis and CRP synergistically repress *fis* gene expression. Notably the activity of the *crp* promoter itself is modulated by the Fis protein where Fis is a transcriptional repressor of *crp* gene expression (Gonzalez-Gil *et al.*, 1998).

Many of the *fis*-related observations made in *E. coli* are also true for *S. typhimurium* (Keane and Dorman, 2003; Osuna *et al.*, 1995). The pattern of Fis protein and mRNA expression in *S. typhimurium* are subject to the same dramatic burst of expression in early exponential phase and rapid decline thereafter to become undetectable in stationary phase following a nutrient upshift. *S. typhimurium* Fis protein levels peak at 40,000 dimers per cell just prior to logarithmic growth (Osuna *et al.*, 1995) and Fis levels appear to be depleted slightly quicker in *E. coli* compared to *S. typhimurium* (Keane and Dorman, 2003). The *fis* promoter of *S. typhimurium* is also negatively autoregulated by Fis protein (Osuna *et al.*, 1995). The Fis binding sites within the *fis* promoter region were identified in *S. typhimurium* and compared to sites I to VI in *E. coli* (Fig. 1.8). Sites I and II are present in *S. typhimurium*, this was expected since this region of the *fis* promoter (-49 to +94) is conserved between the two species. Site IV is missing in *S. typhimurium*, site III is present but is a low affinity site and despite differences in the upstream promoter region, site V is also present in *S. typhimurium* (Osuna *et al.*, 1995). The presence of site VI in *S. typhimurium* remains to be elucidated.

The role of Fis in the cell extends beyond its involvement in DNA inversion (Finkel and Johnson, 1992; Wagner, 2000). Fis was shown to be involved in other cellular processes including bacteriophage λ DNA excision and integration (Ball and Johnson, 1991a, b; Thompson *et al.*, 1987), regulation of initiation of DNA replication at *oriC* (Filutowicz *et al.*, 1992; Gille *et al.*, 1991; Wold *et al.*, 1996), the cold shock response (Brandi *et al.*, 1999), oxidative stress (Weinstein-Fischer *et al.*, 2000), DNA supercoiling (Schneider *et al.*, 1997; Schneider *et al.*, 1999) the catabolism of sugars and nucleic acids (Gonzalez-Gil *et al.*, 1996) and the regulation of gene expression (Finkel and Johnson, 1992). As mentioned above Fis is a repressor of transcription

from its own gene (Walker *et al.*, 1999) (Ball *et al.*, 1992; Ninnemann *et al.*, 1992; Osuna *et al.*, 1995). Fis has been shown to modulate the transcription of many genes including those encoding stable RNA (rRNA and tRNA) in *E. coli*. Upstream of rRNA and tRNA operons are regions of DNA known as Upstream Activating Regions (UARs) or Upstream Activating sequences (UASs), responsible for the high levels of transcription from these operons. Located in these UARs/UASs are Fis-binding sites. Fis has been shown to activate transcription from *rrnB*, *thrU/tufB* and *tyrT* operons (Nilsson *et al.*, 1990; Ross *et al.*, 1990). Fis binds upstream of, and activates, all seven *rrn* P1 promoters (Hirvonen *et al.*, 2001). The mode of action by which Fis activates transcription from the *rrnB* P1 promoter is by stabilizing the interaction between the RNA polymerase and the *rrnB* P1 promoter (Zhi *et al.*, 2003). Fis also activates the *leuV* operon, which encodes three of the four genes for the $tRNA_1^{LEU}$ isoacceptors. A different mode of action is proposed to be responsible for the Fis activation of the *leuV* promoter. It is suggested that upon Fis binding there is a translocation of superhelical energy (negative twist) to the *leuV* promoter, resulting in an increase in open complex formation and the rate of transcription (Opel *et al.*, 2004). Fis also participates in the regulation of chromosomal DNA replication at *oriC*. Fis binds multiple sites within *oriC* (Filutowicz *et al.*, 1992). It is reported that Fis inhibits DNA replication from *oriC* (Hiasa and Marians, 1994; Wold *et al.*, 1996). The degree of Fis inhibition is proportional to the concentration of DnaA protein (the initiator of chromosomal DNA replication) such that as the concentration of DnaA increases Fis repression is relieved (Wold *et al.*, 1998). Fis and IHF carry out opposing activities at *oriC* and increased IHF binding is coincident with decreased Fis binding and the initiation of DNA synthesis (Cassler *et al.*, 1995). Therefore, a dynamic interplay exists between DnaA, IHF and Fis at *oriC*, which is responsible for DNA replication initiation being precisely timed (Ryan *et al.*, 2004).

Fis is emerging as an important regulator of virulence genes in bacterial pathogens. It positively regulates the expression of invasion genes in *Shigella flexneri* (Falconi *et al.*, 2001). Here, *virF* is the first step in a regulatory cascade that activates these virulence genes. H-NS binds two sites in the *virF* promoter region to repress *virF* transcription.

However Fis binding sites in the *virF* promoter region overlap with the H-NS binding sites and Fis binding counters H-NS inhibition (Falconi *et al.*, 2001). Enteroaggregative *E. coli* (EAEC) form thick biofilms on the intestinal mucosa (Sheikh *et al.*, 2001). The Fis protein is a transcriptional activator of *aggR* expression, which encodes the activator AggR of aggregative adherence fimbriae II (AAF/II) required for biofilm formation (Sheikh *et al.*, 2001). Enteropathogenic *E. coli* (EPEC) infection of the gut is characterized by the formation of pedestal structures called attaching and effacing (AE) lesions. These lesions are formed as a result of the coordinated expression of proteins encoded by the chromosomal locus of enterocyte effacement (LEE). Fis was found to be a transcriptional activator of the LEE4 operon and the LEE encoded virulence regulator Ler all of which are essential for AE lesion formation (Goldberg *et al.*, 2001). Also the Fis protein positively regulates the virulence genes of SPI-1 whose expression enables the *S. typhimurium* invasion of intestinal epithelial cells (Wilson *et al.*, 2001). HilA is the SPI-1 encoded activator of SPI-1 genes and Fis is required for the full induction of *hilA* expression (Schechter *et al.*, 2003; Wilson *et al.*, 2001).

1.1.11 Fis and DNA topology

With very few exceptions the DNA isolated from prokaryotes is negatively supercoiled (Drlica, 1992). The level of negative supercoiling is tightly controlled by many factors including DNA binding proteins, transcription, replication and topoisomerase enzymes (Drlica, 1992; Hatfield and Benham, 2002). DNA gyrase introduces negative supercoils into the DNA in an ATP dependent fashion (Fig. 1.9), (Drlica, 1992; Steck *et al.*, 1993). DNA topoisomerase I relaxes negatively supercoiled DNA, it does not require ATP to do this because it can avail of the stored energy in the negatively supercoiled DNA to drive the DNA relaxation reaction (Fig. 1.9), (Drlica, 1992; Steck *et al.*, 1993). Topoisomerase IV can also remove negative supercoils from the DNA but its primary function is resolving DNA knots and catenanes by a process requiring ATP (Deibler *et al.*, 2001). DNA gyrase has an A_2B_2 tetrameric structure, and the A and B subunits are

Fig. 1.9. The homeostatic control mechanism of DNA supercoiling. DNA gyrase is composed of two subunits, GyrA and GyrB, and has an A_2B_2 tetrameric structure.

DNA gyrase requires ATP to supercoil DNA negatively. The *gyrA* and *gyrB* promoters are supercoiling sensitive. They are inhibited by increases in supercoiling levels but are induced when DNA is relaxed. *topA* codes for the monomeric DNA topoisomerase I, and it also has a supercoiling responsive promoter. The *topA* promoter is activated by high levels of DNA supercoiling and inhibited by relaxation of the DNA. Topoisomerase I functions to relax negatively supercoiled DNA. The opposing activities of DNA gyrase and DNA topoisomerase I establish a homeostatic supercoiling mechanism in the cell. The supercoiling sensitive *fis* promoter has maximal activity at high levels of DNA supercoiling. Fis protein acts as a transcriptional repressor of both *gyr* genes and activates *topA* under conditions of oxidative stress. Fis is proposed to 'fine-tune' the homeostatic control mechanism of DNA supercoiling.

DNA gyrase

gyrA and *gyrB*

Fis Protein

ATP/ADP

Negatively
supercoiled DNA

Relaxed DNA

Oxidative stress

Nutritional upshift

fis promoter

Activates
topA promoter

Fis Protein

DNA topoisomerase I
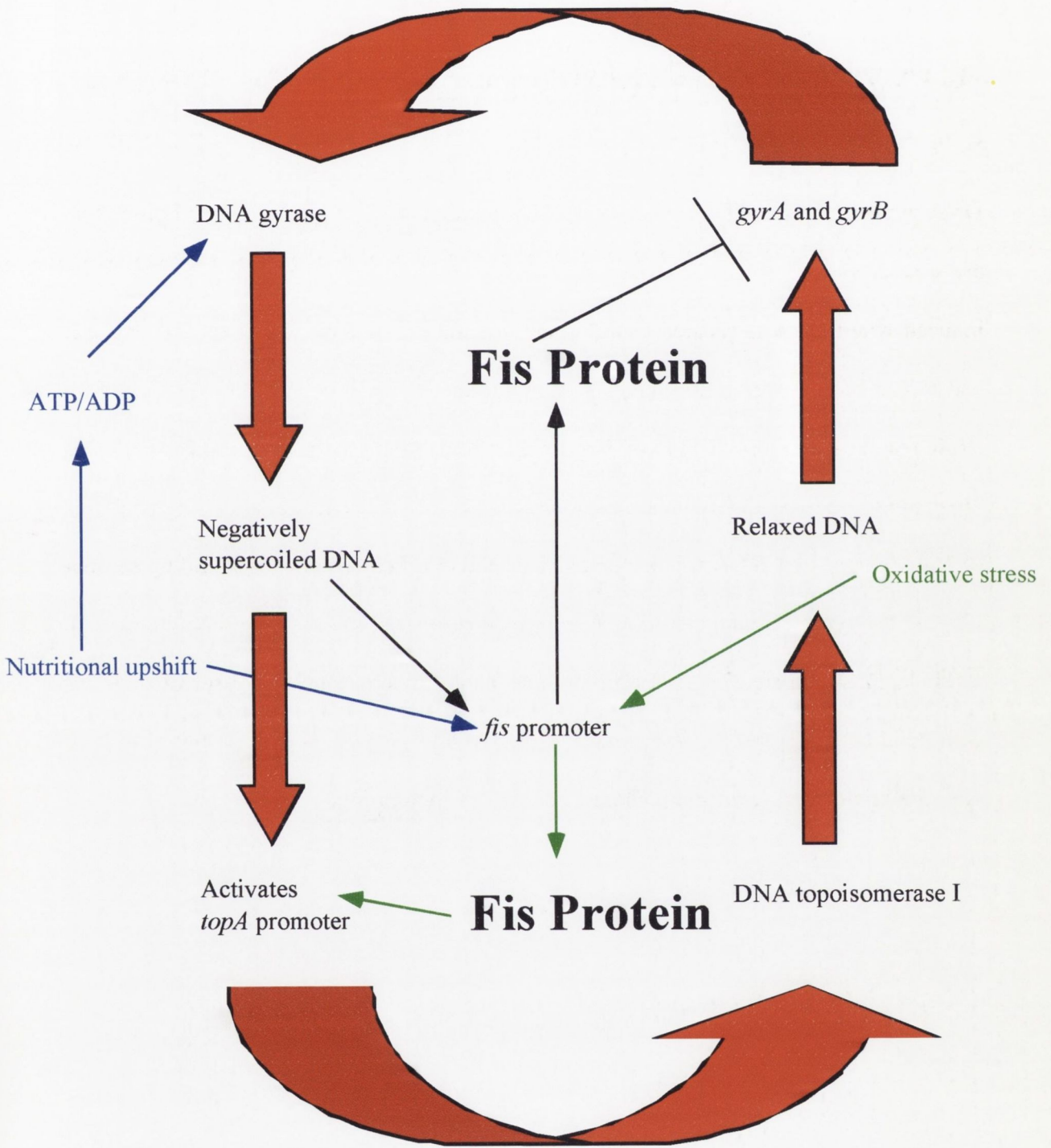
encoded by the *gyrA* and *gyrB* genes, respectively. The promoters of the gyrase genes are supercoiling sensitive (Menzel and Gellert, 1983). They are repressed by increases in the level of negative supercoiling of the DNA and are induced by relaxation of the DNA (Menzel and Gellert, 1983). *topA* encodes the DNA topoisomerase I enzyme and the *topA* promoter is also supercoiling responsive (Menzel and Gellert, 1983). It is activated when DNA supercoiling levels increase and it is inhibited by relaxation of the DNA (the opposite of the *gyr* gene promoters) (Menzel and Gellert, 1983). Therefore the consequences of the activities of DNA gyrase and DNA topoisomerase I feed back on the expression of their genes (Fig. 1.9). It is the opposing activities of these enzymes that establish a homeostatic supercoiling balance of the bacterial DNA (Fig. 1.9).

The activity of DNA gyrase is controlled by the ATP/ADP ratio in the cell, which links it to the physiological state of the cell (Fig. 1.9). Supercoiling imparts free energy to DNA and this drives structural transitions in the DNA helix including open complex formation at certain promoters. Therefore a change in the external environment can influence DNA gyrase activity which in turn can alter the chromosomal superhelical density which can cause many genes in the genome to respond simultaneously (Dorman, 1996). For example, in stationary phase growing cells the ATP/ADP ratio is low. As cells enter log phase after nutrient upshift the ATP/ADP ratio increases, as does the negative superhelical density of the bacterial DNA (Kusano *et al.*, 1996). Physical stresses can also alter the global level of DNA supercoiling. A change from aerobic to anaerobic growth conditions results in a decrease in the ATP/ADP ratio and a decrease in the level of negative supercoiling (Cortassa and Aon, 1993; Dorman *et al.*, 1988). Osmotic stress increases the ATP/ADP ratio and this results in an increase in the superhelical density of the DNA (Higgins *et al.*, 1988). Also it was reported that following the phagocytosis of *S. typhimurium* by macrophage the bacterial DNA is rapidly relaxed (Marshall *et al.*, 2000). The link between an environmental change, the global negative superhelical density of the DNA and transcription means that alterations in the external environment can influence the DNA topology which in turn can trigger the activation of a set of genes for the adaptation to that environment (Dorman *et al.*, 1988; Dorman *et al.*, 1990; Dorman, 1991, 1996). The protein expression profiles of *E.*

coli are altered in the presence of either a non-lethal *topA* or *gyrB* mutant, which render the chromosomal DNA oversupercoiled or undersupercoiled, respectively and therefore the chromosomal superhelical density of the DNA is a global regulator of gene expression (Hatfield and Benham, 2002; Steck *et al.*, 1993).

Another global regulator of gene expression is the nucleoid-associated protein Fis, which also influences the DNA topology (Fig. 1.9), (Schneider *et al.*, 2001). Fis modulates the dynamics of DNA supercoiling during growth phase in *E. coli* (Schneider *et al.*, 1997). After nutrient upshift ATP levels rise, DNA gyrase activity increases and as a result an increase in the DNA superhelicity occurs. Post nutrient upshift Fis protein levels peak and the Fis protein binds the DNA and maintains intermediately supercoiled DNA preventing detrimentally high levels of negative supercoiling (Schneider *et al.*, 1997). In addition, Fis is a transcriptional repressor of the *gyrA* and *gyrB* genes, which code for DNA gyrase (Keane and Dorman, 2003; Schneider *et al.*, 1999). Fis expression correlates with lower levels of both *gyrA* and *gyrB* mRNA and the corresponding proteins, resulting in a reduction in intracellular supercoiling activity. Upon nutrient upshift, Fis is induced, stabilizes intermediately supercoiled DNA and represses *gyrA* and *gyrB* transcription and as a result Fis is proposed to be involved in coupling cellular physiology and DNA topology (Fig. 1.9).

The *E. coli fis* promoter is extremely sensitive to the superhelical density of the DNA (Fig. 1.9), where expression of *fis* requires high levels of negative supercoiling for maximal activity and the GC-rich discriminator of the *fis* promoter is the primary structural element responsible for its supercoiling sensitivity (Schneider *et al.*, 2000). A GC-rich discriminator is also found in the *E. coli tyrT* and *S. typhimurium hisR* promoter regions. Both of these promoters are subject to stringent control and are stimulated by negative supercoiling of the DNA (Figueroa-Bossi *et al.*, 1998; Free and Dorman, 1994; Lamond, 1985). Given that *fis* expression responds to alterations in DNA supercoiling, and that the level of negative supercoiling is maintained by DNA gyrase, whose activity is influenced by Fis protein levels, it is proposed that *fis* is a

component of a network involved in the homeostatic control of DNA supercoiling (Fig. 1.9).

Under conditions of oxidative stress in *E. coli*, Fis is a transcriptional activator of *topA* (Fig. 1.9), (Weinstein-Fischer *et al.*, 2000). The activation of *topA*, which encodes DNA topoisomerase I that relaxes the DNA, leads to a relaxation of the DNA simultaneously stimulating gene expression of select genes important for oxidative stress survival. Fis is responsible for communicating this environmental stimulus (oxidative stress) to the DNA topology. Therefore, Fis is an important component of the *E. coli* response to oxidative stress. This observation supports the proposals that Fis couples cellular physiology to DNA topology and that Fis may be involved in 'fine-tuning' the homeostatic control of DNA supercoiling (Fig. 1.9).

1.1.12 Flagellar gene expression and assembly

S. typhimurium is motile as a result of whip like appendages called flagella on its surface. *S. typhimurium* possesses 6-10 flagella that originate at random points on the cell surface and this is known as peritrichous arrangement of flagella (Bonifield and Hughes, 2003; Soutourina and Bertin, 2003). Flagella move by rotation without bending and bacteria swim due to this propeller like action of their flagella.

The flagellum (Latin for a whip) can be subdivided into a hook-basal body and an external filament (Fig. 1.10). Assembly of the flagellum (Fig. 1.10) begins with an inner membrane anchor called an MS-ring. Then a type III secretion apparatus (similar to the SPI-1 and SPI-2 encoded type III secretion systems in Fig. 1.3) and C-ring are associated with the cytoplasmic face of the MS-ring (Aldridge and Hughes, 2002). A rod structure is built onto the MS-ring, linking the type III secretion apparatus to the external environment (Aldridge and Hughes, 2002). P-rings and L-rings associate with and support the rod at the peptidoglycan layer and the outer membrane, respectively

Fig. 1.10. The flagellar regulatory network. *S. typhimurium* flagellar transcription is subject to a regulatory hierarchy coupled to flagellar assembly. There are over 50 genes involved in flagellar biosynthesis. These genes are divided into operons classified as early, middle and late with respective promoters classified as class 1, class 2 and class 3. The FlhD and FlhC proteins are the master regulators of flagellar gene expression and are encoded by the *flhDC* operon containing the early genes. Transcription of these early genes is essential for the activation of middle genes. Middle gene operons encode structural proteins for the formation of the hook-basal body. Middle genes also encode the σ^{28} sigma factor and the anti-σ^{28} factor, *flgM*. Class 3 promoters that drive late gene transcription are σ^{28} RNA polymerase specific. However the anti-σ^{28} factor binds to σ^{28} directly and prevents transcription from class 3 promoters until hook-basal body completion. When the hook basal body is completed the anti-σ^{28} factor is secreted from the cell and σ^{28} repression is relieved allowing class 3 promoter transcription to occur.

OM, outer membrane, **PG**, peptidoglycan layer and **IM**, inner membrane.

Early genes

Global regulatory signals

flhD flhC

FlhD₂FlhC₂

Transcriptional activation complex

Middle genes

flgAMN

flgBCDEFGHIJKL

fliAZY

fliDST

fliE

fliFGHIJK

fliLMNOPQR

Late genes

flgMN

flgKL

fliC

fliDST

fljBA

motAB cheAW

tar cheRBYZ

tsr

aer

σ₂₈

Flagellar filament

FLgM secreted

hook

Hook-basal body

OM

PG

IM

L-ring

P-ring

rod

MS-ring

Type III secretion system

C-ring

motor force generators

FLgM σ₂₈ **Inactive complex**

σ₂₈ **Active**

Hook-basal body   **Completed flagellum**

(Aldridge and Hughes, 2002). The hook is attached to the rod at the cell surface and hook-associated proteins and the filament cap are built onto the end of the hook (Aldridge and Hughes, 2002). Such distal components are translocated through the type III secretion system. Finally the filament subunits are assembled beneath the cap to complete the filament (Fig. 1.10), (Aldridge and Hughes, 2002). Rotation of the hook-basal body for movement of the flagellum results from the interaction of motor force generators at the C-ring (Aldridge and Hughes, 2002). Motor force generators are proton pumps. Energy to drive this motor is derived from the transmembrane gradient of protons, or proton motive force (Manson *et al.*, 1977). The flagella motor rotates in either a clockwise or counterclockwise direction causing swimming or tumbling, respectively (Berg, 2000). Upon a shift in intracellular pH from 7.0 to 5.0 in the presence of weak acid, the rotational speed of flagella motors significantly decreases (Minamino *et al.*, 2003; Adams *et al.*, 2001). In summary, the entire flagellar structure consists of a long helical filament connected to a short curved structure called a hook, both the filament and hook are extracellular and are anchored to the basal body, a complex structure which is embedded in the cell, spanning the membrane envelope (Fig. 1.10).

There are more than 50 genes involved in flagellar biosynthesis, function and chemotaxis and these genes are divided into at least 17 operons that comprise the coordinately regulated flagellar regulon (Fig. 1.10), (Aldridge and Hughes, 2002; Chilcott and Hughes, 2000; Soutourina and Bertin, 2003). Within the hierarchical regulon the operons are divided into three temporal transcriptional classes, early, middle and late and their respective promoters are class 1, class 2 and class 3 (Fig. 1.10) (Aldridge and Hughes, 2002; Chilcott and Hughes, 2000; Soutourina and Bertin, 2003). However, certain genes within the flagellar regulon are transcribed from multiple promoters of different classes (Aldridge and Hughes, 2002; Chilcott and Hughes, 2000; Soutourina and Bertin, 2003). At the apex of the flagellar gene hierarchy, the class 1 promoter transcribes the *flhDC* operon (early genes), which encodes FlhD and FlhC, the master regulators of the flagellar regulon. FlhD and FlhC are transcriptional activators of class 2 promoters, which transcribe the middle genes (Aldridge and Hughes, 2002;

Chilcott and Hughes, 2000; Soutourina and Bertin, 2003). Middle genes code for the structural proteins required for the morphogenesis of the hook-basal body (Fig. 1.10). Middle genes also code for transcriptional regulators such as FlgM (anti-σ^{28} factor) and FliA (σ^{28}) (Aldridge and Hughes, 2002; Chilcott and Hughes, 2000; Soutourina and Bertin, 2003). Class 3 promoters that transcribe the late genes are σ^{28} RNA polymerase specific. The anti-σ^{28} factor levels are responsible for coupling gene expression and flagellar assembly (Aldridge and Hughes, 2002; Chilcott and Hughes, 2000; Soutourina and Bertin, 2003). The anti-σ^{28} factor binds to the σ^{28} directly and prevents transcription of the class 3 promoters. Upon completion of the hook-basal body the anti-σ^{28} factor is secreted from the cell through the flagellum-specific type III secretion system. This relieves σ^{28} repression and results in σ^{28}-dependent transcription of the late genes. Transcription of late genes, that encode proteins dedicated to completing the flagellar filament (propeller) and rotating it, occur only when the hook basal body is finished, which is a sensible order of events since the filament is polymerized onto the hook-basal body. In this way transcription of flagellar genes is precisely timed and linked to flagellar assembly (Fig. 1.10).

In *S. typhimurium* the flagellar filament is approximately 10 μm in length and composed of approximately 20,000 subunits of flagellin protein (Bonifield and Hughes, 2003). *S. typhimurium* (but not *E. coli*) possesses two antigenically distinct flagellin genes, *fliC* and *fljB* and their expression is subject to phase variation, therefore at any given time, only one of the two genes is expressed (Fig. 1.11), (Bonifield and Hughes, 2003) (Haykinson *et al.*, 1996; Macnab, 1996). A site-specific DNA inversion event in a region of the chromosome flanked by inverted repeats upstream of the *fljBA* operon is responsible for flagellar phase variation (Fig. 1.11), (Bonifield and Hughes, 2003) (Macnab, 1996). The promoter of the *fljBA* operon is flanked by the *hixL* and *hixR* inverted repeats and also located between the *hix* sequences is the *hin* gene that codes for Hin recombinase (Fig. 1.11), (Bonifield and Hughes, 2003; Haykinson *et al.*, 1996; Macnab, 1996). The Hin recombinase carries out a reversible DNA recombination reaction between the *hix* sites in combination with the histone-like proteins, Fis and HU (Bonifield and Hughes, 2003; Haykinson *et al.*, 1996; Macnab, 1996). In one

phase-2 flagellin

FljB flagellin filament

FljA repression

ON promoter

fljB   fljA

ON

P *fliC*

fliC

OFF

Hin   Fis

HU

DNA inversion

OFF promoter

fljB   fljA

OFF

P *fliC*

fliC

ON

FliC flagellin filament
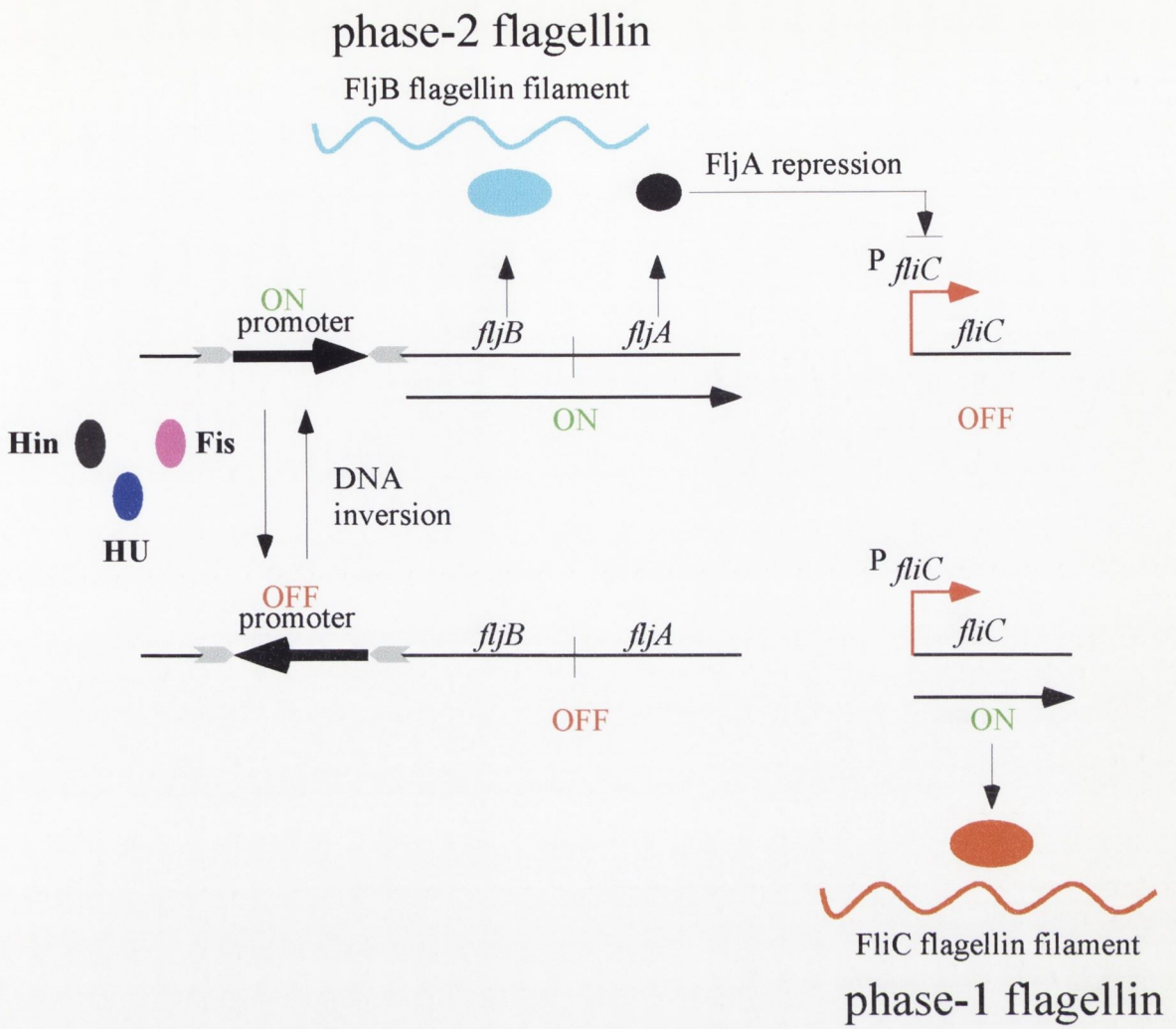
phase-1 flagellin

Fig. 1.11. Mechanism of flagellar phase variation in *S. typhimurium*. FliC and FljB are the two flagellin filaments alternately expressed on the surface of *S. typhimurium*. The genes that encode these flagellin proteins are located at different regions on the chromosome. An inverted repeat flanks the *fljBA* promoter and the *hin* gene. This region of DNA can be inverted by site-specific recombination mediated by the Hin protein and involves the histone-like proteins, HU and Fis. In one direction the promoter drives *fljBA* operon expression and FljB or phase-2 flagllin is produced. FljA protein is also produced and acts as a repressor of FliC/phase-1 flagellin synthesis at the transcriptional and translational levels. In the opposite orientation, the *fljBA* promoter cannot drive *fljBA* operon expression. No FljB flagellin or FljA repressor are produced. Repression of FliC synthesis is alleviated. The cell is now on for FliC or phase-1 flagellin.

orientation the *fljBA* promoter drives transcription of the *fljBA* operon, resulting in flagella composed of FljB flagellin subunits (Fig. 1.11). Transcription at the *fljBA* operon also produces FljA protein, which, acts as a repressor of the unlinked *fliC* gene at the transcriptional and translational levels (Bonifield and Hughes, 2003; Macnab, 1996). Following a further site-specific recombination reaction, the *fljBA* promoter is switched in the opposite direction. In the opposite orientation no transcription occurs at the *fljBA* operon (Bonifield and Hughes, 2003) (Macnab, 1996). This relieves *fliC* repression and results in FliC composed flagella (Fig. 1.11), (Bonifield and Hughes, 2003) (Macnab, 1996).

The production of flagella and the resulting motility represent a significant drain on the cell's resources and may induce a strong immune response in the host organism. The expression of the *flhDC* operon is sensitive to many regulatory signals such as, cAMP-CRP, temperature, heat shock proteins DnaK, DnaJ and GrpE, DNA supercoiling, high concentrations of either inorganic salts, carbohydrates, or alcohols, high temperature, growth phase, surface-liquid transition, phosphatidylethanolamine and phosphatidylglycerol synthesis, cell cycle control and gyrase inhibitors (Adler and Templeton, 1967; Chilcott and Hughes, 2000; Li *et al.*, 1993; Shi *et al.*, 1993). The ability of bacteria to turn off flagellar formation under adverse conditions is a mechanism of conserving energy in order to help them survive. However, motility in pathogenic bacteria is considered a virulence factor providing the ability to swim to food sources and away from deleterious substances (Schmitt *et al.*, 2001; Soutourina and Bertin, 2003). In addition flagella play a role in adhesion, biofilm formation and colonization (Soutourina and Bertin, 2003). Therefore, the decision to initiate flagellar synthesis or prevent it is a crucial and multifaceted one and not surprisingly the master operon (*flhDC* operon) is at the centre of a complex regulatory system.

Under different conditions of growth FlhDC can act as an autogenous repressor or as an autogenous activator of *flhDC* expression (Kutsukake, 1997). DnaK, DnaJ and GrpE protect the cell from a variety of adverse conditions and in addition are required for the synthesis of flagella (Shi *et al.*, 1992). In *E. coli* and *S. typhimurium* the post-

transcriptional regulator CsrA is required for motility (Lawhon *et al.*, 2003; Wei *et al.*, 2001). In *E. coli* the binding of CsrA to the *flhDC* message is thought to stabilize it and allow its translation (Wei *et al.*, 2001). The ClpXP ATP-dependent protease negatively regulates flagellar synthesis in *S. typhimurium* (Tomoyasu *et al.*, 2002; Tomoyasu *et al.*, 2003). A ClpXP mutant is hyperflagellate and hypermotile and like CsrA, ClpXP does not control the *flhDC* operon at the transcriptional level (Tomoyasu *et al.*, 2002). Instead it degrades the $FlhD_2FlhC_2$ activation complex (Tomoyasu *et al.*, 2003). In a ClpXP mutant FlhD and FlhC accumulate to higher levels and the FlhC half-life is fivefold longer so that class 2 and class 3 transcription of flagellar genes increases in the absence of ClpXP (Tomoyasu *et al.*, 2003).

Six transcriptional start sites, P1 to P6, were identified in the *flhDC* operon promoter region of *S. typhimurium* (Yanagihara *et al.*, 1999). P1 and P6 were shown to be transcribed by σ70-RNA polymerase and to be responsible for cAMP-CRP dependent and cAMP-CRP repressible transcriptions, respectively. Mutations in *crp* or in *hns*, the structural genes of CRP and H-NS, respectively results in complete loss of motility and lack of flagella in *E. coli* and *S. typhimurium* (Bertin *et al.*, 1994; Hinton *et al.*, 1992; Silverman and Simon, 1974). *flhDC* transcription is the main target for both regulators (Soutourina *et al.*, 1999). However the findings of another study in *E. coli* disputes the suggestion that the positive regulation of flagella genes by H-NS is direct. Instead it is suggested that the activation of motility genes by H-NS is due to alleviation of repression at *flhDC* by the negative regulator HdfR, which is negatively regulated by H-NS (Ko and Park, 2000b). In addition to positively regulating flagellar biogenesis (directly or indirectly) H-NS is involved in flagellar function (Donato and Kawula, 1998; Ko and Park, 2000a). H-NS binds the flagellar torque-generating protein FliG (located at the cytoplasmic face of the MS-ring, Fig. 5.1) and it is postulated that H-NS is involved in torque generation through protein-protein interactions with FliG (Donato and Kawula, 1998).

HU is a heterodimeric histone-like protein and its subunits are encoded by the *hupA* and *hupB* genes. In *E. coli* mutants deficient in expression of either gene have normal

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motility but a double deletion mutant *hupAhupB* is immotile (Nishida *et al.*, 1997). The HUα and HUβ homodimers can substitute functionally for the HUα- HUβ heterodimers in many cellular processes including the expression of flagella (Nishida *et al.*, 1997). Under conditions that activate the expression of the LEE-encoded type III secretion system, wild-type EPEC (enteropathogenic *E. coli*) and EHEC (enterohaemorrhagic *E. coli*) cells are nonmotile (Yona-Nadler *et al.*, 2003). Lee genes are positively regulated by the integration host factor (IHF) (Friedberg *et al.*, 1999). It was found that an *ihfA* mutant, in EPEC and EHEC bacteria under the same conditions, displayed hypermotility (Yona-Nadler *et al.*, 2003). The small heterodimeric nucleoid-associated protein IHF is composed of the subunits IHFα and IHFβ and the *ihfA* gene encodes the IHFα subunit. The IHF repression of flagellar gene expression in EPEC and EHEC is due to indirect transcriptional repression at *flhDC*. IHF repression of motility is unique to EPEC and EHEC and is not found in all *E. coli* strains. Similarly BipA (a new member of the elongation factor GTPase superfamily) is required for expression of the LEE genes and repression of motility in EPEC (Farris *et al.*, 1998). In *S. typhimurium*, SirA, a component of the SirA/BarA two-component regulatory system, participates in the control of motility and virulence. Like IHF and BipA, this system increases the expression of virulence genes encoded on *Salmonella* pathogenicity island 1 (SPI-1) and decreases the expression of motility genes (Goodier and Ahmer, 2001; Teplitski *et al.*, 2003). A regulatory operon located on a *Salmonella*-specific island near tRNA[PheU] encodes RtsA and RtsB (Ellermeier and Slauch, 2003). RtsA induces expression of SPI-1 genes by directly binding to the SPI-1 located promoter of *hilA* and RtsB transcriptionally represses the flagella operon by binding downstream of the *flhDC* regulatory operon promoter (Ellermeier and Slauch, 2003). It is proposed that repression of the flagella genes in pathogenic bacteria is a mechanism to reduce the host's inflammatory response (Ellermeier and Slauch, 2003).

Flagellin is the major proinflammatory determinant of *S. typhimurium* and without it *S. typhimurium* can go largely undetected in host cells (Zeng *et al.*, 2003). It is reasonable to speculate that flagellar phase variation in *S. typhimurium* is a strategy to evade

immune detection. In addition the movement and spatial distribution of flagella on the surface of *S. typhimurium* influence its invasive ability (Jones *et al.*, 1992).

FimZ is a positive regulator of type 1 fimbrial expression in *S. typhimurium*. The overexpression of FimZ in *S. typhimurium* results in hyperfimbriated nonmotile bacteria (Clegg and Hughes, 2002). This lack of motility is associated with down regulation of the *flhDC* regulatory flagellar operon. Therefore it is proposed that FimZ provides the molecular connection between fimbriated and motile phenotypes of *S. typhimurium* providing coordination between the cells ability to adhere and swim.

1.1.13 Hypothesis

I propose that the Fis nucleoid-associated protein is a global regulator with effects on specific groups of genes in *S. typhimurium*. This thesis was tested in the research project that I now describe.

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, Anzingster. 7, Ebersberg, D-85560, Germany. 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

All the amino acids, nucleotide precursors, vitamins and other metabolites required for growth were supplied in a rich broth such as Luria-Bertani (LB) broth.

Ingredients for preparing growth media were obtained from Bacto, 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$_2$O). Media were supplemented with the appropriate antibiotics as required. Media agar was allowed to cool to 50°C before the addition of the appropriate antibiotic.

LB broth and LB agar plates:

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 LB broth unless otherwise stated.

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

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

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 were added.

No-carbon-E (NCE) medium:

NCE medium (Vogel and Bonner, 1956) (Berkowitz *et al.*, 1968), a minimal medium, was used in this study supplemented with 1,2-propanediol as the sole source of carbon. *S. typhimurium* catabolizes 1,2-propanediol via a pathway that is dependent upon adenosyl cobalamin (AdoCbl), a metabolically active form of vitamin B_{12} (Jeter, 1990). In, this study it was shown that the Fis protein inhibits growth on 1,2-propanediol.

Cells were grown aerobically in NCE medium supplemented with 1,2-propanediol, 50 mM, $MgSO_4$, 1 mM (added after autoclaving), cyanocobalamin (vitamin B_{12}), 150 nM and histidine, 0.5 mM. The *S. typhimurium* strain SL1344 used in this study is a histidine auxotroph and therefore, histidine was added to the NCE medium to support SL1344 growth.

Motility agar:

Motility agar was used to prepare swarm plates for the measurement of bacterial motility. The motile ability of bacteria was characterized using swarm plates. These plates were inoculated centrally with equal numbers of bacteria and incubated at 37°C for 8 hours. The rate of spreading of rings of bacteria ("swarming") on a swarm plate was a measure of chemotaxis and motility.

Motility agar: 3 g agar, 10 g Bacto tryptone and 5 g NaCl.

Green agar:

Green agar plates were routinely used following bacteriophage P22-mediated generalized transduction to obtain isolates of *S. typhimurium* free of phage. 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 and X-Gal

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$_2$O 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.

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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.

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 typically grown by inoculating single colonies into 3 ml of LB broth in a sterile test-tube and incubating at the required temperature overnight. Alternatively, or when larger volumes were required, cultures were grown by inoculating fresh media in a suitably sized conical flask at a dilution of 1:100 from overnight cultures.

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

Strain	Relevant Genotype	Reference/Source
Salmonella typhimurium		
SL1344	Virulent wild type *rpsL hisG*	Hoiseth and Stocker, (1981)
SL1344 *fis*	SL1344 *fis::cat*	Keane and Dorman, (2003)
SL1344 *hns*	SL1344 *hns::kan*	J. Hinton
RG200	14028 *flhD* $^+$ ϕ (*flhD::lacZY*) merodiploid	Goodier and Ahmer, (2001)
RG202	14028 *fliA* $^+$ ϕ (*fliA::lacZY*) merodiploid	Goodier and Ahmer, (2001)
RG207	14028 *fliC* $^+$ ϕ (*fliC::lacZY*) merodiploid	Goodier and Ahmer, (2001)
RG211	14028 *fliE* $^+$ ϕ (*fliE::lacZY*) merodiploid	Goodier and Ahmer, (2001)
RG213	14028 *flgA* $^+$ ϕ (*flgA::lacZY*) merodiploid	Goodier and Ahmer, (2001)
AK01	SL1344 *flhD* $^+$ ϕ (*flhD::lacZY*) merodiploid	This study
AK02	SL1344 *fliA* $^+$ ϕ (*fliA::lacZY*) merodiploid	This study
AK03	SL1344 *fliC* $^+$ ϕ (*fliC::lacZY*) merodiploid	This study
AK04	SL1344 *fliE* $^+$ ϕ (*fliE::lacZY*) merodiploid	This study
AK05	SL1344 *flgA* $^+$ ϕ (*flgA::lacZY*) merodiploid	This study
AK06	SL1344 *fis::cat flhD* $^+$ ϕ (*flhD::lacZY*) merodiploid	This study
AK07	SL1344 *fis::cat fliA* $^+$ ϕ (*fliA::lacZY*) merodiploid	This study

AK08	SL1344 *fis::cat fliC* $^+$ ϕ (*fliC::lacZY*) merodiploid	This study
AK09	SL1344 *fis::cat fliE* $^+$ ϕ (*fliE::lacZY*) merodiploid	This study
AK10	SL1344 *fis::cat flgA* $^+$ ϕ (*flgA::lacZY*) merodiploid	This study
TH2285	LT2 *fis::cat*	K. T. Hughes
TH6232	Δ*hin*7517::FRT *fljBA* off FliC$^+$	K. T. Hughes
TH6233	Δ*hin*7518::FRT *fljBA* on FliC$^-$	K. T. Hughes
AK11	TH6232 *fis::cat*	This study
AK12	TH6233 *fis::cat*	This study
Escherichia coli		
XL-1	Routine cloning strain	Stratagene
CSH50	F *ara* Δ(*lac pro*) *rpsL thi*	K. B. Low
CSH50 *fis*	CSH50 *fis::kan*	Koch et al., 1988

2.3 Plasmids, bacteriophages and oligonucleotides

2.3.1 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.

2.3.2 Bacteriophage

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

2.3.3 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.

2.4 Transformation of bacterial cells 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-triggered 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.

Table 2.2.   Plasmids used throughout this study.

Plasmid	Relevant characteristics	Source/Reference
pQF50	*lacZ* promoterless trap vector. Ap^R	Farinha and Kropinski, (1990)
pQFfis	671 base pair *fis* promoter sequence upstream of promoterless *lacZ* in pQF50	This study
pQFssrA	645 base pair *ssrA* promoter sequence upstream of promoterless *lacZ* in pQF50	Ronan Carroll
pQFssaG	580 base pair *ssaG* promoter sequence upstream of promoterless *lacZ* in pQF50	Ronan Carroll
pBAD24	pMB1 replicon, Ap^R	Guzman *et al.*, (1995)
pBADfis	*fis* ORF downstream of the arabinose inducible promoter in pBAD24	This study
pFis349	Ap^R, pGS349 containing the *yhdG fis* operon	Wilson *et al.*, (1990)
pUC18	ColE1 replicon, Ap^R	Yanisch-Perron *et al.*, (1985)
pUCflhd	725 base pair *flhD* promoter sequence in polylinker region of pUC18	This study
pBR322	pMB1 replicon, Ap^R Tet^R	Bolivar *et al.*, (1977)
pACYC177	p15A replicon, Ap^R Kan^R	Chang and Cohen, (1978)

Ap^R=Ampicillin resistant.
Tet^R=Tetracycline resistant.
Kan^R=Kanamycin resistant.

Table 2.3. Oligonucleotide primers used throughout this study.

Primer Name	Sequence[a]
pfisfw[b]	5'-CGA T<u>GG ATC C</u>TA GCC TTT TCT CCA CAC AAC TGC C-3'
pfisrv[c]	5'-CGA T<u>GG TAC C</u>TA GCG ATC ACG CGA TTT CTG ACG-3'
fisfw	5'-TTC GAA CAA CGC GTA AAT TCT G-3'
fisrv[d]	5'-CGA C<u>AA GCT T</u>GC TCC TGA GGT TCA CAT TCC GC-3'
fisseq	5'-GGT ATC TCC AGG AGC ACG-3'
BSflhDfw	5'-GCG CTA ATG CCA CAT TAA TG-3'
BSflhDrv	5'-GTT CCC ATC CAG ATT AAC C-3'
BSfliAfw	5'-CGG GCC GTA AGT AAC GAA-3'
BSfliArv	5'-GCG GTA TAC AGT GAA TTC AC-3'
BSfliCfw	5'-CGG TAA GTT TGA TCC CAC-3'
BSfliCrv	5'-TTA ATG ACT TGT GCC ATG ATC-3'
SeqBSflhDfw[e]	5'-GGC C<u>GA ATT C</u>GC GCT AAT GCC ACA TTA ATG-3'
SeqBSflhDrv[d]	5'-GGC C<u>AA GCT T</u>GT TCC CAT CCA GAA TAA CC-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'
ssrAfw[b]	5'-ATA C<u>GG ATC C</u>GA ATT CGT CGA CGG CAA GAC AAG GCT TAG GTA AGC-3'
ssrArv[c]	5'-ATT A<u>GG TAC C</u>GG ATC CGC CTG ATT ACT AAA GAT GTT TGC-3'
ssaGfw[b]	5'-CGC <u>GGA TCC</u> GGA TTG GCC TTG CTA TTG C-3'
ssaGrv[c]	5'-CGG <u>GGT ACC</u> GGG TTG AGC AAA TCA TTA CC-3'
pe-fw	5'- GCT AGA AGC TTC TAG AGA TCC CCG GGT ACC-3'

a. Restriction enzyme cleavage sites are underlined.

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

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

d. Primer tailed with restriction site *Hind*III

e. Primer tailed with restriction site *Eco*RI

2.4.1 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 (Sorvall® RC5C Plus) at 6,000 r.p.m. 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 pH 8.0). The cells were subjected to centrifugation (Sorvall® RC5C Plus) again for 10 min at 6,000 r.p.m, 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 or in a 37°C water bath for 5 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.

2.4.2 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 (Sorvall® RC5C Plus) at 10,000 r.p.m. for 10 min and the bacterial pellet resuspended in 100 ml of sterile, ice-cold ddH_2O and incubated on ice for 20 min. The cells were then pelleted by centrifugation (Sorvall® RC5C Plus) at 6,000 r.p.m. for 10 min and resuspended in 100 ml cold sterile ddH_2O. The previous centrifugation step was repeated and cells were resuspended in 500 μl of ice-cold 10% (v/v) glycerol. Aliquots (120 μl) 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 plasmid-borne antibiotic resistance 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, transformants were single colony purified.

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.

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 (MSE Mistral 2000) at 6,000 r.p.m. 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 (Eppendorf Centrifuge 5415R) for 4 min at 14,000 r.p.m. 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 Assays based on spectrophotometry

2.6.1 Monitoring bacterial growth

The growth of bacterial cultures was monitored by measuring the optical density of the culture at a wavelength of 600 nm (OD_{600nm}). For routine measurement of OD_{600nm}, including estimation of cell quantity for β-galactosidase assays (section 2.6.2), 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.

2.6.2 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 activity of transcription from the promoter is 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 (*o*-nitrophenol) 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 (Eppendorf Centrifuge 5415R) at 14,000 r.p.m 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 (1 litre):   60 mM Na_2HPO_4, 40 mM $NaH_2PO_4.2H_2O$, 50 mM β-mercaptoethanol, 10 mM KCl, 1 mM $MgSO_4.7 H_2O$

2.6.3 Determination of nucleic acid concentration

The concentration of DNA and RNA samples was determined spectrophotometrically by measuring the absorbance at 260 nm. Samples were diluted (typically 1:500) in ddH_2O 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 $A_{260}nm$ to $A_{280}nm$ is 2 (Sambrook *et al.*, 1989).

2.6.4 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.

2.7 Preparation of plasmid DNA, chromosomal DNA and RNA

2.7.1 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$_2$O.

2.7.2 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 2.7.1, 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 100 μl ddH$_2$O.

2.7.3 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

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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$_2$O.

2.7.4 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$_2$O (1 ml DEPC/L ddH$_2$O, mixed overnight then autoclaved), which inactivates RNases by covalent modification 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 and Northern blotting was prepared using the FastRNA® Pro Blue Kit (BioGene) and contaminating DNA was removed using Ambion's DNA-*free*™ Kit. While RNA for use in microarray analysis was prepared using the Promega SV Total RNA isolation kit (see section 2.20.1).

Total RNA was extracted from 10 ml samples of exponential phase cultures (OD_{600nm} of 0.6) by the, FastRNA® Pro Blue Kit (BioGene). The FastRNA® Pro Blue Kit is a single reagent extraction method designed to quickly and efficiently isolate total cellular RNA from Gram positive and Gram negative bacteria. The RNApro™ Solution included in the kit is designed to inactivate efficiently cellular RNases during cell lysis to prevent RNA degradation. During use the RNApro™ Solution was mixed with the bacterial sample in a tube containing a specifically selected lysing matrix. The tube was then processed in the FastPrep® Instrument for 40 seconds to release total cellular RNA, DNA and proteins. Following the FastPrep® homogenization the RNA was purified and isolated by chloroform extraction and ethanol precipitation. Following resuspension, RNA concentration and purity was assessed by A_{260nm} and A_{280nm} measurements as described in section 2.6.3.

2.8 Manipulation of DNA *in vitro*

2.8.1 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 and/or purification (see section 2.8.2) of the DNA between digestions. Reactions were incubated at 37°C for 1–2 h unless otherwise recommended.

2.8.2 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.

2.8.3 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. 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.

2.8.4 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 (Eppendorf Centrifuge 5415R) for 4 min at 14,000 r.p.m. 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 (Eppendorf Centrifuge 5415R) at 14,000 r.p.m. 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 (Eppendorf Centrifuge 5415R) at 14,000 r.p.m. for 10 min. The pellet was washed in 500 μl of 70% ethanol, air-dried and resuspended in a suitable volume of ddH$_2$O.

2.9 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.

2.9.1 Amplification of DNA

Two different thermostable polymerases were used in this study. *Taq* DNA polymerase (Roche Molecular Biochemicals) is a highly processive 5'–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'–3'-DNA polymerase, free of endo- or exo-nucleases. *Pfx* harbours a 3'–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 sterile 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 $Tm = 2 \times (A+T) + 4 \times (G+C) -2$, 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 (see section 2.7). 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$_2$O, boiled for 3 min and 2 μl used for each PCR reaction.

2.10 Gel electrophoresis

2.10.1 Agarose gel electrophoresis

Analysis of DNA and RNA samples was performed on 1% (w/v) agarose gels. Agarose gels were prepared as follows: 1 g 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. Ethidium bromide intercalates the DNA and fluoresces strongly in long wavelength UV light. Samples were prepared by adding 1μl of Promega's Blue/Orange 6 X loading dye (10 mM Tris pH 7.5, 15% Ficoll® 400, 0.03% bromophenol blue, 0.03% xylene cyanol FF, 0.4%

orange G and 50 mM EDTA) to 5 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.

2.10.2 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 identical 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$_2$O. 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 (see section 2.12.1) were denatured at 90°C for 10 min and centrifuged (Eppendorf Centrifuge 5415R) at 14,000 r.p.m. for 1 min. Electrophoresis was performed at 150 V for 60–90 min.

2.10.3 Staining of proteins

Gels were washed in ddH$_2$O 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.

2.11 Antiserum

Immunising a New Zealand White rabbit with MBP-labelled purified SsrB (a gift from R. Carroll) generated antibodies specifically reactive against SsrB. The initial injection was with 300 μg of SsrB 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-SsrB 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.

2.12 Western immunoblot analysis

Western immunoblotting is a sensitive technique whereby proteins (antigens) are (i) solubilized with SDS and β-mercaptoethanol (section 2.12.1) and separated by SDS-PAGE (section 2.10.2), (ii) irreversibly transferred to nitrocellulose membrane (section 2.12.2), (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 2.12.3).

2.12.1 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 50 μl B-PER reagent (Pierce) supplemented with lysozyme (500 μg/ml) and 50 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.

2.12.2 Transfer of proteins to nitrocellulose membrane

Following SDS-PAGE (section 2.10.2), 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 (0.2% (w/v) Ponceau S (Sigma), 3% (v/v) trichloroacetic acid) for 5 min followed by extensive washing with ddH$_2$O.

2.12.3 Detection of bound antigens

Nitrocellulose membranes were blocked for 1 hour in blocking buffer (10% (w/v) nonfat dry milk, in phosphate-buffered saline (PBS)). Antisera were diluted appropriately in blocking buffer, 1:1000 for the Fis antisera (Keane and Dorman, 2003) and incubated with the membrane overnight at 4°C, 1:1000 for the SsrB antisera and 1:1000 for the FliC antisera (Becton Dickinson) and incubated with the membrane for 1 hour 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 (Cell Signalling) for 1 hour. The blot was washed as before, and in the presence of a suitable chemiluminescent substrate (Pierce SuperSignal), the horseradish-peroxidase-mediated enzymatic 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.

2.13 Northern blotting

The principle of Northern blotting is that a transcript within immobilized RNA can be quantified after hybridization with a specific probe. The procedure involves (i) resolution of an RNA sample by denaturing gel electrophoresis (section 2.13.1), (ii) transfer of the resolved RNA to nylon membrane (section 2.13.2), (iii) synthesis of the DIG-labelled probes (section 2.13.3) and (iv) hybridization and detection of the RNA with labelled probes (section 2.13.4).

2.13.1 Denaturing electrophoresis of RNA

RNA molecules can contain a high degree of secondary structure and are consequently electrophoresed under denaturing conditions. In this study the denaturant used was formaldehyde for agarose gel electrophoresis. Electrophoresis was done using pre-cast denaturing 1.25% Reliant agarose gels (FMC). Samples of total RNA (5 µg), prepared as described in section 2.7.4 were denatured prior to loading in 3 volumes of denaturing solution (50% formamide, 1 x MOPS) at 70°C for 5 min, transferred to ice for 5 min, then loaded with 0.1 volumes of loading buffer. Gels were run in 1 X MOPS buffer at 100 V for 1–3 h.

2.13.2 Transfer of the resolved RNA to nylon membrane

After electrophoresis the RNA was transferred by overnight capillary transfer to Biodyne B nylon membrane (Pall) according to the method outlined in Sambrook *et al* (1989). RNA was immobilized on the membrane after transfer by UV-crosslinking.

2.13.3 DIG (Digoxigenin) probe synthesis

A DIG-labelled probe was synthesized by the polymerase chain reaction (section 2.9) using the PCR DIG Labelling Mix (Roche Molecular Biochemicals). The mix contains 2 mM dATP, 2 mM dCTP, 2 mM dGTP, 1.9 mM dTTP and 0.1 mM DIG-11-dUTP. Of this 5 µl was added to a 50 µl volume PCR reaction instead of standard dNTP mix. The PCR products synthesized, incorporate the DIG-labelled nucleotides. The labelled probes were purified from an agarose gel slice as described in section 2.8.2, and eluted in 100 µl ddH$_2$O. Typically 5-25 ng of probe was used per 1 ml hybridization solution. The probe was denatured by heating to 95 °C for 10 min then incubated on ice prior to use.

2.13.4 Hybridization and detection of DIG probe

Hybridization and detection of DIG labelled DNA probes were carried out following the detailed instructions in the DIG Systems User's Guide for Filter Hybridization (1995). Briefly, the procedure involves overnight incubation of the probe with the nylon membrane at 50 °C. Non-specific hybridized probe is removed by a series of stringency washes (decreasing salt concentration, increasing temperature). Following 30 min incubation with blocking solution (DIG Wash and Block Buffer Set, Roche Molecular Biochemicals), the membrane is then incubated with an Anti-DIG alkaline phosphatase-antibody conjugate (Anti-DIG-AP-conjugate, Roche Molecular Biochemicals), which recognises the immobilised DIG moiety that is incorporated into the probe. In the

presence of a suitable chemiluminescent substrate (CDP-Star, Roche Molecular Biochemicals), the alkaline phosphatase-mediated enzymatic dephosphorylation reaction results in a luminescent signal that visualises hybridized molecules by autoradiography (section 2.14).

2.14 Autoradiography

In this study autoradiography was used to visualize and quantitate on X-ray film; (i) radio-emissions from molecules that incorporated $[\alpha\text{-}^{35}S]dATP$ or $[\gamma\text{-}^{32}P]ATP$ (sequencing reactions, primer extension products, DNase I footprints and gel mobility shift assays); (ii) non-radioactive chemiluminescent emissions derived from alkaline phosphatase or horseradish peroxidase cleavage of chromogenic reagents (Northern and 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 Gel mobility shift analysis of protein: DNA interactions

The association of purified Fis protein with the flagella and SPI2 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

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template. Amplified probes were gel purified as detailed in section 2.8.2, and labelled as described in section 2.17.1. Approximately 5 ng of probe was incubated with increasing concentrations of His-tag purified Fis (O. Keane) from 0–60 ng for 20-30 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 (section 2.14).

A second gel mobility shift procedure was also used to examine binding of H-NS to the *flhDC* promoter region. This procedure involves the use of an excess of competitor DNA (Bertin *et al.*, 1999). Plasmid pBR322 was digested into 8 fragments with *Taq*I and *Ssp*I restriction enzymes. One restriction fragment is 217 bp, and contains the sequence of the curved *bla* promoter. This promoter is bound efficiently by H-NS due to the preference of H-NS for curved DNA (Bracco *et al.*, 1989). *flhD* was amplified by PCR with *Pfx* polymerase (Gibco-BRL), using *S. typhimurium* SL1344 chromosomal DNA as template. Amplified *flhD* was purified from an agarose gel slice (section 2.8.2). The *flhD* probe and the digested pBR322 were incubated with 0-5 μM H-NS in the reaction buffer 40 mM HEPES (pH 8.0), 100 mM potassium glutamate, 10 mM magnesium aspartate, 0.022% NP_40 and 0.1 mg/ml BSA. The products were electrophoresed through 3% molecular screening agarose (Roche Molecular Biochemicals) for 5 hours at 100 V. The gel was stained with 1 μg/ml ethidium bromide and viewed under UV illumination.

2.16 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 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. In each reaction, a mixture of fragments was generated, each terminated with the particular ddNTP present in that reaction. 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 (see section 2.17.3) 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 Primer extension

Primer extension analysis was used to map the *Salmonella typhimurium flhD, ssaG, sseA and ssaB* transcription start sites. 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.

2.17.1 5'-end labelling of DNA using γ-³²P-ATP

Primers were labelled with [γ-³²P]ATP 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. 200 pmoles of primer (100 pmol/µl) was mixed with 50 µCi of [γ-³²P]ATP (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. Following incubation at 37°C the reaction was heated to 90°C for 2 min to inactivate the T4 PNK.   90 µl of nuclease free water was then added producing a final concentration of 1 pmol/µl of labelled primer.

2.17.2 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 2.7.4. Approximately 10 µg of each RNA sample in a 5 µl volume was mixed with 2 pmol of ³²P-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 ddH₂O, 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. The reaction was then precipitated with 3 µl sodium acetate (3.5 M pH5.2) and 500 µl 100% ethanol at −20°C for 30 min. Following centrifugation, the pellet was resuspended in 5 µl of loading dye. The samples were heated to 90°C for 10 min and analysed by denaturing polyacrylamide gel electrophoresis alongside the DNA sequencing reactions.

2.17.3 Denaturing polyacrylamide gel electrophoresis

The products from sequencing, primer extension reactions and DNase I footprinting 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 80 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, primer extension products and DNase I digestion products in a denatured state. Prior to loading, the samples were heated to 90°C. The gel was then run at 80 W for 90–120 min, dried under vacuum and exposed to X-ray film.

2.18 Mapping Fis binding sites

Deoxyribonuclease I (DNase I) footprinting was introduced by Galas and Schmitz (1978) to identify the DNA sequences that constitute binding sites for site-specific DNA-binding proteins. The basis of footprinting techniques is that DNA-bound proteins protect the phosphodiester backbone of the DNA from cleavage by DNase I. The DNase I reactions are inactivated, the DNA denatured, cleavage products separated by sequencing gel electrophoresis (2.17.3) and visualised by autoradiography (2.14).

2.18.1 Synthesis of probes for DNase I footprinting

DNA probes were amplified by PCR with *Pfx* polymerase (Gibco-BRL), using *S. typhimurium* SL1344 chromosomal DNA as template. The PCR amplified probes were purified as described in section 2.8.2 and end-labelled according to the principle described in section 2.17.1 with the exceptions that the labelling reaction was incubated at 37°C overnight and then purified as described in section 2.8.2. The probes were then digested by a specific restriction endonuclease enzyme into two fragments labeled at one extremity differing significantly in size. The fragment required as the DNA probe for DNase I footprinting was purified by extraction from a 6% TBE polyacrylamide gel following electrophoresis. The desired DNA fragment was visualised by autoradiography (section 2.14), cut out using a surgical blade and eluted in 3 ml elution buffer (10 mM Tris-HCl, (pH 8.0), 1 mM EDTA, 300 mM Sodium acetate (pH 5.2), 0.2% SDS at 37°C overnight. The eluted probes were the extracted once with an equal volume of phenol: chloroform and ethanol precipitated. The probes were then resuspended in 100 µl ddH$_2$O.

2.18.2 DNase I footprinting

5 µl of labelled probe was incubated with increasing concentrations of His-tag purified Fis (O. Keane) in each footprinting experiment. Protein-DNA complexes were formed in 50 µl of footprinting buffer (20 mM Tris-HCl (pH 7.5), 80 mM NaCl, 1 mM EDTA, 100 µg/ml BSA, 10% glycerol and 1 mM DTT) at 37°C for 60 min. 50 µl of 10 mM MgCl$_2$, CaCl$_2$ was then added and incubation continued for a further 10 min. 2 µl of DNase I (0.001 U Roche Molecular Biochemicals) was then added and digestion allowed proceed for 1 min before the reaction was stopped by the addition of 90 µl Stop solution (200 mM NaCl, 30 mM EDTA (pH 8.0), 1% SDS, 100 µg/ml yeast tRNA). Samples were then extracted once with an equal volume of phenol: chloroform, ethanol precipitated and resuspended in 6 µl of PE loading dye (95% (v/v) formamide, 20 mM EDTA, 0.05% (w/v) bromophenol blue, 0.05% (w/v) xylene cyanol FF). After

denaturation of DNase I digestion products at 90°C for 3 min, samples were subjected to electrophoresis on a 7% urea-polyacrylamide gel (section 2.17.3) alongside the appropriate sequencing reactions.

2.19 Analysis of DNA topoisomer distributions

Closed circular DNA molecules extracted from enteric bacteria are generally negatively supercoiled with a mean superhelical density (σ) of around –0.065 (Schneider *et al.*, 2000). Molecules with different linking numbers form a Gaussian distribution about the mean in a wild-type cell and are known as topoisomers. Plasmid topoisomers can be separated and their distribution analysed by agarose gel electrophoresis in the presence of the intercalating agent chloroquine. The distribution of topoisomers reflects the *in vivo* levels of supercoiling in the cell population from which the plasmid was extracted. Alterations in the distribution between different bacterial strains or under different bacterial conditions reflect altered *in vivo* DNA supercoiling levels. Analysis of DNA topoisomer distribution in this study was carried out by a method based on that outlined by Higgins *et al* (1988) as described below.

Plasmid DNA was isolated by Qiagen preparation (section 2.7.2) and quantified by OD_{260} reading as described in section 2.6.3. The DNA was then electrophoresed through a 1.2% agarose gel containing 2.5 µg/ml chloroquine. At low concentrations of chloroquine, each type of molecule takes up equal amounts of the drug. This allows separation of individual topoisomers with negatively supercoiled molecules remaining supercoiled and relaxed topoisomers remaining relatively more relaxed. Thus at low chloroquine concentrations, the DNA that migrates fastest was highly negatively supercoiled before it left the bacterial cell. The samples were electrophoresed for 20 hours at 3 V/cm in TBE containing the same concentration of chloroquine as the gel itself. Following electrophoresis the gel was washed in distilled water for 4 hours to remove the chloroquine. The gel was then stained with ethidium bromide (5 µg/ml) and the topoisomers visualised by illumination with UV light.

2.20 DNA microarray analysis

A DNA microarray analysis was carried out to determine the *fis* regulon of *S. typhimurium* during growth in LB broth and was performed as described previously (Clements *et al.*, 2002). A PCR product *S. typhimurium* genome array was used courtesy of J. Hinton (Lucchini *et al.*, 2001; Thompson *et al.*, 2001). 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.

2.20.1 RNA isolation

Volumes (100 ml) of LB broth in 250 ml flasks were inoculated from overnight cultures of SL1344 and SL1344*fis::cat* and grown at 37°C with shaking. At 1 and 4 hours post subculture bacterial RNA was extracted. RNA for use in microarray analysis was isolated from a volume of culture corresponding to 4 OD_{600} units (i.e. 4 ml of OD_{600} = 1 or 8 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 (Tedin and Blasi, 1996). After 30 mins samples were pelleted by centrifugation (Jouan MR23i) at 14,000 rpm. RNA was isolated from these pellets using the Promega SV Total RNA isolation kit according to the manufacturer's instructions and as described at www.ifr.ac.uk/safety/microarrays/protocols.html. After elution the RNA was quantified as described in section 2.6.3, precipitated and resuspended at a concentration of 3 µg/µl in RNase free water (Sigma).

2.20.2 cDNA synthesis and Cy5-dye labelling

In a sterile microfuge tube 10 μg of RNA was incubated with 5 μg of random hexamer primers in a total volume of 15 μl using RNase free water (Sigma). 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 12 μl of the following RT mix was added: 5.0 μl 10 X Reverse Transcriptase buffer, 0.84 μl 25 mM dNTPs, 5.0 μl 0.1 M DTT, 5.0 μl RNase free water (Sigma). 100 U Stratascript Reverse Transcriptase and 2 μl of Cy5-dCTP (1mM stock, Amersham Pharmacia) were then added and samples were incubated at 37°C overnight.

Following overnight incubation, 1.5 μl of 20 mM EDTA was added to stop the RT reaction and 15 μl of 0.1 M NaOH was added and samples heated to 70°C for 15 min to hydrolyse the RNA. 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 2.8.2).

The above describes Cy5 labelling of enough cDNA for one array. Microarrays were carried out in quadruplicate and therefore the final eluate of 100 μl from the Wizard PCR Prep DNA purification system contained enough Cy5 labelled cDNA for 4 arrays (see section 2.20.5).

2.20.3 Cy3-dye labelling of genomic 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 labelled red with Cy5-dCTP. Both Cy-labelled 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

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binding of the cDNA (red) is compared. The BioPrime DNA Labelling Kit (Gibco/BRL) was used for the labelling of genomic DNA outlined below.

4 μg of genomic DNA (to be labelled with Cy3) isolated using the Qiagen 'Genomic DNA' Kit and quantified as described in section 2.6.3 was added to a sterile microfuge tube and the volume adjusted to 21 μl with RNase free water (Sigma). To this, 20 μl of 2.5 X Random primer/reaction buffer mix from the 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. On ice 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 Cy3-dCTP (1mM stock, Amersham), 1 μl of Klenow form the kit and incubation at 37°C was carried out overnight. Reactions were stopped by the addition of 5 μl of 0.5 M EDTA, purified using the Wizard PCR Prep DNA purification system (see section 2.8.2) and eluted in 100 μl nuclease free H_2O. The 100 μl provides enough of Cy3 labelled genomic DNA for eight arrays (see section 2.20.5).

2.20.4 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-methylimidazol 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 96% ethanol for 1 min and then centrifuged (Jouan MR23i) at 1200 rpm for 5 min to dry.

2.20.5 Hybridizations

Hybridization mix for 4 arrays was set up as follows. 50 μl of eluted Cy3 labelled genomic DNA (see section 2.20.3) was added to the 100 μl of eluted Cy5 labelled cDNA (see section 2.20.2), ethanol precipitated and resuspended in, 38 μl RNase free water (Sigma). To this the following were added: 6.0 μl of 50 X Denhardts, 9.0 μl of 20 X SSC, 4.5 μl of Yeast tRNA, 1.5 μl of 1M HEPES pH 7.0 and 1.48 μl of 10% (w/v) SDS. Samples were incubated at 100°C for 2 min then cooled to 20°C for 5 min. Samples were subjected to centrifugation in a microfuge at full speed for 5 min, transferred to a clean tube and the centrifugation step repeated. To set up the hybridizations, array slides were placed into the hybridization chambers (Die-Tech). The hybridization solution (15 μl per array) was applied towards one edge of the array. The edge of a 22 mm X 22 mm glass coverslip was placed on the edge of the array. A fine nosed forceps was positioned under the coverslip. Using the forceps the coverslip was gently lowered onto the hybridization solution on the array. The coverslip was lowered very gently to cover the array taking care to exclude air bubbles and ensuring even distribution of the solution across the array. 4 X 5 μl drops of 3 X SSC were placed on the four corners of the glass slide to maintain the correct 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 coverslips. 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 (Jouan MR23i) at 1200 rpm for 5 min at room temperature.

In this study all RNA samples were hybridized to microarrays in quadruplicate and two biological replicates were performed.

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2.20.6 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. Lucchini), 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 program GeneSpring 6.0 (Silicon Genetics). All array analysis was carried out using GeneSpring 6.0 and graphs of relevant data produced within the program (see chapter 4).

2.21 The *flhDC* promoter region was tested for curvature

In silico analysis of promoter curvature was calculated with the BEND.IT server (http://www2.icgeb.trieste.it/), using the DNase I-based bendibility parameters of (Brukner *et al*, 1995). The result of this analysis suggested that regions of the *flhDC* promoter were sharply curved.

2.22 Phase contrast microscopy

Phase contrast microscopy of bacterial samples was carried out using the Olympus BX51 microscope. Fixing and preparation of bacterial samples was performed as previously described (Bongaerts *et al.*, 2002).

Chapter 3

DNA supercoiling and Fis in

Salmonella enterica serovar Typhimurium

3.1 Introduction

It is thought that the countervailing activities of the DNA topoisomerase I and DNA gyrase establish a homeostatic balance of DNA supercoiling in the cell (Fig. 1.9), (Dorman, 1996; Hatfield and Benham, 2002; Menzel and Gellert, 1983). It is proposed that Fis is another component of the homeostatic control mechanism (Fig. 1.9). The activity of the *fis* promoter is highly sensitive to alterations in the superhelicity of the DNA, being maximal at slightly higher than optimal levels of DNA supercoiling (Fig. 1.9), (Schneider *et al.*, 2000). The Fis protein acts as a transcriptional repressor of the *gyrA* and *gyrB* genes in *E. coli* and *S. typhimurium* (Keane and Dorman, 2003; Schneider *et al.*, 1999). Therefore Fis can respond to deviations from physiological DNA supercoiling levels by either reinforcing or relieving repression at *gyrA* and *gyrB* (Fig. 1.9). Fis also mediates the induction of *topA* expression under conditions of oxidative stress (Weinstein-Fischer *et al.*, 2000).

Since, DNA supercoiling has an important influence on the ability *S. typhimurium* to adapt to an intracellular environment (Marshall *et al.*, 2000) and the Fis protein has been found to influence expression of the virulence genes of the SPI-1 pathogenicity island (Wilson *et al.*, 2001) and the *gyr* genes of *S. typhimurium* (Keane and Dorman, 2003), these findings provided a good reason to investigate further the effect of Fis on DNA topology.

The aim of this work was to examine a *fis* mutant in *S. typhimurium* for phenotypic differences compared to the wild-type, examine *fis* promoter activity, *fis* mRNA levels and Fis protein levels during growth and to investigate the effect of the Fis protein on DNA topology in *S. typhimurium*.

3.2 Results

3.2.1 Confirmation of a *fis* mutation

A *fis* knockout mutant, SL1344*fis::cat* (Keane and Dorman, 2003), was constructed by transducing the *fis::cat* lesion from *S. typhimurium* LT-2 strain TH2285 to the *S, typhimurium* strain used in this study SL1344 by bacteriophage P22 generalized transduction.

An initial investigation to confirm the *fis* mutation was undertaken. It was deduced by DNA sequence analysis that the 297 bp *fis* gene had undergone a deletion of 151 bp, leaving 65 bp at the 5' end and 81 bp at the 3' end and a chloramphenicol acetyltransferase gene had been inserted in place of the 151 bp of deleted *fis* DNA. The custom automated sequencing was carried out at MWG Biotech using the primers fisseq and fisrv (Table 2.3). fisseq binds 138 bp upstream from the *fis* ORF and fisrv binds 109 bp downstream of the *fis* ORF.

The *fis* mutation in *S. typhimurium* was also confirmed by PCR amplification (Fig. 3.1A) using the primer pair fisfw and fisrv (Table 2.3) and genomic DNA prepared from SL1344 and SL1344*fis::cat*. The primer fisfw binds the DNA directly downstream of the ATG translational start codon in the *fis* ORF and as mentioned above fisrv binds 109 bp downstream of the translational stop site in the *fis* ORF. Where wild-type SL1344 genomic DNA was used as template for the PCR reaction, the primers produced an amplimer of 403 bp in size (Fig. 3.1A). This size of amplimer represented the intact *fis* gene. The primer pair produced an amplimer of approximately 2 kb in size from SL1344*fis::cat* genomic DNA, consistent with the disruption of the *fis* gene (Fig. 3.1A).
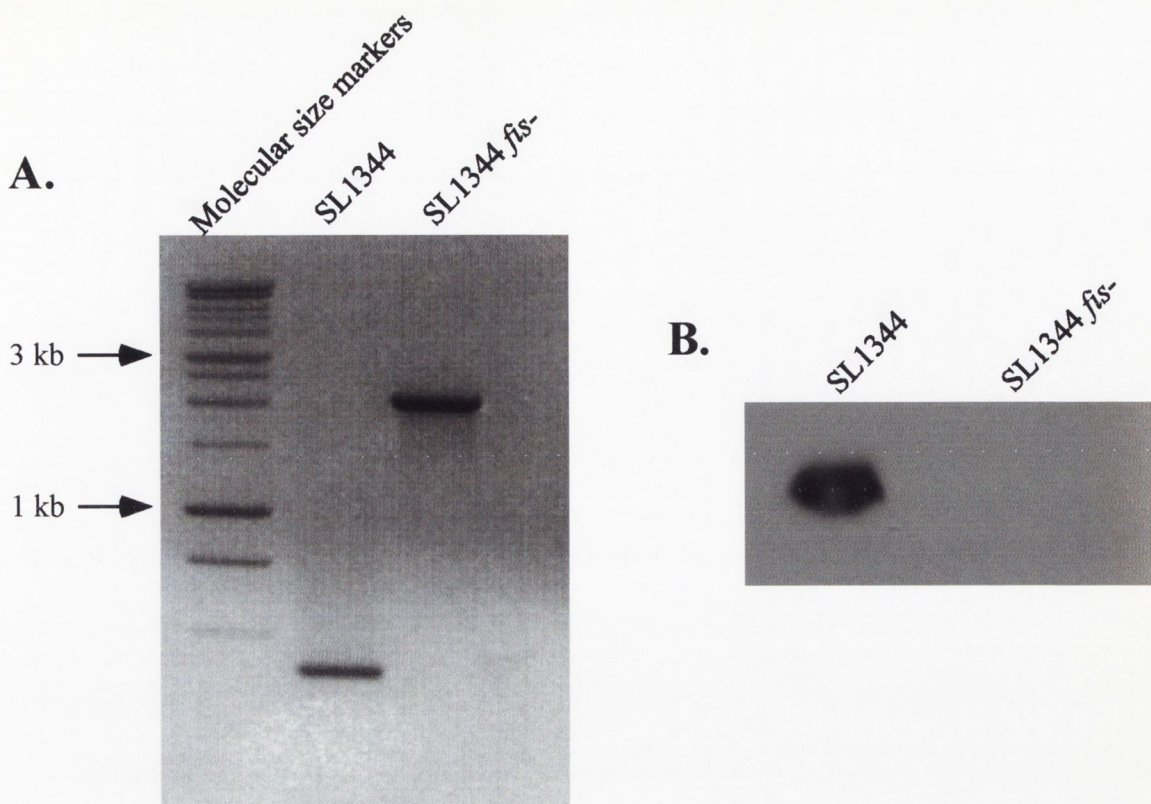
Fig. 3.1. Investigation of the *fis* mutation. **(A)** PCR analysis confirmed the disruption of the *fis* gene with a chloramphenicol cassette in SL1344*fis::cat*. The *fis* gene was amplified by PCR using genomic DNA from SL1344 and SL1344*fis::cat*. An amplimer of 403 bp was produced in the wild-type background compared to an amplimer of approximately 2 kb in the *fis* mutant background. These results verify the insertion of a chloramphenicol cassette in the *fis* gene of SL1344*fis::cat*.

(B) The absence of the Fis protein in SL1344*fis::cat* was confirmed by Western blotting. Protein samples were harvested from SL1344 and SL1344*fis::cat* after 90 min growth in LB broth at 37° C. Equal quantities of protein were loaded to an SDS PAGE gel and analysed by Western blotting with anti-Fis antiserum. The Fis protein present in the wild-type strain SL1344, is absent in the *fis* mutant background, SL1344*fis::cat*.

Sequencing and PCR analysis of SL1344*fis::cat* demonstrated the disruption of the *fis* gene with the insertion of a chloramphenicol cassette. To find out if Fis protein was produced from SL1344*fis::cat*, Western immunoblots were performed. 3 ml overnight LB broth cultures of SL1344 and SL1344*fis::cat* were subcultured into fresh 100ml volumes of LB broth in 250 ml non-baffled conical flasks and grown at 37°C with shaking for 90 min. Crude protein extracts were prepared from a volume of cells that corresponded to 2 ml of culture per 1.0 OD_{600nm} unit. Equal volumes of each extract were loaded to a 16% SDS-PAGE gel and analysed by Western blot using Fis-antiserum. Results clearly established the absence of the Fis protein in the SL1344 *fis* knockout mutant and the presence of an abundance of Fis protein in the wild-type background (Fig. 3.1B).

3.2.2 Growth of *S. typhimurium* in LB broth

Having confirmed that *S. typhimurium* SL1344*fis::cat* had undergone a 151 bp deletion of the *fis* gene, and that a chloramphenicol acetlytransferase gene replaced the deleted DNA, and having established the absence of Fis protein in the mutant strain, a phenotypic analysis of the *fis* mutant was carried out. Firstly growth rates of the wild-type and *fis* mutant in rich medium were compared. 100 ml fresh LB broth in 250 ml non-baffled conical flasks were inoculated from 3 ml overnight cultures of *S. typhimurium* SL1344 and SL1344*fis::cat* and grown at 37°C with shaking during which time the cell density was monitored at OD_{600nm}. The OD_{600nm} values were plotted against time and form the growth curve shown in Fig. 3.2. All the amino acids, nucleotide precursors, vitamins and other metabolites required for growth are supplied in the rich LB broth and both SL1344 and SL1344*fis::cat* grew rapidly. The wild-type and *fis* mutant doubled approximately every 30 minutes in logarithmic growth (Fig. 3.2). The *fis* mutant displayed a slightly longer lag phase prior to exponential growth but eventually grew to higher OD_{600nm} values in late stationary phase (Fig. 3.2). No
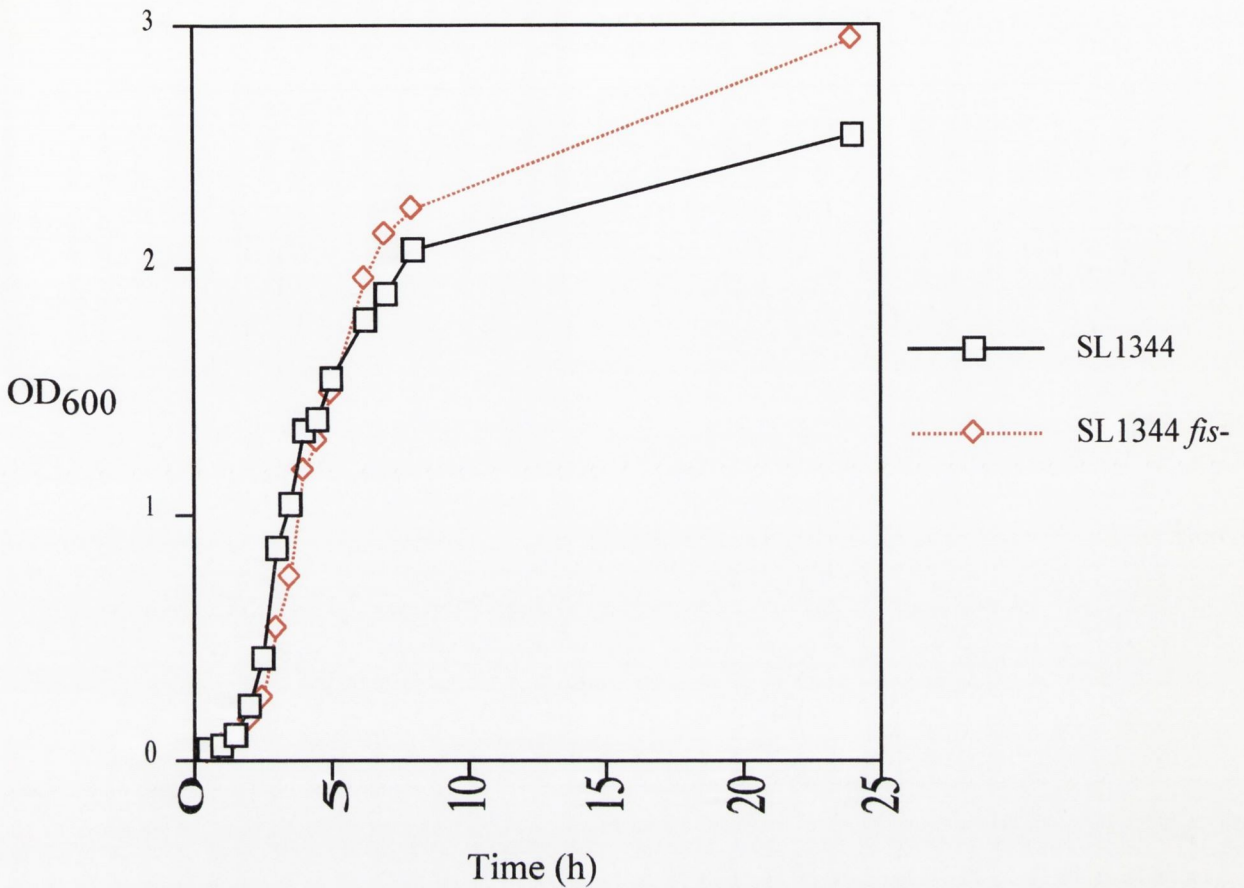
Fig. 3.2. Growth of SL1344 and SL1344*fis::cat* in LB broth. *S. typhimurium* SL1344 and SL1344*fis::cat* were grown overnight in 3 ml LB broth. Following overnight incubation they were subcultured into 100 ml volumes of fresh LB broth in 250 ml non-- baffled conical flasks and grown at 37°C with shaking. Growth of the two strains was monitored by OD_{600nm} readings at various time points post inoculation. The OD_{600nm} values were plotted against time. The wild-type and *fis* mutant displayed similar doubling times. The *fis* mutant had a slightly longer lag phase prior to exponential growth and reached slightly greater OD_{600nm} values in stationary phase, compared to the wild-type. The above experiment was performed at least three times and representative data are shown.

difference in cell viability was observed between the *fis* mutant and the wild-type (data not shown).

3.2.3 Phase contrast microscopy of SL1344 and SL1344*fis::cat*

In a further examination of *fis* mutant phenotypes, phase contrast microscopy was carried out to examine if there were any morphological differences between SL1344 and SL1344*fis::cat*. Wild-type and *fis* mutant SL1344 overnight cultures which were grown in LB broth at 37°C were examined by phase contrast microscopy. Wild-type cells were short and rod shaped whereas approximately 50% of *fis* mutant cells deviated from this and displayed significant filamentation (Fig. 3.3). This is in keeping with a previous report that describes *fis* null mutants forming filamentous cells at 32°C, 37°C and 44°C (Filutowicz *et al.*, 1992).

3.2.4 Construction of a *fis* transcriptional fusion

The level of Fis in the cell is subject to complex and multifactorial control. Transcription of the *fis* gene is influenced by the stringent response, is autoregulated by Fis protein and is controlled by the intracellular concentration of cytosine triphosphate (Ball *et al.*, 1992; Walker *et al.*, 1999). The *fis* promoter is stimulated by negative supercoiling of the DNA (Schneider *et al.*, 2000). Many of the Fis-related observations made in *E. coli* are also true in *S. typhimurium* (Keane and Dorman, 2003; Osuna *et al.*, 1995). Some differences in expression that have been reported reflect differences in the promoter sequence between the species (Osuna *et al.*, 1995). An investigation was carried out to study the *S. typhimurium fis* promoter activity in the wild-type and *fis* mutant backgrounds under conditions of growth in LB broth. To this end, a *fis* transcriptional fusion was constructed.

SL1344
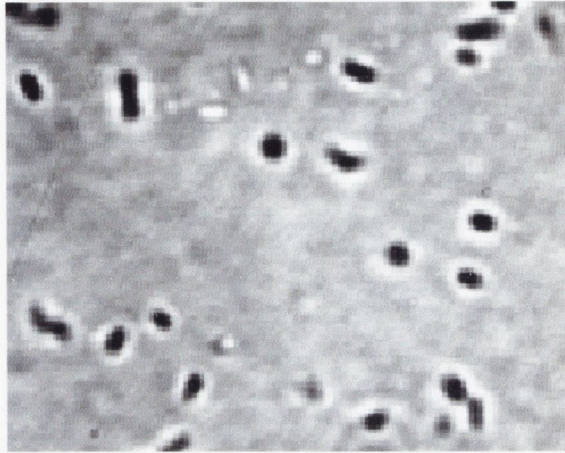
SL1344 *fis::cat*

Fig. 3.3. Phase contrast microscopy of the *S. typhimurium* **wild-type strain (SL1344) and the isogenic** *fis::cat* **mutant (SL1344***fis::cat***).** SL1344   and SL1344*fis::cat* were grown overnight in LB broth at 37° C with shaking prior to examination by phase contrast microscopy. The results indicate that the *fis* mutant has a propensity to form filamentous cells.

The *fis* gene is positioned downstream of *yhdG (orf 1)*, a gene with which it forms an operon. A 671 bp DNA fragment corresponding to the regulatory region upstream of the *fis* operon and the 5' region of the *yhdG* gene was amplified by PCR using the oligonucleotides pfisfw and pfisrv (Table 2.3) and cloned into the plasmid pQF50 to create plasmid pQFfis (Table 2.2, Fig 3.4). The amplified *fis* 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 the *Bam*HI/*Kpn*I digested pQF50. The order of these restriction sites in the plasmid pQF50 ensured the correct orientation of the *fis* promoter. Ligated plasmids were transformed into *E.coli* XL-1 cells and selected on LB agar containing ampicillin. Plasmid DNA was harvested and sequenced from the colonies recovered, to confirm that the *fis* promoter was inserted correctly into pQF50. PCR analysis and restriction enzyme digests were also employed to confirm pQFfis.

3.2.5 β-galactosidase activity of *fis* promoter

In an analysis of *fis* expression, β-galactosidase assays were performed on SL1344 and SL1344*fis* harbouring pQFfis at intervals after subculture into fresh LB broth. The results of the assay confirmed that there is a burst of *fis* expression immediately after subculture (lag phase/early exponential phase) and a steep decline in promoter activity soon after, confirming that *fis* expression is subject to growth phase regulation (Fig. 3.5). Also, the results verify *fis* expression to be negatively autoregulated by Fis protein, as *fis* promoter activity was higher in the *fis* mutant than in the wild-type (Fig. 3.5). Maximum *fis* promoter activity in the *fis* mutant, in early exponential phase, was 5 fold higher than the promoter activity in the *fis*+ background, at the same cell density. Fig 3.5 suggests that the induction of the *fis* promoter occurred earlier in the wild-type cells relative to the *fis* mutant cells. *fis* promoter activity in this study followed the same regulation pattern as was observed for *E. coli* (Ball *et al.*, 1992) and for *S. typhimurium* (Osuna *et al.*, 1995), in response to nutrient upshift.
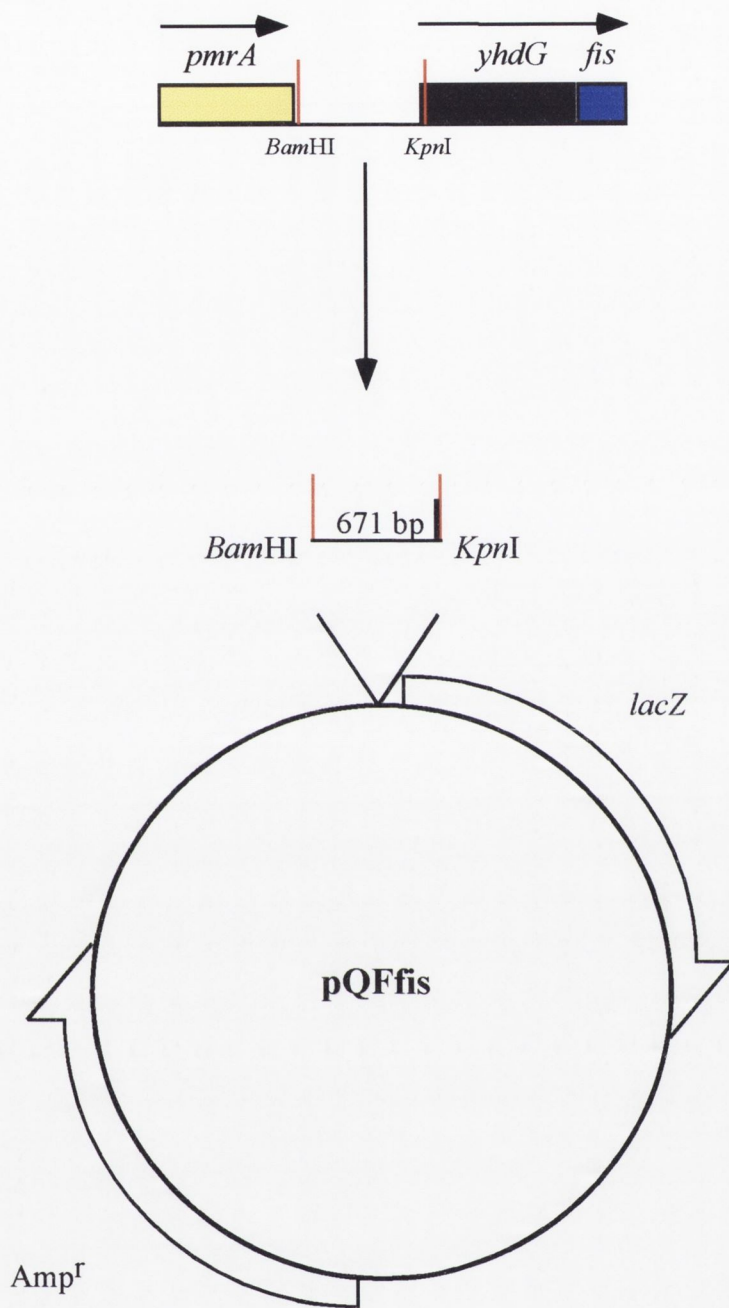
Fig. 3.4. Cloning of the *fis* operon promoter region into the plasmid pQF50. A 671 bp

section of DNA, incorporating the 5' region of *yhdG* and the *fis* operon regulatory region 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 pQFfis.
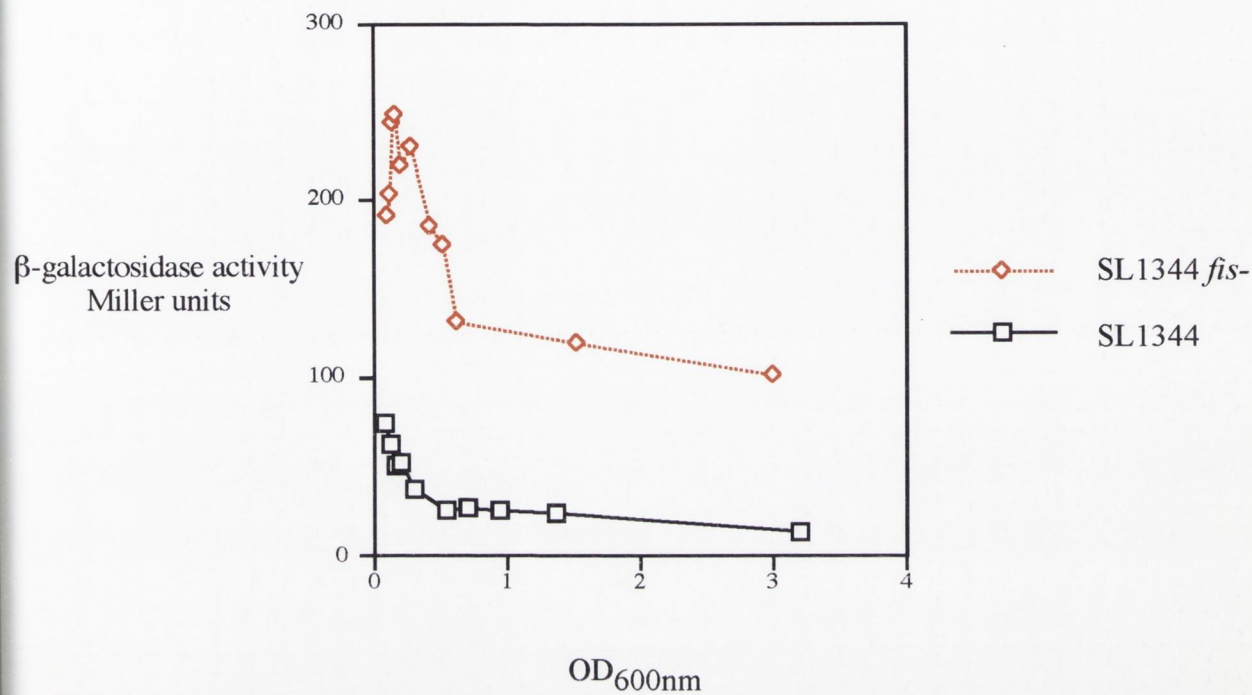
Fig. 3.5. **β-galactosidase activity of the *fis* promoter.** SL1344 and SL1344*fis-*

harbouring pQFfis were harvested for β–galactosidase analysis of the *fis* promoter at fixed

points during growth in LB at 37° C. The *S. typhimurium fis* promoter activity is subject

to growth phase regulation and autoregulation. The data shown are an average of two

independent experiments and standard deviation is less than 10%.

3.2.6 *fis* mRNA levels in *S. typhimurium*

A stationary phase overnight culture of SL1344 was used to inoculate 100 ml of fresh LB broth and total RNA was extracted from the bacterial culture at fixed time points during growth (Fig. 3.6A). The RNA was subjected to electrophoresis and probed for the presence of *fis* mRNA by Northern analysis. The result showed *fis* mRNA levels peaked at 30 minutes post nutrient upshift and were barely detectable at 1 and 2 h (Fig. 3.6B). Expression of *fis* mRNA was rapid and transient which is in good agreement with previous reports of *fis* mRNA expression in *E. coli* (Ball *et al.*, 1992; Ninnemann *et al.*, 1992) and *S. typhimurium* (Osuna *et al.*, 1995).

3.2.7 Fis protein levels in *S. typhimurium*

A stationary phase overnight culture of SL1344 was used to inoculate 100 ml of fresh LB broth and total protein was extracted from the bacterial culture at fixed time points during growth (Fig. 3.6A). Protein samples were electrophoresed on a polyacrylamide gel. A specific antibody to the Fis protein was used to monitor Fis protein expression in the wild-type strain throughout the growth cycle by Western blotting (Fig. 3.6C). It was found that peak expression of Fis protein occurred 1h after diluting the overnight culture into fresh medium. No Fis protein was detectable by 3 h which correlates with reports in *E. coli* and *S. typhimurium* on the growth phase-dependent expression of Fis (Ball *et al.*, 1992; Keane and Dorman, 2003; Osuna *et al.*, 1995). The similarity in the expression profile of the Fis protein (Fig. 3.6C) and the *fis* transcript (Fig. 3.6B) is also in good agreement with previous reports (Ball *et al.*, 1992; Osuna *et al.*, 1995). Thus, the synthesis of the *fis* mRNA and protein under these culturing conditions appears to be efficiently switched off as cells enter exponential growth.

Fig. 3.6. Expression patterns of *fis* mRNA and Fis protein throughout growth.

(A) Growth curve of *S. typhimurium* strain SL1344 during growth in LB broth.

(B) Northern blot analysis of SL1344 *fis* mRNA levels during growth in LB broth. Total RNAs were extracted at various time points throughout the growth curve (A) and hybridized with a *fis*-specific probe. A burst of *fis* mRNA expression occurred immediately after nutrient upshift and then declined suddenly, with only faint traces of the *fis* message at 1 h and 2 h.

(C) Fis protein levels as a function of growth phase. Total protein was extracted from SL1344 during growth in LB broth at time points indicated and Western blotting was performed to examine the Fis protein expression profile. The Fis protein levels peaked at 1 h and were undetectable by 3 h.

A.

B.

TIME (h)	1/2	1	2	3	4	24

fis mRNA

OD$_{600nm}$	0.054	0.094	0.53	1.03	1.33	2.97

C.

TIME (h)	1/2	1	2	3	4	24

Fis protein

OD$_{600nm}$	0.051	0.082	0.382	0.97	1.2	3.13
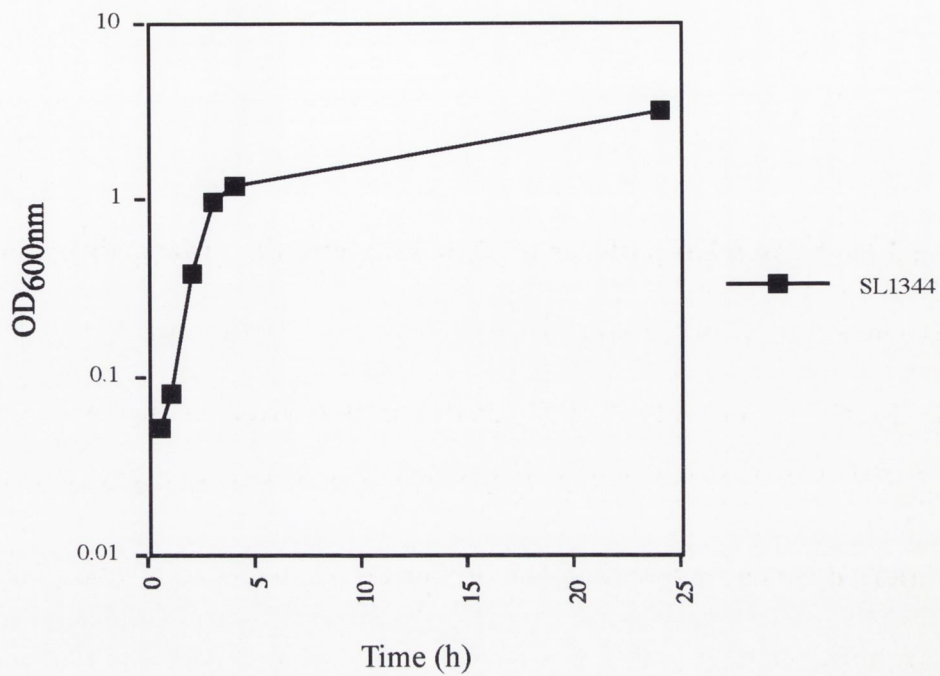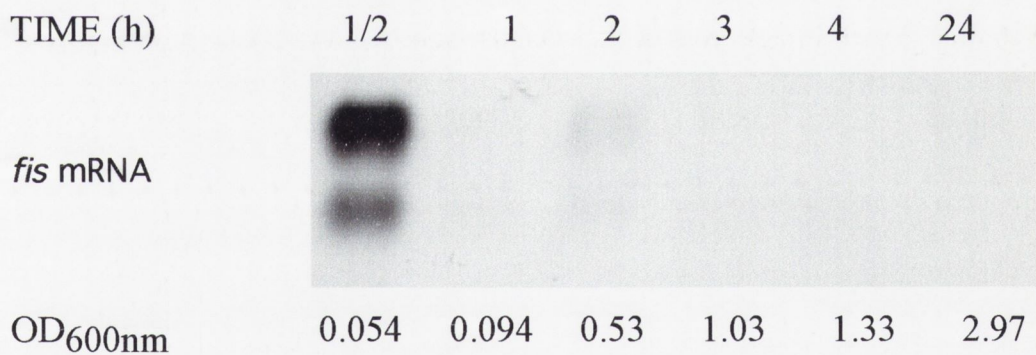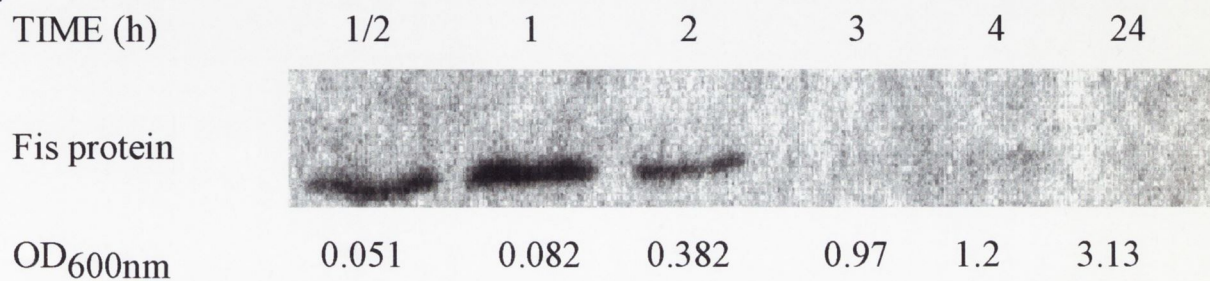
3.2.8 Fis and DNA supercoiling in *S. typhimurium*

To investigate the effect of the architectural protein Fis on DNA topology, pUC18 was used as a reporter of DNA topology. Overnight cultures of *S. typhimurium* strains, SL1344 and SL1344*fis::cat* and *E. coli* strains, CSH50 and CSH50*fis::kan*, harbouring the reporter plasmid pUC18, were inoculated into 100 ml fresh LB broth and grown at 37°C with shaking. pUC18 was harvested from these cells during early exponential phase at a cell density of OD_{600nm} 0.5. The reporter plasmid DNA was isolated by Qiagen preparation, quantified by OD_{260nm} reading and equal amounts electrophoresed through an agarose gel containing 2.5 μg/ml chloroquine to resolve topoisomers. At this concentration of chloroquine the most negatively supercoiled topoisomers migrate fastest. A difference in the DNA topology between *fis+* and *fis-* cells was observed. The topological profiles for pUC18 in the wild-type background of *S. typhimurium* and *E. coli* were predominantly negatively supercoiled, whereas in the *fis-* background of *S. typhimurium* and *E. coli*, pUC18 was present as a broader range of topoisomers (Fig. 3.7A). When Fis was present, the population of topoisomers was predominantly highly negatively supercoiled and in the absence of Fis, the DNA topology consisted of highly negatively supercoiled and more relaxed topoisomers. The effect of Fis on DNA topology was also evident when different reporter plasmids, pACYC177 and pBR322 were used (Fig. 3.7A). Thus the effect of Fis on DNA topology is not plasmid specific.

Following overnight culturing to an OD_{600nm} reading of 2.5 pUC18 was once again isolated and subjected to agarose gel electrophoresis in the presence of 2.5 μg/ml chloroquine. The Fis+ cells of *S. typhimurium* and *E. coli* had similar pUC18 topological profiles composed of negatively supercoiled and intermediately supercoiled topoisomers (Fig. 3.7B). However, in the absence of Fis the pUC18 topological profiles differed between *S. typhimurium* and *E. coli* (Fig. 3.7B). In *S. typhimurium* pUC18 was maintained as negatively supercoiled and intermediately supercoiled topoisomers, but in *E. coli* pUC18 was present only as negatively supercoiled topoisomers (Fig. 3.7B). Fis protein levels are significantly reduced in overnight cultures of *S. typhimurium* (Fig.
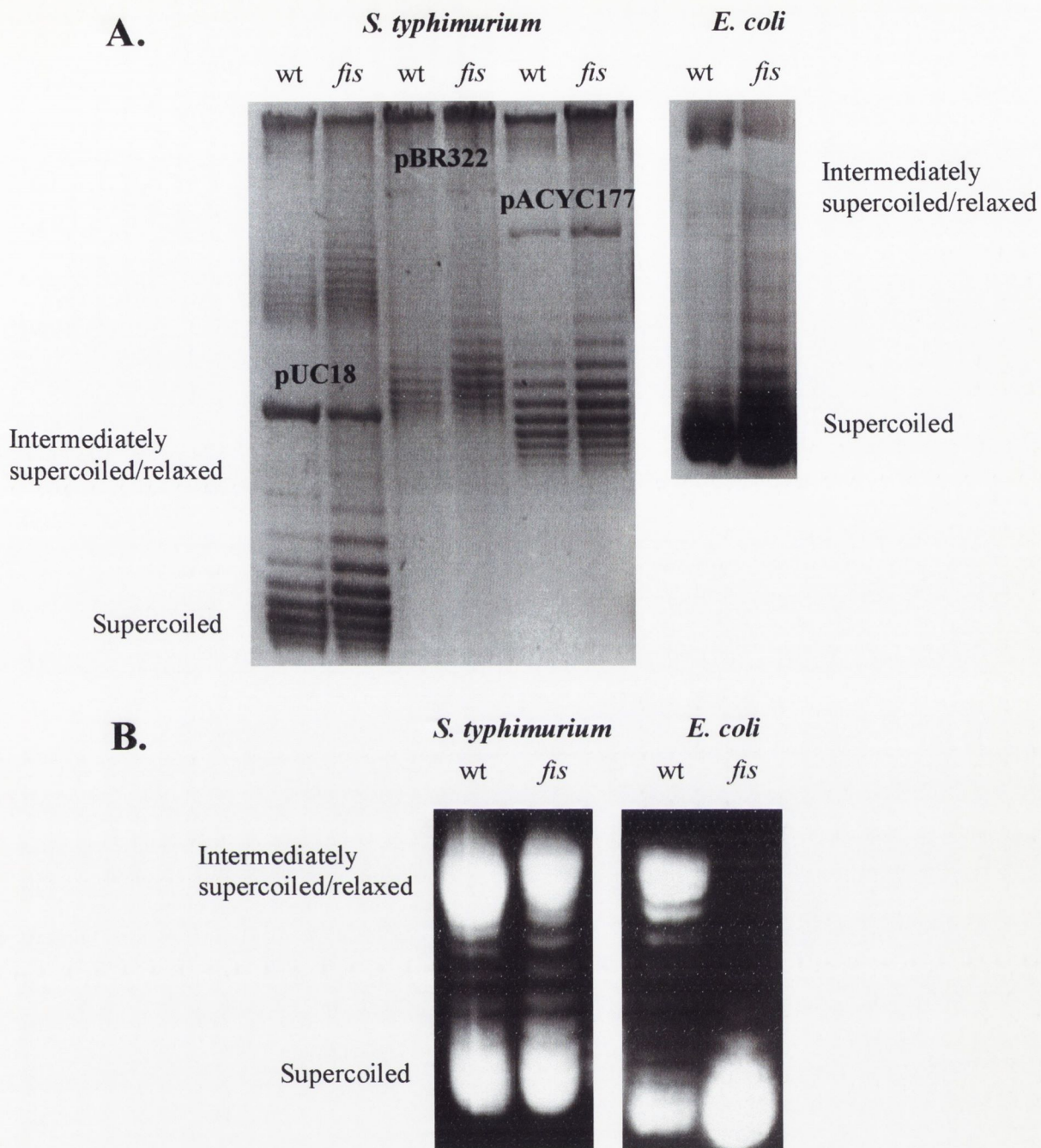
Fig. 3.7. Effect of Fis on topoisomer distribution in *S. typhimurium* and *E. coli*. Reporter plasmids were isolated from wild-type and *fis* mutant backgrounds of *S. typhimurium* and *E. coli* in early exponential phase of growth (A) and following overnight culturing (B). Equal concentrations of plasmids were subjected to agarose gel electrophoresis in the presence of 2.5 µg/ml chloroquine to resolve topoisomers.

3.6C), (Keane and Dorman, 2003) and *E. coli* (Ball *et al.*, 1992). Therefore no difference in the topological profiles between wild-type and *fis-* backgrounds of *S. typhimurium* and *E. coli* was expected. In agreement with this, pUC18 extracted from SL1344 *fis+* and *fis-* cells displayed identical topological profiles. However, the topological profiles of *fis+* and *fis-* overnight cultures of *E. coli* were different. pUC18 topoisomers were composed of negatively supercoiled DNA and intermediately supercoiled DNA in the wild-type background, but only negatively supercoiled topoisomers were present in the *fis* mutant background. Differences in the superhelical densities of DNA extracted from *fis+* and *fis-* *E. coli* backgrounds were reported previously (Schneider *et al.*, 1997). Clearly the Fis protein can influence DNA topology and the ability to do this differs between *S. typhimurium* and *E. coli*.

To continue the investigation of the effect of Fis on DNA topology, the pUC18 plasmid was isolated at different intervals after subculturing SL1344 and SL1344*fis* into fresh medium. This is referred to as nutritional shift-up and is responsible for the dramatic induction of *fis* expression. Overnight cultures consisted of intermediately supercoiled and negatively supercoiled topoisomers (Fig. 3.8). After nutritional shift-up the level of negative supercoiling increased and intermediately supercoiled DNA slowly disappeared, a process that occurred at the same rate in the wild type and *fis* backgrounds. However, when this experiment was carried out in *E. coli* it was observed that the increase in supercoiling after nutritional shift-up was slower to occur in the wild type than in the *fis* background and Fis was proposed to be responsible for binding to and preserving intermediately supercoiled DNA after nutritional shift-up (Schneider *et al.*, 1997).
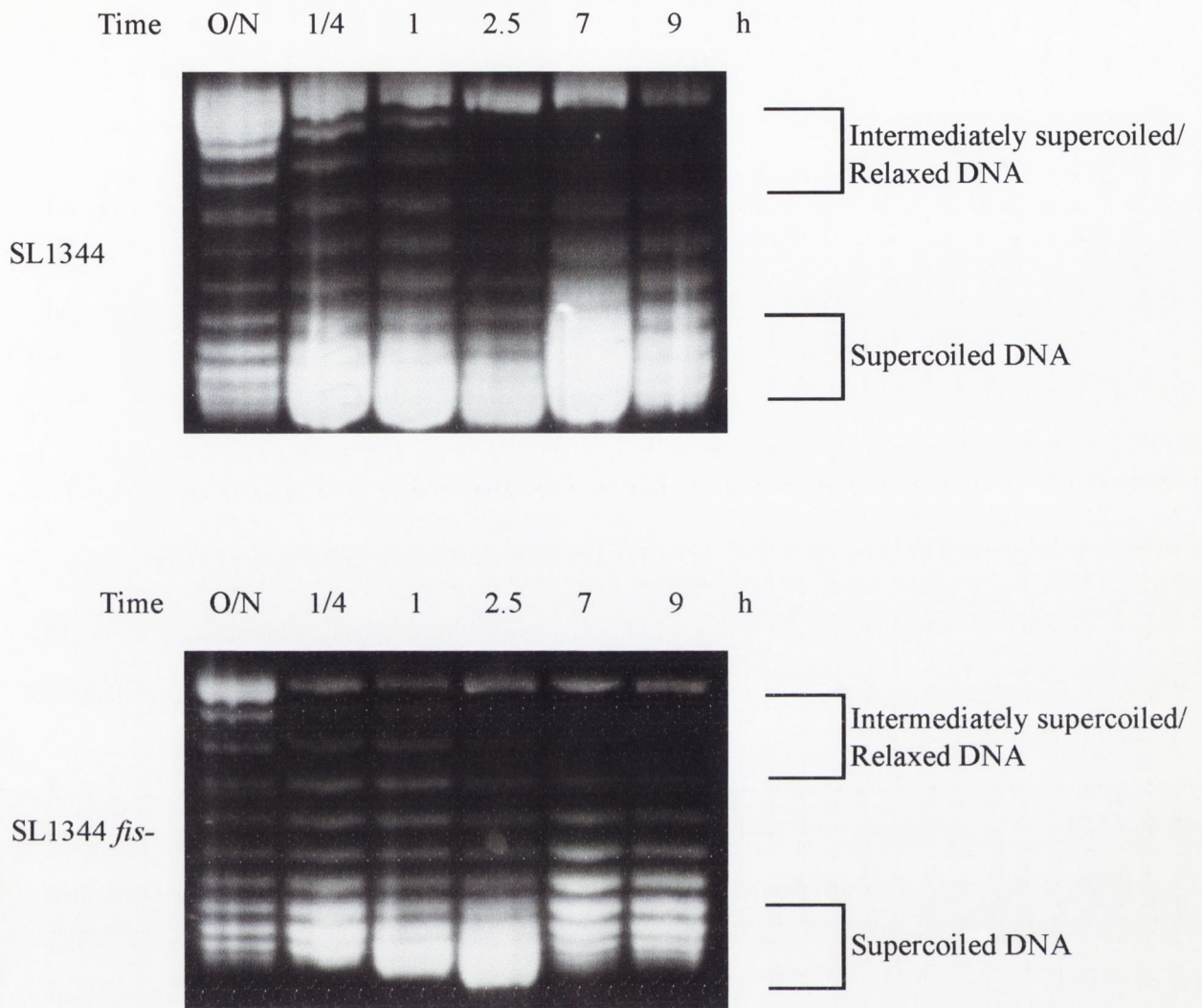
Fig. 3.8. Plasmid supercoiling in SL1344 and SL1344*fis-* **cells during growth phase.** pUC18 plasmid DNA was isolated at different intervals after subculturing the overnight cultures of SL1344 and SL1344 *fis-* into fresh LB broth. pUC18, a reporter of DNA topology was subjected to agarose gel electrophoresis in the presence of 2.5 µg/ml chloroquine, to resolve topoisomers. 2.5 µg/ml chloroquine allows the most negatively supercoiled topoisomers to migrate fastest.

3.2.9 Construction of pBADfis

In order to see more clearly the effect of Fis on DNA supercoiling levels in *S. typhimurium*, high levels of Fis protein were artificially induced in a *fis* mutant background. To this end, the *fis* open reading frame (ORF) was put under the control of the P_{BAD} promoter in the vector pBAD24 (Table 2.2). A 403 bp fragment corresponding to the 297 bp *fis* ORF and a downstream regulatory region between *fis* and *yhdJ* was amplified by PCR using the oligonucleotides fisfw and fisrv (Table 2.3), and cloned into the plasmid pBAD24 to generate pBADfis (Table 2.2, Fig. 3.9). The amplified *fis* ORF fragment was extended with a *Hind*III site at the 3' end. After digestion with *Hind*III, the fragment was cloned into pBAD24, linearized with *Hind*III and *Nco*I. Following *Nco*I digestion and prior to *Hind*III digestion, Klenow polymerase was employed to create a blunt end at the *Nco*I cut sites. The relative locations of the blunt end and *Hind*III cut end of pBAD24 and the DNA fragment ensured that the *fis* ORF was cloned into pBAD24 in the correct orientation. Ligated plasmids were transformed into *E. coli* XL-1, and selected for on agar containing ampicillin. Plasmid DNA was harvested and sequenced from transformants to confirm that the *fis* ORF had inserted correctly into pBAD24. PCR analysis and restriction enzyme digests were also employed to verify the structure of pBADfis.

The plasmid pBADfis was confirmed as containing the *fis* ORF cloned downstream of the P_{BAD} promoter of the *araBAD* (arabinose) operon. The pBAD vector also contains the regulatory gene *araC*. The AraC protein is a positive and negative regulator of the P_{BAD} promoter. In the presence of arabinose, transcription from the P_{BAD} promoter is turned on, in its absence, transcription occurs at very low levels and in the presence of glucose transcription is repressed. This tightly regulated system was used in this study to control the expression of Fis protein in an experiment to investigate further the effect of Fis on DNA topology.
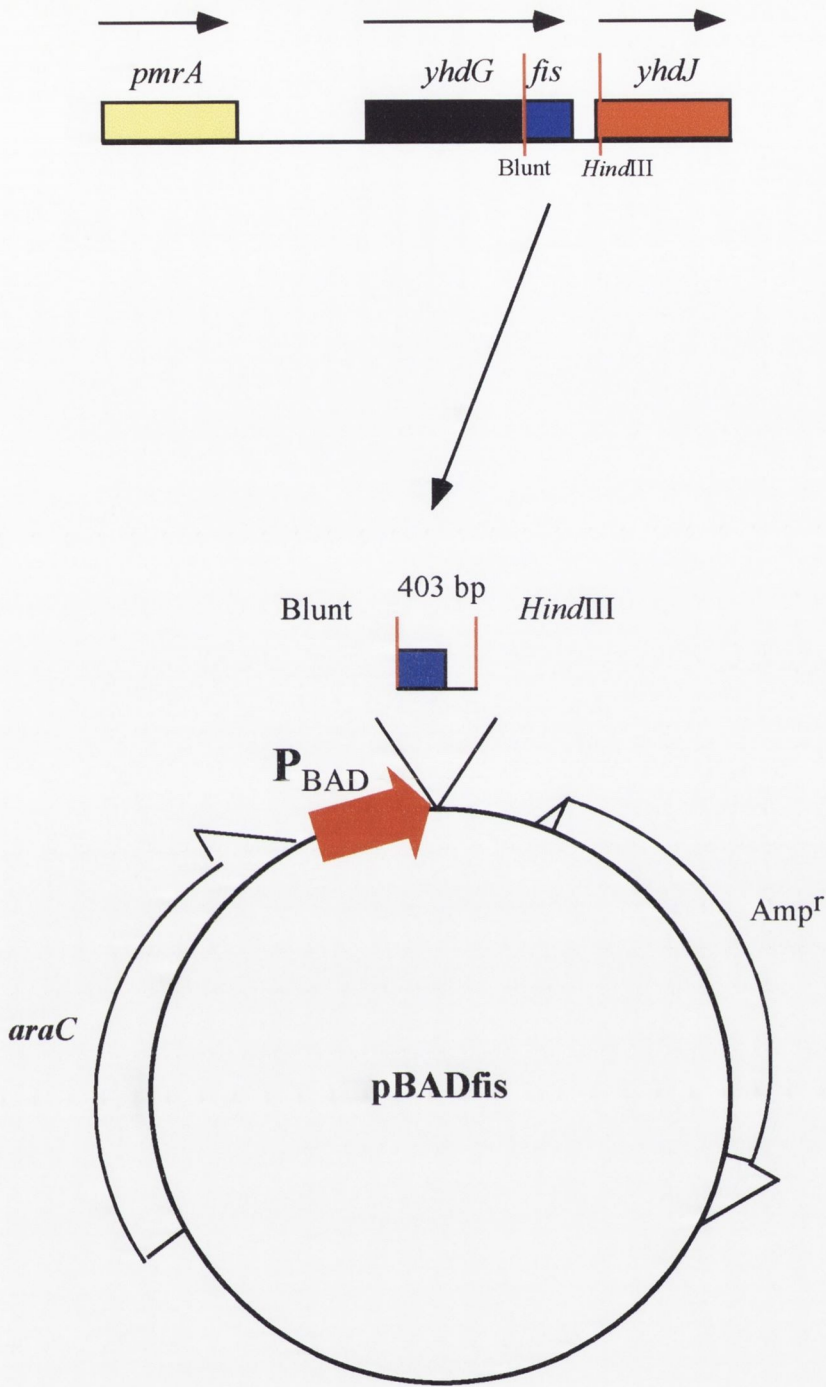
Fig. 3.9. Cloning of the *fis* open reading frame (ORF) into plasmid pBAD24. A 403 bp region of DNA, incorporating the *fis* ORF was amplified by PCR and ligated into the multiple cloning site of the plasmid pBAD24 downstream of the arabinose inducible promoter P_{BAD}. The resulting plasmid was named pBADfis.

3.2.10 Controlled expression of Fis and its effect on DNA topology

Plasmid pBADfis was introduced into SL1344*fis*. SL1344*fis* harbouring pBADfis was grown overnight in LB broth at 37°C with shaking in the absence or presence (0.2% or 1.0%) of arabinose. Following overnight culturing pBADfis was isolated and subjected to electrophoresis in the presence of 2.5 µg/ml chloroquine to resolve topoisomers. pBADfis was used as a reporter of DNA topology (Fig. 3.10A). Also following overnight culturing, protein samples were harvested to monitor the controlled induction of Fis protein by Western immunoblotting (Fig. 3.10B). Figure 3.10 illustrates Fis protein levels at 0%, 0.2% and 1.0% arabinose with the corresponding topoisomer distributions of pBADfis at these levels of Fis protein. Topoisomers of pBADfis at 0% arabinose were composed largely of negatively supercoiled DNA (Fig 3.10A). Western blotting did not detect Fis protein at 0% arabinose (Fig 3.10B). At 0.2% arabinose, an induction of Fis protein was observed by Western blotting (Fig. 3.10A) and the topological profile of pBADfis was altered only by a slight increase in negatively supercoiled topoisomers and a disappearance of the faint intermediately supercoiled topoisomers (Fig. 3.10A). A more dramatic change in the topological profile of pBAD*fis* was observed at 1.0% arabinose. 1.0% arabinose induced the greatest level of Fis protein (Fig. 3.10B) and the topological profile of pBADfis consisted of a broad range of intermediately supercoiled/relaxed DNA and negatively supercoiled plasmids (Fig. 3.10A). The greatest level of Fis protein was accompanied by a dramatic relaxation of the DNA. This is in keeping with the role of Fis as a transcriptional repressor of the genes that encode DNA gyrase. It is proposed that at these high levels of Fis protein the supercoiling activity of DNA gyrase is greatly reduced and therefore the DNA topology consists of less highly negatively supercoiled topoisomers and more intermediately supercoiled and relaxed topoisomers.

A.
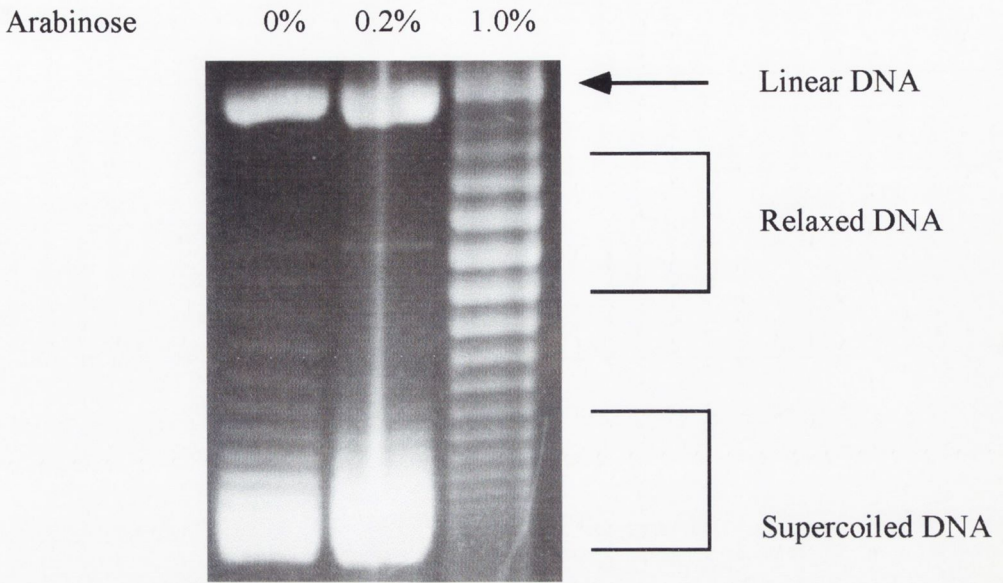
B.
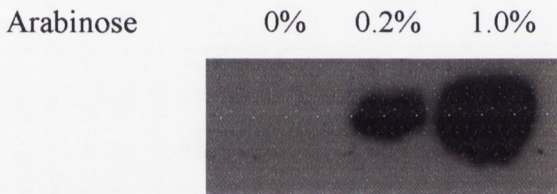
Fig. 3.10. Controlled induction of Fis and its effect on DNA topology in *S. typhimurium.*
SL1344*fis*- harbouring the pBADfis plasmid was cultured overnight in the absence (0%) or presence (0.2% or 1.0%) of arabinose. Following overnight culturing pBADfis was isolated and used as a reporter of DNA topology (A), and total protein samples were harvested to show the controlled induction of Fis protein by Western blotting (B). Plasmid DNA topoisomers were resolved by agarose gel electrophoresis in one dimension in the presence of 2.5 µg/ml chloroquine. The topoisomers that migrated fastest were negatively supercoiled before they left the bacterial cell. Fis protein levels increased with increasing arabinose levels. The greatest induction of Fis protein correlated with relaxation of the superhelical density of the DNA.

3.3 Discussion

Custom automated sequencing and PCR analysis confirmed that the *S. typhimurium fis* knockout mutant, SL1344*fis::cat,* had undergone a 151 bp deletion of the *fis* gene and the deleted DNA was replaced with the chloramphenicol acetyltransferase gene (Fig. 3.1A). The absence of Fis protein in SL1344*fis::cat* was established by Western blotting (Fig 3.1B). Having confirmed the presence of a functional *fis* gene in SL1344 and a *fis* knockout mutation in SL1344*fis::cat*, the growth of SL1344 and SL1344*fis::cat* in LB at 37°C was monitored (Fig. 3.2). During exponential growth the wild-type and *fis* mutant cells both doubled approximately every 30 minutes. Therefore Fis does not appear to influence the growth rate of *S. typhimurium* during rapid growth. The *fis* mutant demonstrated an extended lag phase prior to exponential growth compared to the wild-type (Fig. 3.2), in good agreement with a previous study (Osuna *et al.*, 1995). Despite this the *fis* mutant cells grew to greater cell densities in stationary phase (Fig. 3.2). SL1344 and SL1344*fis::cat* cells growing in LB at 37°C were examined by phase contrast microscopy for morphological differences (Fig. 3.3). It was observed that the *fis* mutant cells have a propensity to filament, while the wild-type does not. Filamentation is a previously observed phenotype for *fis* mutant *S. typhimurium* and *E. coli* cells (Filutowicz *et al.*, 1992; Osuna *et al.*, 1995). Filamentation of an *E. coli fis* mutant was observed at 32°C, 37°C and 44°C in LB medium (Filutowicz *et al.*, 1992). In the same study the Fis protein was shown to function in the initiation of replication of the *E. coli* chromosome. Therefore it is possible that, under conditions of rapid growth, *fis* mutants defective in DNA replication manifest this defect by filamenting.

A study of the regulation of *fis* promoter activity, RNA expression and protein production as a function of growth phase was carried out. β-galactosidase assays were performed on SL1344 and SL1344*fis::cat* harbouring pQFfis at intervals after subculture into fresh LB broth to measure *fis* operon promoter activity during the growth of the bacteria following nutrient upshift. The results of the assay confirmed

that there is a burst of *fis* expression immediately after subculture into fresh medium and a decline in promoter activity occurs during exponential growth phase and is minimal during stationary phase, confirming *fis* expression to be subject to growth phase regulation (Fig. 3.5). Also the results verify that the *fis* promoter is negatively autoregulated, as promoter activity was higher in the *fis* mutant background than in the wild-type background (Fig. 3.5). Maximum *fis* promoter activity in the *fis* mutant, in early exponential phase, was 5 fold higher than the promoter activity in the wild-type background, at the same cell density. The magnitude of *fis* promoter activity was greater in the *fis* mutant compared to the wild-type background throughout the growth curve, but the *fis* promoter was still subject to the same growth phase regulation in the absence of Fis. Therefore growth phase regulation of the *fis* promoter is not Fis dependent. *fis* promoter activity in this study was subject to growth phase regulation and autoregulation as was observed in *E. coli* (Ball *et al.*, 1992; Nilsson *et al.*, 1992; Ninnemann *et al.*, 1992) and *S. typhimurium* (Osuna *et al.*, 1995).

Overnight cultures of *S. typhimurium* were inoculated into fresh LB broth and total RNA and protein were harvested at fixed time points during growth for Northern and Western analysis, respectively (Fig. 3.6). Correlating with previous *fis* regulation studies, the Fis protein and mRNA patterns of expression were similar to each other. *fis* mRNA levels peaked 30 min post nutrient upshift and by 1 h had already declined rapidly. Fis protein levels peaked, later than the *fis* mRNA, at 1 h after inoculation into fresh medium and were undetectable by 3 h. The Fis protein levels peaked later than the mRNA levels but lasted in the cell for longer. Although Fis protein levels decline dramatically and are undetectable at 3 h by Western blotting, they are thought to reach a low but non-zero value, rather than vanishing from the cell (Azam *et al.*, 1999; Walker *et al.*, 1999). It is suggested that at very low cellular concentrations of the Fis protein, it is associated to high affinity binding sites while low affinity sites are vacant (Muskhelishvili and Travers, 2003).

pUC18 was used as a reporter of the superhelical density of the DNA in *S. typhimurium* and *E. coli* cells. Overnight cultures of *S. typhimurium* strains, SL1344 and

SL1344*fis::cat* and *E. coli* strains, CSH50 and CSH50*fis::kan*, harbouring the reporter plasmid pUC18, were inoculated into 100 ml fresh LB broth and grown at 37°C with shaking. pUC18 was isolated during early exponential phase. A difference in the DNA topology between *fis+* and *fis-* cells was observed. The topological profiles for pUC18 in the wild-type background of *S. typhimurium* and *E. coli* was predominantly negatively supercoiled, whereas in the *fis-* background of *S. typhimurium* and *E. coli*, pUC18 was present as a broader range of topoisomers (Fig. 3.7A). When Fis is present, the population of topoisomers is predominantly highly negatively supercoiled and in the absence of Fis, the DNA topology consists of highly negatively supercoiled and more relaxed topoisomers. The effect of Fis on DNA topology is not plasmid specific because the same difference in DNA topology between wild-type and *fis-* cells is observed when different reporter plasmids were used (Fig. 3.7A). Since Fis is proposed to be a component of the homeostatic control mechanism of DNA supercoiling (Schneider *et al.*, 2000) perhaps it is the absence of Fis from this network that is responsible for the topological differences observed between wild-type and *fis* mutant cells. Since Fis is a transcriptional repressor of the *gyrA* and *gyrB* promoters (Keane and Dorman, 2003; Schneider *et al.*, 1999), the absence of Fis would relieve this repression and thus lead to an increase in gyrase activity. An increase in gyrase activity would increase negative supercoiling levels (Drlica, 1992; Hatfield and Benham, 2002). High levels of DNA supercoiling activate the *topA* promoter (Drlica, 1992; Hatfield and Benham, 2002). An increase in DNA topoisomerase I activity, relaxing negatively supercoiled DNA, would occur to counter the increase in gyrase activity (Drlica, 1992; Hatfield and Benham, 2002). Possibly it is an increase in DNA topoisomerase I activity in the *fis-* cells that is responsible for their DNA topology being composed of more intermediately supercoiled and relaxed DNA in comparison to the wild-type cells which are composed largely of only negatively supercoiled DNA (Fig. 3.7A).

The difference in the DNA topology between wt and *fis-* cells was observed in early exponential phase when Fis protein levels are relatively abundant in the wild-type cells (Fig. 3.6C). The experiment was repeated, this time isolating pUC18 from overnight/stationary phase cultures when Fis protein levels are reduced in the wild-type

background of *S. typhimurium* (Fig. 3.6C) and *E. coli* (Keane and Dorman, 2003). In *S. typhimurium* wild-type and *fis* mutant backgrounds, pUC18 was maintained as negatively supercoiled and intermediately supercoiled topoisomers (Fig. 3.7B). The Fis+ cells of *E. coli* had similar pUC18 topological profiles composed also of negatively supercoiled and intermediately supercoiled topoisomers (Fig. 3.7B). However, *E. coli fis-* cells were composed only of highly negatively supercoiled topoisomers (Fig. 3.7B). Since Fis protein levels are depleted in stationary phase cultures no difference in the topological profiles between wt and *fis-* backgrounds of *S. typhimurium* and *E. coli* was expected. In agreement with this, pUC18 extracted from *S. typhimurium fis+* and *fis-* cells displayed identical topological profiles. However, the topological profiles of *fis+* and *fis-* overnight cultures of *E. coli* were dramatically different and such differences in the superhelical densities of DNA extracted from *fis+* and *fis- E. coli* backgrounds was reported previously (Schneider *et al.*, 1997). Differences in the overall DNA topology between wild-type and *fis* mutant backgrounds of *E. coli* cells in stationary phase is in good agreement with Fis protein concentrations being at a low but non-zero value in stationary phase (Azam *et al.*, 1999; Walker *et al.*, 1999).

Bacterial DNA supercoiling levels increase upon nutrient upshift (Balke and Gralla, 1987). Fis was shown to bind and stabilize intermediately supercoiled DNA upon nutrient upshift in *E. coli* to prevent detrimentally high levels of negative supercoiling (Schneider *et al.*, 1997). In *E. coli* the absence of Fis resulted in a rapid transition from intermediately and negatively supercoiled DNA to a highly negatively supercoiled population of topoisomers upon nutrient upshift and the presence of Fis in *E. coli* slowed down this process delaying the disappearance of the intermediate supercoils (Schneider *et al.*, 1997). In this study, Fis protein did not appear to carry out this function in *S. typhimurium,* or at least not exclusively (Fig. 3.8). Nutritional shift-up increased the level of negative supercoiling and intermediate supercoils slowly disappeared, a process that occurred at the same rate in the wild type and *fis-* backgrounds of *S. typhimurium* (Fig. 3.8). Therefore the absence of Fis had no

significance on the rate of increasing supercoiling levels upon nutrient upshift in *S. typhimurium*.

Common to both *S. typhimurium* and *E. coli* is the role of Fis as a transcriptional repressor of the *gyr* genes that code for DNA gyrase (Keane and Dorman, 2003; Schneider *et al.*, 1999). This repression is clearly observed when Fis protein levels are artificially induced to high levels in a *fis* mutant background resulting in the concomitant relaxation of the DNA (Fig. 3.10). The relaxation of the DNA is perhaps due to an overwhelming repression of the supercoiling activity of DNA gyrase. It is interesting to speculate that at these high levels of Fis protein the homeostatic control mechanism cannot compensate for the Fis mediated repression of DNA gyrase. Also since the induction of Fis protein is from the arabinose inducible promoter P_{BAD} in a *fis* mutant background there is no negative autoregulation of Fis expression to try and relieve the repression at the *gyr* gene promoters. In addition in the absence of a functional *fis* gene downstream of the native *fis* promoter means any feedback from the supercoiling levels on the *fis* promoter activity is useless.

Clearly the Fis protein can influence DNA topology and the ability to do this differs between *S. typhimurium* and *E. coli*. Differences observed in the DNA superhelicity between *E. coli* wild-type and *fis* mutant cells in this study and in previous studies discussed seem more pronounced than the differences in the DNA topology between *S. typhimurium* wild-type and *fis* mutant cells. It is proposed, since alterations in DNA topology may influence virulence gene expression in *S. typhimurium*, a facultative intracellular pathogen (Marshall *et al.*, 2000), that tighter control is maintained over its DNA supercoiling levels compared to *E. coli*.

Why the topological profiles of pUC18 extracted from wild-type and *fis* mutant cells are similar in *S. typhimurium* but very different in *E. coli* is difficult to explain (Fig. 3.7B and Fig. 3.8), (Schneider *et al.*, 1997). It is interesting to speculate it is somehow related to the fact that Fis protein levels are depleted quicker in *E. coli* than in *S. typhimurium* (Keane and Dorman, 2003).

It is important to note that wider influences than DNA gyrase, DNA topoisomerase I and Fis affect DNA supercoiling and that these factors may vary between *E. coli* and *S. typhimurium*. The nucleoid-associated proteins each have very distinct expression profiles when growing in batch culture (Azam *et al.*, 1999) and they contribute to the overall DNA supercoiling level in the cell. The HU protein one of the most abundant nucleoid-associated proteins can constrain negative supercoils in the DNA (Broyles and Pettijohn, 1986; Rouviere-Yaniv *et al.*, 1979). *hupAhupB* double mutants have an altered chromosome structure and lose approximately 10% of plasmid supercoiling, where *hupA* and *hupB* encode the subunits of the heterodimeric HU protein (Oberto and Rouviere-Yaniv, 1996). The histone-like protein H-NS that binds preferentially to curved DNA has the ability to compact the DNA when overexpressed (Dame *et al.*, 2000; Spurio *et al.*, 1992; Ussery *et al.*, 1994). It was also reported that the linking number of plasmid DNA is altered in *hns* mutants (Dorman *et al.*, 1990; Higgins *et al.*, 1988; Hinton *et al.*, 1992). The cyclic AMP receptor protein CRP positively regulates the expression of *gyrA* (Gomez-Gomez *et al.*, 1996). The situation is complicated further since Fis can also indirectly influence the DNA topology as it is a transcriptional activator of *hns* (Falconi *et al.*, 1996) and it activates *hupA* and represses *hupB* expression (Claret and Rouviere-Yaniv, 1996). Fis is also a transcriptional repressor of *crp* expression (Gonzalez-Gil *et al.*, 1998) and CRP in the absence of Fis activates *fis* expression but in the presence of Fis, together Fis and CRP synergistically repress *fis* transcription (Nasser *et al.*, 2001). In addition CRP is a positive regulator of both *hupA* and *hupB* genes (Claret and Rouviere-Yaniv, 1996). It is interesting given the differences between the overall DNA topology of *E. coli* and *S. typhimurium* in the absence of Fis that the abundance of *E. coli* H-NS protein is reported to increase in stationary phase (Dersch *et al.*, 1993) but a study in *S. typhimurium* found no significant increase in the abundance of the H-NS protein as cells entered stationary phase (Hinton *et al.*, 1992).

Chapter 4

The Fis regulon of *Salmonella enterica* serovar Typhimurium

4.1 Introduction

The factor for inversion stimulation (Fis) is an 11.2 kDa DNA-binding protein comprising 98 amino acids. Fis was originally identified because of its ability to stimulate DNA inversion at the *hin* invertible DNA element in *S. typhimurium* (Finkel and Johnson, 1992; Heichman and Johnson, 1990). This small, basic, nucleoid-associated protein binds the DNA as a dimer at a degenerate consensus sequence and can upon binding induce a bend between 40° and 90° in the DNA (Finkel and Johnson, 1992; Hengen *et al.*, 1997; Wagner, 2000). The gene that encodes Fis is part of an operon with one open reading frame of unknown function preceding it (Fig. 1.8), (Ball *et al.*, 1992; Ninnemann *et al.*, 1992). Transcription of the *fis* gene is subject to growth phase regulation, stringent control, is negatively autoregulated by Fis protein and is controlled by the intracellular level of cytosine triphosphate (Ball *et al.*, 1992; Walker *et al.*, 1999). The *fis* gene is positively regulated by the histone-like protein IHF (Pratt *et al.*, 1997) and by increasing DNA negative supercoiling levels (Schneider *et al.*, 2000). CRP activates *fis* expression in the absence of Fis but in its presence, Fis and CRP synergistically repress *fis* transcription (Nasser *et al.*, 2001). It was reported recently that *fis* is also regulated at the translational level by BipA, a novel member of the ribosome binding GTPase superfamily (Owens *et al.*, 2004). When bacteria are inoculated into fresh medium there is a dramatic burst of Fis expression producing between 50,000 and 100,000 dimers per cell. Thereafter, this high level of Fis protein declines rapidly as the cells divide until there are fewer than 500 dimers at the onset of stationary phase (Appleman *et al.*, 1998; Azam *et al.*, 1999; Ball *et al.*, 1992). In the absence of BipA *fis* transcripts are abundant but Fis protein is barely detectable (Owens *et al.*, 2004).

The role of Fis in the cell extends beyond DNA inversion events (Finkel and Johnson, 1992; Wagner, 2000). Fis is required for *oriC*-directed DNA replication (Filutowicz *et al.*, 1992; Gille *et al.*, 1991), to modulate the transcription of genes including those coding for stable RNA (rRNA and tRNA) (Nilsson *et al.*, 1992; Ross *et al.*, 1990), the

cold shock response (Brandi *et al.*, 1999), the catabolism of sugars and nucleic acids (Gonzalez-Gil *et al.*, 1996) and to be involved in the life cycle of bacteriophage lambda (Finkel and Johnson, 1992). Fis influences the DNA topology directly by binding and preserving intermediately supercoiled DNA and indirectly by regulating the expression of the genes that encode DNA gyrase and DNA topoisomerase I (Fig. 1.9), (Schneider *et al.*, 1997; Schneider *et al.*, 1999; Weinstein-Fischer *et al.*, 2000). Recently, Fis has been implicated in controlling the virulence genes in *Shigella flexneri* (Falconi *et al.*, 2001), pathogenic strains of *E. coli* (Goldberg *et al.*, 2001; Sheikh *et al.*, 2001) and in *S. typhimurium* where Fis is required for the full expression of genes in the SPI-1 pathogenicity island (Wilson *et al.*, 2001).

In this study the *fis* regulon of *S. typhimurium* was elucidated by a DNA microarray analysis that determined fully the extent of Fis involvement in the regulation of gene expression in *S. typhimurium*. DNA microarrays, also called DNA chips, consist of either oligonucleotide or PCR (polymerase chain reaction) products corresponding to every gene in a given genome, deposited in an ordered grid onto specially coated glass microscope slides (Lucchini *et al.*, 2001). DNA microarrays are used for the assessment of transcription at the genomic level. Comparison of gene expression profiling under various conditions can reveal how the organism responds to those particular conditions. Also the gene expression profiles between two genotypes can be compared using DNA microarrays. The wild-type expression profile is compared with the expression profile of its isogenic mutant derivative to reveal how the particular mutation influences global transcription. The DNA microarray analysis in this study was performed in collaboration with Dr. Jay Hinton at the Institute of Food Research (IFR), Norwich, UK. The genes most strongly upregulated by Fis were involved in virulence and motility. Genes contributing to metabolism were also Fis-regulated, usually negatively. The microarray data highlight the role played by Fis in coordinating the expression of both housekeeping genes and virulence genes in *S. typhimurium* to facilitate the dynamic life of a facultative intracellular pathogen.

4.2. Results

4.2.1 DNA microarray analysis to determine the Fis regulon

Gene expression technology is a powerful new tool, which we employed to test the global effects on *S. typhimurium* gene expression induced by the loss of *fis*. A DNA microarray analysis was carried out to elucidate the *fis* regulon of *S. typhimurium* during growth in LB broth. DNA probes used in this study consisted of cDNA (PCR products) and were printed on Corning CMT-GAPS™-coated glass slides by a robotic DNA arrayer built in-house by Arthur Thompson, Sacha Lucchini and Bruce Pearson at the IFR, according to the step-by-step plans for the construction of a DNA arrayer developed and published on the internet by Pat Brown and Joe de Risi at Stanford University (http://cmgm.stanford.edu/pbrown/mguide/index.html). Each microarray consisted of 4414 coding sequences and was based on the *S. typhimurium* LT-2a genome sequence (McClelland *et al.*, 2001).

The optimum time points for transcriptional profiling of SL1344 and its isogenic *fis* mutant derivative SL1344*fis::cat*, were determined by monitoring Fis protein levels in the wild-type strain during growth in LB broth by Western blot analysis (Fig. 3.8C). Fis protein levels were found to peak at 1 h after the overnight culture was diluted into fresh medium and were undetectable by 3 h. The time points 1 h and 4 h were chosen as they represent samples where the cells contained maximum and minimum levels of Fis protein, respectively. Overnight cultures of SL1344 and SL1344*fis::cat* were subcultured into 100 ml LB broth in 250 ml flasks and grown at 37°C with shaking. At 1 h and 4 h post subculture total RNA was harvested from SL1344 and SL1344*fis::cat*. The RNA was fluorescently labelled during reverse transcription into cDNA. The fluorescently labelled cDNA was hybridized to the microarray slide. Comparison of the fluorescent intensity of the cDNA between the wild-type and *fis* mutant backgrounds indicated the effect of Fis on the expression of every gene in the cell at 1h and 4h.

Genomic DNA from SL1344 was fluorescently labelled, a different colour to the cDNA, hybridized to all the microarray slides and used as a reference channel in each experiment. All cDNA samples were hybridized to microarrays in quadruplicate and two biological replicates were performed. Once data had passed statistical analysis (statistical filtering with an FDR of $\leq 0.05\%$) they were imported into the GeneSpring 6.0 software program where all further analysis was carried out. Microsoft Excel files containing the results for all the genes that passed statistical analysis were created from the GeneSpring data. These are presented as supplementary data on a CD-ROM at the back of this manuscript. The microarray results provided a wealth of gene expression data. Genes were ranked in order according to their expression ratio. The expression ratio is a numerical value representing the fold increase or decrease of gene expression in the absence of Fis and was calculated by dividing the average expression value for a gene in the wild-type background into the average expression value for the same gene in the *fis* mutant background. Genes with an expression ratio less than 1.0 were deemed to be activated by Fis and genes with a ratio greater than 1.0 were repressed by Fis. Only genes whose expression increased or decreased by twofold or greater in the absence of Fis were considered as being significantly affected by the *fis* mutation.

4.2.2 The global transcriptional profile

From the microarray analysis, 2041 of the 4414 SL1344 coding sequences passed the statistical filter, set with an FDR $\leq 0.05\%$. Of the 2041 coding sequences, 291 showed $\geq$ twofold changes in expression at the 1 h time point. Of these, 167 displayed higher levels of expression in the *fis* mutant while 124 displayed lower levels of expression. At the 4 h time point 844 genes were differentially expressed in the *fis* mutant compared to the wild-type. Of these, 356 genes showed increased expression in the absence of Fis and 488 genes showed decreased expression. Of the 167 genes that displayed increased expression in the absence of Fis at 1 h, 78 were downregulated at 4 h. Also, of the 124 genes that showed lower expression in the *fis* mutant at 1 h, 97 had elevated expression

by 4 h. Therefore the response of over half of the genes to the absence of Fis at 1 h was transient which may reflect the fact that the abundance of Fis protein at 1 h is only short-lived and Fis protein is undetectable by 3 h post subculture as measured by Western blotting (Fig. 3.6C). Interestingly, a greater number of genes were Fis-regulated at 4 h than at 1 h post subculture. This apparent anomaly will be discussed later.

An indication of the types of genes regulated by Fis was obtained by defining functional categories of genes based on the Kyto Encyclopedia of Genes and Genomics (KEGG; www.genome.ad.jp/kegg/kegg2.html). Functional categories with a high proportion of Fis-dependent genes were identified (Fig. 4.1). From this analysis, kindly carried out by Martin Goldberg at the IFR, Norwich, it was evident that the greatest number of Fis-regulated genes were genes involved in virulence and motility/chemotaxis.

4.2.3 Fis and virulence gene expression

To date a total of five pathogenicity islands SPI-1 to SPI-5 have been characterised in *S. typhimurium* and mutations in any of these pathogenicity islands result in attenuation of virulence (Marcus *et al.*, 2000). Among the most strongly Fis-activated genes were the virulence genes encoded on these pathogenicity islands (Fig. 4.2 {sections I and II} and Fig. 4.3). The genes that were most downregulated in the *fis* mutant at 1 h were those in SPI-2 (Fig 4.2 {section II}). SPI-2 is required for survival and replication inside macrophages. The two-component signal transduction system PhoP/PhoQ is induced inside macrophages and PhoP activated genes, *pag* genes, are also switched on inside macrophages and many of these are required for intracellular survival (Miller *et al.*, 1989). A number of *pag* genes displayed decreased expression in the absence of Fis as did the macrophage-induced genes *mig-3* and *mig-14* (Table 4.1). In addition to the *pag* genes and *mig* genes some other chromosomal virulence genes located outside of the pathogenicity islands were regulated by Fis (Table 4.1). The SPI-1 genes also displayed
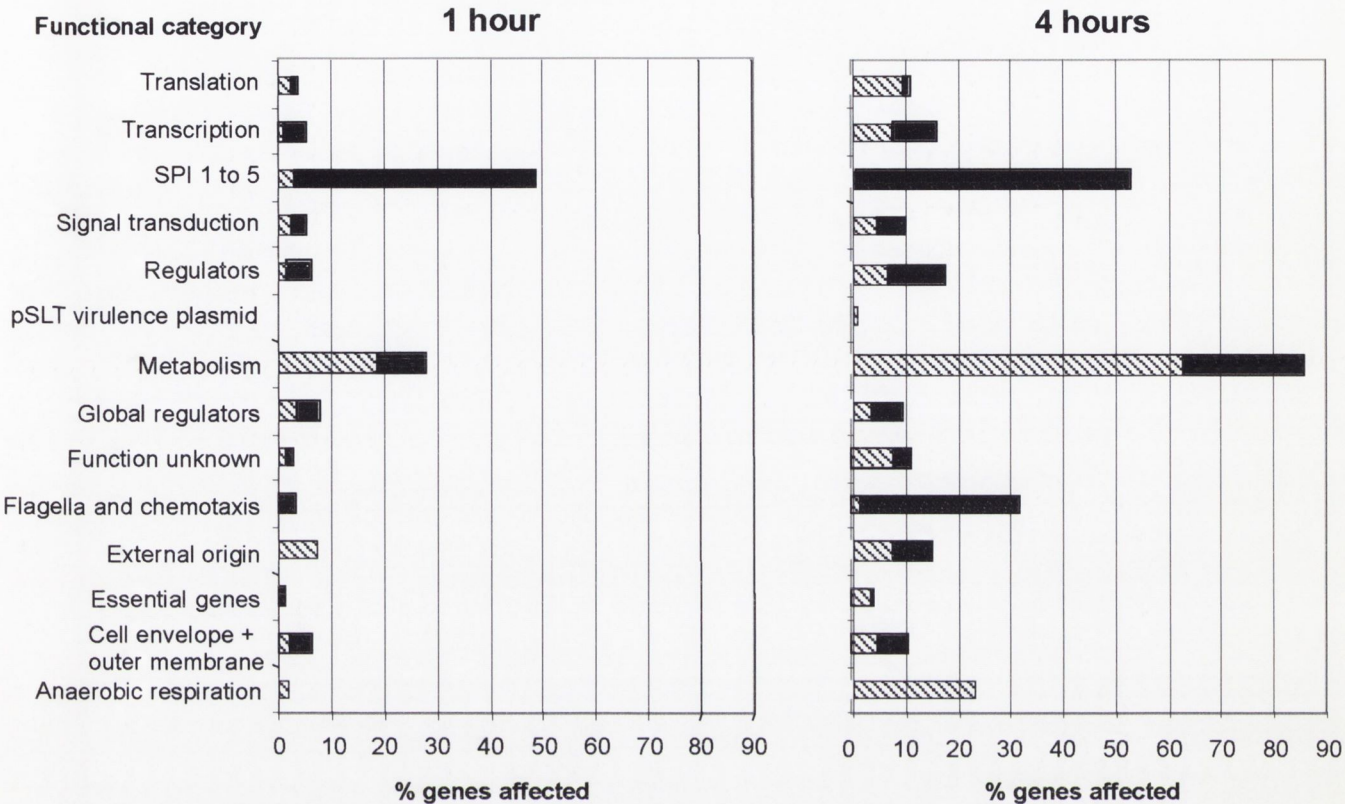
Fig. 4.1. Functional categories of genes regulated by Fis . The Kyto Encyclopedia of
Genes and Genomics (KEGG) was used to sort the Fis-regulated genes as determined from
the DNA microarray analysis into functional categories. The histograms represent the per-
centage of genes from a functional category that are either activated or repressed by Fis at 1
h and 4 h. The filled bars indicate the percentage of genes more highly expressed in
SL1344 than in the *fis* mutant. Hatched bars represent the percentage of genes more highly
expressed in the *fis* mutant than in the wild-type. It is apparent that the majority of genes
regulated by Fis in *S. typhimurium* are involved in virulence and motility/chemotaxis.

decreased expression in the absence of Fis protein (Fig. 4.2 {section I}), which is in good agreement with previous findings (Wilson *et al.*, 2001). The SPI-1 genes were most severely affected by the *fis* mutation at 4 h (Fig. 4.2 {section I}). The SPI-1 genes were the most Fis-dependent class of genes. The gene with the strongest Fis-dependence was *pipC*, which is encoded on SPI-5 (Fig. 4.3). Other genes within SPI-5 were also positively regulated by Fis (Fig. 4.3). SPI-5 genes are suggested to encode effector proteins, secreted by the SPI-1 type III secretion system and are indicated to play a role in the invasion of epithelial cells (Galyov *et al.*, 1997). Genes within SPI-3 and SPI-4 also displayed Fis-dependence (Fig. 4.3). Specific genes within SPI-3 and SPI-4 are required for intramacrophage survival (Blanc-Potard and Groisman, 1997) (Blanc-Potard *et al.*, 1999) (Baumler *et al.*, 1994). From these results it would appear that Fis coordinates the expression of virulence genes in SPI-1, SPI-2 and some in SPI-3, SPI-4 and SPI-5. This is in keeping with a previous finding which demonstrated a link between the expression of SPI-5 with SPI-1 and SPI-2 genes (Knodler *et al.*, 2002).

Transcriptional fusions to selected SPI-2 genes, including the regulatory gene *ssrA* and the structural gene *ssaG*, were tested for Fis activation (Carroll, 2003; Kelly *et al.*, 2004). The results showed that SPI-2 genes were significantly less active in the absence of Fis, in full agreement with the DNA microarray data. The Fis protein was subsequently shown to interact with the promoter regions of *ssrA* and *ssaG* indicating that Fis activation of these SPI-2 genes is a direct effect (Carroll, 2003; Kelly *et al.*, 2004).

Not all *S. typhimurium* virulence genes were regulated by Fis. The *S. typhimurium* virulence plasmid is approximately 90 kb in size and contributes to systemic infection in the mouse by increasing the replication rate of the bacteria inside host cells (Gulig and Doyle, 1993). The <u>S</u>almonella <u>p</u>lasmid <u>v</u>irulence genes, *spv* genes were not affected by the *fis* mutation (supplementary data).

Fig. 4.2 section I and II. The effect of the *fis* mutation on SPI-1 (section I) and SPI-2 (section II) virulence gene expression. (A) Graphical representation from GeneSpring 6.0 of the fold difference in gene expression of the SPI-1/SPI-2 genes in the *fis* mutant compared to the wild-type at 1 h and 4 h. Gene expression values were normalized to the wild-type and fold differences are indicated on the Y-axis. An increase from left to right indicates higher expression in the absence of Fis, while a decrease from left to right indicates a decrease in expression.

(B) Expression data of selected SPI-1/SPI-2 genes and effectors (bold type) in the absence of Fis. Expression data is normalized to the wild-type for the 1 h (filled bars) and 4 h (open bars) time points.

The expression data in (A) and (B) are the ratios of gene expression in the mutant/wild-type calculated for SPI-1/SPI-2 genes. Expression ratios less than 1.0 represent genes activated by Fis. It is apparent that SPI-1 and SPI-2 genes of *S. typhimurium* are activated by Fis.

Fig. 4.2 section I
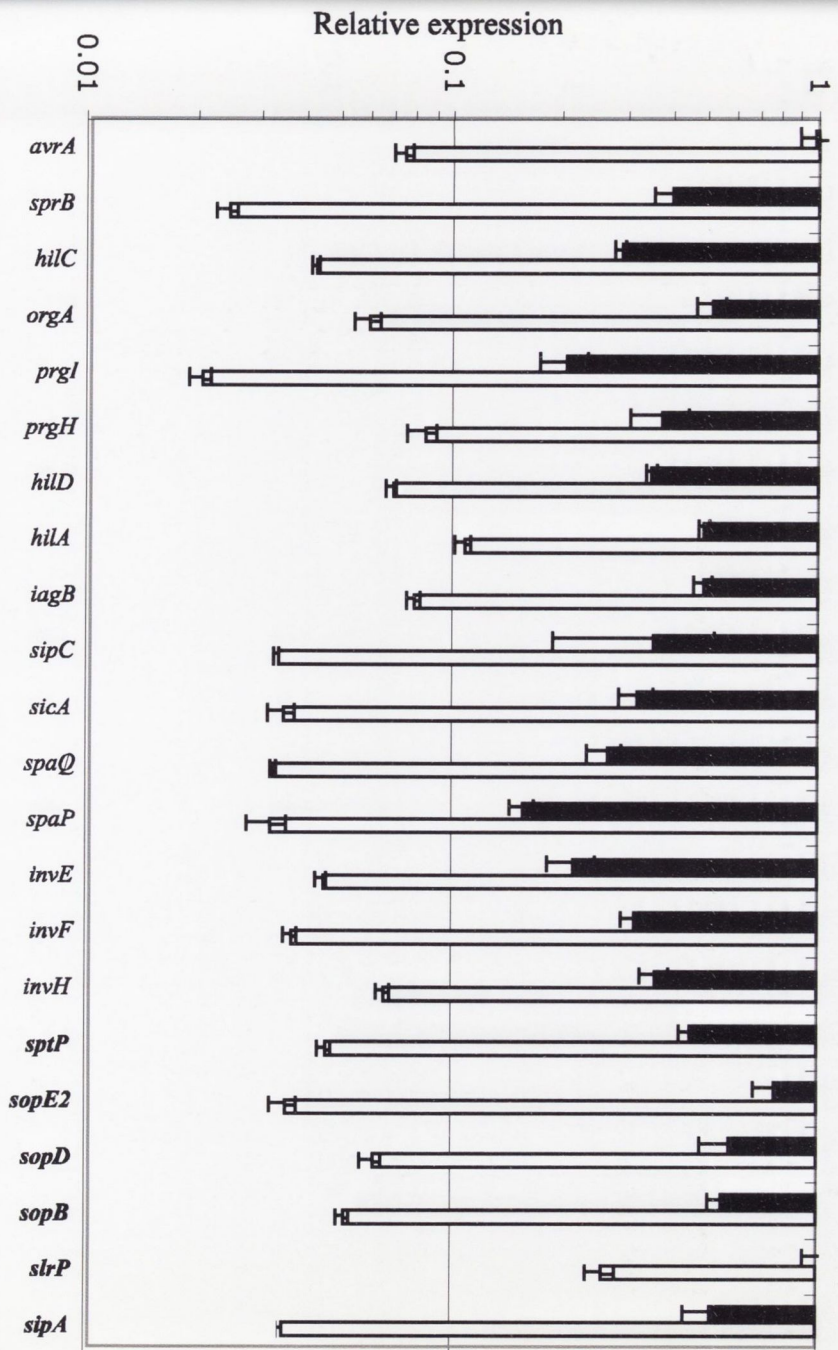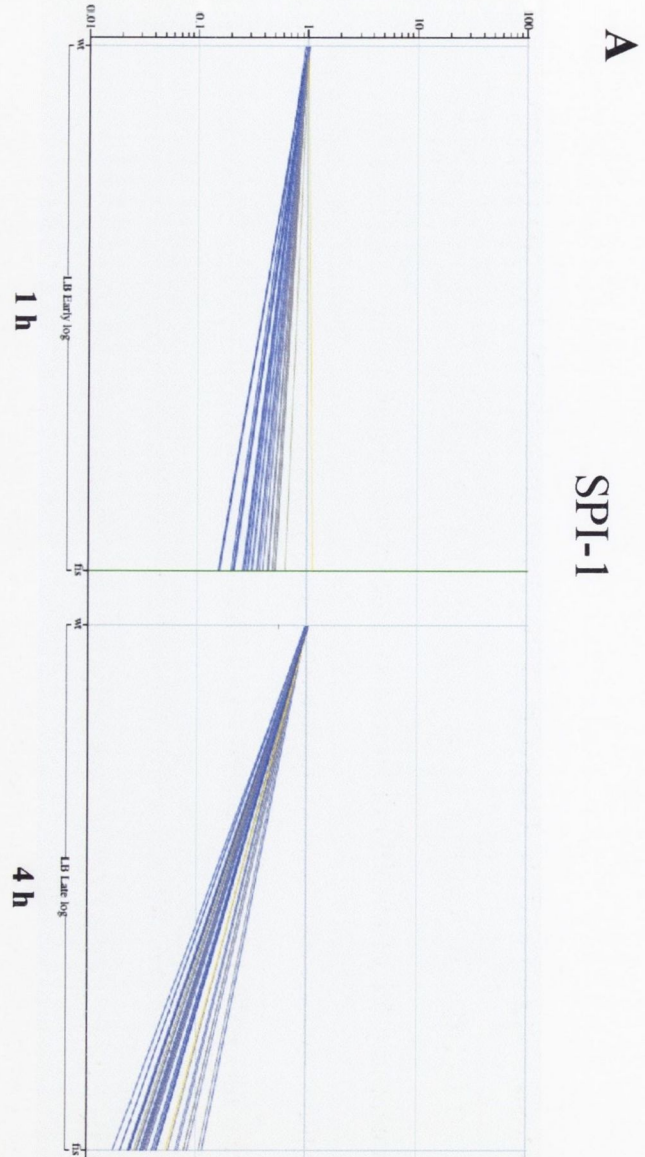
Fig. 4.2 section II
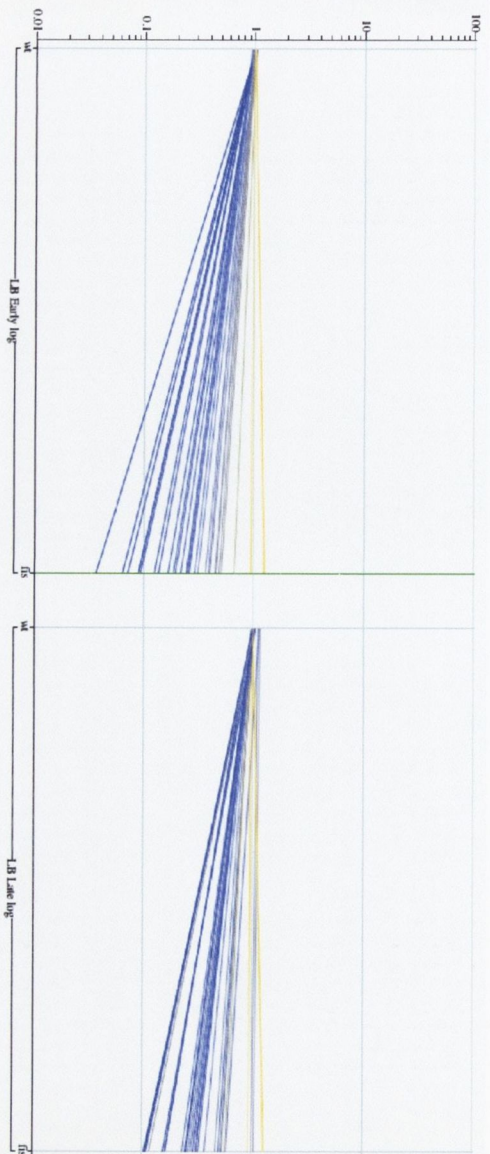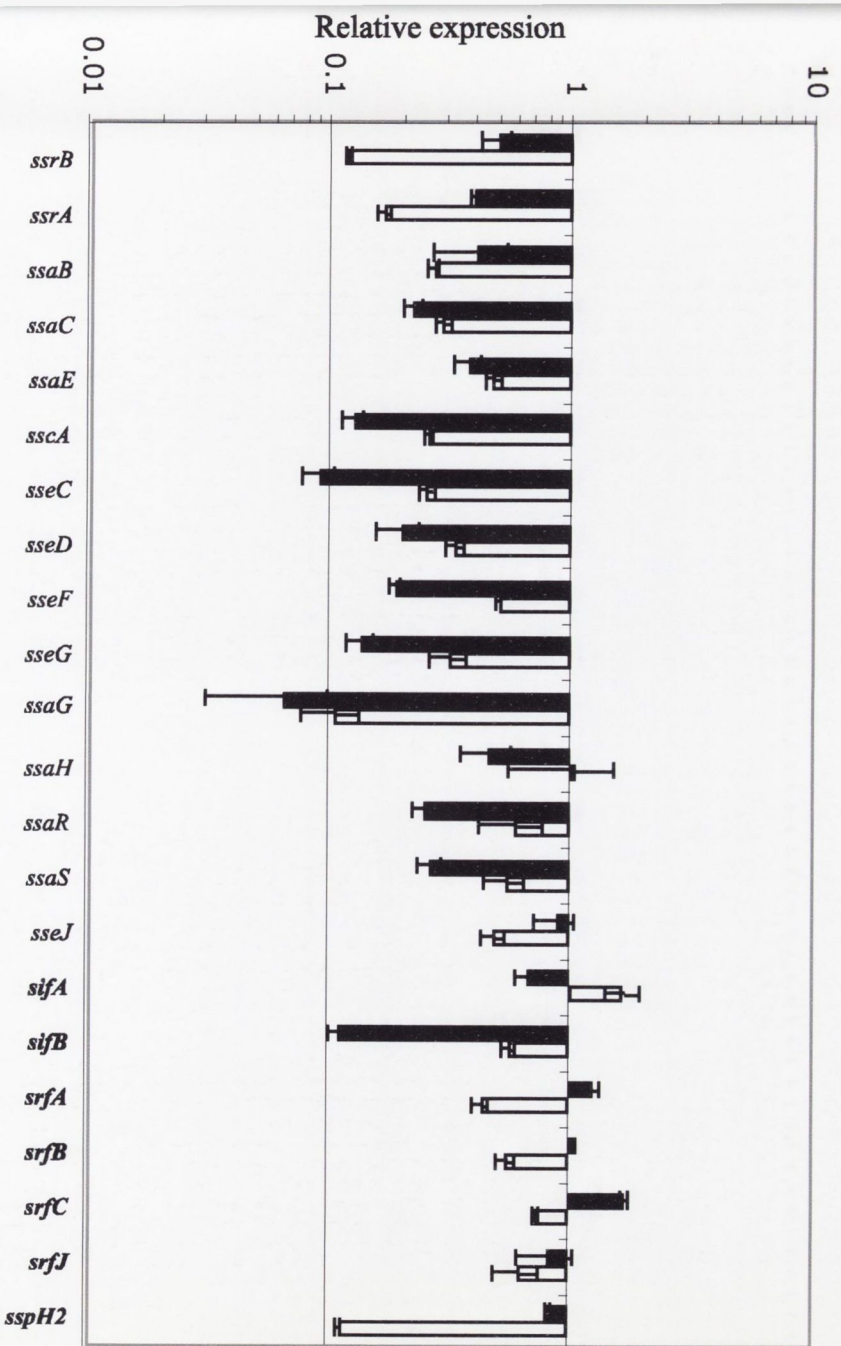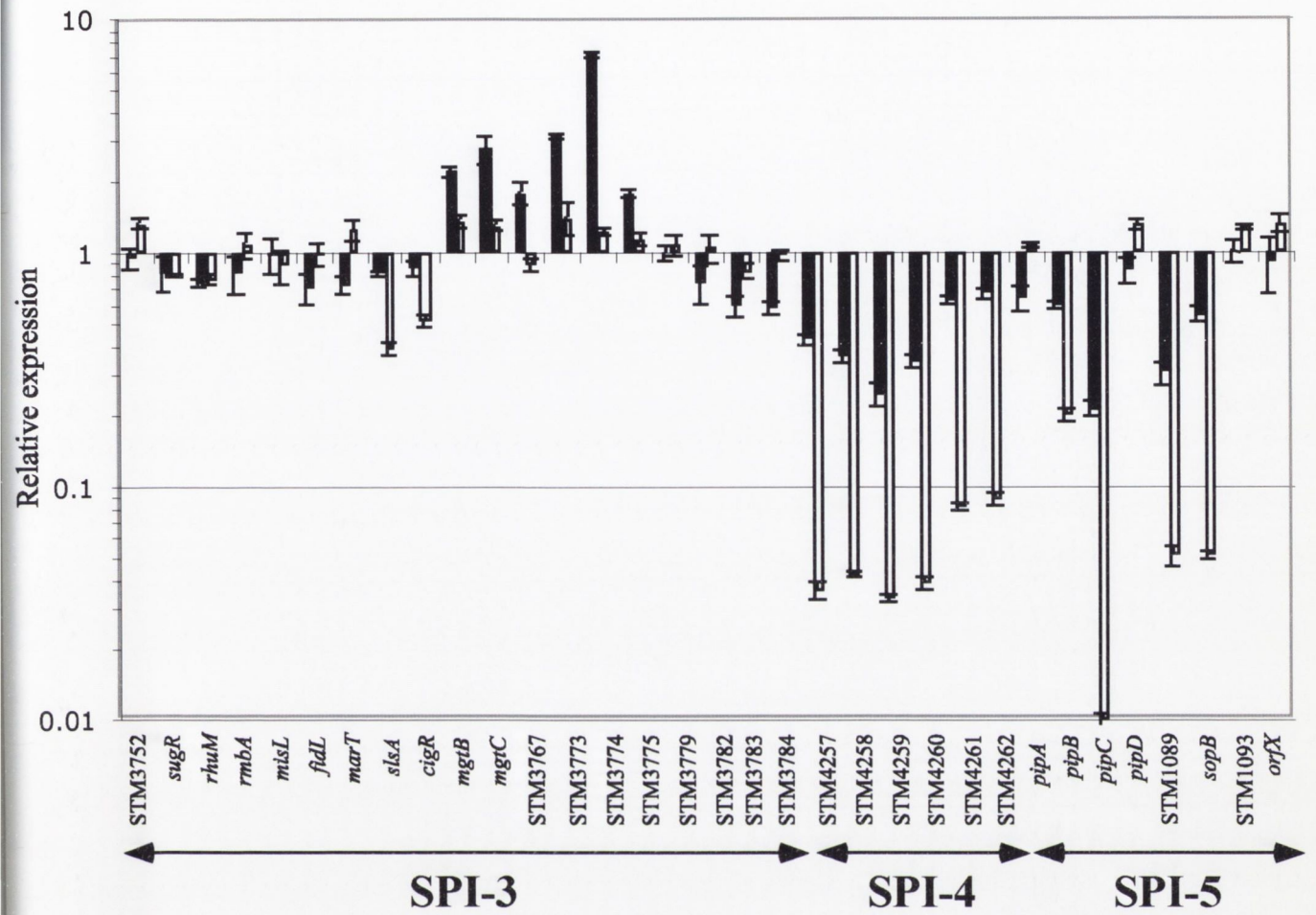
Fig. 4.3. Effect of the *fis* mutation on selected SPI-3, SPI-4 and SPI-5 genes.
Expression data were normalized to the wild-type for the 1 h (filled bars) and 4 h
(open bars) time points and the ratio of the mutant/wild-type was calculated. Expres-
sion ratios less than one indicate genes activated by Fis. The fold difference in gene
expression in the *fis* mutant compared to the wild-type is indicated on the Y-axis. The
data indicate that some genes encoded on the *S. typhimurium* pathogenicity islands
SPI-3, SPI-4 and SPI-5 are Fis-regulated.

Table 4.1. Other virulence genes regulated by Fis

Gene	Function	*fis* mutant/Wild type expression ratio	
		1 h	4 h
Chromosomal genes outside pathogenicity islands			
iagB	cell invasion protein	0.49	0.08
mig-3	macrophage-induced gene	0.98	0.44
mig-14	macrophage-induced gene	0.15	0.36
pagC	PhoPQ-regulated, macrophage survival	0.29	0.03
pagD	PhoPQ-regulated	0.67	0.31
pagK	PhoPQ-regulated	0.63	0.11
pagO	PhoPQ-regulated	0.53	0.24
sopD	secreted; transferred to eukaryotes	0.56	0.06
sopE2	type III secreted protein effector; invasion-associated	0.67	0.03
virK	homologue of *virK* in *Shigella*	0.33	0.31

4.2.4 Motility genes

Flagellar/chemotaxis genes were also among the most strongly Fis-activated genes. *S. typhimurium* has a peritrichous arrangement of flagella on its cell surface. There are over 50 genes involved in flagellar biosynthesis and function (Chilcott and Hughes, 2000). These genes are divided into at least 17 operons, which are subdivided into three temporally regulated transcriptional classes, early, middle and late. The early genes *flhD* and *flhC* encode the transcriptional activators of the middle genes. Middle genes encode the structural components of the hook-basal body an intermediate structure in flagellar assembly. The middle genes also code for regulatory proteins required to activate the transcription of the late genes whose products include proteins for synthesis of the external flagellar filament. *S. typhimurium* can produce two immunologically distinct, phase variable flagellin proteins FliC and FljB (Macnab, 1996, 2003).

The microarray analysis showed that flagellar genes belonging to all three transcriptional classes early, middle and late including regulatory and structural genes displayed significantly decreased expression in the absence of Fis at 4 h (Fig. 4.4). Few of these genes were affected by the *fis* mutation at 1 h. Expression of the lipoprotein gene *lpp* was also reduced in the *fis* mutant (Table 4.2), which is relevant here since lipoprotein affects flagellar assembly (Dailey and Macnab, 2002).

4.2.5 Metabolism and transport

Not all of the genes revealed by the DNA microarray analysis to be regulated by Fis were affected positively. The most strongly Fis-repressed genes were those involved in metabolism and transport (Table 4.2). This confirmed the ability of Fis to act as a transcriptional repressor as well as an activator. Many of these Fis-repressed genes are likely to be used by *S. typhimurium* in the intestinal milieu (Badia *et al.*, 1985; Bry *et*
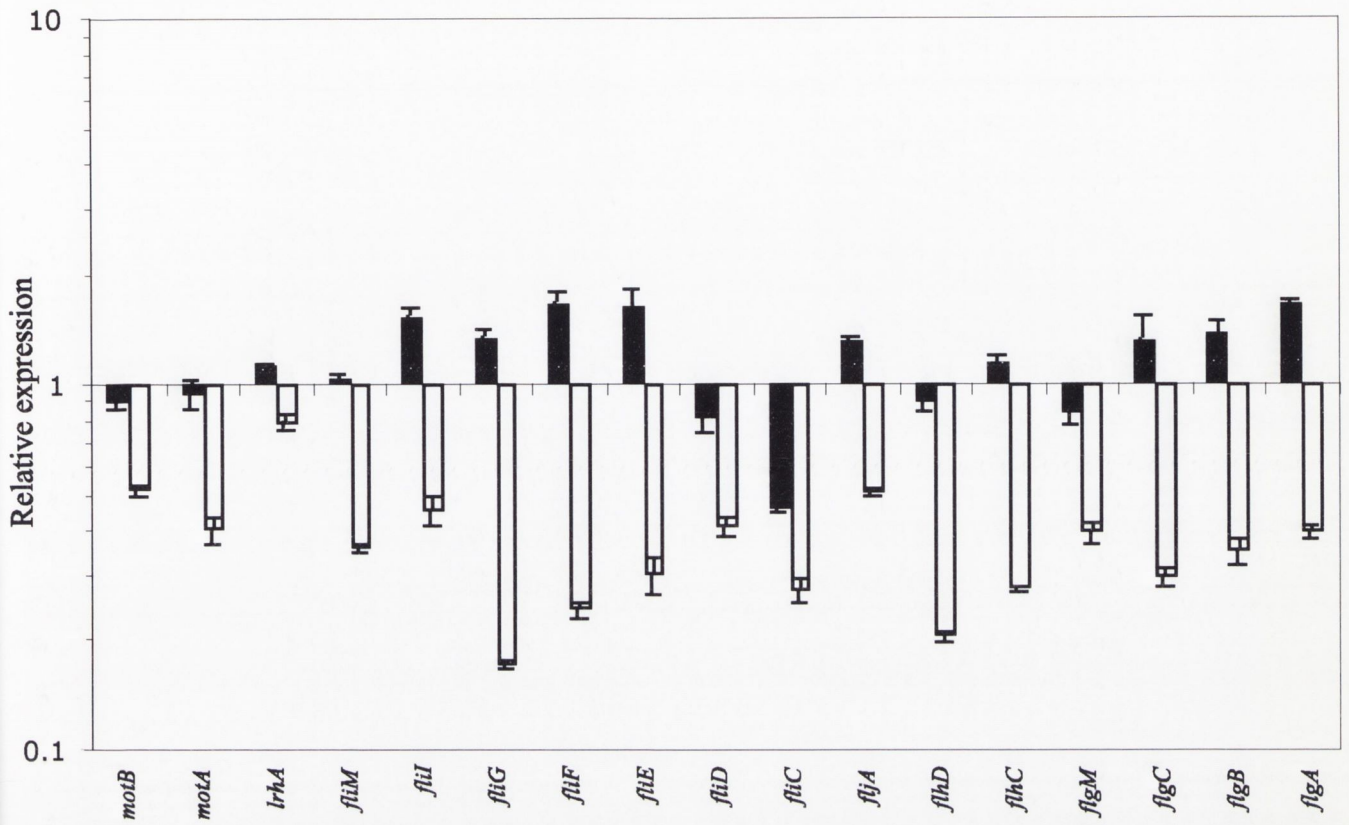
Fig. 4.4. The effect of the *fis* mutation on the expression of selected flagellar genes.

Expression data were normalized to the wild-type for the 1 h (filled bars) and 4 h (open

bars) time points and the ratios of the mutant/wild-type were calculated. Expression

ratios less than 1.0 indicate a decrease in gene expression in the absence of Fis. It is

apparent from the data that *S. typhimurium* flagellar genes are activated by Fis at 4 h.

Table 4.2. Metabolism and transport genes regulated by *fis*

Gene	Function	*fis* mutant/Wild type expression ratio	
		1 h	4 h
aceB	malate synthase A	1.52	3.3
aldB	aldehyde dehydrogenase B	1.49	5.88
btuB	outer membrane receptor for vitamin B12, E colicins	1.1	3.45
btuC	vitamin B12 ABC transporter	1.79	2.08
cadA	lysine decarboxylase I	1.45	2.94
cdB	lysine/cadaverine transport	1.22	3.45
citC	citrate lyase synthetase	1.64	2.44
citD	citrate lyase acyl carrier protein	1.02	2.78
citF	citrate lyase alpha chain; citrate-ACP transferase	1.59	5.56
citT	citrate:succinate antiporter	1.67	3.85
csgF	transport and assembly of curli	1.04	2.7
cysP	thiosulphate ABC transporter	1.28	3.85
dadA	D-amino acid dehydrogenase	0.55	2.56
eutA	chaperonin in ethanolamine utilization	1.33	0.4
eutB	ethanolamine ammonia lyase, heavy chain	1.32	0.59
eutC	ethanolamine ammonia lyase, light chain	1.35	0.36
eutD	putative phosphotransacetylase	1.47	0.37
eutE	putative aldehyde oxidoreductase	1.1	0.25
eutH	putative transport protein	1.6	0.4
eutJ	putative heat shock protein	1.89	0.26
eutK	putative carboxysome structural protein	1.25	0.29
eutL	putative carboxysome structural protein	1.54	0.33
eutM	putative detoxification protein	1.18	0.24
eutN	putative detoxification protein	1.35	0.33
eutP	putative ethanolamine utilization protein	1.43	0.37
eutQ	putative ethanolamine utilization protein	1.2	0.41
eutR	putative transcription regulator (AraC/XylS-like)	1.09	0.37
eutS	putative carboxysome structural protein	0.99	0.49
eutT	putative cobalamin adenosyltransferase	0.74	0.42
fabB	3-oxo acyl synthase I	0.85	2.5
fabD	malonyl-CoA transacylase	1.56	2.44
fhuE	outer membrane receptor for Fe III siderophores	0.88	4.55
fumB	fumarase B	0.94	2.56
garK	glycerate kinase	1.72	3.13
glnH	glutamine high affinity ABC transporter	0.39	2.33
glnP	glutamine high affinity ABC transporter	0.41	2.63
gltI	glutamate/aspartate ABC transporter	0.69	2.94
gltJ	glutamate/aspartate ABC transporter	1.18	2.17
gltK	glutamate/aspartate ABC transporter	0.83	2.5
gltS	glutamate transport protein	3.23	2.5
lpp	murein lipoprotein, links inner and outer membranes	0.25	0.36
marA	regulator of multiple antibiotic resistance	1.33	2.86
ndk	nucleoside diphosphate kinase	0.86	4.0
nupG	nucleoside transport	1.08	2.94
potB	spermidine/putrescine ABC transporter	0.71	2.56
potC	spermidine/putrescine ABC transporter	0.92	2.78
psd	phosphatidylserine decarboxylase	0.96	2.5
rbsC	D-ribose ABC transporter	1.22	2.44
sbp	sulphate ABC transporter	0.62	2.94
sdhC	succinate dehydrogenase; cytochrome b556	0.47	6.67
sdhD	succinate dehydrogenase hydrophobic subunit	0.38	4.0
speD	s-adenosylmethionine decarboxylase	1.47	3.23
tctD	regulator of tricarboxylic transport	1.05	2.94

al., 1996; Obradors *et al.*, 1988). The greatest increase in gene expression in the absence of Fis was displayed by the genes involved in biotin biosynthesis (*bioB*, *bioC* and *bioF*) at 1 h (Fig. 4.5). Biotin (vitamin H) is a critical cofactor required by many enzymes involved in various metabolic pathways where it acts as a carrier of carbon dioxide, such enzymes include biotin carboxylase involved in an early step of lipid biosynthesis (Cronan and Rock, 1996). From the microarray analysis genes involved in lipid biosynthesis were also found to be repressed by Fis (Table 4.2). These included *fabB* encoding β-ketoacyl-ACP synthase I (KAS I), which converts malonyl-ACP to acetoacetyl-ACP, *fabD*, the gene encoding malonyl-CoA:ACP transacylase, and *psd* which encodes phosphatidylserine decarboxylase (Cronan and Rock, 1996).

Also the microarray analysis highlighted the role of Fis as a repressor of genes involved in carbon utilization and energy generation. This included genes encoding enzymes of the citric acid cycle and its glyoxylate bypass, glycolysis and anaerobic respiration (Table 4.2 and supplementary data).

Genes involved in propanediol utilization were affected by the *fis* mutation. Of 18 *pdu* (propanediol utilization) genes for which data were available, 17 showed significantly increased expression in the *fis* mutant, at the 4 h time point (Fig. 4.5). These results indicate Fis is a repressor of *pdu* genes. To test this, wild-type *S. typhimurium* strain SL1344, its *fis* mutant derivative SL1344*fis::cat* and the complemented *fis* mutant strain SL1344*fis::cat* harbouring the plasmid pFis349, were grown aerobically at 37°C with 1,2-propanediol as the sole source of carbon (Fig. 4.6). Minimal medium with propanediol as a carbon source was composed of NCE medium supplemented with 1,2-propanediol (50 mM), $MgSO_4$ (1 mM), cyanocobalamin/vitamin B_{12} (150 nM) and histidine (0.5 mM), (section 2.1.2). Vitamin B_{12} and histidine were added to the medium because *S. typhimurium* catabolizes 1,2-propanediol via a pathway that is dependent on vitamin B_{12} (Jeter, 1990) and SL1344 is a histidine auxotroph. Following overnight culturing of these strains in LB broth at 37°C equal numbers of bacteria corresponding to 1 ml of culture per 1.0 OD_{600nm} unit were harvested, subjected to centrifugation and resuspended in 100 μl of the 1,2-propanediol medium. 10 μl of each
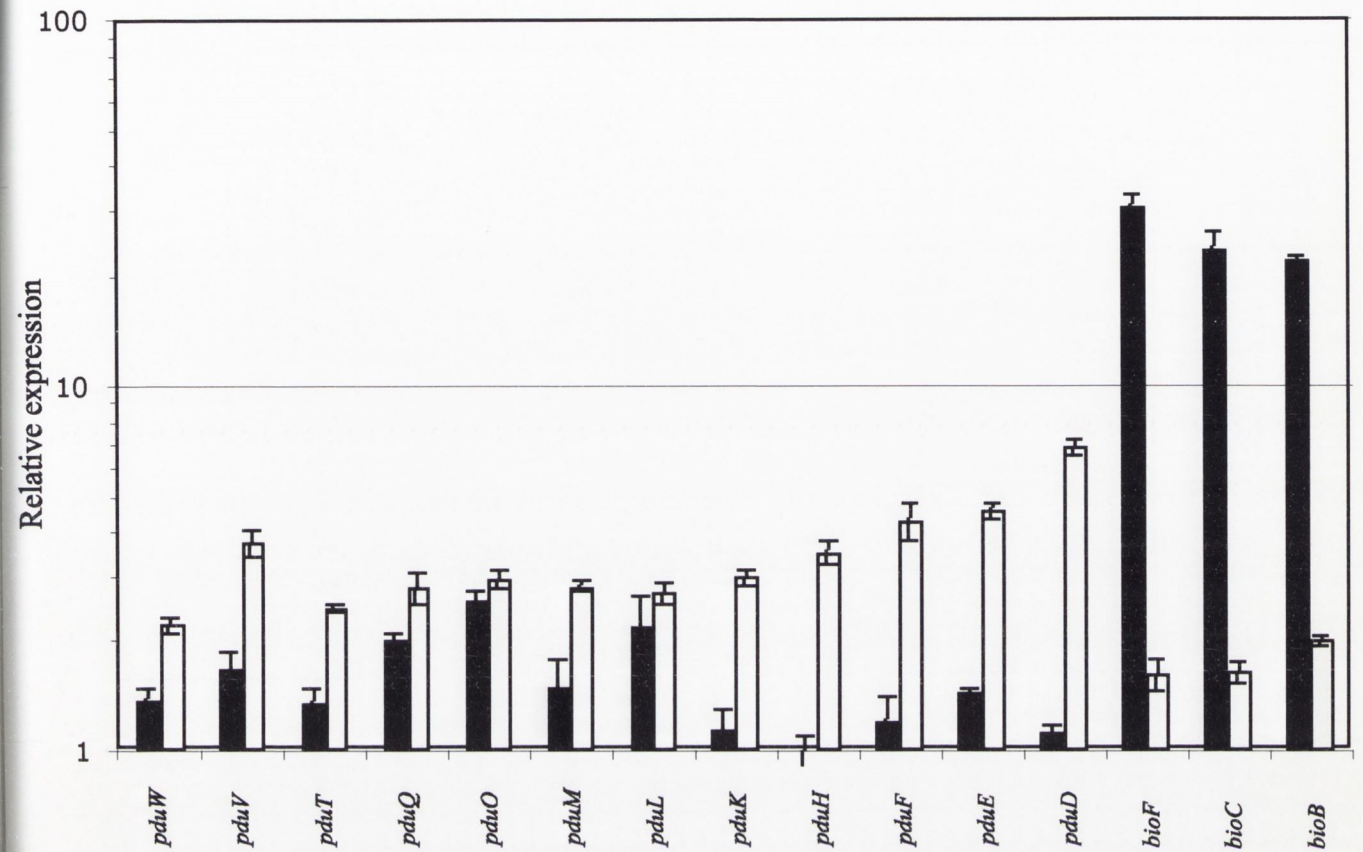
Fig. 4.5. Effect of the *fis* mutation on the *pdu* and *bio* metabolic genes. Expression data were normalised to the wild-type for the 1 h (filled bars) and the 4 h (open bars) time points and the expression ratios for the mutant/wild-type were calculated. Fis is an activator of genes with a relative expression value below 1.0 and a repressor of genes with values above 1.0. The DNA microarray data suggest Fis is a repressor of genes involved in propanediol utilization and biotin synthesis in *S. typhimurium*.
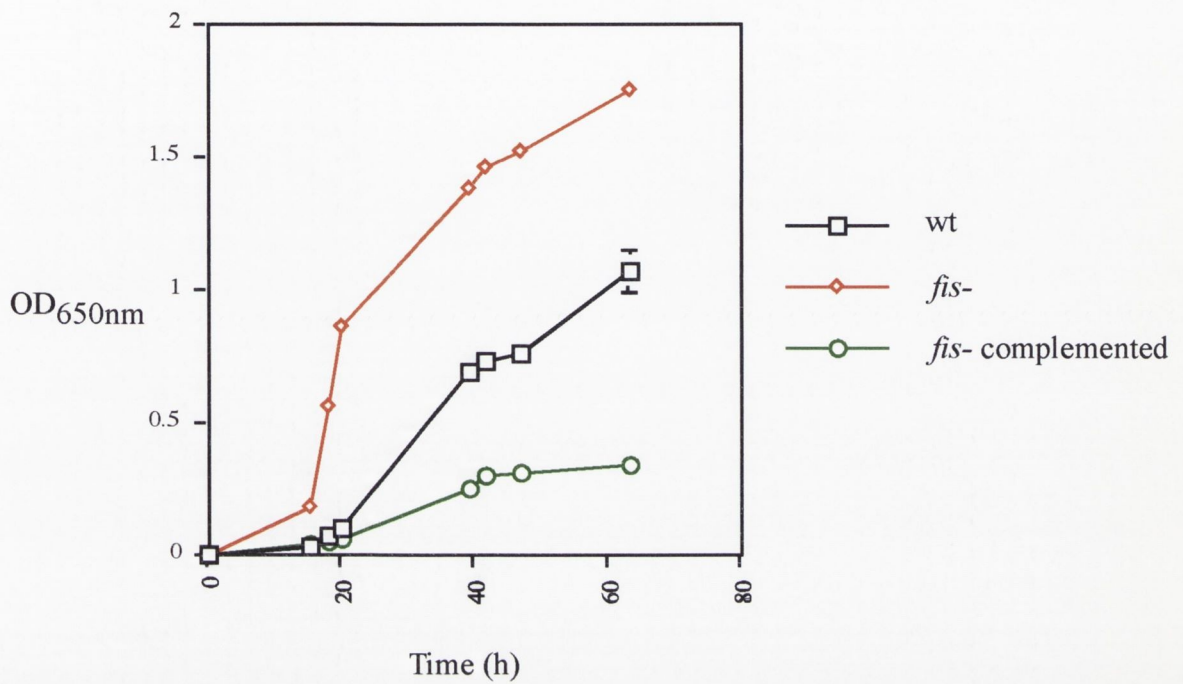
Fig. 4.6. Fis represses the growth of *S. typhimurium* on 1,2-propanediol. *S. typhimurium* wild-type strain SL1344, the *fis* mutant SL1344*fis::cat* and the complemented mutant SL1344*fis::cat* (pFis349) were grown aerobically at 37°C in NCE minimal medium containing 1,2-propanediol (50 mM), $MgSO_4$ (1 mM), cyanocobalamin (150 nM) and histidine (0.5 mM). Growth was monitored by measuring the optical density at 650 nm. The *fis* mutant grew more rapidly than the wild-type and the complemented *fis* mutant.

strain was then subcultured into 10 ml fresh 1,2-propanediol medium in 250 ml flasks and grown at 37°C with shaking. The growth of the bacterial cultures was monitored by measuring the optical density of the culture at a wavelength of 650 nm (OD_{650nm}). Consistent with the microarray data the *fis* mutant grew more rapidly than the wild-type and the complemented *fis* mutant.

The utilization of both propanediol and ethanolamine requires vitamin B_{12} as a cofactor, its synthesis being encoded by the *cob* operon. CsrA positively regulates both the utilization of ethanolamine and propanediol and induces the *cob* operon (Lawhon *et al.*, 2003). Fis was found to have a positive role in the expression of ethanolamine utilization (*eut*) genes at the 4 h time point (Table 4.2). This was in contrast to the negative role Fis was shown to have on the regulation of the *pdu* genes. Although the *fis* mutation had no effect on the *cob* operon for vitamin B_{12} synthesis, Fis was found to repress the *btuB* and *btuC* genes involved in the uptake of vitamin B_{12} at the 4 h time point (Table 4.2). The *aldB* gene encodes aldehyde dehydrogenase, an enzyme that links propanediol and glyoxylate metabolism (Lin, 1996). The *aldB* gene was repressed by Fis (Table 4.2) in good correlation with previous data from *E. coli* (Xu and Johnson, 1995a, b).

Several genes involved in polyamine metabolism were found to have elevated expression in the *fis* mutant (Table 4.2). These genes encoded lysine decarboxylase (*cadA*), which is required for the conversion of lysine to cadaverine, cadaverine transport (*cadB*), S-adenosylmethionine decarboxylase (*speD*), which feeds S-adenosylmethionine into the spermidine biosynthetic pathway, and putrescine/spermidine transport (*potB* and *potC*).

Fis also repressed transcription of *ndk*, the gene that encodes nucleoside diphosphate kinase which catalyses the interconversion of GDP and GTP (Table 4.2). The involvement of Fis in the negative regulation of *ndk* was of interest given that it similarly repressed the transcription of *nupG* and *rbsC* nucleoside transport genes

(Table 4.2), suggesting Fis coordinates the expression of genes involved in pyrimidine metabolism.

4.2.6 Stress response genes and global regulators

In response to deleterious environments bacterial cells induce the expression of stress response genes as part of their defense mechanisms. Few classical stress response genes were affected by the *fis* mutation at the 1 h time point but by the 4 h time point several genes involved in adaptation to a stressful environment showed decreased expression in the *fis* mutant (Table 4.3). Genes induced in response to heat shock (*htrA*) (Wick and Egli, 2004) or cold shock (*cspC*) (Polissi *et al.*, 2003) were Fis-activated at 4 h. The genes, *proV* and *proX* belonging to the *proU* osmotic stress response (Cairney *et al.*, 1985) locus were also Fis-activated. Also found to be Fis-dependent at the 4 h but not the 1 h time point were the *dsbA* (Miki *et al.*, 2004) gene encoding the periplasmic protein disulphide isomerase, the *sodC* gene encoding the Cu-Zn-containing superoxide dismutase (Fang *et al.*, 1999), and the *sodA* (Pomposiello and Demple, 2000; Tsolis *et al.*, 1995) and the *sodB* genes encoding the Mn- and Fe- containing superoxide dismutases, respectively.

The nucleoid-associated proteins or histone-like proteins contribute to the control of global gene expression, as well as being involved in other DNA transactions such as recombination, DNA replication and as contributors to the overall structure of the bacterial nucleoid (Dame *et al.*, 2000; Dorman and Deighan, 2003; Ussery *et al.*, 2001). Fis, H-NS, HU and IHF are the best characterised nucleoid-associated proteins in Gram-negative bacteria (Dorman and Deighan, 2003). A number of genes encoding nucleoid-associated proteins were affected by the *fis* mutation in this study (supplementary data). In keeping with previous data (Dersch *et al.*, 1994) (Falconi *et al.*, 1996) the cold shock-responsive *hns* gene was found to be Fis-activated at the 1 h and 4 h time points (supplementary data). The *hha* gene, whose product can form heteromeric complexes

Table 4.3. Stress response genes regulated by Fis

Gene	Function	*fis* mutant/Wild type expression ratio	
		1 h	4 h
cspC	cold shock protein	0.56	0.42
dsbA	periplasmic protein disulphide isomerase I	0.77	0.34
htrA	periplasmic heat shock protein; serine protease	0.77	0.36
katE	hydroperoxidase HPII; catalase	1.0	0.49
osmE	osmotic stress; activator of *ntrL* transcription	0.77	0.42
osmY	osmotic stress; periplasmic protein	1.05	0.17
proV	osmotic stress response	0.59	0.3
proX	osmotic stress response	0.63	0.36
psiF	phosphate starvation induced gene	0.67	0.43
sodA	superoxide dismutase (Mn)	0.43	0.42
sodB	superoxide dismutase (Fe)	0.63	0.4
sodC	superoxide dismutase (Cu-Zn)	0.71	0.36

with H-NS and whose function (like that of H-NS), includes the regulation of virulence genes in response to temperature (Madrid *et al.*, 2002; Nieto *et al.*, 2002) was also Fis-activated but only at the 1 h time point (supplementary data). The *stpA* gene that encodes a paralogue of H-NS and also forms heteromers with it (Deighan *et al.*, 2003; Free *et al.*, 2001; Johansson *et al.*, 2001; Williams *et al.*, 1996) was reported previously in *E. coli* to be unaffected by Fis at 30 min post subculture (Free and Dorman, 1997). In this study *stpA* transcription was unaffected by the *fis* mutation at 1 h although it was Fis-activated by the 4 h time point (supplementary data). The heterodimeric HU protein is formed by two different subunits, the products of genes *hupA* and *hupB* (Hillyard *et al.*, 1990). HU contributes to the osmotic response of the cell and the regulation of transcription of the *proU* operon (Manna and Gowrishankar, 1994). In good agreement with a previous report (Claret and Rouviere-Yaniv, 1996) the *hupA* gene showed a significant decrease in expression at the 1 h time point in the absence of Fis (supplementary data). The repressive effect of Fis on *hupB* (Claret and Rouviere-Yaniv, 1996) was not detected under the conditions used in this study (supplementary data). IHF is another heterodimeric nucleoid-associated protein. It is encoded by the genes *himA* and *himD* (Ali *et al.*, 2001) and the expression of these genes displayed no dependence on the Fis protein (supplementary data).

RtsA and RtsB are two regulatory proteins of *S. typhimurium* encoded in an operon located on an island integrated at tRNA^{PheU} (Ellermeier and Slauch, 2003). RtsA belongs to the AraC/XylS family of regulators and the RtsB is a helix-turn-helix DNA binding protein. RtsA activates expression of SPI-1 while RtsB represses the expression of flagellar genes. Specifically, RtsA binds to the SPI-1 encoded *hilA* promoter region and RtsB binds to the regulatory region of the *flhDC* operon at the apex of the flagellar regulon (Ellermeier and Slauch, 2003). RtsA and RtsB coordinate the expression of SPI-1 virulence genes and the repression of motility genes in *S. typhimurium*. *rtsA* (STM4315) and *rtsB* (STM4314) were among the most strongly Fis activated genes detected in this microarray study (supplementary data). In the absence of Fis *rtsA* expression was severely decreased at the 4 h time point only and *rtsB* expression was significantly decreased at the 1 h and 4 h time points. Therefore Fis was

found to activate the expression of these two important *S. typhimurium* regulatory proteins.

4.3 Discussion

The DNA microarray study determined the membership for the Fis regulon and revealed that the majority of genes regulated positively by Fis are involved in virulence or motility/chemotaxis (Fig. 4.1). Fis was found to coordinate the activation of genes in the pathogenicity islands SPI-1, SPI-2 and some in SPI-3, SPI-4 and SPI-5 (Fig. 4.2 and Fig. 4.3), and also positively regulated chromosomal virulence genes located outside the pathogenicity islands (Table 4.1). Fis was found to activate both structural and regulatory genes involved in all aspects of flagellar assembly and function, including genes of the early middle and late stages of the flagellar regulon at 4 h (Fig. 4.4). Flagella gene expression is low in early exponential phase, increases sharply with a peak at mid to late exponential phase and declines slowly thereafter (Amsler *et al.*, 1993). It is perhaps this growth phase regulation of flagellar genes that is the underlying reason why the expression of these genes was significantly decreased in the *fis* mutant at 4 h (late exponential phase) but not at 1 h (early exponential phase) (Fig. 4.4). SPI-1, SPI-2 and the flagellar regulon encode the three type III secretion systems of *S. typhimurium*. Therefore, Fis regulates specifically the type III secretion systems and their associated effector proteins to coordinate invasion of host cells, intramacrophage survival and locomotion. Also worthy of note here is the ability of Fis to activate the expression of *lpp* (Table 4.2), which encodes Braun's lipoprotein, a murein lipoprotein that is a structural component of the cell envelope. It is appealing to propose that through the positive regulation of *lpp* that Fis maintains the integrity of the cell envelope to ensure the correct assembly and function of these three type III secretion systems.

Other examples of regulators that coordinate the expression of virulence genes and flagellar genes are the regulatory proteins RtsA and RtsB, the two component regulatory system SirA/BarA and the post-transcriptional regulator CsrA, and outside of *S. typhimurium* in pathogenic strains of *E. coli*, the nucleoid-associated protein IHF and BipA (a member of the elongation factor GTPase superfamily) (Ellermeier and Slauch,

2003; Farris *et al.*, 1998; Goodier and Ahmer, 2001; Grant *et al.*, 2003; Lawhon *et al.*, 2003; Yona-Nadler *et al.*, 2003). It is interesting to note that in contrast to Fis the majority of these regulators activate the expression of virulence genes and repress flagellar genes. However, the CsrA protein of *S. typhimurium*, like Fis, positively regulates the expression of SPI-1 genes and flagellar genes (Lawhon *et al.*, 2003).

The pleiotropic nature of BipA mutants suggest a model in which BipA regulates the expression of one or more global regulatory proteins (Owens *et al.*, 2004). The results of the DNA microarray analysis presented here confirm that Fis is a global regulator of *S. typhimurium* with wide-ranging effects on gene expression. Owens *et al* (2004) demonstrated that BipA is a translation factor required specifically for the expression of Fis and its downstream targets. It was revealed that the BipA positive regulation of virulence genes, *espC* and LEE (locus of enterocyte effacement) gene clusters of enteropathogenic *E. coli* (Grant *et al.*, 2003) is mediated through the Fis protein (Owens *et al.*, 2004). In good agreement with this Fis was found to positively regulate the virulence genes located within the five *Salmonella* pathogenicity islands in this study. In *S. typhimurium* loss of BipA leads to a decrease in flagella synthesis and decreased motility (David O'Connor, personal communication) in good agreement with the DNA microarray analysis, which revealed Fis positively regulates the expression of the *S. typhimurium* flagellar genes. Interestingly BipA negatively regulates motility in enteropathogenic *E. coli* (Grant *et al.*, 2003), regulation that is perhaps not mediated through the Fis protein.

The most strongly Fis-repressed genes are involved in aspects of metabolism and transport that are pertinent to life in the gut (Fig. 4.5, 4.6 and Table 4.2). These are genes involved in propanediol utilization, ethanolamine utilization, acetate and fatty acid utilization. The *pdu* (propanediol utilization) genes were repressed by Fis (Fig. 4.5 and Fig. 4.6). It is worthy of note that the *pdu* genes have been shown to be important for growth in host tissues and competitive index studies in mice have shown that *pdu* mutations confer a virulence defect (Conner *et al.*, 1998; Heithoff *et al.*, 1999). It is apparent that Fis regulates house-keeping genes and genes involved in virulence and

motility and this implies Fis plays an important regulatory role in adapting the cell to the free living environment of the gut and to intracellular niches.

Relevant to metabolism and virulence is the elevated expression of *cadA* in the *fis* mutant (Table 4.2). *cadA* encodes lysine decarboxylase and is repressed by Fis. The absence of the *cadA* gene from *Shigella* is important for full virulence in that pathogen. As bacterial pathogens evolved they not only gained virulence genes but also shed genes via deletions (Maurelli *et al.*, 1998). *Shigella* lost its lysine decarboxylase activity by a genome deletion event in order to acquire greater virulence, the rationale for this being that cadaverine, a product of lysine decarboxylation, acts as an inhibitor of *Shigella* enterotoxin activity and thus attenuates virulence (Maurelli *et al.*, 1998). Although it is interesting to speculate that Fis acts as a repressor of the *cadA* gene in order to enhance *S. typhimurium* virulence, it is not known if lysine decarboxylase activity plays any role in *S. typhimurium* pathogenicity. However, it has been shown that *cadA* contributes to acid tolerance in *S. typhimurium* (Park *et al.*, 1996).

Fis was shown to repress transcription of *ndk*, which encodes nucleoside diphosphate kinase (Table 4.2). The *ndk* gene product catalyses the interconversion of GDP and GTP, which regulate the size of the pppGpp and ppGpp pools that underlie the stringent response (Godfrey *et al.*, 2002). The expression of the *fis* gene is regulated by the stringent response (Ninnemann *et al.*, 1992) (Walker *et al.*, 1999) and therefore one might hypothesize that the regulation of *ndk* by Fis is an indirect way in which the Fis protein can autoregulate *fis* expression.

Fis regulates the expression of *S. typhimurium* global regulators. A number of genes that encode nucleoid-associated proteins with global regulatory roles were shown to be Fis-regulated (supplementary data). The *hns*, *hha*, *stpA* and *hupA* genes were all Fis-activated. The Hha protein was reported to affect *hilA* expression by directly binding to its promoter region (Fahlen *et al.*, 2001). The regulatory proteins RtsA and RtsB also have widespread effects on gene expression in *S. typhimurium* and they too were Fis-activated (supplementary data). Specifically RtsA binds the *hilA* regulatory region to

modulate SPI-1 expression and RtsB binds the *flhDC* operon promoter region to influence flagellar gene expression. This suggests Fis may influence regulatory hierarchies at multiple levels.

None of the Fis-dependent genes revealed in this study is regulated by Fis alone. In order for Fis to modulate and fine-tune gene expression it must act either in synergy with or in opposition to the action of other regulators. For example Fis and H-NS act antagonistically to each other in the regulation of the *hns* (Falconi *et al.*, 1996), *cspA* (Brandi *et al.*, 1999), *rrnB*P1 (Afflerbach *et al.*, 1999; Tippner *et al.*, 1994) and *virF* promoters (Falconi *et al.*, 2001).

Fewer genes were Fis-dependent at 1 h than at 4 h, which seemed paradoxical given the concentrations of Fis protein present in the cell at those times. Fis protein levels were found to peak at 1h and were undetectable by 3 h (Fig. 3.8C). Although the Fis levels decline dramatically they are thought to reach a low but non-zero value (Azam *et al.*, 1999) (Walker *et al.*, 1999). When there is only a small quantity of the protein in the cell it is proposed that binding would only be to preferred sites with a high affinity for Fis (Keane and Dorman, 2003; Muskhelishvili and Travers, 2003). The influence of Fis on gene expression being more pronounced in late exponential and stationary phase rather than early exponential phase is not unique. Studies in enteropathogenic *E. coli* revealed Fis-dependent virulence genes that were maximally expressed in late stationary phase (Goldberg *et al.*, 2001). Other studies revealed the effect of Fis on the expression of *gyr* genes was also most acute in stationary phase (Keane and Dorman, 2003) (Schneider *et al.*, 1999). The fact that a greater number of genes were Fis-dependent at 4 h than at 1h may also reflect the input of additional regulators at different stages during the growth cycle.

The ability of Fis to bind a wide range of sites with different affinities probably results from the fact that Fis binds to a degenerate consensus sequence. The *S. typhimurium* pathogenicity islands were acquired by horizontal gene transfer (Galan, 2001; Groisman and Ochman, 1993, 1996, 1997; Hensel *et al.*, 1999b; Hensel, 2000) and the degeneracy

associated with Fis binding sites may have assisted in its recruitment as a regulator of these regions of foreign DNA.

The data presented here show that Fis influences the transcription of a wide range of genes in *S. typhimurium*. This study highlighted the ability of Fis to act as a repressor as well as an activator of gene expression and one must also bear in mind the possibility that the Fis effects observed at either time point may be indirect.

Chapter 5

The regulation of flagella gene expression by Fis

in *Salmonella enterica* serovar Typhimurium

5.1 Introduction

Bacterial flagella facilitate the mobility of *S. typhimurium* allowing it to move towards attractants and away from repellants (Macnab, 1996). *S. typhimurium* is peritrichously flagellated possessing 6-10 individual flagella per cell. Each flagellum is composed of a long helical filament connected to a short curved structure called a hook, both the filament and hook are extracellular and are anchored to the basal body, a complex structure embedded in and spanning the membrane envelope (Fig. 1.10), (Aldridge and Hughes, 2002; Macnab, 2003). The flagellar filament is approximately 10 μm in length and is made up of approximately 20,000 subunits of flagellin protein. Flagella rotate in either a clockwise or counterclockwise direction causing swimming or tumbling, respectively (Berg, 2000).

There are more than 50 genes involved in flagellar biosynthesis function and chemotaxis and these genes are divided into at least 17 operons (Fig.1.10), (Aldridge and Hughes, 2002; Chilcott and Hughes, 2000; Soutourina and Bertin, 2003). The promoters of the flagellar regulon are organized into three classes that determine their temporal expression. At the apex of this hierarchical regulon the class 1 promoter transcribes the *flhDC* regulatory operon. FlhD and FlhC form a heterotetrameric complex which activates the expression of the flagellar genes under the control of the class 2 promoters including *fliA*, which encodes an alternative sigma factor necessary for the transcription of the class 3 promoters. This cascade serves to link flagellar gene expression with the assembly of the flagellar apparatus. During assembly, distal components are secreted by the flagellum-specific type III secretion system, which is assembled at the base of the flagellum.

S. typhimurium possesses two immunologically distinct flagellin genes, *fliC* and *fljB* that are at distinct locations on the chromosome (Fig. 1.11), (Macnab, 1996). The expression of these flagellin genes is subject to phase variation mediated by a DNA

inversion reaction (Fig. 1.11), (Bonifield and Hughes, 2003; Macnab, 1996). Therefore at any given time only one flagellin protein is expressed on the cell surface.

The production of flagella and the resulting motility represent a significant drain on the cell's resources (Chilcott and Hughes, 2000). Also flagellin is the major proinflammatory determinant of *S. typhimurium* (Zeng *et al.*, 2003). Consequently the synthesis of flagella is highly regulated by many growth conditions and by mutations in a variety of genes, which are unrelated to the flagellar regulon. The expression of the *flhDC* regulatory operon is affected by DNA supercoiling, high concentrations of inorganic salts and carbohydrates, growth phase, alcohols and by the heat shock proteins DnaK, DnaJ and GrpE (Chilcott and Hughes, 2000; Li *et al.*, 1993; Shi *et al.*, 1992; Shi *et al.*, 1993). The two component regulatory system SirA/BarA acts to repress the expression of most flagellar regulons including *flhDC* (Goodier and Ahmer, 2001). In *E.coli* and *S. typhimurium* the post-transcriptional regulator CsrA is required for motility (Lawhon *et al.*, 2003; Wei *et al.*, 2001). CsrA binds to the *flhDC* transcript, protecting it from degradation. BipA, belonging to a family of ribosome binding GTPases, is involved in controlling the motility of enteropathogenic *E. coli* and *S. typhimurium* (Farris *et al.*, 1998).

A *fis* mutant of *S. typhimurium* has been described as having reduced motility (Osuna *et al.*, 1995), and a proteome analysis of a *S. typhimurium fis* mutant revealed flagella protein expression was affected by Fis (Yoon *et al.*, 2003), although in neither case was the underlying reason established.

This study elucidated the direct regulatory pathway of the Fis protein on flagella gene expression in *S. typhimurium*.

5.2 Results

5.2.1 A *fis* mutant displays reduced motility

Previous studies suggested the possibility of a role for Fis in *S. typhimurium* motility (Osuna *et al.*, 1995; Yoon *et al.*, 2003). It was discovered from the DNA microarray experiment (Chapter 4), that genes involved in flagellar biosynthesis, motility and chemotaxis were downregulated in the *fis* mutant. The effect of Fis on the motility phenotype was established by tests performed on semi-solid motility agar plates. The *fis* mutant displayed significantly reduced motility compared to the wild-type (Fig. 5.1). Moreover, full motility was restored when the *fis* lesion was complemented *in trans* using a (pFis349) that carries a functional *fis* gene (Fig. 5.1).

5.2.2 Fis activates the expression of flagellar genes

Genes involved in flagellar biosynthesis, function and chemotaxis are organised into a hierarchical regulon divided into three transcriptional classes, early, middle and late and their respective promoters are class 1, class 2 and class 3 (Fig. 1.10), (Aldridge and Hughes, 2002; Chilcott and Hughes, 2000; Soutourina and Bertin, 2003). To examine the effect of Fis on the expression of these genes, merodiploid chromosomal *lacZY* transcriptional fusions (Goodier and Ahmer, 2001) to genes representing each promoter class of the regulon, kindly donated by Goodier and Ahmer, were transduced by bacteriophage P22 generalised transduction into the wild-type *S. typhimurium* strain SL1344 and its *fis* mutant derivative SL1344*fis::cat*, generating strains, AK01-AK10 (Table 2.1). The genes for analysis chosen were *flhD* (the regulator of Class 2 flagella operons), *fliA* (the sigma factor for class 3 operon expression), *flgA* (assembly of the

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wt   *fis-*   complemented
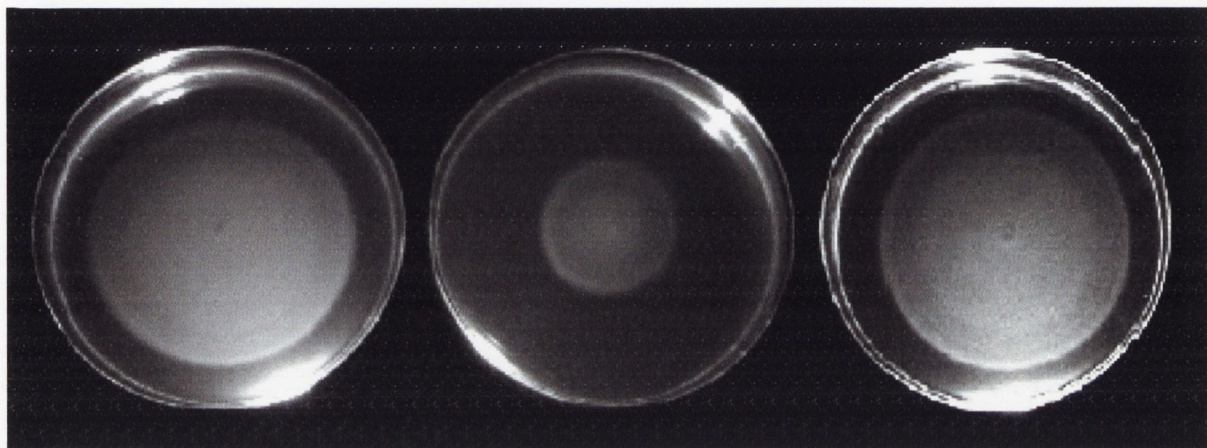
Fig. 5.1. Effect of a *fis* mutation on the motility of *S. typhimurium*. The wild-type strain SL1344, the *fis-* mutant strain SL1344*fis::cat* and the complemented mutant SL1344*fis::cat* (pFis349) were compared for motility. Semi-solid swarming agar plates were inoculated centrally with equal numbers of bacteria and incubated at 37° C for 8 hours. The rate of spreading of rings of bacteria ("swarming") on a swarm plate was a measure of chemotaxis and motility.

flagellar basal body P ring), *fliE* (the MS ring/rod adapter in the basal body) and *fliC* (phase 1 flagellin) (Macnab, 1996).

β-galactosidase analysis was employed to determine flagella promoter activity in the presence and absence of Fis throughout the growth curve. All five flagellar genes showed a similar pattern of expression in the wild-type strain (Fig. 5.2). Following inoculation of fresh LB broth, expression declined rapidly to a minimum value at approximately 2.5 h. Thereafter, there was a strong increase in flagellar gene expression leading to a peak at approximately 5 h. Expression then declined as the bacteria entered stationary phase. Flagellar gene expression in the absence of Fis was still subject to the same growth phase regulation. The effect of the *fis* knockout mutation was negative in all cases and resulted in a reduction in expression of approximately twofold (Fig. 5.2).

5.2.3 Reduced production of the flagellin protein FliC in a *fis* mutant

S. typhimurium motility was severely reduced in the absence of Fis and flagellar gene expression was shown to be positively regulated by Fis. FliC flagellin protein production was also investigated for Fis dependence. Western immunoblotting was performed to determine the levels of the FliC phase 1 flagellin protein in the absence of Fis. Total protein was isolated from wild-type and *fis* mutant cultures following 4 h growth in LB broth at 37°C. Protein samples were also harvested for Western blotting from TH6233 and TH6232 *S. typhimurium* strains locked on and locked off, respectively for flagellar phase variation, and used as negative and positive controls for FliC protein expression. Equal protein concentrations were loaded on an SDS-PAGE gel and analysed by Western blot using anti-FliC antiserum (Fig. 5.3). Results demonstrated that the level of FliC protein was strongly reduced in the *fis* mutant. This finding was fully consistent with the results of the motility assays (Fig. 5.1), β-galactosidase assays (Fig. 5.2) and the microarray data (Chapter 4).

Fig. 5.2. The effect of Fis on flagellar gene fusions. β–galactosidase assays were performed to measure the expression of *lacZ* in the wild-type and *fis* mutant strains of *S. typhimurium* harbouring fusions to a selection of flagellar genes representative of the three classes of promoter in the flagellar regulon. A typical growth curve is shown for SL1344 and and its *fis* mutant derivative, SL1344*fis::cat* (a). The flagella gene expression data throughout the growth curve are presented for *flhD* (b), *fliA* (c), *fliC* (d), *fliE* (e) and *flgA* (f), in the presence and absence of Fis.
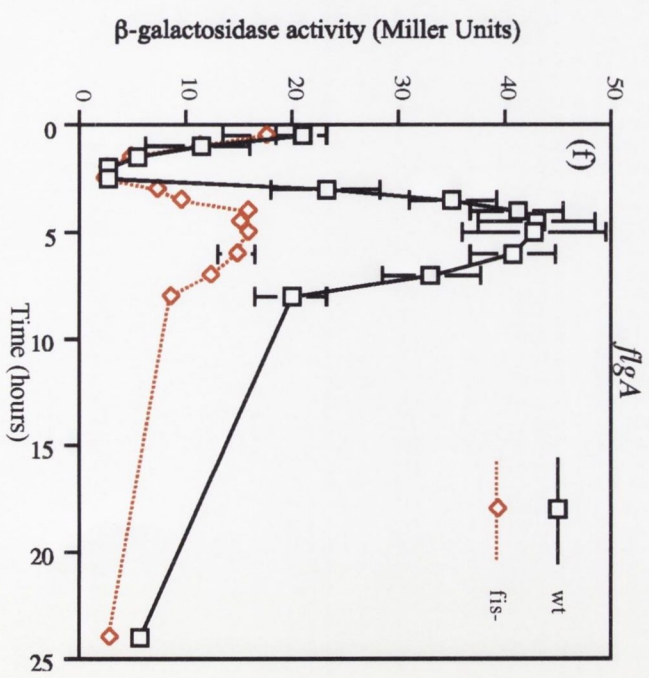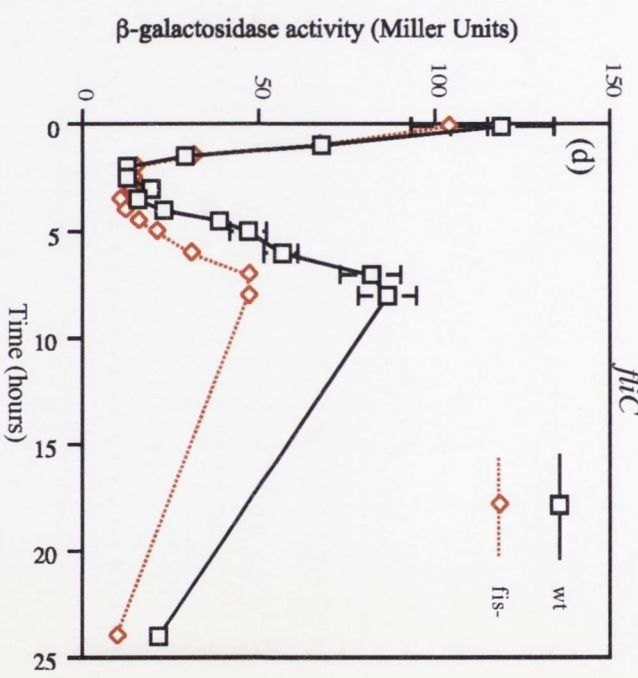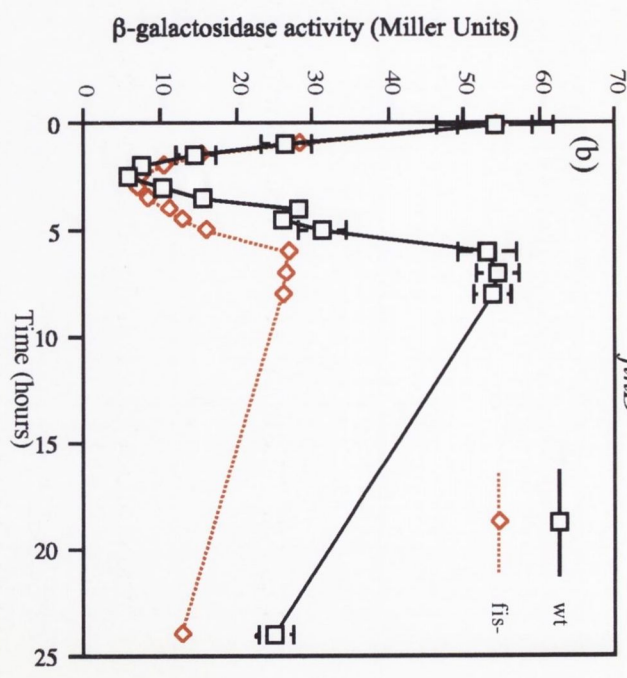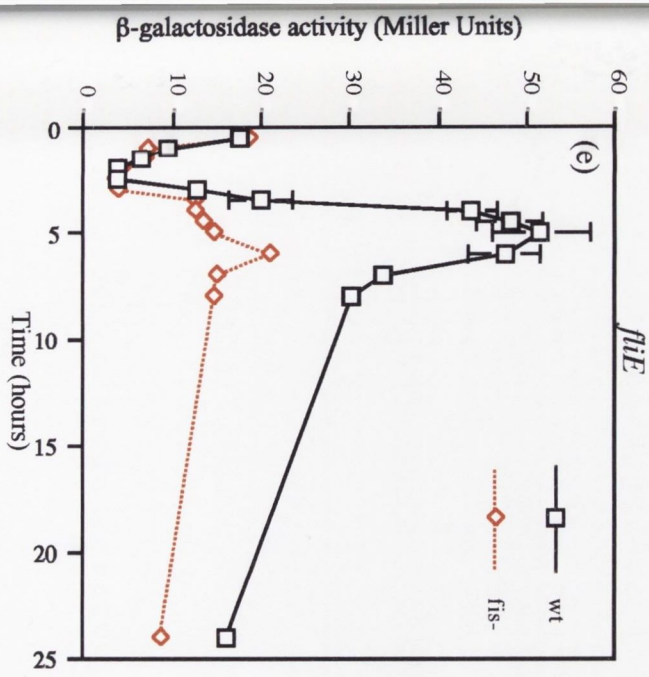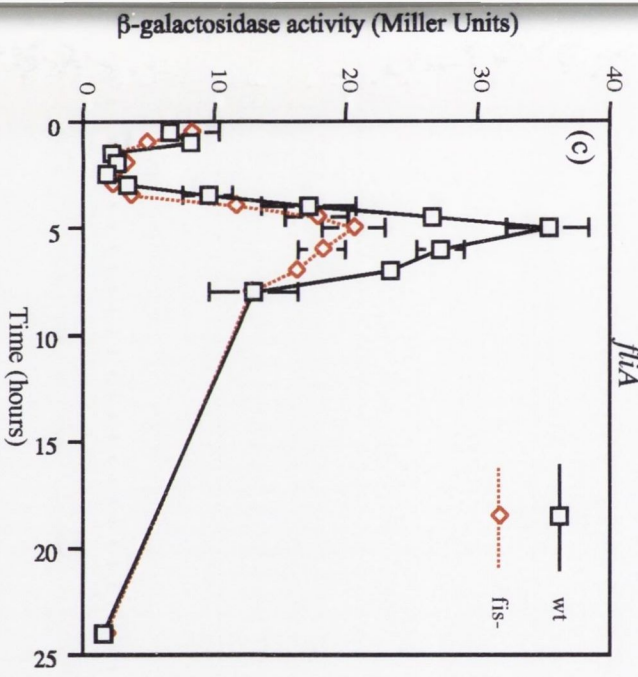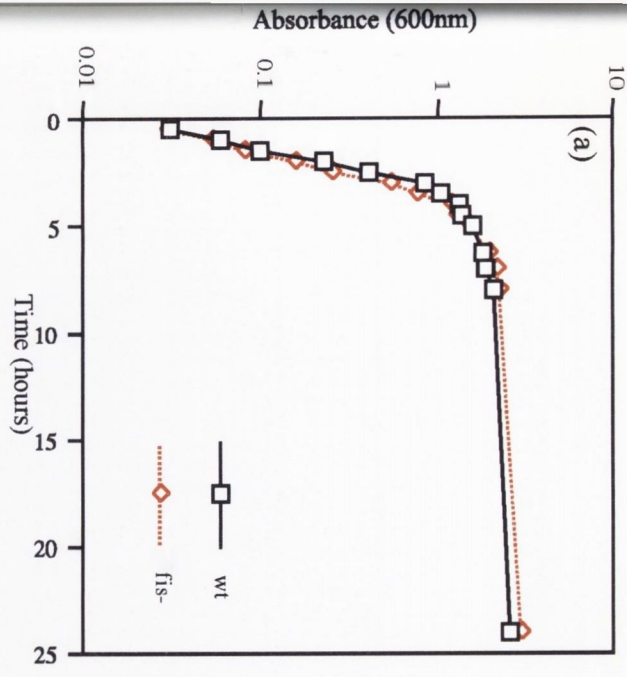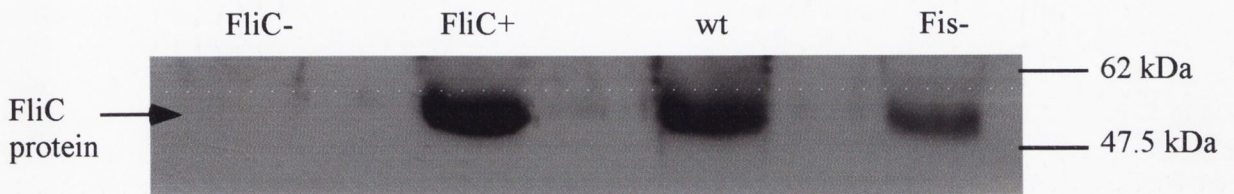
Fig. 5.3. Western immunoblot analysis of FliC protein levels in the presence and absence of Fis. Total protein from *S. typhimurium* wild-type strain SL1344 and its *fis* mutant derivative SL1344*fis::cat* were harvested following 4 h growth in LB broth at 37° C and analysed by Western blot. FliC protein levels were reduced in the *fis* knockout mutant strain. Strains TH6233 (locked on, negative control; FliC-) and TH6232 (locked off, positive control; FliC+) were included for comparison. The migration positions of molecular mass markers are indicated at the right hand side of the gel.

5.2.4 Fis directly binds flagellar promoters

Motility, flagella gene expression and phase 1 flagellin protein expression were all shown to be Fis regulated. In order to establish if the observed effect of Fis on flagellar biosynthesis and motility was a direct one the possibility of Fis binding to flagellar gene promoters was investigated. Electrophoretic mobility shift assays were carried out to study the interaction of Fis protein with the regulatory regions of the flagella genes *flhD*, *fliA* and *fliC* (Fig. 5.4). The flagellar genes selected were from the early (*flhD*), middle (*fliA*) and late (*fliC*) stages of flagellar biosynthesis. DNA fragments corresponding to the promoters of *flhD*, *fliA* and *fliC* were amplified by PCR (using the primer pairs BSflhDfw and BSflhDrv, BSfliAfw and BSfliArv and BSfliCrv and BSfliCfw (Table 2.3), generating fragments 723 bp, 301 bp and 314 bp in size, respectively), gel purified and radiolabelled with $[\gamma$-^{32}P]ATP. The *spvR* promoter region was used as a negative control since the *spvR* gene appears not to be Fis-regulated (supplementary data). The *spvR* gene from the 90 kb virulence plasmid was amplified by PCR using the primers spvR11 and spvR14 (Table 2.3), generating a 645 bp DNA fragment that was also gel purified and radiolabelled with $[\gamma$-^{32}P]ATP. The radiolabelled DNA fragments were incubated with increasing concentrations of Fis protein. Purified Fis protein containing an N-terminal six-histidine tag was a gift from O. Keane (Keane and Dorman, 2003). Bovine serum albumin and poly-[d(I-C).(dI-dC)] were used as competitors (section 2.15). The DNA protein mixtures were then resolved on a non-denaturing 7% polyacrylamide gel. The binding of protein to the DNA retards the mobility of the DNA fragment through the gel resulting in a "shift" in the electrophoretic mobility of the DNA visualised by autoradiography.

In each of the flagellar genes that was used a shift in electrophoretic mobility was seen at the lowest concentration of Fis (Fig. 5.4). By comparison, the negative control only underwent a weak shift at the highest Fis concentration. These results show that Fis interacts directly with *flhD* (the regulator of Class 2 flagella operons), *fliA* (the sigma factor for class 3 operon expression) and *fliC* (phase 1 flagellin) flagellar promoters.


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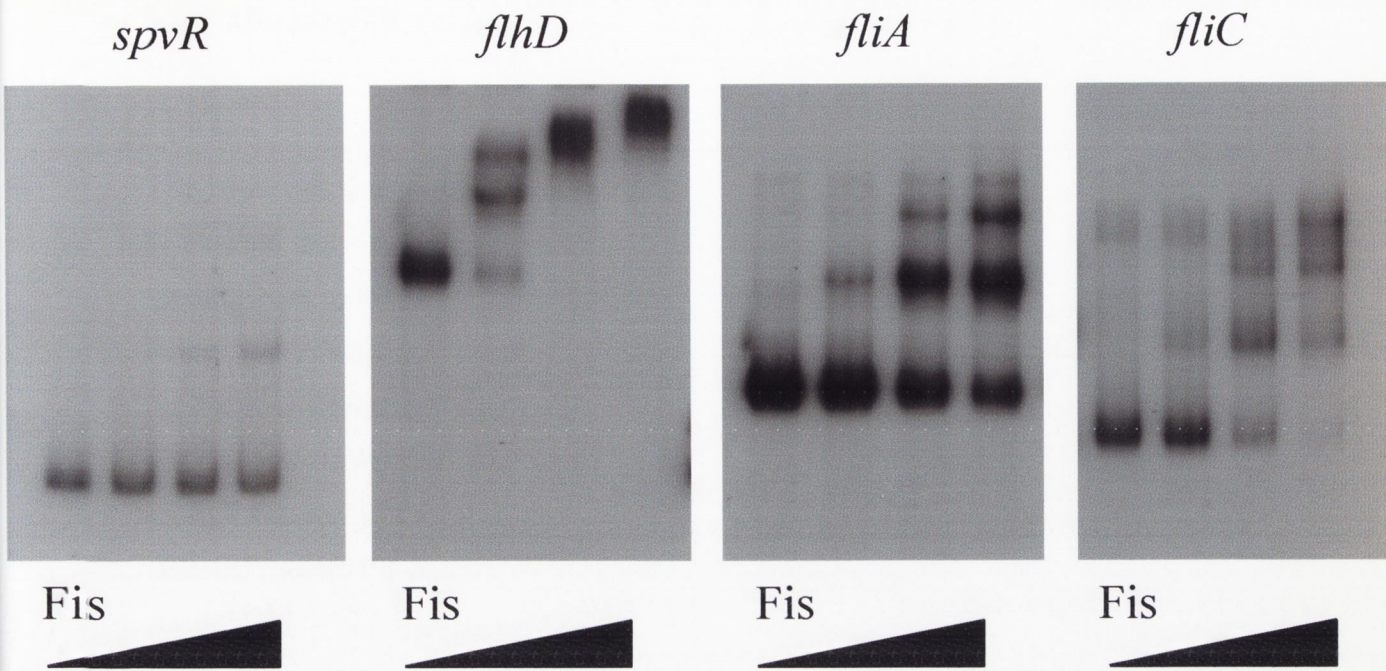
Fig. 5.4. Binding of the Fis protein to flagellar gene promoter regions. The interaction of the Fis protein with the transcription regulatory regions of three flagellar genes representing each level of the hierarchical flagellar regulon was assessed by DNA mobility shift assay. The regulatory sequences were amplified by PCR, radiolabelled with [γ-^{32}P]ATP and incubated with 0, 4, 20 or 60 ng of purified Fis protein and electrophoresed. Samples were resolved by electrophoresis in 7% polyacrylamide gels. Mobility of the DNA fragments was visualised by autoradiography. The *spvR* promoter was used as a negative control. Fis appears to bind to three sites within the regulatory regions of the class 1, class 2 and class 3 flagella promoters, *flhD*, *fliA* and *fliC* respectively.

5.2.5 Mapping Fis binding sites at the *flhDC* promoter

The bandshift data indicated that Fis interacts with the regulatory region of *flhDC* at more than one site (Fig. 5.4). DNase I footprinting was performed to map the Fis binding sites upstream of the *flhDC* operon. The footprinting technique is based on the theory that DNA sites bound by protein are protected from DNase I degradation. The region of DNA being examined is incubated with increasing protein concentrations and the phosphodiester backbone of the DNA is then subjected to DNase I treatment. The phosphodiester backbone is cleaved in regions where no protein is bound. DNA-bound proteins protect the backbone from cleavage and with increasing protein concentrations the protection becomes more effective (Galas and Schmitz, 1978).

The promoter region of *flhDC* from *S. typhimurium* SL1344 was amplified by PCR using the primers BSflhDfw and BSflhDrv (Table 2.3) generating a 723 bp fragment, the exact same region of DNA that was used in the gel shift assays that determined Fis binding the *flhDC* promoter region (Fig. 5.4). The PCR product was gel purified and end-labelled at both 5' ends using T4 polynucleotide kinase and $[\gamma\text{-}^{32}P]ATP$. Only one 5' end is required to be labelled for this technique. Therefore, following the labelling reaction the PCR product was digested with *Ssp*I, cleaving the 723 bp fragment into 203 bp and 515 bp fragments. The 203 bp fragment incorporated the forward primer and the 515 bp fragment incorporated the reverse primer. The reverse primer in the PCR reaction annealed to the start of the *flhD* coding sequence and the forward primer annealed upstream of *flhD*. Therefore the 515 bp fragment is composed of the beginning of the *flhD* coding sequence and the upstream regulatory region. The desired 515 bp fragment was purified after separation on a 7% polyacrylamide gel. This fragment was then incubated with increasing amounts of Fis protein at 37°C for 1 h in order to allow Fis protein to interact with the DNA. Following the 1 h incubation DNase I was added for 1 min and the reaction was stopped. The resulting ladder of DNA fragments were resolved by electrophoresis on a 7% acrylamide gel. The 723 bp fragment corresponding to the *flhDC* regulatory region was amplified again by PCR but

this time using the oligonucleotides SeqBSflhDfw and SeqBSflhDrv (Table 2.3) in order to flank the DNA fragment with *Eco*RI and *Hind*III restriction sites. The digested PCR product was cloned into plasmid pUC18 generating plasmid pUCflhD (Table 2.2). The products of a DNA sequencing reaction, carried out using plasmid pUCflhD and the oligonucleotide BSflhDrv, were run alongside the DNase I reactions on a 7% acrylamide gel (Fig 5.5A).

Fis protected regions of the *flhDC* promoter region were identified (Fig. 5.5A). Since the *flhDC* promoter region has more than one transcriptional start site (Yanagihara *et al.*, 1999) and for the purpose of relating the Fis binding sites, the A of the ATG translational start site of *flhD* will be referred to as +1 and the Fis sites will be mapped relative to +1. Fis protected two regions upstream of +1 extending from –80 to –104 and from –149 to –188. These sequences are underlined in Fig. 5.5 (C). A sequence displaying good homology with the Fis consensus sequence (5'-KNNYRNNWNNYRNNM -3') (Finkel and Johnson, 1992; Hengen *et al.*, 1997), identified within the protected regions is indicated in blue type (Fig. 5.5C).

Previously six transcriptional start sites P1 to P6 were identified for the *flhDC* operon (Yanagihara *et al.*, 1999). P1 is indicated at –203 bp in Fig. 5.5 (C). The other start sites P2-P6 would be located at –255, -296, -358, -530 and –278, respectively but are not included in Fig. 5.5 (C). The Fis protein binding sites were mapped downstream of all these transcriptional start sites (Fig. 5.5 C). In this study primer extension analysis was performed to determine independently the *flhDC* transcriptional start site(s). Total RNA was isolated from *S. typhimurium* containing the pUCflhD plasmid. Primer extension analysis was carried out using the RNA as template and the radiolabelled oligonucleotide BSflhDrv. The primer extension products were run on a 7% acrylamide gel alongside DNA sequencing reactions generated using the same oligonucleotide BSflhDrv and pUCflhD plasmid (Fig. 5.5B). In this way the primer extension product(s) could be mapped to the sequence and the transcription start site(s) identified.

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Fig. 5.5. Mapping the Fis binding sites upstream of *flhDC* and determination of *flhDC* transcriptional start sites. (A) DNase I footprint of Fis binding the upstream regulatory region of *flhDC*. The radiolabelled *flhDC* regulatory region was incubated with increasing concentrations of Fis protein. Fis protected the two regions indicated as protected region I and protected region II. A DNA sequencing ladder generated with the primer BSflhDrv that had been used to map the Fis binding sites was run alongside the DNase I reactions.

(B) Primer extension analysis was performed with RNA extracted from *S. typhimurium* strain SL1344 harbouring pUCflhD to determine the *flhDC* transcriptional start sites. A DNA sequencing ladder was obtained using the same primer that was used for the primer extension reactions. The putative transcriptional start sites revealed are indicated as P1 and P.

(C). Sequence of the *flhDC* regulatory region. The A residue of the *flhD* translational start codon is numbered +1. The regions protected by Fis are underlined and labelled region I and region II corresponding to the protected regions I and II indicated in section A. Sequences within the protected regions I and II with good homology to the Fis consensus sequence are in blue type. The putative transcriptional start sites P1 and P identified in section B are highlighted in red and labelled P1 and P. Potential -10 and -35 hexamers are highlighted in orange.

A

A C G T

Fis

0 25 50 100 150 200 250 300 nm Fis

protected region I

protected region II

B

A C G T

P1
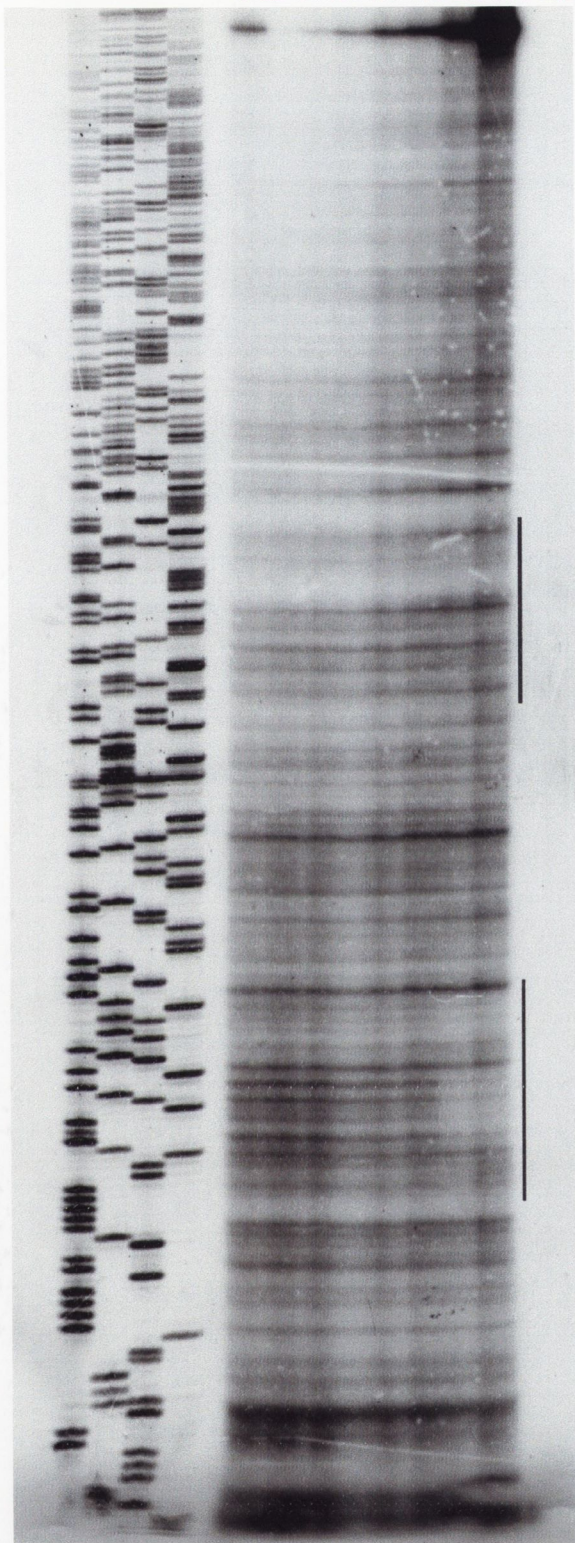
flhDC operon transcription start sites

P

C
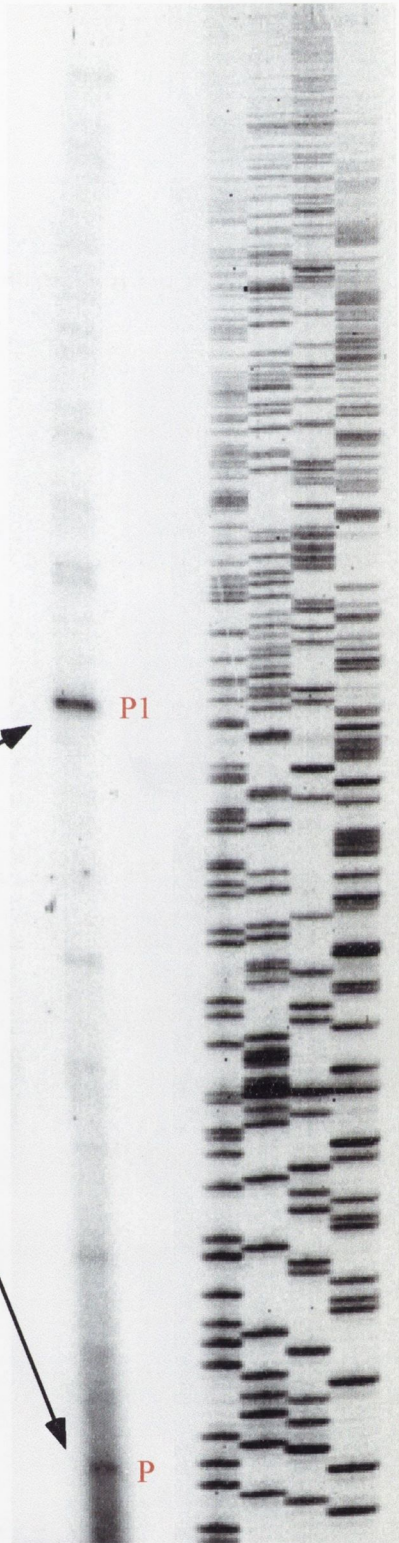
```
       -35                              -10        P1
-240 CGTTGTATGT  CACGAAGCTG  ACGAGTAGAG  TTGCGTCGAA  TTAGGAAAAA  TCTTAGGCAT
                                                                            -35
-180 TTGTAAAAAT  TGATGTAAAC  GTGTAAGGCG  AATCTCAGTG  GGAGGCTGCG  TTATACGTCA
                    region II
                 -10              P
-120 CAATGTCCAT  AATGTCTGAG  CGCTGCTATG  CATTTGACCT  TTTTGCTTCT  TTTACCGGGC
                                 region I
-60  CTTCCCGGCG  ACATCACGGG  GTGCGGCTAC  GTCGCACAAA  AATAAAGTTG  GTTATTCTGG

+1   ATGflhD
```

In this case two transcriptional start sites were identified mapping to an A residue and a G residue 93 bp and 203 residues upstream of the ATG translational start codon, respectively (referred to as P and P1, respectively in Fig. 5.5B+C). The G residue 203 bp upstream of ATG was identical to the P1 transcriptional start site identified previously (Yanagihara *et al.*, 1999). The other start sites P2-P6 were not clear from this experiment and further investigation will be required to identify them.

The putative transcriptional start site (P) mapped to an A residue at −93 bp which was not identified previously (Fig. 5.5B+C). Fis protected region II was located upstream of this putative transcriptional start site P and P was found to overlap with the Fis protected region I.

Due to the degenerate nature of the Fis consensus sequence it is proposed that the intrinsic curvature of the DNA may help define a Fis-binding site (Finkel and Johnson, 1992; Hengen *et al.*, 1997). Given this, a feature of Fis-binding sites is a propensity for A/T-rich regions, often consisting of runs of 3-6 A's or T's flanking the core sequence (Finkel and Johnson, 1992). Protected region I displays a run of 5 T's downstream of the core sequence and upstream of the core sequence at region II there is a run of 2 T's and 5 A's (Fig. 5.5C).

5.2.6 Fis activation of motility is independent of flagellar phase variation

S. typhimurium has two genes (*fliC* and *fljB*) that code for antigenically distinct flagellin proteins (Fig. 1.11). In *S. typhimurium,* flagellin genes are subject to phase variation and therefore at any given time only one flagellin gene is expressed (Fig. 1.11), (Bonifield and Hughes, 2003) (Macnab, 1996). Fis is involved in the Hin mediated DNA inversion mechanism of flagellar phase variation (Fig. 1.11), (Bonifield and Hughes, 2003). Flagellar phase variation is severely reduced in the absence of Fis (Osuna *et al.*, 1995). Therefore it was necessary to investigate the effect of the *fis*

knockout mutation on *S. typhimurium* motility independent of phase variation. The *fis::cat* lesion from SL1344*fis::cat* was transduced by bacteriophage P22 generalised transduction into *S. typhimurium* strains TH6232 and TH6233, locked off and locked on for flagellar phase variation, respectively, generating the strains AK11 and AK12 (Table 2.1). Following overnight culturing of strains AK11 and AK12, semi-solid swarming plates were inoculated centrally with equal numbers of bacteria and incubated at 37°C for 8 h (Fig. 5.6). In the locked on background, cells produce only FljB flagellin protein and in the locked off background, cells express only FliC flagellin protein. The absence of Fis had a negative effect on motility regardless of the flagellin protein expressed. Since the reduced motility phenotype of *fis* mutants occurs in the absence (Fig. 5.6) and presence (Fig. 5.1) of flagellar phase variation, the phenotype is not attributable to the role of Fis in flagellar phase variation.

5.2.7 H-NS regulates motility through a different mechanism to Fis

Fis binds to sites that display a somewhat degenerate consensus sequence and intrinsic DNA curvature (Finkel and Johnson, 1992; Hengen *et al.*, 1997). The 723 bp *flhDC* regulatory region amplified by PCR for the DNA electrophoretic mobility shift assays and DNase I analysis was tested for curvature using the BEND-IT computer program. This revealed regions of intrinsic curvature (Fig. 5.7).

The motility of an *hns* mutant was tested by inoculating soft agar motility plates with overnight cultures of the wild-type and *hns* mutant of *S. typhimurium* and incubating them at 37°C. The wild-type and *hns* mutant were compared for motility and the *hns* mutant was found to be nonmotile (Fig. 5.8A). The result that HN-S is required for motility in *S. typhimurium* correlates with previous findings. (Hinton *et al.*, 1992).

The major histone-like protein H-NS does not have a consensus DNA binding sequence but binds preferentially to curved DNA (Dorman, 2004). This study showed Fis

TH6232
wt

TH6232*fis::cat*
fis-

Locked off

TH6233
wt

TH6233*fis::cat*
fis-
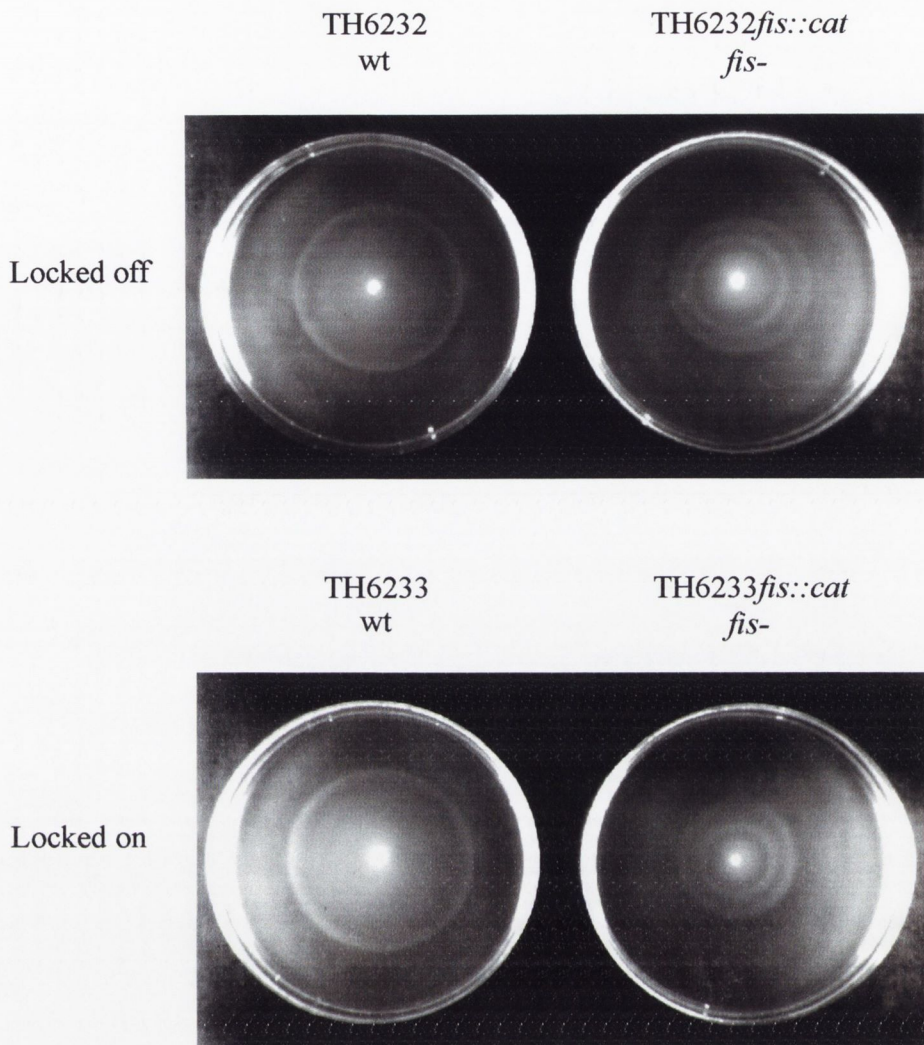
Locked on

Fig. 5.6. Effect of a *fis* mutation on *S. typhimurium* motility in the absence of flagellar phase variation. The *fis::cat* lesion from SL1344*fis::cat* was transduced into the *S. typhimurium* strains TH6232 and TH6233, locked off and locked on for flagellar phase variation, respectively. The locked off and locked on, wild-type and *fis* mutant strains were compared for motility. Equal numbers of bacteria were used to inoculate the centres of semi-solid swarming agar plates and incubated at 37°C for 8 h. The absence of Fis had a negative affect on motility that was independent of phase variation.

interacts strongly with the *flhDC* promoter region and directly activates flagellar gene expression. It was interesting to speculate that Fis and H-NS might both bind the *flhDC* promoter region and interact there in a synergistic fashion to activate *flhDC* expression. However the relationship between Fis and H-NS at promoter regions has only ever been reported to be antagonistic and such a relationship is involved in regulating the *hns* (Falconi *et al.*, 1996), *cspA* (Brandi *et al.*, 1999) and *rrnB*P1 promoters (Afflerbach *et al.*, 1999; Tippner *et al.*, 1994).

A competitive gel shift assay was carried out to investigate if indeed the HN-S protein binds to the intrinsically curved *flhDC* promoter region. A DNA fragment corresponding to the *flhDC* promoter regulatory region was amplified by PCR, using the primer pair BSflhDfw and BSflhDrv (Table 2.3), generating a fragment of 723 bp in size. The *Taq*1-*Ssp*1 digested pBR322 (providing an excess of competitor DNA and the *bla* promoter, a positive control) and the PCR amplimer of the *flhDC* promoter region were incubated with increasing HN-S protein concentrations, and resolved by electrophoresis through a 3% molecular screening agarose gel (section 2.15). H-NS binds to the *bla* promoter and shifts this curved piece of DNA (Fig. 5.8B). The electrophoretic mobility of the *flhDC* promoter region was not affected by HN-S protein (Fig. 5.8B).

The results in this study show H-NS clearly is involved in the regulation of motility in *S. typhimurium* but this regulation does not appear to result from an interaction between H-NS and the intrinsically curved *flhDC* promoter region. Further investigation is required to elucidate the mechanism of H-NS-dependent motility in *S. typhimurium*.
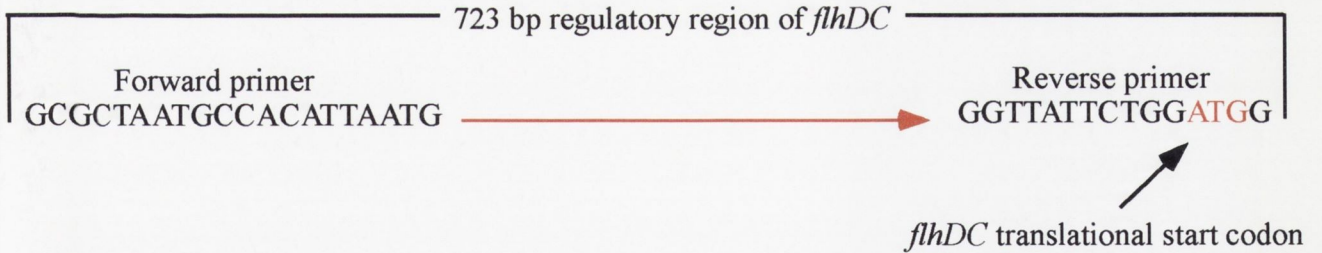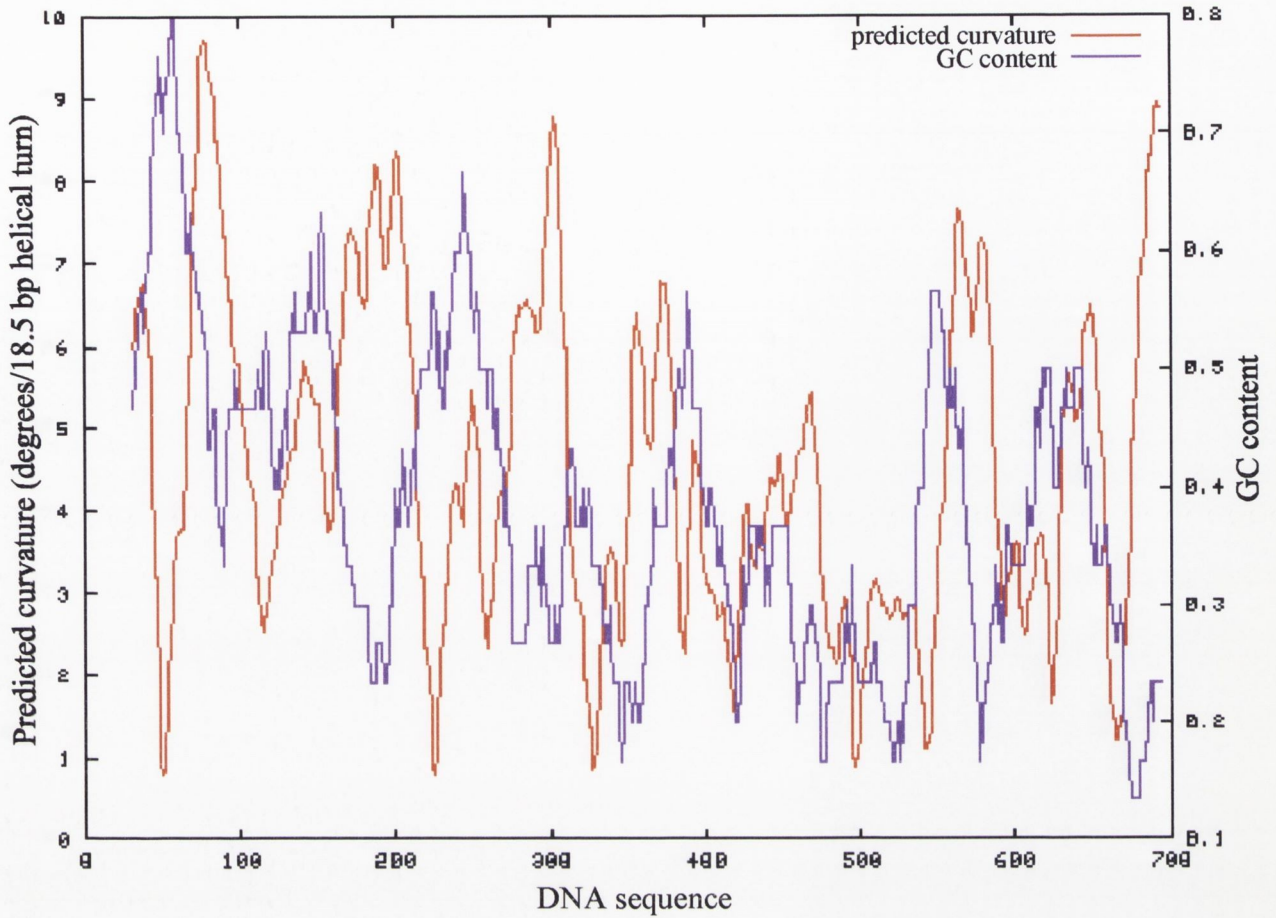
Fig. 5.7. The *flhDC* promoter region is intrinsically curved. The 723 bp promoter region used in DNA mobility shift assays and DNase I footprinting experiments was the regulatory region of *flhDC* tested for curvature. The red line plots the predicted curvature. Significant curvature is regarded as a value greater than 5.

Fig. 5.8. The role of H-NS in the motility of *S. typhimurium.* (A) The effect of an *hns* mutation on *S. typhimurium* motility. SL1344 and SL1344*hns::kan* were compared for motility on semi-solid swarming agar plates. Following 8 hours growth at 37° C the wild-type swarm ring is large but the *hns* mutant has not swarmed. The motility of *hns* mutants were also examined by phase contrast microscopy. In good agreement with the results of the semi-solid swarming agar plates, *S. typhimurium* in the absence of H-NS was nonmotile.

(B) Competitive gel retardation assay examining the possibility of an interaction between HN-S and the *flhDC* promoter region. The plasmid pBR322 was digested with *Taq1* and *Ssp1*, and the resulting DNA fragments were mixed with a 723-bp PCR amplimer of the *flhDC* promoter region. The mixture was incubated with increasing H-NS protein concentrations as indicated. The free DNA and DNA protein complexes were resolved by electrophoresis through a 3% molecular screening agarose gel. The migration of *bla* and *flhDC* DNA fragments are indicated. The intrinsically curved *bla* fragment was used as a positive control. The migration of the *flhDC* promoter is unaltered in the presence of H-NS, strongly suggesting that H-NS does not interact with the *flhDC* promoter region.

A

wt   *hns-*

B

μM H-NS

0   0.5   1.0   2.0   3.0   4.0   5.0

flhDC promoter region

500-bp

bla fragement

200-bp

100 bp ladder   100 bp ladder
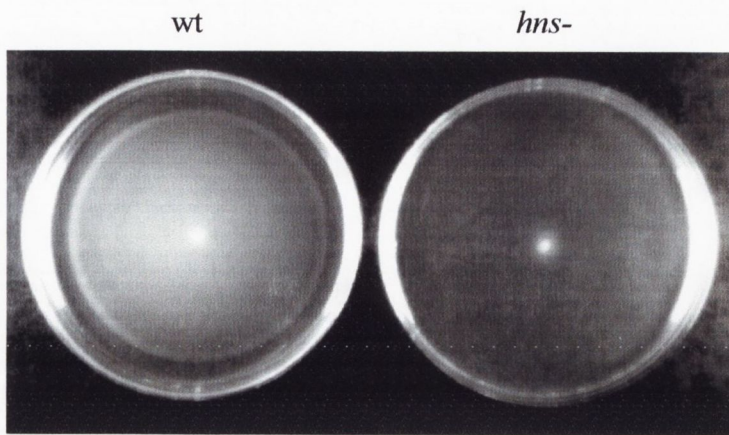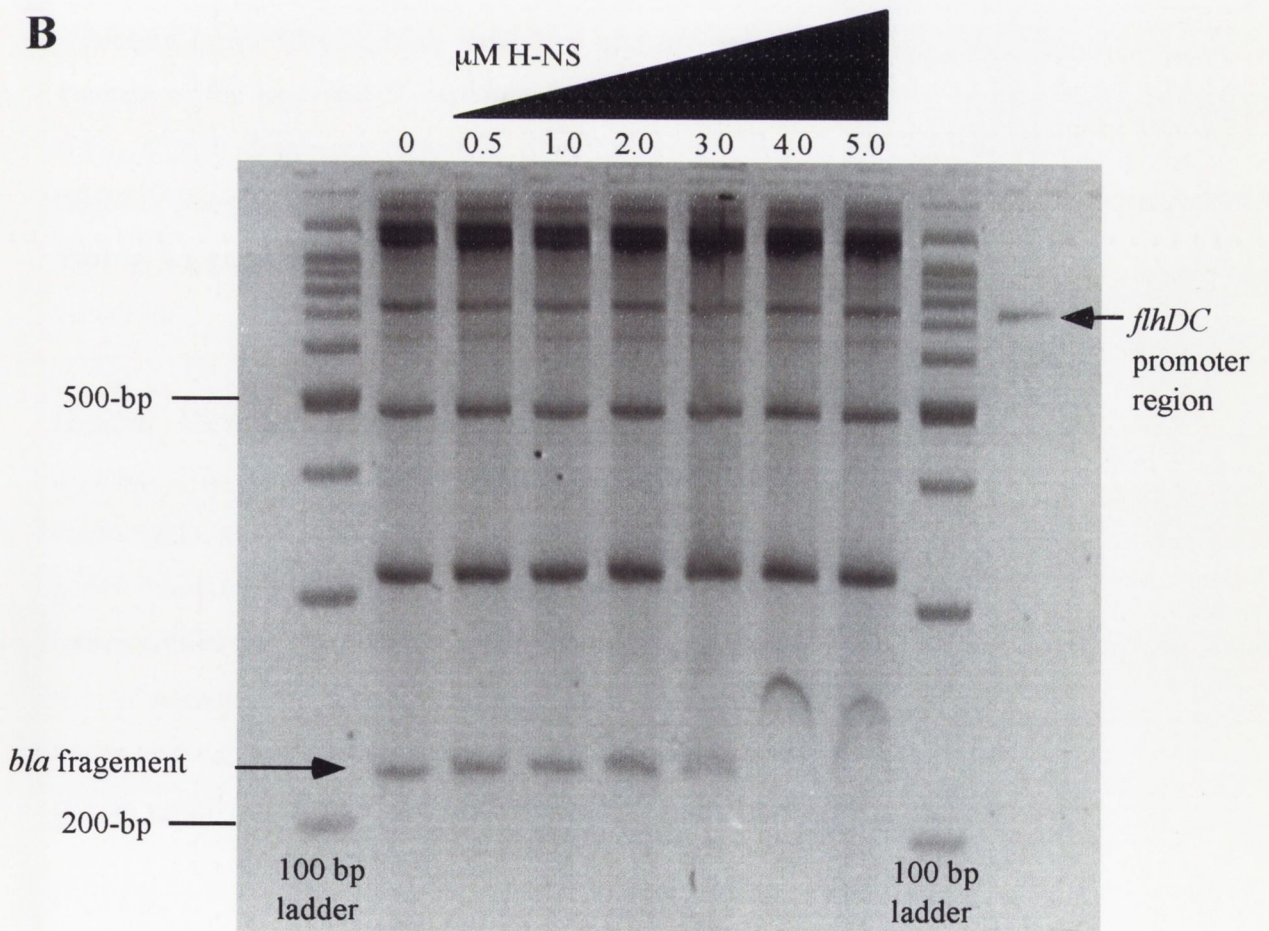
5.3 Discussion

The microarray data presented in Chapter 4 show that Fis exerts widespread effects on gene expression in *S. typhimurium* fully qualifying it as a global regulator. Genes involved in flagellar biosynthesis, chemotaxis and motility were the most strongly downregulated in the absence of Fis. Genes from the early, middle and late stages of flagellar gene expression, which included regulatory and structural genes, were affected by the *fis* mutation. Such an extensive effect on flagellar gene expression deserved to be investigated further.

Bacterial motility was examined on semi-solid agar plates and the *fis* mutant was clearly less motile than the wild-type in good correlation with the array data and with the motility phenotype of a *fis* mutant of *S. typhimurium*, previously described (Osuna *et al.*, 1995). Further analyses of motility using wild-type and *fis* mutant *S. typhimurium* strains locked on and locked off for flagellar phase variation indicated the same reduction in motility in both locked on and off backgrounds in the absence of Fis. Therefore, Fis activates *S. typhimurium* motility independently of phase variation. Also, *E. coli* expresses only one flagellin protein and therefore does not undergo flagellar phase variation. An E. coli *fis* mutant also has reduced motility (data not shown) in good agreement with Fis influencing motility independently of phase variation.

Hitherto, there has been no explanation for the significantly reduced motility of *fis* mutants. A defect in the swarming behaviour could arise from a disruption in chemotaxis, a defect in flagella assembly or be due to a decrease in the expression of genes involved in flagellar biogenesis (Macnab, 1996). The microarray data already indicated that the latter could explain the lack of *fis* mutant motility. Here, the effect of the *fis* mutation on the expression of specific flagella genes was investigated. The genes chosen represented the three temporal stages of flagellar gene expression, early (*flhD*), middle (*fliA*, *flgA*, *fliE*) and late (*fliC.*) genes, typically driven by class 1, class 2

and class 3 promoters. Chromosomal transcriptional fusions of all five flagellar gene promoters were analysed in the presence and absence of Fis during growth. The absence of Fis was negative and expression of all the flagella genes was reduced approximately two fold. An *E. coli hns* mutant, aflagellate and nonmotile, displayed a threefold and fourfold reduction in *flhD* and *fliA* expression, respectively (Bertin *et al.*, 1994). *E. coli* cells grown in the presence of 100 mM D-glucose are also nonmotile and lack flagella (Shi *et al.*, 1993) (Shi *et al.*, 1992). Under these conditions a less than twofold reduction in *flhD* expression and a threefold reduction in *fliA* expression was observed (Shi *et al.*, 1993) (Shi *et al.*, 1992). Therefore the reduction in activity of the five flagella gene transcriptional fusions observed in this study is definitely sufficient to account for the decreased motility of a *S. typhimurium fis* mutant.

In agreement with the motility assays, the β-galactosidase assays and the DNA microarray data the FliC or phase-1 flagellin protein levels were reduced in the *fis* mutant. In *S. typhimurium* the ClpXP protease is also involved in the regulation of flagella biosynthesis. A *clpP* mutant displays a hyperflagellated phenotype, which reflects an overproduction of FliC and FljB flagellin proteins (Tomoyasu *et al.*, 2002). By the same rationale perhaps the reduced FliC protein levels reflect fewer flagellar filaments on the bacterial surface of *fis* mutants. This might be revealed by electron microscopic analysis in the future.

The flagella genes were shown to be Fis regulated but was this regulation due to Fis interacting with flagella gene promoter regions and modulating gene expression directly or due to an indirect effect? Electrophoretic mobility shift assays were performed to determine the interaction of Fis with *flhD* (early), *fliA* (middle) and *fliC* (late) promoter regions. It was demonstrated that Fis binds to multiple distinct sites at each promoter. DNase I footprinting analysis identified two Fis binding sites at the *flhDC* promoter region located between −80 to −104 and −149 to −188 relative to the translational start site of *flhD*. Six transcriptional start sites (P1-P6) were previously identified (Yanagihara *et al.*, 1999) for the *flhDC* operon regulatory region and were located 203, 255, 278, 296, 350 and 530 bp upstream of the translational start site of *flhD*. Another

transcriptional start site identified in this study was found to be located at –93. The location of the Fis binding sites downstream of the six transcriptional start sites P1 to P6 is atypical for an activator. The Fis binding site extending from –149 to-188 is upstream of the transcriptional start site (P) at –93 and the other Fis binding site overlaps this start site. It is thought that most prokaryotic activators have at least one binding site 30-80 nucleotides upstream of the transcription start site. In previous reports of Fis activating transcription at the *leuV*, *proP*, and *rrn* P1 promoters Fis was found to bind three to four sites with the most downstream site mapping at least 70 bp upstream of the transcription start site (Appleman *et al.*, 1998; Hirvonen *et al.*, 2001; Opel *et al.*, 2004; Xu and Johnson, 1995c). Rns, an AraC-like protein is a transcriptional activator of the CS1 and CS2 pili of enterotoxigenic *E. coli* and is also an activator of its own expression (Caron *et al.*, 1989; Munson and Scott, 2000). However, Rns binds only to a single site upstream and to two sites downstream of the *rns* transcriptional start site (Munson and Scott, 2000). The position of the Rns binding sites appears inconsistent with its function as an activator of transcription. However, binding at the upstream binding site and at least one downstream binding site is necessary for the positive autoregulation of the *rns* promoter (Munson and Scott, 2000). DNase I footprinting further upstream of the transcriptional start sites P1-P6 might reveal more Fis binding sites within the *flhDC* regulatory region in the future. Also repeating the primer extension analysis using an oligonucleotide that anneals further downstream than the one used in this study might elucidate other potential transcriptional start sites located closer to the translational start site. Mutating the Fis binding site(s) would confirm its/their role in *flhDC* activation.

A *S. typhimurium hns* mutant tested for motility was nonmotile in agreement with studies performed with *E. coli* and *S. typhimurium* that examined motility in the absence of H-NS (Bertin *et al.*, 1994; Hinton *et al.*, 1992; Kutsukake, 1997). Electrophoretic mobility shift assays found the H-NS protein did not interact with the *flhDC* promoter region despite the presence of regions of intrinsic DNA curvature. In *E. coli*, the H-NS protein was shown to interact with the *flhDC* promoter region (Soutourina *et al.*, 1999). However, another study (also in *E. coli*) suggests that the H-NS-dependent regulation of

flagellar genes is indirect and mediated through the negative regulator of flagellar gene expression HdfR (Ko and Park, 2000b). In addition, H-NS has been shown to modulate flagella motor function (Donato and Kawula, 1998; Ko and Park, 2000a). Further investigation is required to define the underlying mechanism of how H-NS activates motility in *S. typhimurium*.

Fis is a direct transcriptional activator of all three levels of the flagellar regulon and hence in its absence *fis* mutants display a reduced motility phenotype. In addition the microarray data from Chapter 4 revealed that the lipoprotein gene *lpp* was downregulated in the *fis* mutant. This is relevant since lipoprotein is a structural component of the cell envelope that affects flagellar assembly (Dailey and Macnab, 2002). It is interesting to speculate that Fis activates genes required for motility and in concert maintains the cell surface integrity for the assembly and function of the flagellar specific type III secretory apparatus and the hook-basal body, in addition to the type III secretory systems of the SPI-1 and SP1-2 *Salmonella* pathogenicity islands (Chapter 4).

Chapter 6

The role of the nucleoid-associated protein Fis in

Salmonella pathogenicity island-2 (SPI-2) gene regulation

6.1 Introduction

S. typhimurium is dependent upon the products of a large number of genes to cause infection (Finlay and Brumell, 2000; Lucas and Lee, 2000; Marcus *et al.*, 2000). Some of these virulence genes are located on the 90 kb *Salmonella* large virulence plasmid (Fig. 1.2 and Fig. 1.7). However, the majority of virulence genes are located within the *Salmonella* pathogenicity islands (Fig. 1.2) of which SPI-1 and SPI-2 are the best characterised (Fig. 1.4 and Fig. 1.5) and encode two of the three type III secretion systems (Fig. 1.3 and Fig. 1.10) of *S. typhimurium*. SPI-1 encodes the Inv/Spa type III secretion system (Fig. 1.3 and Fig. 1.4) and is required by *S. typhimurium* for epithelial cell invasion (Hardt *et al.*, 1998; Mills *et al.*, 1995; Wood *et al.*, 1998).

SPI-2 (see section 1.1.5 for a detailed review on SPI-2) is a 40 kb insertion at 31 centisomes (cs) on the *S. typhimurium* chromosome adjacent to the tRNA^{ValV} gene (Fig. 1.5) (Hensel *et al.*, 1997a; Ochman *et al.*, 1996; Shea *et al.*, 1996). *S. typhimurium* requires SPI-2 for intracellular replication in host cells such as the macrophage (Cirillo *et al.*, 1998; Hensel *et al.*, 1998; Hensel, 2000; Ochman *et al.*, 1996) and for systemic infection (Fig. 1.1) (Hensel *et al.*, 1995; Shea *et al.*, 1996). Within SPI-2 are the genes encoding a type III secretion system, effector, chaperone and regulatory proteins (Fig. 1.5) (Cirillo *et al.*, 1998). Functionally related genes in SPI-2 are organised together into four groups, regulatory, structural I, effector/chaperone and structural II (Fig. 1.5) (Cirillo *et al.*, 1998). Studies detailing the organisation of the transcriptional units within SPI-2 are confusing and often contradictory. It seems likely that the regulatory region is transcribed from the *ssrA* and *ssrB* promoters (Feng *et al.*, 2003) and that the structural I and effector/chaperone regions are transcribed from the *ssaB* and *sseA* promoters, respectively (Cirillo *et al.*, 1998). However, the transcriptional units within the structural II region is not fully understood (Cirillo *et al.*, 1998; Hensel *et al.*, 1997b). Unlike SP1-1 little is known about the environmental or genetic regulation of SPI-2 genes. While there are reports that concluded low pH, Mg^{2+} deprivation and phosphate starvation activate SPI-2 gene expression there are also studies that refute this (Beuzon

et al., 1999; Cirillo *et al.*, 1998; Deiwick *et al.*, 1999; Lee *et al.*, 2000; Miao *et al.*, 2002; Rathman *et al.*, 1996). Therefore the environmental conditions that influence SPI-2 gene expression is a contentious topic of investigation. SPI-2 genes are preferentially expressed in the intracellular environment of the macrophage and the two-component regulatory system SsrA/SsrB encoded on SPI-2 is necessary for this induction (Cirillo *et al.*, 1998; Valdivia and Falkow, 1997). Besides SsrA/SsrB the only other known regulator of SPI-2 is another two-component signal transduction system OmpR/EnvZ (Feng *et al.*, 2003; Lee *et al.*, 2000).

SPI-2 encoded genes of *S. typhimurium* have been shown in this study to be regulated by the nucleoid-associated protein Fis (Kelly *et al.*, 2004). A DNA microarray analysis comparing a *S. typhimurium* wild-type strain with its isogenic *fis* mutant derivative revealed the Fis-regulation of SPI-2 genes under conditions of batch culture in LB broth (Chapter 4). The aim of this study was to investigate further the regulation of SPI-2 genes by Fis under the same conditions of the DNA microarray analysis and under conditions of limiting oxygen. This study also aimed to clarify the transcriptional start site(s) along the structural II region of SPI-2.

6.2 Results

6.2.1 The effect of the *fis* mutation and oxygen on specific SPI-2 genes

Up to now the only known regulators of SPI-2 were the two component regulatory systems SsrA/SsrB and OmpR/EnvZ (Cirillo *et al.*, 1998; Lee *et al.*, 2000) and the environmental cues that induce the expression of SPI-2 genes is a contentious topic of investigation. The regulon of genes whose expression is influenced by Fis in *S. typhimurium* was defined by DNA microarray analysis (Chapter 4). Among the genes that were most strongly upregulated by Fis were SPI-2 genes and some SPI-2 effectors encoded outside of SPI-2 (Chapter 4). *ssrA* (encodes the sensor kinase of the SsrA/SsrB two-component regulatory system), *ssaG* (encodes a putative structural component of the type III secretory apparatus) and *sseA* (encodes a chaperone for SseB and SseD), were all Fis-activated at the 1 h and 4 h time points after subculture as determined from the DNA microarray analysis (Chapter 4 and supplementary data).

β-galactosidase analysis was employed to determine the activity of *ssrA*, *ssaG* and *sseA* in the presence and absence of Fis, under conditions of moderate aeration (100 ml LB broth in a 250 ml flask) and poor aeration (200 ml LB broth in a 250 ml flask). The region upstream of *ssrA* known to harbour the *ssrA* promoter (Carroll, 2003; Feng *et al.*, 2003) and regions upstream of *ssaG* and *sseA* harbouring putative promoters were cloned upstream of the promoterless *lacZ* reporter gene in the plasmid pQF50 generating the plasmids pQFssrA, pQFssaG and pQFsseA (Carroll, 2003). These plasmids harbouring *lacZ* transcriptional fusions to *ssrA*, *ssaG* and *sseA*, were kindly donated by Ronan Carroll and transformed into *S. typhimurium* strain SL1344 and its isogenic *fis* mutant derivative SL1344*fis::cat*. SL1344 and SL1344*fis::cat* harbouring pQFssrA, pQFssaG and pQFsseA were grown overnight at 37°C with shaking in 100 ml and 200 ml volumes of LB broth in 250 ml conical flasks to provide varying levels of oxygenation to the cultures. The DNA microarray analysis was also conducted in 100

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ml volumes of LB broth in 250 ml flasks (Chapter 4). Following overnight culturing 10 µl samples of the stationary phase cultures were harvested for β-galactosidase analysis.

In good correlation with the DNA microarray data, the results showed that SPI-2 promoters following overnight culturing in 100 ml LB broth (moderate aeration) were significantly less active in the absence of Fis compared with the wild-type, with a reduction in the expression of *ssrA*, *ssaG* and *sseA* of approximately 2-fold, 16-fold, and 20-fold, respectively (Fig. 6.1). Overnight 200 ml cultures in 250 ml flasks were more depleted of oxygen compared to the 100 ml overnight cultures in 250 ml flasks. The SPI-2 promoters displayed greater activity under these conditions of reduced oxygenation (Fig. 6.1). *ssrA*, *ssaG* and *sseA* expression following overnight culturing in 200 ml LB broth in the presence of Fis was increased 70-fold, 12-fold and 11-fold, respectively compared to their expression in 100 ml overnight cultures in the presence of Fis (Fig. 6.1). The activity of *ssrA*, *ssaG* and *sseA* after overnight culturing in 200 ml LB broth in the absence of Fis was increased approximately 146-fold, 5-fold and 3-fold compared to the *fis* mutant 100 ml overnight cultures (Fig. 6.1). The Fis-dependence of the *ssaG* and *sseA* promoters was greater following overnight culturing in 200 ml compared to 100 ml. The *sseA* and *ssaG* promoters were 39-fold and 88-fold less active in the absence of Fis but the *ssrA* promoter was not significantly affected by the *fis* mutation (Fig. 6.1). It is apparent that limiting oxygen induces increased expression from these SPI-2 promoters, a response that is more pronounced in the wild-type background than in the *fis* mutant background except in the case of *ssrA*. These findings are in good correlation with reports of increased Fis protein levels under conditions of poor aeration (Tadhg O'Cróinin, unpublished data). *ssrA* promoter activity is subject to a greater increase in expression in response to reduced oxygenation in the absence of Fis.
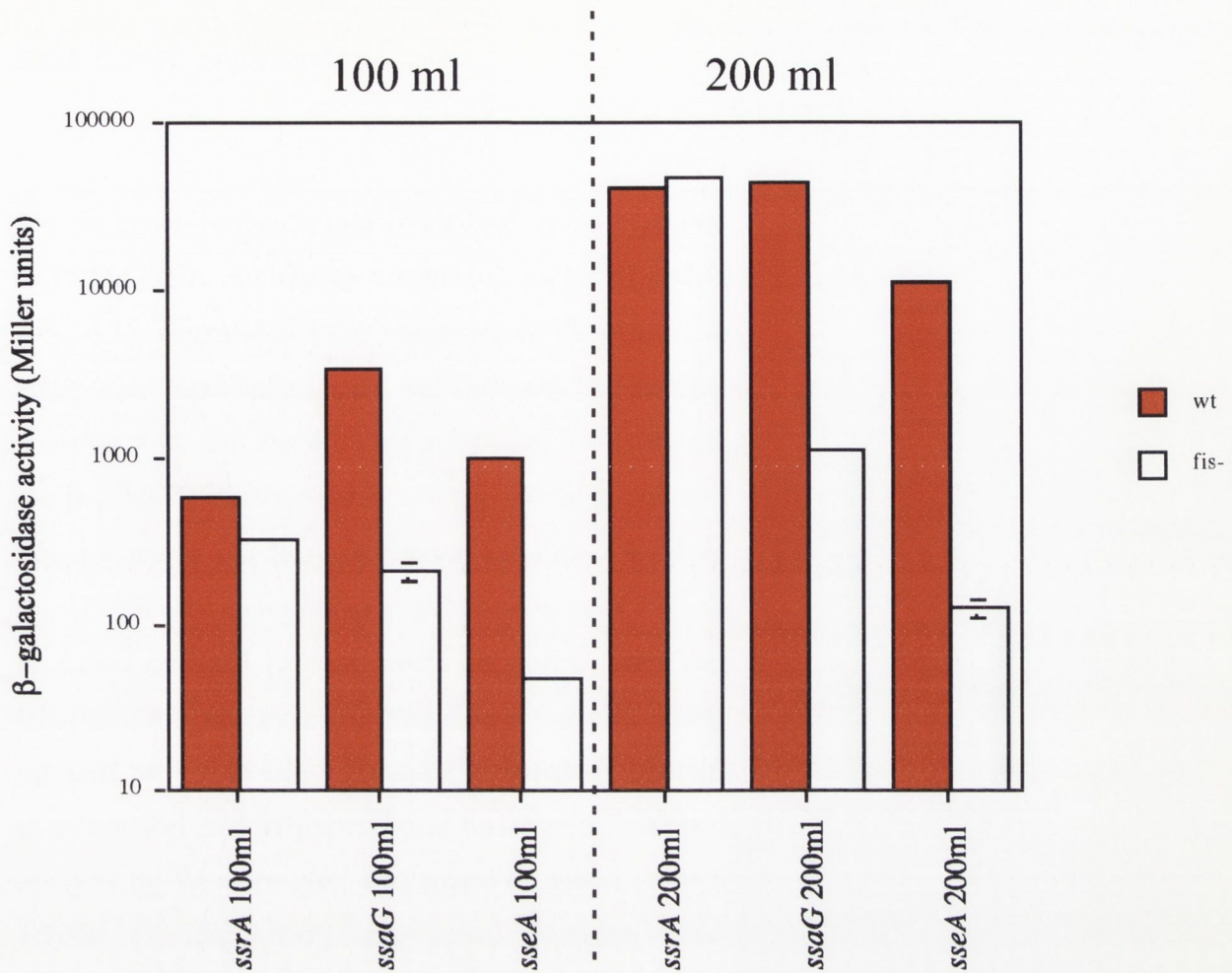
Fig. 6.1. Expression of SPI-2 gene fusions in the presence and absence of Fis under conditions of moderate aeration and poor aeration. β-galactosidase assays were performed to measure the expression of *lacZ* in SL1344 and SL1344*fis::cat* strains harbouring fusions to a selection of SPI-2 genes following overnight culturing at 37°C with shaking in 100 ml and 200 ml volumes of LB broth in 250 ml flasks. 100 ml and 200 ml volumes of culture provided moderate and poor oxygenation levels. The results indicated an increase in *ssrA*, *ssaG* and *sseA* expression under conditions of poor aeration in both the presence and absence of Fis. However, the SPI-2 promoters display decreased activity in the absence of Fis which is more pronounced under the conditions of reduced oxygenation.

6.2.2 SsrB protein levels

The *fis* knock-out had a negative effect on the expression of SPI-2 genes as determined from the DNA microarray analysis (Chapter 4) and from the β-galactosidase assays (Fig. 6.1). Specifically, the expression of the sensor kinase *ssrA* of the SsrA/SsrB two-component regulatory system was shown to be Fis-dependent from the DNA microarray analysis at the 1 h and 4 h time points and it was shown again to be Fis-dependent from the β-galactosidase assays in 100 ml overnight culture conditions of moderate aeration but not under conditions of poor aeration. SsrB is the response regulator of the SsrA/SsrB two-component regulatory system. To investigate the effect of the *fis* mutation on SsrB protein levels antibodies were raised against the purified MBP-labelled SsrB protein (a gift from Ronan Carroll). Three injections, each containing 300 µg SsrB 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 blot, and found to contain anti-SsrB antibodies at a dilution of 1:5000. The rabbit was exsanguinated and serum stored at 4°C.

Stationary phase SsrB protein levels in the wild-type and *fis* mutant backgrounds of *S. typhimurium* were determined by Western blot (Fig. 6.2). Following overnight culturing at 37°C with shaking in 100 ml and 200 ml LB broth in 250 ml conical flasks (the same conditions as the β-galactosidase assays), equal quantities of SL1344 and SL1344*fis::cat* were harvested corresponding to 2 ml of culture per 1.0 OD_{600nm} for the preparation of crude protein extracts. Equal volumes of each extract were loaded on an SDS-PAGE gel and analysed by Western immunoblotting using the SsrB antiserum.

Results demonstrated that the level of SsrB was strongly repressed in the *fis* mutant under moderate and poor oxygenation levels and greater SsrB protein levels were detectable from the wild-type 200 ml overnight culture compared to the wild-type 100 ml overnight culture (Fig. 6.2). The finding that reduced oxygenation increases SsrB protein levels in the wild-type background is fully consistent with the β-galactosidase
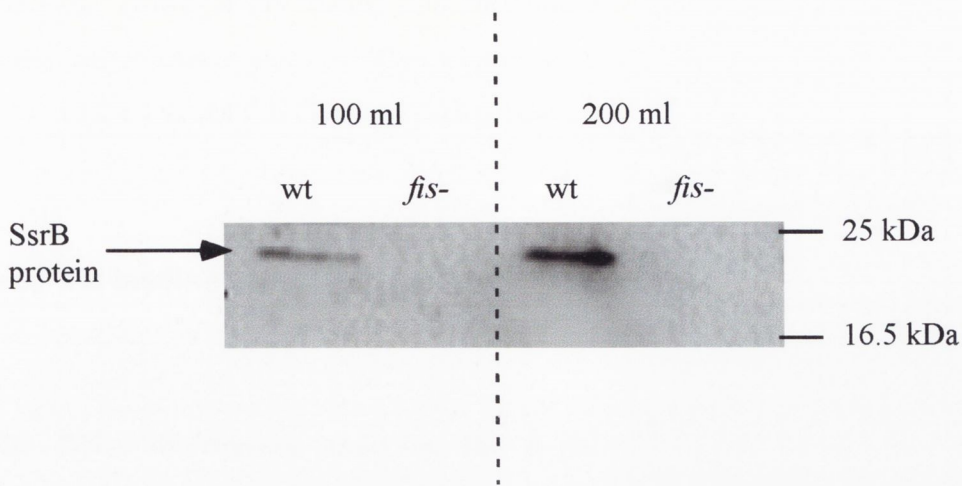
Fig. 6.2. Western immunoblot analysis of SsrB protein levels in the presence and absence of Fis and under conditions of moderate and poor aeration. Protein samples were harvested from overnight cultures of SL1344 and SL144*fis::cat* growing at 37°C with shaking in 100 ml (moderate aeration) and 200 ml (poor aeration) volumes of LB broth in 250 ml conical flasks. The results indicated SsrB was undetectable in the absence of Fis. In the presence of Fis, poorly aerated 200 ml overnight cultures displayed greater levels of SsrB protein compared to the 100 ml overnight cultures subject to increased aeration.

analysis. However the finding that SsrB protein levels (Fig. 6.2) were Fis-dependent under conditions of poor aeration was in contrast to the *ssrA* promoter activity (Fig. 6.1), which was not found to be Fis-dependent in poor aeration.

6.2.3 Fis binding to the *ssrA* promoter

The DNA microarray analysis, the β-galactosidase assays and the Western immunoblotting had indicated a role for Fis in the regulation of SPI-2. To determine if the observed effects of Fis on SPI-2 were as a result of Fis directly interacting with SPI-2 promoters and modulating promoter activity, electrophoretic mobility shift assays were performed. The interaction of purified Fis protein with the regulatory region of *ssrA* was investigated. The regulatory region of *ssrA* was amplified by PCR using the oligonucleotide pair ssrAfw and ssrArv (Table 2.3), generating a DNA fragment 647 bp in size (The same 647 bp of *ssrA* promoter region cloned upstream of the promoterless *lacZ* in pQF50 generating pQFssrA, used in the β-galactosidase analysis in section 6.2.1). The DNA fragment was gel purified and radiolabelled with $[\gamma\text{-}^{32}P]ATP$. The *spvR* promoter region was used as a negative control since the *spvR* gene appears not to be Fis-regulated as determined from the DNA microarray analysis (supplementary data). The *spvR* gene from the 90 kb virulence plasmid was amplified by PCR using the primers spvR11 and spvR14 (Table 2.3), generating a 645 bp DNA fragment that was also gel purified and radiolabelled with $[\gamma\text{-}^{32}P]ATP$. The radiolabelled DNA fragments were incubated with increasing concentrations of Fis protein. Purified Fis protein containing an N-terminal six-histidine tag was provided as a gift from O. Keane (Keane and Dorman, 2003). Bovine serum albumin and poly-[d(I-C).(dI-dC)] were used as competitors (section 2.15). The DNA-protein mixtures were then resolved on a non-denaturing 7% polyacrylamide gel. The binding of protein to the DNA forms a complex that runs more slowly through the gel resulting in a "shift" in the electrophoretic mobility of the DNA visualised by autoradiography.

Results of the DNA mobility shift assays revealed a shift in the electrophoretic mobility of the *ssrA* promoter (Fig. 6.3). In addition Fis appeared to bind three distinct sites at the *ssrA* promoter since three distinct complexes can be seen (Fig. 6.3). By comparison, the negative control only underwent a weak shift at the highest Fis concentration (data not shown), in good correlation with the previous DNA mobility shift assays examining the interaction of Fis and *spvR* in Chapter 5 (Fig. 5.4). The results indicate that Fis interacts directly with the promoter region of *ssrA*. This finding is in agreement with studies that revealed Fis binding to the *ssrA*, *ssaG*, *sseA* and *ssaH* promoter regions but not to the *spvR* promoter region (Carroll, 2003).

6.2.4 Mapping Fis binding sites at the *ssrA* promoter

The DNA mobility shift assay revealed Fis binds the *ssrA* promoter region at multiple sites. In order to map the Fis binding sites DNase I footprinting was performed. The DNase I footprinting technique was discussed in detail previously (section 5.2.5). Briefly, the footprinting technique is based on the theory that sites of the DNA bound by protein are protected from DNase I degradation. The promoter region of *ssrA* was amplified by PCR using the oligonucleotide pair ssrAfw and ssrArv (Table 2.3), generating the same 647 bp DNA fragment used in the β-galactosidase analysis (section 6.2.1) and in the electrophoretic mobility shift assays (section 6.2.3). The PCR product was gel purified and end-labelled at both 5' ends using T4 polynucleotide kinase and [γ-^{32}P]ATP. Only one 5' end is required to be labelled for this technique and therefore following the labelling reaction the PCR product was digested with *Eco*RV, cleaving the 647 bp fragment into 136 bp and 511 bp fragments, both labelled at one end only. The 511 bp DNA fragment was purified after separation on a 7% acrylamide gel. The 511 bp DNA fragment composed largely of regulatory region upstream of *ssrA* and including the first 53 bp of the *ssrA* ORF was then incubated with increasing amounts of Fis protein at 37°C for 1 h in order to allow Fis protein to interact with the DNA. After the 1 h incubation DNase I was added for exactly 1 min and the reaction was then
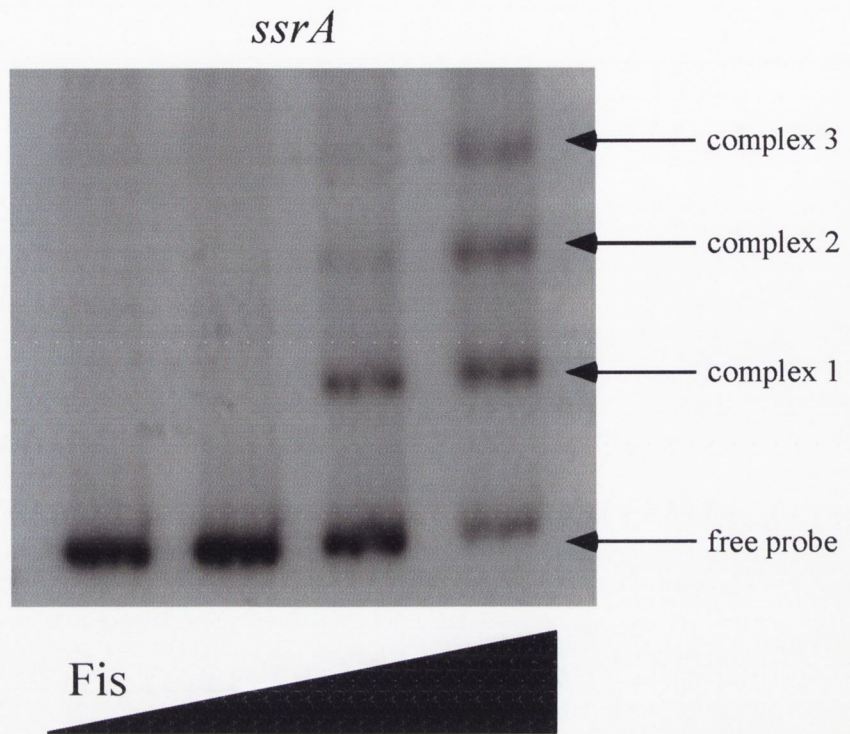
Fig. 6.3. Interaction of Fis protein with the *ssrA* promoter region. The ability of Fis to bind the *ssrA* promoter region was assessed by electrophoretic mobility shift assay. The PCR amplified, gel purified and radiolabelled *ssrA* regulatory sequence was incubated with 0, 4, 20 or 60 ng of purified Fis protein and subjected to electrophoresis in a 7% polyacrylamide gel. Three distinct DNA-protein complexes were formed indicating three possible Fis binding sites at the *ssrA* regulatory region. The *spvR* promoter from the 90 kb virulence plasmid was used as a negative control and in contrast it only underwent a weak shift at the highest Fis concentration (Fig. 5.4).

stopped immediately. The resulting fragments were then resolved by electrophoresis on a 7% acrylamide gel. The products of a DNA sequencing reaction, carried out using the plasmid pQFssrA and the oligonucleotide ssrArv, were run alongside the DNase I reactions on a 7% acrylamide gel (Fig. 6.4B).

The Fis binding sites within the *ssrA* regulatory region can be seen in Fig. 6.4 (B). Fis protects 3 regions of the *ssrA* regulatory region that extend from −24 to −17, +43 to +56 and +74 to +154 relative to the transcriptional start site elucidated by Feng *et al* (2003). These regions protected by Fis from DNase I cleavage are underlined in Fig. 6.4 (A). A sequence that has good homology with the Fis consensus sequence (5'-KNNYRNNWNNYRNNM -3') (Finkel and Johnson, 1992; Hengen *et al.*, 1997), identified within the Fis-protected regions is indicated in blue type (Fig. 6.4A). Binding of Fis to the *ssrA* promoter region also induced a hypersensitive site to DNase I cleavage (Fig. 6.5B) at position +143, indicated in yellow type (Fig. 6.4A).

6.2.5 Identification of the *ssaG* transcription start site

At present, the transcriptional organization of the structural II region of SPI-2 is not clear. A previous report described at least two operons within the structural II region, *ssaK/U* was one and the *ssaJ* gene upstream of this was described as the terminal gene of another operon, which was not defined, thus suggesting a promoter was located somewhere between *ssaJ* and *ssaK* (Hensel *et al.*, 1997b). Another report proposed the structural II promoter was positioned further upstream between *sseG* and *ssaH* driving the *ssaH/V* operon (Cirillo *et al.*, 1998). However, this report failed to take into account the *ssaG* gene, which lies between *sseG* and *ssaH*.

β-galactosidase assays performed in section 6.2.1 confirmed that the region upstream of *ssaG* contained a promoter because this region when cloned upstream of the promoterless *lacZ* in pQFssaG produced high quantities of *lacZ* gene product. Primer

Fig. 6.4. Mapping the Fis binding sites within the *ssrA* regulatory region.

(A) Sequence of the *ssrA* regulatory region. The transcriptional start site is at a C residue, in red type and numbered +1. The three regions of Fis-protection are underlined and labelled region I, region II and region III corresponding to the regions of protection determined in section B. A sequence within the protected regions with good homology to the Fis binding consensus sequence is indicated in blue type. A site of DNase I hypersensitivity resulting from Fis binding is indicated in yellow type.

(B) DNase I footprint of Fis binding the *ssrA* regulatory region. Radiolabelled *ssrA* regulatory region was incubated with increasing concentrations of purified Fis protein prior to DNase I digestion. A DNA sequencing ladder generated with the oligonucleotide ssrArv was run alongside the DNase I reactions to map regions of Fis-protection. Fis protected three regions upstream of *ssrA* that are indicated by brackets and labelled regions I to III. Dots within brackets more accurately indicate Fis protection.

A

TAAAATTCACAATTACATTTTCAGCATTGACATAAAAACTTACAATTTGAAAAAT

$+1$

TATTTATTAAATAAACTGTTACGATGTTTTTACATCGCCATCTTATTAAAAAGTAA
region I

TTGTAGTACTCGACTGGGTTATATATGAAGAAATTTATCTTCCTAATGAT
region II

AACACCATCGATTAATCTTCTGATGAAACTATATGTACTGCGATAGTGAT

CAAGTGCCAAAGATTTTGCAACAGGCAACTGGAGGGAAGCATTATG*ssrA*
region III

B

Fis

0 25 50 100 150 200 250 300   nm Fis

A C G T

+1

protected
region I

protected
region II

protected
region III

hypersensitivity

extension analysis was performed to locate the exact transcriptional start site(s) for *ssaG*.

A 580 bp DNA fragment was amplified by PCR using the oligonucleotides ssaGfw and ssaGrv (Table 2.3) and cloned upstream of the promoterless *lacZ* in the plasmid pQF50 (Table 2.2) generating pQFssaG (Table 2.2). pQFssaG was transformed into the *S. typhimurium* strain SL1344. Total RNA was isolated from SL1344 harbouring the pQFssaG plasmid. Primer extension analysis was carried out using the RNA as template and the radiolabelled oligonucleotide pe-fw (Table 2.3). The primer extension products were run on a 7% acrylamide gel alongside DNA sequencing reactions generated using the same oligonucleotide pe-fw, and the plasmid pQFssaG. In this way the primer extension product(s) could be mapped to the sequence and the transcription start site(s) identified.

The putative *ssaG* transcription start sites identified are indicated in Fig. 6.5. Two transcription start sites were identified mapping to an A residue and a G residue 18 bp and 37 residues upstream of the *ssaG* translational start codon, respectively (Fig. 6.5A+B). When the primer extension analysis was repeated using total RNA isolated from wild-type *S. typhimurium* SL1344 and its *fis* mutant derivative SL1344*fis::cat* both harbouring the plasmid pQFssaG, the intensity of the transcriptional start sites were decreased in the *fis* mutant background compared to the wild-type (data not shown).

A

B

STOP*sseG*TAATCGTTTTCAGGTATATACCGGATGTTCATTGCTTTCTAAATTTTGCTATG

TTGCCAGTATCCTTACGATGTATTTATTTTAAGGAAAAGCATTATG*ssaG*

Transcription start sites
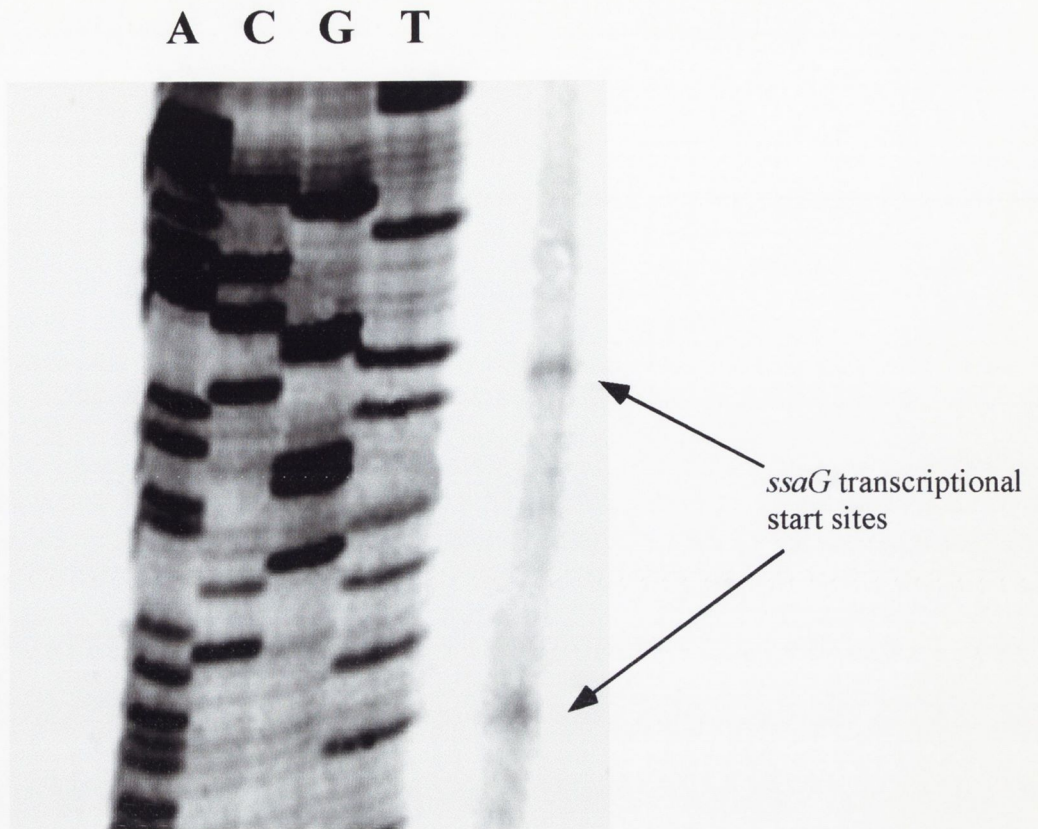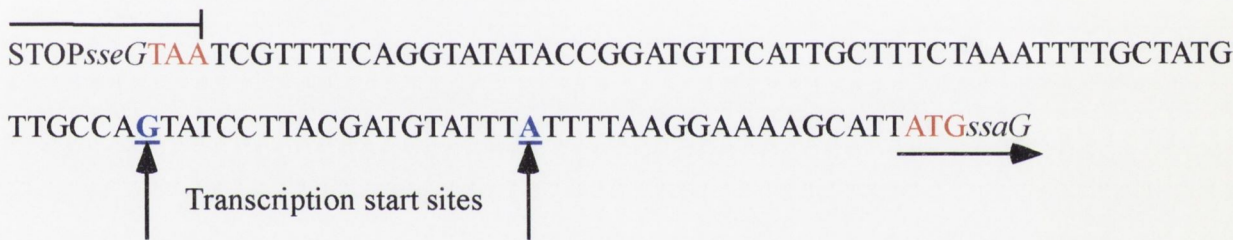
Fig. 6.5. Determination of the *S. typhimurium* ssaG transcription start site(s) by primer extension analysis. (A) Primer extension analysis was performed using total RNA isolated from SL1344 harbouring the plasmid pQFssaG and using the radiolabelled oligonucleotide pe-for. DNA sequencing reactions were prepared using the same oligonucleotide pe-for and electrophoresed in parallel in order to identify the transcription start site(s). DNA sequencing reactions are labelled ACGT. The *ssaG* transcription start sites are indicated.

(B) Sequence of the *ssaG* upstream region. The two transcription start sites that mapped to an A and a G residue are indicated in blue type and with arrows. The translational start codon for *ssaG* and the translational stop codon for *sseG* are indicated in red type.

6.3 Discussion

The DNA microarray analysis to determine the Fis regulon (Chapter 4) revealed that Fis exerts wide-ranging effects on gene expression in *S. typhimurium.* It was revealed that the *fis* mutation influenced the expression of virulence genes located in the *Salmonella* pathogenicity islands SPI-1, SPI-2, SPI-3, SPI-4 and SPI-5 and virulence genes located outside the pathogenicity islands. Up to now Fis was implicated in the regulation of virulence in *S. flexneri* (Falconi *et al.*, 2001), enteropathogenic *E.coli* (Goldberg *et al.*, 2001), enteroaggregative *E. coli* (Sheikh *et al.*, 2001) and *S. typhimurium* (Wilson *et al.*, 2001). In the case of *S. typhimurium* Fis was reported to be required for the full expression of SPI-1 virulence genes (Wilson *et al.*, 2001). Mutations in *fis* were also shown to attenuate the virulence of *S. typhimurium* in mice infected by the oral route (Wilson *et al.*, 2001). To date Fis has influenced the expression of the virulence genes associated with the invasion of epithelial cells in these enteric pathogens. Earlier in this study the DNA microarray implicated Fis in the regulation of SPI-2 virulence genes, concerned with bacterial proliferation in macrophages (Cirillo *et al.*, 1998; Hensel *et al.*, 1998; Hensel, 2000; Ochman *et al.*, 1996) and systemic infection in mice (Hensel *et al.*, 1995; Shea *et al.*, 1996). Here the role for Fis as a positive regulator of SPI-2 gene expression was confirmed.

lacZ transcriptional fusions to the SPI-2 genes, *ssrA*, *ssaG* and *sseA* were used to investigate the effects of the *fis* mutation. β-galactosidase analysis was carried out on 100 ml LB broth overnight wild-type and *fis* mutant cultures harbouring these fusions (Fig. 6.1). The *fis* mutation resulted in a 2-fold, 16-fold and 20 fold reduction in expression of *ssrA*, *ssaG* and *sseA*, respectively (Fig. 6.1). This was in good agreement with results that found that an intact *fis* gene was necessary for full induction of the SPI-2 genes, *ssrA*, *ssaG*, *sseA*, *ssaB*, *ssaK* and *ssaH* in macrophage (Carroll, 2003). Also when the *ssaG* transcription start site was determined by primer extension using total RNA from the wild-type (Fig. 6.5) and the *fis* mutant the intensity of the two bands that

corresponded with the start sites was decreased in the *fis* mutant background, providing further evidence of *ssaG* Fis-dependence (data not shown).

It was also found that under conditions of poor aeration SPI-2 gene expression was higher in the wild-type and *fis* mutant backgrounds compared to the same strains grown under conditions of moderate aeration (Fig. 6.1). It was observed that the full induction of the SPI-2 genes, *sseA* and *ssaG* in response to limiting oxygen required a fully functional *fis* gene (Fig. 6.1). For example in response to reduced aeration the activity of the *sseA* promoter increased 11-fold in the presence of Fis but only 3 fold in the absence of Fis (Fig. 6.1). It is interesting to speculate that the increased Fis protein levels observed under conditions of reduced aeration (Tadhg O'Cróinin, unpublished data) are responsible, at least in part for the increased expression of the *sseA* and *ssaG* genes under the conditions of limiting oxygen in this study. In addition it was observed that under limiting oxygen *ssrA* was not Fis-regulated which suggests that the Fis-regulation of *sseA* and *ssaG* under these conditions is not mediated through the SsrA/SsrB two component regulatory system. In support of this, Fis was observed to interact with the DNA sequences upstream of *sseA* and *ssaG*, which implies that Fis directly regulates these promoters (Carroll, 2003).

SsrB protein levels were investigated in the presence and absence of Fis under conditions of moderate and poor aeration, the same conditions that the β-galactosidase assays were performed under. As with the β-galactosidase assays in the presence of Fis SsrB protein levels were induced under conditions of reduced aeration (Fig. 6.2). Results also indicated that SsrB protein levels were strongly repressed in the absence of Fis in both moderate and poor aeration (Fig. 6.2). In contrast to this *ssrA* promoter activity was not dependent on Fis under conditions of poor aeration (Fig. 6.1). OmpR activates separately the transcription of *ssrA* and *ssrB* (Feng *et al.*, 2003). It is suggested from these results that Fis, like OmpR, may regulate *ssrA* and *ssrB* expression separately. However an investigation of *ssrB* promoter activity in the presence and absence of Fis under conditions of moderate and poor oxygenation is needed to complete this story.

An investigation further into the Fis regulation of *ssrA* using DNA electrophoretic mobility shift assays (Fig. 6.3) and DNase I footprinting (Fig. 6.4) revealed that Fis interacts directly with three sites at the promoter region of *ssrA*. Multiple complexes appear to be the norm at promoters dependent on Fis for full activation (Appleman *et al.*, 1998; Hirvonen *et al.*, 2001; Opel *et al.*, 2004; Travers *et al.*, 2001; Xu and Johnson, 1995c), and at promoters subject to Fis repression (Keane and Dorman, 2003; Schneider *et al.*, 1999). One site was mapped upstream and two were located downstream of the *ssrA* transcriptional start site, which is an atypical binding pattern for a transcriptional activator (Fig. 6.4). Similarly phosphorylated OmpR was found to bind upstream and downstream of the *ssrA* transcription start site (Feng *et al.*, 2003). To date, OmpR and Fis have only ever been associated with the activation of *ssrA* transcription and never with its repression. Recently it has become more apparent that transcriptional activators can bind downstream of the transcription start site and still play a role in activation. For example, Rns of the AraC family is a transcriptional activator of CS-1 and CS-2 pili in enterotoxigenic *E. coli*. Rns is also an activator of its own expression, and the Rns binding sites are located both upstream and downstream of the *rns* promoter (Munson and Scott, 2000). The molecular mechanism behind this type of binding and transcriptional activation is not fully understood. It is interesting to speculate that in the case of Fis, its downstream binding sites serve to compete with and displace a transcriptional repressor, as is the case at the *virF* promoter where Fis and H-NS interact antagonistically (Falconi *et al.*, 2001). Interestingly, an unidentified repressor of *ssrA* transcription has been suggested previously (Lee *et al.*, 2000). Future work aims to explore this possibility and to investigate the interaction of Fis with the *ssrB* promoter region recently defined (Feng *et al.*, 2003).

The SsrA/SsrB two component signal transduction system controls the expression of genes encoding the components of the SPI-2 type III secretion system and SPI-2 effectors located in and outside of the pathogenicity island (Beuzon *et al.*, 2000; Brumell *et al.*, 2003; Cirillo *et al.*, 1998; Knodler *et al.*, 2002; Miao and Miller, 2000; Worley *et al.*, 2000). The OmpR/EnvZ two-component regulatory system is in addition to Fis a positive regulator of SPI-2 (Feng *et al.*, 2003; Lee *et al.*, 2000) but its effect on

SPI-2 gene expression is mediated through *ssrAB* (Garmendia *et al.*, 2003). Given Fis binds directly at *ssrA* (this study), *sseA* and *ssaG* (Carroll, 2003) promoter regions it is likely that Fis is perhaps the first example of a regulator other than SsrA/SsrB that makes its contribution to SPI-2 gene expression not just at *ssrAB* but also directly at other SPI-2 promoters.

Fis protein levels are high immediately after sub-inoculation and then decline rapidly as the culture begins to divide and are undetectable by Western immunoblotting before the culture enters stationary phase (Fig. 3.6C), (Ball *et al.*, 1992; Keane and Dorman, 2003; Ninnemann *et al.*, 1992). The expression of the SPI-2 genes *ssrA*, *ssaG*, *sseA* and the SsrB protein levels were severely reduced in a *fis* mutant compared to the wild type in stationary phase. This striking feature where Fis-regulation occurs at a stage in growth where Fis protein has become undetectable by Western blot is not unique. It has been observed previously in enteropathogenic *E. coli* where Fis-dependent virulence genes encoded on the Locus for Enterocyte Effacement (LEE) are maximally expressed in late stationary phase (Goldberg *et al.*, 2001). Similarly the transcriptional repression that Fis exerts on the *gyr* genes that encode DNA gyrase is most severe in stationary phase in both *S. typhimurium* (Keane and Dorman, 2003) and *E. coli* (Schneider *et al.*, 1999). Also the DNA microarray analysis performed to elucidate the Fis regulon found a greater number of Fis-dependent genes in late rather than early exponential phase (Chapter 4). It is apparent however that although Fis levels dramatically decline during stationary phase they reach a low but non-zero value and therefore the cell is not entirely depleted of Fis (Azam *et al.*, 1999; Walker *et al.*, 1999). It is proposed that as Fis levels decline it is only sites with a high affinity for the Fis protein that will continue to be occupied by Fis when lower affinity sites are vacant (Muskhelishvili and Travers, 2003). Perhaps SPI-2 promoters along with these promoters previously found to be occupied by Fis when Fis levels are in decline belong to this privileged group.

In this chapter the Fis-regulation of SPI-2 was confirmed and the Fis binding sites at the *ssrA* promoter region were defined. Within the Fis binding sites was a region with some homology to the degenerate Fis binding consensus sequence (Fig. 6.4A). The *S.*

typhimurium pathogenicity islands were acquired by horizontal gene transfer (Galan, 2001; Groisman and Ochman, 1993, 1996, 1997; Hensel *et al.*, 1999b; Hensel, 2000) and perhaps it is the degeneracy associated with Fis binding that made it an ideal candidate for recruitment as a regulator at these regions of foreign DNA.

It is interesting to speculate that since Fis contributes to the activation of SPI-2 (this study), SPI-1 (Wilson *et al.*, 2001), SPI-3, SPI-4 and SPI-5 gene expression (Chapter 4) that it is involved in coordinating the activation of the virulence genes in these distinct pathogenicity islands, which is definitely an issue that warrants further study.

Chapter 7

General Discussion

The main focus of this work was to determine and explore the Fis regulon of *S. typhimurium* by combining a DNA microarray analysis with more specific assays. In the microarray work, *S. typhimurium* Fis-regulated genes were identified by comparing the gene expression profile in a wild-type strain with a *fis* knockout mutant. This assessment of transcription at the genomic level revealed that the most strongly Fis-activated genes were those located within the five *Salmonella* pathogenicity islands and the genes involved in motility and chemotaxis (Fig. 7.1). Among the most strongly Fis-repressed genes were genes involved in metabolism and transport (Fig. 7.1).

Genes involved in flagellar biosynthesis, chemotaxis and motility were among the most severely downregulated in the *fis* mutant. Genes from the early, middle and late stages of flagellar gene expression, which included regulatory and structural genes were affected by the *fis* mutation. Such a widespread effect was deemed to be worthy of further investigation. Chromosomal *lacZ* transcriptional fusions to five representative flagellar genes (*flhD, fliA, flgA, fliE* and *fliC*) were analysed in the presence and absence of Fis. The absence of Fis had a negative effect on the expression of all five flagellar genes during growth. It is likely that Fis makes its contribution to the transcription of these genes directly as it was discovered that the Fis protein binds to the sequences located upstream of *flhD, fliA* and *fliC*. The specific Fis binding sites at the *flhDC* promoter region were determined by DNase I footprinting analysis and the *flhDC* transcriptional start sites were determined by primer extension. The locations of the Fis binding sites were atypical for a transcriptional activator. Given that Fis interacts directly with flagellar gene promoters to activate transcription, the effect of Fis on the motility phenotype was investigated. The *fis* mutant was much less motile than the wild-type and motility that was fully restored when the *fis* lesion was complemented *in-trans*. *S. typhimurium* expresses alternately two unlinked genes, *fliC* and *fljB* that encode two antigenically distinct flagellin proteins (Bonifield and Hughes, 2003; Haykinson *et al*., 1996; Macnab, 1996). Fis is involved in the DNA inversion reaction that is the mechanism of flagellar phase variation (Bonifield and Hughes, 2003; Osuna *et al*., 1995). However, the reduced motility phenotype displayed by the *fis* mutant was found to be independent of flagellar phase variation and the expression of normal phase

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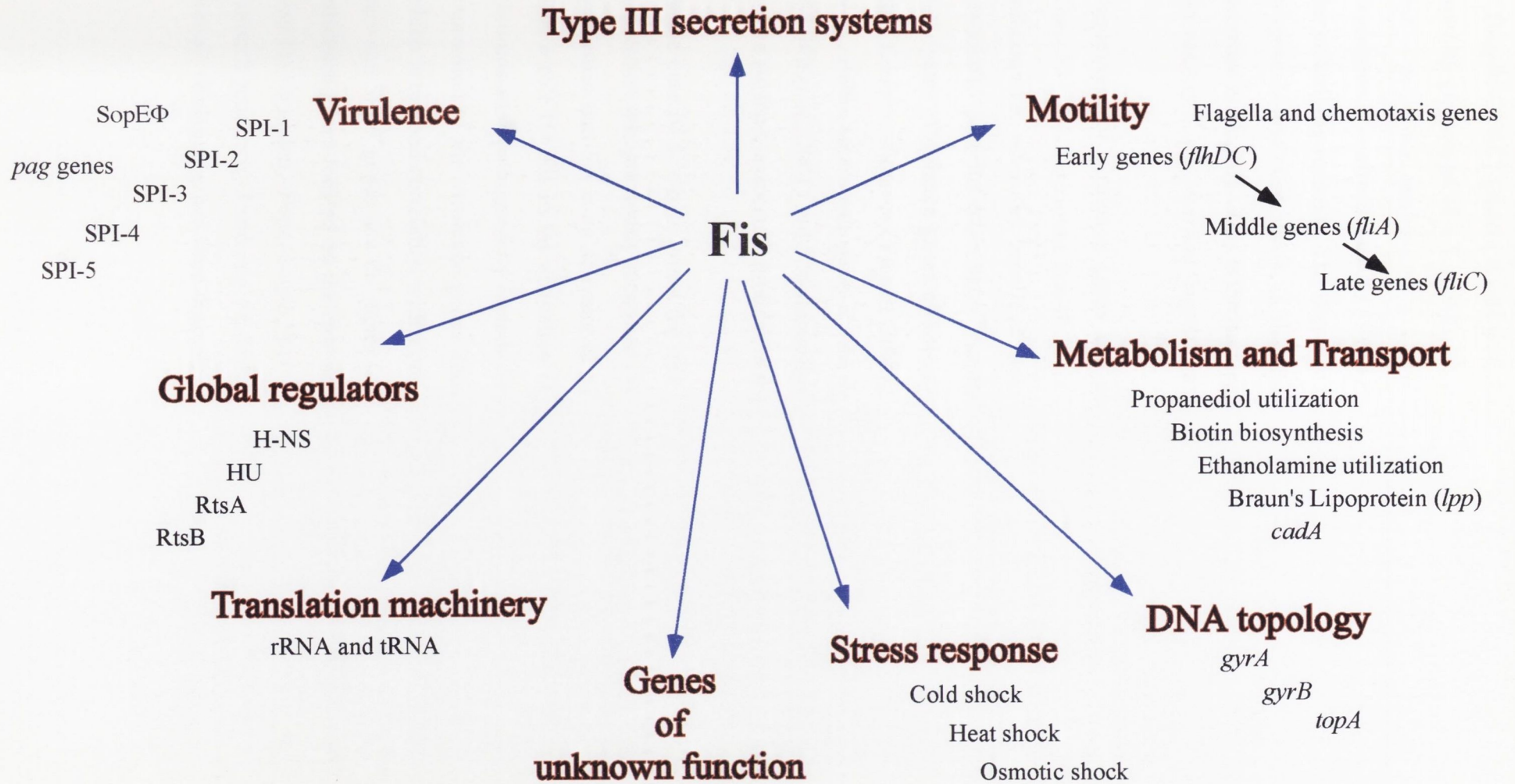
Fig. 7.1. Summary diagram of the major processes and systems that Fis regulates in *S. typhimurium*.

l flagellin (FliC) protein levels was also shown to be Fis-dependent. Previously the motility phenotype of a *S. typhimurium fis* mutant was described (Osuna *et al.*, 1995) and a proteome analysis of a *S. typhimurium fis* mutant revealed flagella protein expression was affected by Fis (Yoon *et al.*, 2003) but my study is the first to establish the underlying reasons. The decrease in the expression of genes involved in flagellar biogenesis and chemotaxis, in the absence of Fis is responsible for the *fis* mutant's decrease in motility. This is the first report to identify the role of Fis as a transcriptional activator of *S. typhimurium* flagellar genes.

Expression of virulence genes can place 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. Nucleoid-associated proteins have been recruited as regulators of virulence genes in many pathogens. Virulence genes of *Shigella flexneri* (Dorman *et al.*, 2001; Falconi *et al.*, 2001), *Vibrio cholerae* (Yu and DiRita, 2002), *Pseudomonas aeruginosa* (Delic-Attree *et al.*, 1996), enteropathogenic *E. coli* (Goldberg *et al.*, 2001), enteroaggregative *E. coli* (Sheikh *et al.*, 2001) and *S. typhimurium* (Schechter *et al.*, 2003; Wilson *et al.*, 2001) all utilize nucleoid-associated proteins in their regulation.

In the case of *S. typhimurium* the role of Fis in the positive regulation of SPI-1 gene expression was previously identified (Wilson *et al.*, 2001). The DNA microarray study confirmed that Fis is an activator of SPI-1 gene expression and revealed for the first time a role for Fis in the activation of the virulence genes located in the four other chromosomal pathogenicity islands SPI-2-5. This suggests that Fis coordinates the expression of the virulence genes encoded in SPI-1, SPI-2, SPI-3, SPI-4 and SPI-5, which is in good correlation with a previous study that showed a link in the expression between SPI-5 genes and the genes in SPI-1 and SPI-2 (Knodler *et al.*, 2002). Some virulence genes located on the chromosome outside of the pathogenicity islands were also Fis-regulated. For example PhoP activated genes (*pag* genes) and the macrophage induced genes *mig-3* and *mig-14* displayed decreased expression in the absence of Fis. Not all virulence genes were dependent on Fis. The *spv* genes on the 90 kb virulence

plasmid were not affected by the *fis* mutation. Interestingly the *cadA* gene that was lost from *Shigella* by a genome deletion event in order to acquire greater virulence (Maurelli *et al.*, 1998) was Fis-repressed in *S. typhimurium*. It is interesting to speculate that the Fis-repression of *cadA* is a mechanism to enhance *S. typhimurium* virulence but it is not known if the *cadA* gene product plays any role in the virulence of *S. typhimurium*. It is known however that *cadA* contributes to acid tolerance in *S. typhimurium* (Park *et al.*, 1996).

In this study the role of the nucleoid-associated protein Fis in SPI-2 virulence gene regulation was examined further. β-galactosidase assays were performed to determine the activity of representative SPI-2 genes (*ssrA*, *ssaG* and *sseA*) in the presence and absence of Fis. This study confirmed Fis is required for the full expression of SPI-2 genes during batch culture in LB broth as indicated from the DNA microarray analysis. This finding was in good agreement with results from a previous study that found the presence of Fis to be necessary for the full induction of SPI-2 genes in macrophage (Carroll, 2003). Fis directly activates the transcription of *ssrA* by binding to the promoter sequence at three specific sites. Up to now the two known regulators of SPI-2 gene expression are the two component regulatory systems SsrA/SsrB (Cirillo *et al.*, 1998) and OmpR/EnvZ (Feng *et al.*, 2003; Lee *et al.*, 2000). The regulation of SPI-2 by OmpR/EnvZ is mediated through SsrA/SsrB (Garmendia *et al.*, 2003). Since Fis was found to bind the *ssrA* promoter region the regulation of SPI-2 gene expression by Fis is also mediated, at least partly through SsrA/SsrB.

A study to investigate the effect of reduced aeration on the activity of the representative SPI-2 promoters in the presence and absence of Fis was carried out. Reduced aeration was discovered to induce the expression of the SPI-2 promoters in the presence and absence of Fis. Under conditions of poor aeration the expression of the *ssaG* and *sseA* genes were induced compared to conditions of moderate aeration but the upregulation of these genes in response to limiting oxygen was significantly greater in the presence of Fis than in its absence. The expression of *ssrA* under conditions of poor aeration was also induced but was not Fis dependent. These results imply that Fis activates the

expression of *ssaG* and *sseA* independently of SsrA/SsrB and also imply that Fis itself plays a role in the induction of SPI-2 genes in response to reduced aeration. In keeping with this are the findings that Fis interacts with the promoter regions of *sseA* and *ssaG* (Carroll, 2003; Kelly *et al.*, 2004) and that in response to poor aeration Fis protein levels are elevated (Tadhg O'Cróinin, unpublished data). Therefore it is proposed that Fis can activate SPI-2 virulence genes both directly by binding upstream of *ssaG* and *sseA* and through the SPI-2 regulatory system SsrA/SsrB by binding upstream of *ssrA*. It also proposed that the elevated Fis protein levels seen in response to reduced aeration are partly responsible for the induction of *ssaG* and *sseA* expression under the same conditions of reduced aeration. The level of the sensor kinase SsrB was reduced in the absence of Fis under conditions of poor and moderate aeration and was significantly increased under conditions of poor aeration compared to conditions of moderate aeration. SsrB still displayed Fis-dependence under conditions of poor aeration in contrast to the *ssrA* promoter activity, which was not Fis-regulated under conditions of poor aeration. This result suggests Fis regulates *ssrA* and *ssrB* expression separately, which is consistent with a study that found that OmpR activates separately the transcription of *ssrA* and *ssrB*. The environmental stimuli that induce SPI-2 gene expression are subjects of much debate and before now the effect of varying oxygen levels has not been considered. This study suggests that reduced oxygen levels induce SPI-2 gene expression and that this induction is mediated partly through the action of the Fis protein.

The Fis binding sites determined by DNase I footprinting at the *flhDC* operon and *ssrA* promoter regions were atypical for a transcriptional activator and also atypical for Fis. Promoters described previously as being activated by Fis contain three to four binding sites with the most promoter proximal site located at least 70 bp upstream of the transcription start site (Appleman *et al.*, 1998; Hirvonen *et al.*, 2001; Opel *et al.*, 2004; Xu and Johnson, 1995c). The *flhDC* regulatory region was found to contain a total of seven transcription start sites located from 93 to 530 bp upstream of the translational start site (this study)(Yanagihara *et al.*, 1999). In my study Fis protected two regions of the *flhDC* regulatory region extending from 80 to 104 bp and from 149 to 188 bp

upstream of the translational start site. Therefore the two Fis binding sites were located downstream of all the transcription start sites with the exception of the one at 93 bp. One Fis-binding site was located just upstream of the transcription start site at 93 bp and the other Fis-binding site was centered on the transcription start site at 93 bp. Three regions of Fis protection were observed at the *ssrA* promoter region. One site mapped upstream and the other two mapped downstream relative to the transcription start site previously identified (Feng *et al.*, 2003). The Rns transcription activator, an AraC-like protein that positively autoregulates *rns* transcription, binds upstream and downstream of the *rns* promoter (Munson and Scott, 2000). Although the distribution of Rns binding sites appears inconsistent with its role as a transcriptional activator, the upstream binding site and at least one downstream binding site are required for positive autoregulation of *rns* (Munson and Scott, 2000). MetR, PhoP and DnaA are also able to use binding sites downstream of transcription start sites when they activate transcription (Cowan *et al.*, 1993; Liu *et al.*, 1998; Szalewska-Palasz *et al.*, 1998). It is therefore becoming more apparent that transcriptional activators can bind downstream of transcription start sites and still function in activating transcription. In addition phosphorylated OmpR was found to bind to sites upstream and downstream of the *ssrA* transcription start site and OmpR (like Fis) has only ever been reported to activate the transcription of *ssrA* (Feng *et al.*, 2003). The molecular mechanism underlying Fis activation of *flhDC* and *ssrA* transcription is unknown and is an issue for future study.

This is the first report of Fis activating the expression of the three type III secretion systems of *S. typhimurium* (Fig. 7.1). SPI-1 encodes a type III secretory system required for invasion of epithelial cells of the small intestine, SPI-2 encodes a type III secretory apparatus for survival in macrophages and systemic infection and the third type III secretion system in *S. typhimurium* is concerned with the production and deployment of flagella. It is appropriate to mention here the decrease in expression of the *lpp* gene in the *fis* mutant. The Lpp lipoprotein is a structural component of the cell envelope (Hayashi and Wu, 1990) and affects flagellar assembly (Dailey and Macnab, 2002). It is attractive to postulate that Fis coordinates the activation of expression of *lpp* with that of the type III secretion systems in order to maintain the integrity of the

cell envelope for the correct assembly and function of the three type III secretion systems.

Several studies have reported that the expression of pathogenicity island genes is coordinated with that of genes involved in motility. *S. typhimurium* regulators that have been reported to contribute to this coordination of virulence and motility genes include RtsA and RtsB, the SirA/BarA two-component regulatory system, HilA, and CsrA (Ellermeier and Slauch, 2003; Goodier and Ahmer, 2001; Lawhon *et al*., 2003; Lucas *et al*., 2000). The coordination of virulence and motility gene expression is also a theme that has been reported in bacteria other than *S. typhimurium* (Goodier and Ahmer, 2001; Merrell *et al*., 2002). The RtsA and RtsB proteins are global regulators of *S. typhimurium* gene expression (Ellermeier and Slauch, 2003). RtsA shows homology to AraC like proteins and RtsB contains a helix-turn-helix motif that is characteristic of DNA binding proteins. These regulatory proteins coordinately regulate the expression of SPI-1 and flagellar genes (Ellermeier and Slauch, 2003). Specifically RtsA induces the expression of SPI-1 genes via a direct interaction with the *hilA* regulatory gene promoter located in SPI-1 and RtsB represses the expression of flagellar genes through an interaction at the *flhDC* regulatory operon promoter. The DNA microarray data revealed that the genes encoding these proteins, STM4315 (*rtsA*) and STM4314 (*rtsB*) were among the most strongly Fis-activated genes. This is an example of one global regulator regulating another global regulator and its regulon of genes. The Fis-activation of *rtsA* and *rtsB* gene expression shows how Fis can influence a regulatory hierarchy at multiple levels (Fig. 7.2). For example RtsB levels are Fis-dependent, RtsB binds to the *flhDC* promoter region, as does Fis, which also interacts with other flagellar promoters at lower levels of the flagellar regulatory hierarchy (Fig. 7.2).

None of the Fis-responsive genes found in this study is regulated by Fis alone. Each has at least one and frequently more than one, additional regulator. It is proposed that Fis interacts with other regulators in perhaps a synergistic or antagonistic fashion in order to modulate and fine tune gene expression. An example of this is the interaction of Fis and RtsB at the *flhDC* regulatory operon promoter (Fig. 7.2). RtsB was found to
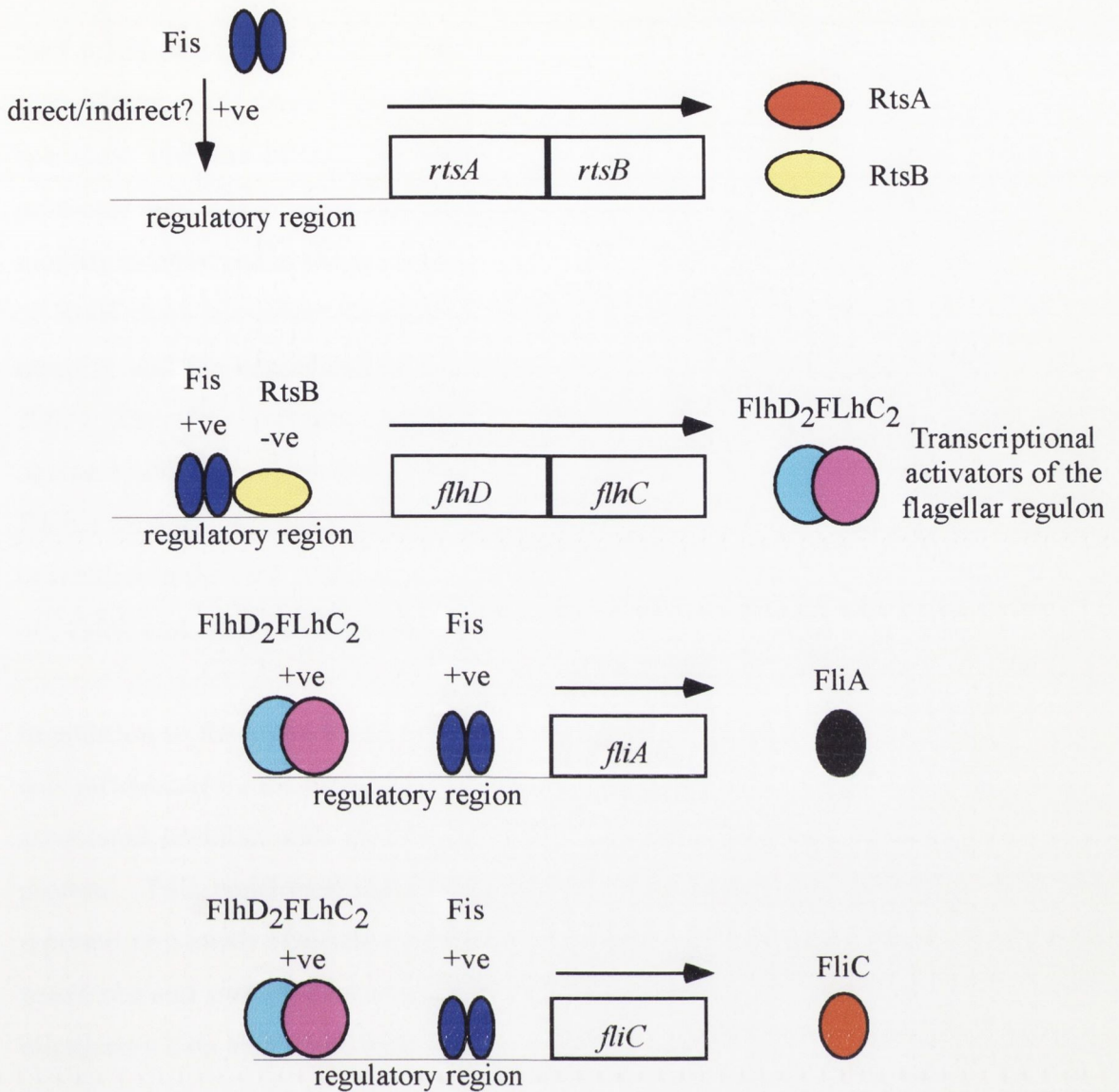
Fig. 7.2. Fis can act at multiple levels within a regulatory hierarchy. RtsB expression is dependent on Fis as determined from the DNA microarray analysis. RtsB interacts at the *flhDC* regulatory operon promoter, as does Fis. Fis and RtsB bind to approximately the same region of the *flhDC* promoter region. Fis also interacts with the *fliA* and *fliC* promoters at the lower levels of the flagellar gene regulatory hierarchy as does the $FlhD_2FlhC_2$ heterotetrameric activation complex.

bind a region of the *flhDC* regulatory region (Ellermeier and Slauch, 2003) that is approximately the same region Fis was found to bind (this study). It is interesting to speculate that the interaction between these two regulatory proteins at the *flhDC* promoter region is antagonistic. Consistent with this hypothesis is the fact that greater motility is observed in the presence of Fis than in its absence (this study). The absence of RtsB does not affect flagellar gene expression but when RtsB is overexpressed motility and the expression of flagellar genes are repressed (Ellermeier and Slauch, 2003). Therefore, it is proposed that in the presence of Fis RtsB cannot bind the *flhDC* operon regulatory region but when it is overexpressed it can compete successfully with Fis for binding. Fis has previously been shown to interact antagonistically with H-NS in regulating the *virF* (Falconi *et al.*, 2001), *hns* (Falconi *et al.*, 1996), *cspA* (Brandi *et al.*, 1999) and *rrnB* P1 (Afflerbach *et al.*, 1999; Tippner *et al.*, 1994) promoters.

In addition to RtsA and RtsB, the expression of other *S. typhimurium* global regulators was influenced by the *fis* mutation (Fig. 7.1). A number of genes encoding nucleoid-associated proteins with global regulatory roles was affected by the absence of Fis protein. This confirmed the Fis-activation of the cold shock-responsive *hns* gene reported previously (Dersch *et al.*, 1994; Falconi *et al.*, 1996). It was revealed that the genes *hha* and *stpA*, the latter a paralogue of H-NS, were both Fis-activated. The DNA microarray data also confirmed the dependency of *hupA* on Fis for full expression, as reported previously in *E. coli* (Claret and Rouviere-Yaniv, 1996). The *hupA* and *hupB* genes encode the subunits of the heterodimeric DNA-binding protein HU (Hillyard *et al.*, 1990). The Fis-regulation of *hupB* (Claret and Rouviere-Yaniv, 1996) was not detected under the conditions used in this study. In addition to *hns* (which responds to cold shock) many other genes involved in adaptation to stresses such as cold shock and osmotic stress were discovered to be Fis-activated.

It is apparent form this DNA microarray study that many genes have a negative response to Fis confirming that this protein can act as a transcriptional repressor as well as a transcriptional activator. The most strongly Fis-repressed genes identified were involved in metabolic and transport processes, many of which are required for

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colonization of the gut (Fig. 7.1). The most strongly Fis-repressed genes were those involved in biotin synthesis (*bioB*, *bioC* and *bioF*). Biotin is a crucial cofactor of the early enzymatic steps in lipid biosynthesis and interestingly genes involved in lipid biosynthesis were also repressed by Fis. Several sets of genes involved in carbon utilization were Fis-repressed including genes encoding enzymes of the citric acid cycle and its glyoxylate bypass, glycolysis and anaerobic respiration. 17 of the 18 genes for which data were available and that belonged to the *pdu* (propanediol utilization) operon displayed increased expression in the *fis* mutant. Consistent with this was the ability of the *fis* mutant to grow more rapidly than the wild-type in minimal media supplemented with propanediol as the sole carbon source. It is reported that the utilization of propanediol is important for growth in host tissues and that *pdu* mutations confer a virulence defect (Conner *et al*., 1998; Heithoff *et al*., 1999). Fis had a positive role in the expression of the *eut* genes concerned with the utilization of ethanolamine, another metabolic pathway likely to be used in the intestinal milieu. CsrA, a global regulator of *S. typhimurium* induces the expression of both the *eut* and *pdu* genes (Lawhon *et al*., 2003). The reasons why Fis represses the *pdu* genes and activates the *eut* genes are unknown. Given this newly discovered role for Fis in the regulation of genes concerned with metabolism and transport it is hypothesized that Fis coordinates the expression of house-keeping and motility genes with that of virulence genes to allow for the transition from a free-living mode in the gut lumen to an intracellular niche during *S. typhimurium* infection.

Interestingly, the *pdu* operon was acquired by horizontal gene transfer (Bobik *et al*., 1999). The pathogenicity islands regulated by Fis are also regarded as having been acquired by horizontal gene transfer. It is appealing to contemplate the lack of sequence specificity in the Fis binding site has aided its recruitment as a regulator of these horizontally acquired genes.

This study confirmed that Fis protein levels are high immediately after subculture into fresh medium and then decline rapidly as the culture begins to divide. Fis protein was undetectable 3 h after sub-inoculation as determined by Western immunoblotting. A

remarkable feature of this study is the fact that fewer genes were Fis-dependent at 1 h than at 4 h, which seemed paradoxical given the concentration of Fis present in the cell at those times. In addition, the β-galactosidase assays carried out to confirm the effect of the *fis* mutation on the flagellar genes and SPI-2 genes showed that the flagella genes displayed some degree of Fis-dependency from approximately 3 h post subculture to 24 h post-subculture and that the SPI-2 genes displayed significantly reduced expression in the absence of Fis after overnight culturing. Fis-regulation occurring at a stage of growth where Fis protein is undetectable by Western blot is not unique, it has been observed in enteropathogenic *E. coli* (Goldberg *et al.*, 2001), *S. typhimurium* (Keane and Dorman, 2003) and in *E. coli* K-12 (Schneider *et al.*, 1999) previously. Although Fis protein levels decline dramatically during growth it is thought they reach a low but non-zero value where the cell is not depleted of Fis protein (Azam *et al.*, 1999; Walker *et al.*, 1999). At low concentrations of Fis protein it is hypothesized that only sites with a high affinity for Fis will maintain Fis binding and that sites with a low affinity for Fis will be vacant. Such promoters with high affinity sites are regarded as privileged during stages of growth when Fis concentrations are low. Perhaps flagellar and SPI-2 promoters belong to this privileged group. It must also be considered that growth-phase-dependent factors may be responsible for the increase in Fis-dependent genes at 4 h compared to 1h.

Many of the effects of the *fis* mutation on gene expression may be indirect. For example it has been reported that Fis can influence transcription in a supercoiling-dependent manner (Travers *et al.*, 2001). Chromosomal superhelicity has been reported to be a global regulator of gene expression (Hatfield and Benham, 2002; Steck *et al.*, 1993). An example of how DNA topology influences transcription is the rapid relaxation of DNA that accompanies engulfment of *S. typhimurium* by macrophage (Marshall *et al.*, 2000). The sudden relaxation of the DNA is proposed to upregulate the expression of genes involved in intramacrophage survival. In good correlation with this, the *ssrA* promoter is activated by relaxation of the DNA (Carroll, 2003). It is reported that Fis modulates the topology of DNA by constraining intermediately supercoiled DNA and by acting as a regulator of the genes encoding DNA

topoisomerase I and DNA gyrase (Keane and Dorman, 2003; Schneider *et al.*, 1997; Schneider *et al.*, 1999; Weinstein-Fischer *et al.*, 2000). Fis is a transcriptional repressor of the *gyr* genes that encode DNA gyrase and in this study a high level of Fis protein was shown to decrease the superhelicity of reporter plasmids. It was apparent from this study that Fis influences the DNA topology but its ability to do this was observed to differ between *S. typhimurium* and *E. coli*. It is interesting to speculate that the ability of Fis to modulate the DNA topology differently between *S. typhimurium* and *E. coli* is linked to the fact that *S. typhimurium* is adapted to intracellular life while *E. coli* K-12 is not.

It is worthy of note that many of the Fis-regulated genes are genes of unknown function and until the function of these genes is established one cannot fully understand all the roles Fis carries out in *S. typhimurium* (Fig. 7.1). Also the genome sequence of SL1344, the *S. typhimurium* strain used throughout this study is incomplete and therefore the entire regulon of genes whose expression is influenced by Fis has yet to be fully determined.

The virulence of a *S. typhimurium fis* mutant is attenuated 100-fold when administered orally, but has wild-type virulence when administered intraperitoneally (Wilson *et al.*, 2001). The Fis-regulation of SPI-1 results in *fis* mutants unable to invade epithelial cells and so explains the severe attenuation in virulence when a *fis* mutant is administered orally (Wilson *et al.*, 2001). Since it was mutants identified by signature-tagged mutagenesis that displayed attenuated virulence when administered intraperitoneally that gave rise to the discovery of SPI-2 (Hensel *et al.*, 1995; Ochman *et al.*, 1996; Shea *et al.*, 1996), it is surprising and largely unexplainable that a *fis* mutant would have wild-type virulence when administered intraperitoneally since the work here has shown Fis is required for the full expression of SPI-2 genes. In this work it was shown that a *fis* mutant produces less flagellin protein and is was speculated that perhaps this results in fewer flagellin subunits on its cell surface. Given flagellin is the major proinflammatory determinant of *S. typhimurium* (Zeng *et al.*, 2003) it is attractive to speculate that a *fis* mutant with potentially less flagella stimulates a relatively mild

immune response following intraperitoneal inoculation, which perhaps compensates for the reduced SPI-2 gene expression, thus allowing *fis* mutants to maintain wild-type virulence.

The finding that Fis activates the expression of genes encoded in SPI-1, SPI-2 and some genes in SPI-3, SPI-4 and SPI-5 suggests Fis coordinates the expression of these virulence genes located in distinct pathogenicity islands required by *S. typhimurium* at different stages of infection. This is an important issue for future study. Many of the effects of the *fis* mutation may be direct or indirect and determining this especially in the case of the pathogenicity islands SPI-3, SPI-4 and SPI-5, would be interesting future research. Electron microscopy to determine the number of flagella on the surface of a *fis* mutant compared to the wild-type would determine if there is a link between flagella number and the *fis* mutant motility phenotype. Fis-binding sites were identified at the *flhDC* operon promoter region and at the *ssrA* promoter region by DNase I footprinting. Mutating these Fis-binding sites to eliminate Fis binding with a view to determining sites important for the Fis-activation of transcription would also be important future work given the unique pattern of Fis-binding sites found at the *ssrA* and *flhDC* promoter regions. It would especially be of interest to investigate the mechanism of Fis activation at *flhDC* considering the possibility that Fis may be functioning there to overcome the influence of the repressor RtsB. To confirm further that Fis can activate the transcription of flagellar genes and SPI-2 genes independently of the regulatory proteins FlhD and FlhC (in the case of flagellar genes) and SsrA/SsrB (in the case of SPI-2 genes), β-galactosidase assays could be performed in the absence of Fis in combination with *flhDC* and *ssrB* mutations. A *S. typhimurium hns* mutant was found to be non-motile. Dissecting the mechanism by which H-NS influences motility in *S. typhimurium* is potentially an interesting research project for the future.

In summary, this work has identified novel roles for the nucleoid-associated protein Fis in regulating the transcription of flagellar genes, SPI-2 genes, genes encoded in SPI-3, SPI-4 and SPI-5, genes involved in metabolism and transport, stress response genes and genes encoding global regulators (Fig. 7.1). Also results from this study provide

evidence that limiting oxygen is an environmental cue that induces the expression of SPI-2 genes. This study confirmed the Fis-regulation of SPI-1 and confirmed the role of Fis in modulating the topology of DNA. It is now apparent that Fis plays a bigger role in virulence gene regulation than previously thought. In conclusion, Fis is hypothesized to coordinate motility and metabolism with virulence to help *S. typhimurium* adapt to the variety of distinct environments associated with its pathogenic lifestyle.

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