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Sustained expression of *fis*, the gene coding for the Fis nucleoid-associated protein, during the stationary phase of growth in *Salmonella enterica*.

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

May 2013

by

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Declarations

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Summary

The Factor for Inversion Stimulation (Fis) is a global regulator of virulence genes in *Salmonella enterica* serovar Typhimurium. Previously, the Fis protein was throught to be expressed only during the early exponential phase of growth, but work in our research group has shown that *fis* gene expression can be sustained into the stationary phase when the bacteria are grown under conditions of low aeration. This finding is significant because Fis is known to be a global regulator of many *Salmonella* virulence genes and low aeration growth is used routinely to maximize the infectivity of *Salmonella* during *in vitro* infection studies. The underlying molecular mechanism responsible for this unusual pattern of *fis* gene expression has now been investigated.

The effect of the aeration growth regime on *fis* gene expression was further tested. In addition to microaerobic experiments, a protocol was also designed to test the effect of fully-anaerobic growth on the activity of the *fis* promoter throughout growth.

Furthermore, it was found that elimination of the global regulator DksA resulted in strong up-regulation of *fis*. Similarly, double knockout mutations that inactived the *relA* and *spoT* genes, both required for the production of the alarmone guanosine tetraphosphate (ppGpp) also up-regulated *fis* transcription throughout growth. These results establish a role for both the stringent response and the synergistically-acting DksA protein as components of the mechanism responsible for sustained *fis* gene transcription. DNA supercoiling is known to vary with the degree of aeration of the bacterial culture, therefore the effect of supercoiling changes on the expression pattern of *fis* was also studied.

Progressive inhibition of DNA gyrase activity with the drugs novobiocin or coumermycin A1 altered the expression of the *fis* gene in both high- and low-aeration cultures. This showed that the topoisomerase that introduces negative supercoiling into DNA (i.e. gyrase) is required for the normal transcription pattern of *fis*. The possibility that DNA topoisomerase I also played a role was tested genetically by making a mutation in the *Salmonella topA* gene. The elimination of the topoisomerase that relaxes negatively supercoiled DNA resulted in

enhanced *fis* transcription at late phases of growth, a finding that was consistent with the data from the DNA gyrase inhibition experiments. These results establish a role for DNA negative supercoiling in the maintenance of *fis* transcription into stationary phase.

Moreover, the regulatory role of the Fis protein on *fis* and several other genes, including SPI-1 genes *hilA* and *orgA,* as well as SPI-2 gene *ssrA*, during transcription specially during the aeration growth regime where *fis* sustained expression is observed, were also investigated. Studies were done in mid-exponential and late-stationary phase culture samples grown under aerated and non-aerated (microaerobic) conditions, to measure Fis enrichment in Fis-binding targets by real-time quantitative PCR (Polymerase Chain Reaction), and to determine where Fis-binding is mostly enriched (peaks) on gene regions that are occupied by Fis by using genome-wide Chromatin Immunoprecipitation Microarray Technology (ChIP-chip). Results revealed patterns of Fis protein binding in bacterial cells grown under microaerobic conditions different to those patterns in bacterial cells grown aerobically. These findings are consistent with the presence of more Fis protein in the bacteria grown microaerobically.

This research investigation points to central roles for DNA negative supercoiling, the DksA protein, the elements of the stringent response, and the regulatory function of Fis-binding patterns, in setting and resetting the activity of the *fis* gene and other involved promoters as a function of the growth conditions experienced by *Salmonella*.

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*I know that I am mortal by nature and ephemeral, but when I trace at my pleasure, the windings to and fro of the heavenly bodies [*or of microbial bodies*], I no longer touch earth with my feet. I stand in the presence of Zeus himself, and take my fill of ambrosia.*

Claudius Ptolemy, A.D. 150

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List of Abbreviations

AFR	Aerobic Fluorescence Recovery
ChIP-chip	Chromatin Immunoprecipitation in a chip (Microarray Technology)
CbpA	Curved-DNA-binding protein A, NAP
Dps	DNA protection from starvation NAP
FIS	Factor for Inversion Stimulation NAP
Fis	Factor for Inversion Stimulation protein
fis	Factor for Inversion Stimulation gene
GFP	Green Fluorescent Protein
gfp	Green Fluorescent gene
GyrA/B	Gyrase complex A/B proteins
H-NS	Histone-like-nucleoid structuring NAP
HU	Heat-Unstable NAP
IHF	Integration Host Factor NAP
Lrp	Leucine-responsive regulatory protein, NAP
NAPs	Nucleoid Associated Proteins
PCR	Polymerase Chain Reaction
ppGpp(p)	Guanosine tetra (penta) phosphate
qPCR	Quantitative (Real–Time) PCR
ROS	Reactive Oxygen Species
RNA P	RNA Polymerase
RNS	Reactive Nitrogen Species
SCV	*Salmonella* Containing Vacuole
sIDD	supercoiling-Induced Duplex Destabilization
SPI-1	Salmonella Pathogenicity Island-1
SPI-2	Salmonella Pathogenicity Island-2
StpA	Salt tolerance protein A, NAP
TopA	Topoisomerase A
T3SS	Type Three Secretion System
$\dot{O}_2$	Microaerobic growth regime/conditions

Chapter 1

Introduction

1.1 *Salmonella*

The bacterial genus *Salmonella* was named after its discoverer, American microbiologist D.E. Salmon, who isolated the first organism, *Salmonella choleraesuis*, from the intestine of a pig (Thomson *et al.,* 2009). *Salmonella* are rod-shaped, Gram-negative bacteria that belong to the family *Enterobacteriaceae*, as they generally inhabit the gastrointestinal tract of animals (Thomson *et al.,* 2009). During the course of evolution, *Salmonella* have adapted their physiology to the aerobic, microaerobic and anaerobic conditions encountered in the gastrointestinal tract of the host for which they have become a facultative intracellular anaerobic pathogen (Coburn *et al.,* 2007).

The genus *Salmonella* is divided into the two species *Salmonella enterica* and *Salmonella bongori*, which in turn subdivide into subspecies or serovars. Serovars are differentiated by their flagellar, carbohydrate and lipopolysaccharide (LPS) structures. *Salmonella enterica* serovars infect humans and cause disease in several animals including cattle, poultry, pigs and mice, while *Salmonella bongori* serovars infect mostly cold-blooded invertebrates (Ohl and Miller, 2001; Silva and Wiesner, 2009).

1.1.1 *Salmonella enterica* as a model for microbial pathogenesis

Salmonella enterica species are usually foodborne pathogens and cause one of four major syndromes: enteric fever (typhoid), enterocolitis/diarrhoea, bacteraemia and chronic asymptomatic carriage (Coburn *et al.,* 2007; Ohl and Miller, 2001). The illness progresses depending on the host susceptibility and *Salmonella enterica* infectivity. *Salmonella enterica* serovar Typhimurium generates a typhoid-like systemic illness in mice but it is also associated with human gastroenteritis, and is an ideal model for human typhoid (Coburn *et al.,* 2007; Kelly *et al.,* 2004; McClelland *et al.,* 2004; Menendez *et al.,* 2009; Silva and Wiesner, 2009). Human typhoid fever and intestinal/diarrheal illnesses comprise *S. enterica* mechanism of pathogenesis, as diverse virulence mechanisms are employed by the bacteria (Coburn *et al.,* 2007). Typhoid fever is a frequent and very serious problem in the tropics (Crump *et al.,*

2004) but so is Salmonellosis, as it is frequently associated with a foodborne disease in humans (Coburn *et al.,* 2007; Ohl and Miller, 2001; Fierer and Guiney, 2001). Furthermore, *S. enterica* serovar Dublin not only infects humans, but it also produces intestinal inflammatory disease, bacteraemia and abortion in cows, while serovar Choleraesuis causes septicemia in pigs (Baulmer *et al.,* 1998). In fact, *Salmonella* cases have become a serious economic problem as *Salmonella*-related illness have an annual cost of more than $2.6 billion in the USA alone (Herrick et al., 2011).

S. enterica serovar Typhimurium (*S.* Typhimurium) is the *Salmonella* serotype used throughout this research investigation. *S.* Typhimurium virulence depends on the expression of several crucial factors to generate a multi-stage systemic infection in mammalian hosts. In order to become systemic, *S. enterica* infection goes from invasion of the epithelium, to crossing the epithelial cell barrier, to intracellular replication within macrophages (Fig. 1.1). *S. enterica* generates an infection-associated cross-talk with host cells dictated by bacterial type III secretion systems (T3SSs) common in enteric bacteria (Hansen-Wester and Hensel, 2001; Ghosh, 2004). T3SSs allow bacteria to translocate effector/virulence proteins from the bacteria into the host cell in order to interfere with host cell functions such as actin polymerization, signal transduction and apoptosis (Hansen-Wester and Hensel, 2001). *S. enterica* is equipped with three T3SSs, two of which are encoded by *Salmonella* Pathogenicity Island 1 (SPI-1) (section 1.3.1), and *Salmonella* Pathogenicity Island 2 (SPI-2) (section 1.3.2), (Hansen-Wester and Hensel, 2001). The third T3SS is responsible for the flagellar motility of the pathogen (Macnab, 2004; McCarter, 2006).

Actually, virulence factors in *Salmonella* are governed by genes located in all known five SPIs, and the *Salmonella* 90 kb virulence plasmid pSLT (Lee *et al.,* 2000; Marcus *et al.,* 2000; Waterman and Holden, 2003), which houses the *spv* genes (Holden, 2002; Libby *et al.,* 2000, 2002; Paesold *et al.,* 2002). SPIs have been acquired by horizontal gene transfer as they have incorporated into tRNA loci, and possess DNA sequences that differ in their G+C content and codon usage from the rest of the *Salmonella* genome (Blanc-Potard et al., 1999).

In order to produce an infection *S.* Typhimurium rely on their virulence factors, which comprise about 4% (~200 genes) of its chromosome (Finlay and Brumell, 2000). Most of

these factors are located within the SPIs on the chromosome (Galán, 2001; Groisman and Ochman, 1993, 1997; Hacker and Kaper, 1999; Hensel, 2000; Hensel *et al.,* 1999). SPI-1 and SPI-2 have been the most studied as they encode two of the three T3SS of *S.* Typhimurium (Ochman and Groisman, 1996; Ohl and Miller, 2001).

In this research investigation, the focus will be mainly in SPI-1 (section 1.3.2) and SPI-2 (section 1.3.3) as they play important roles in our journey to understand *fis* gene sustained expression in *Salmonella enterica*. The SPI-1 gene arsenal enables *Salmonella* to invade the epithelial cells of the small intestine (Hardt *et al.,* 1998; Lee *et al.,* 2000; Waterman and Holden, 2003), while the SPI-2 gene set enables survival and replication inside macrophages (Hensel, 2000; Waterman and Holden, 2003) and the generation of a systemic infection in mice or, a systemic and/or chronic infection in humans (Coburn et al., 2007) (Fig. 1.1).

S. enterica has been frequently used as a model for bacterial infection because its complex regulatory networks can be better understood as their implicated genes are frequently studied in several pathogenic studies (Jarvik *et al.,* 2010; Pullinger *et al.,* 2010). The growth and infection process of *Salmonella*, like many other microbial pathogens in nature, relies on the activation and promotion of genetic changes induced by environmental cues (Yoon *et al.,* 2009; Garner *et al.,* 2009). Furthermore, a recent description of the transcriptional map of SL1344 has significantly advanced our understanding of *S.* Typhimurium. According to the authors, this bacterium has given us the most important bacterial infection model yet (Kröger *et al.,* 2012).

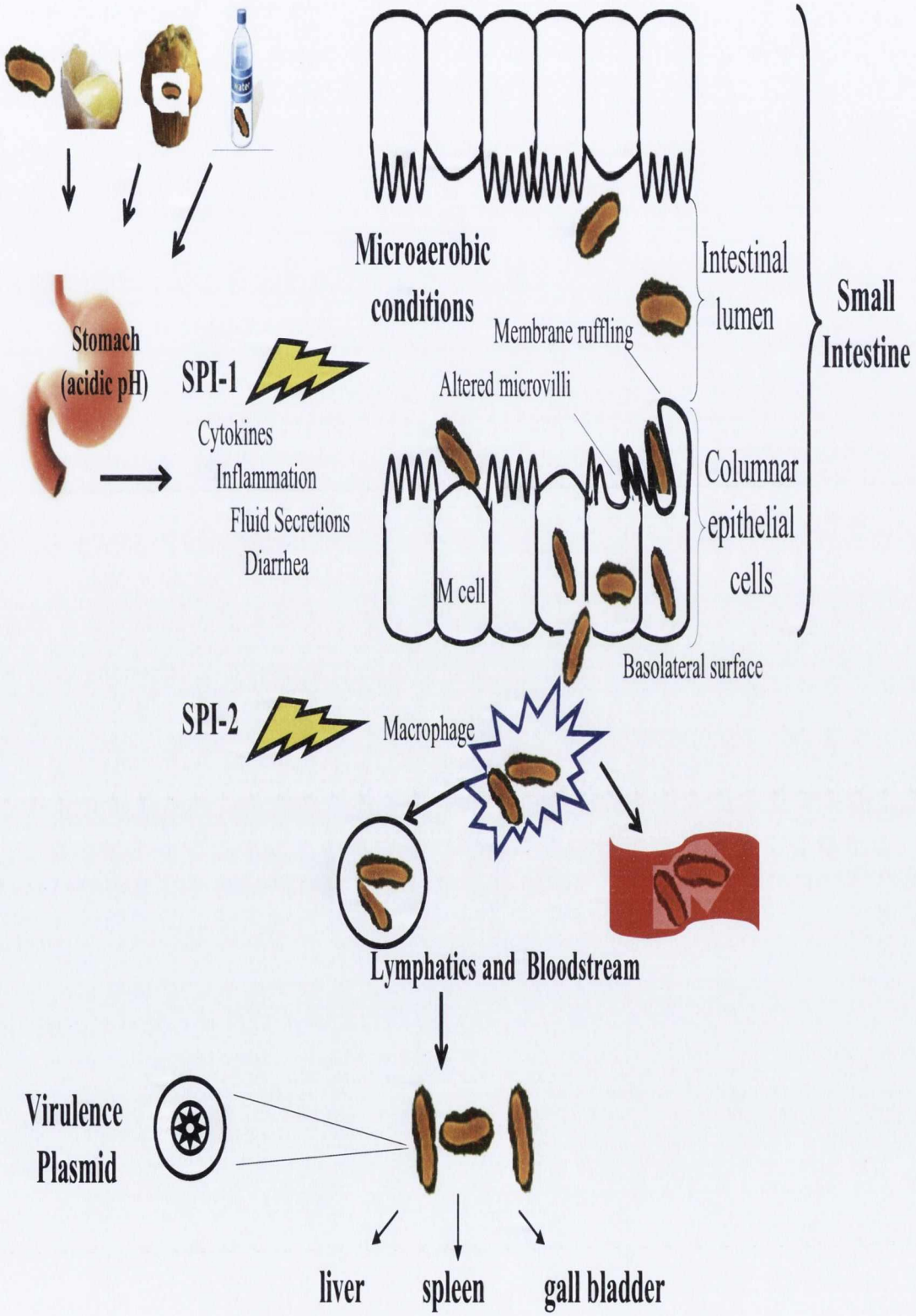
1.1.2 *Salmonella* host infection

As in all bacteria, *Salmonella* must adapt their physiology to the existing environmental conditions in order to thrive. *S.* Typhimurium is a pathogen that has evolved a facultative and intracellular invasive ability to circumvent host barriers and thus thrive in diverse environments. Several studies have shown that following ingestion and passage through the stomach, *Salmonella* cross the intestinal lining by invading the epithelium prior to macrophage engulfment in the mesenteric lymph nodes, and then replicate intracellularly

before entering the blood stream where they disseminate to the spleen and liver to establish a systemic infection (Buchmeier & Heffron, 1991; Galan, 2001; Holden, 2002; Jones & Falkow, 1990; Scherer & Miller, 2001). It is so that the *Salmonella* species causes a gastrointestinal disease called Salmonellosis. It is acquired by ingesting contaminated food or water (Giannella *et al.,* 1972; Kingsley and Baumler, 2000). Several studies have shown that following ingestion and passage through the stomach, the bacteria cross the intestinal lining by invading first the epithelium. There, bacteria are engulfed by macrophages before entering the bloodstream and establishing a systemic infection (Giannella *et al.,* 1972; Kingsley and Baumler, 2000; Ohl and Miller, 2001; Detweiler *et al.,* 2003; Yoon *et al.,* 2009). During the infection process, the host imposes varying stressful conditions to which *Salmonella* must adapt to survive. The bacteria endure the acidic pH of the stomach using a suite of acid survival systems, prior to attaching with their fimbriae to the cells that cover the epithelium of the small intestine and invading them (Foster, 1991; Fang *et al.,* 1992; Ohl and Miller, 2001; Coburn *et al.,* 2007; Yu *et al.,* 2010). *Salmonella* specifically invades specialized M cells in the intestinal lumen by promoting its own uptake by mediated endocytosis (Jones *et al.,* 1994; Ohl and Miller, 2001; Kelly *et al.,* 2004) (Fig 1.1). The microaerobic or non-aerated conditions of the intestinal lumen facilitate SPI-1 (section 1.3.1) mediated invasion of the adjacent epithelial cells (Ó Cróinín and Dorman, 2007), as it has been found to occur in *Shigella flexneri* (Marteyn et al., 2010). SPI-1 uses T3SS effector proteins to penetrate epithelial cells (Lee *et al.,* 2000; Zhou *et al.,* 2001). Specifically, these secretions induce the cytoskeleton to rearrange, the microvilli to alter and the membrane to ruffle, allowing bacterial uptake (Zhou *et al.,* 2001). Cytokines are also produced by the same proteins causing inflammation and fluid secretions in the host, which results in gastroenteritis (Coburn *et al.,* 2007; Ohl and Miller, 2001). *Salmonella* uses the basolateral surface of the epithelial cells to exit, and reaches the Peyer's patches where it is engulfed by phagocytic macrophages (Detweiler *et al.,* 2003). This generates *Salmonella*-containing vacuoles (SCVs) which are hideaways for intracellular bacteria to survive and reproduce far from host defenses (Ohl and Miller, 2001). Nevertheless, effectors translocated by the second T3SS provide the SVC enough protection against host immunity, including recruitment of reactive oxygen (ROS) and reactive nitrogen species (RNS) (Vazquez-Torres *et al.,* 2000; Chakravortty *et al.,* 2001). SPI-2 (section 1.3.2) secreted effector proteins interfere with the antimicrobial properties of the

Fig. 1.1: Diagram depicting of a *Salmonella* host infection. Salmonellosis is the gastrointestinal disease caused by *Salmonella.* The pathogenic bacterium is found in contaminated food or water. During the infection process, bacteria first need to survive the acidic pH of the stomach before attaching and invading the cells in the small intestine. M cells in the intestinal lumen are invaded by promoting its own uptake by mediated endocytosis. SPI-1 mediated invasion of the adjacent epithelial cells is induced by the microaerobic conditions in the intestinal lumen. The T3SS found in SPI-1 secretes effector proteins that penetrate epithelial cells. These secretions re-arrange the cytoskeleton to rearrange, alter the microvilli and ruffle the membrane, facilitating bacterial uptake. Gastroenteritis onsets as cytokines are also produced by these proteins generating inflammation and fluid secretions in the host. Bacteria exit through the basolateral surface of the epithelial cells to be then engulfed by phagocytic macrophages. *Salmonella*-containing vacuoles (SCV) are generated to allow intracellular growth and replication. This intracellular proliferation is facilitated by SPI-2 genes in order to evade the host immune response and eventually mount a systemic infection. The systemic infection starts commonly at the lymphatics, then it proceeds into the bloodstream, liver and spleen. The *Salmonella* virulence plasmid supports cell proliferation within these organs. However, once bacterial cells establish in the gall bladder, a chronic infection onsets.

Salmonella in contaminated food or water



macrophage and thus facilitate *Salmonella* intracellular proliferation to mount a systemic infection (Lee *et al.,* 2000; Kelly *et al.,* 2004). Furthermore, the SPI-2 T3SS is equipped with a pH sensor that has been proposed to undergo a conformational change when exposed to neutral pH in order to transduce a dissociation signal to a translocon (SsaL/SsaM/SpiC) regulatory complex, eventually enabling effector delivery by the intravacuolar bacteria in SCVs (Yu *et al.,* 2010). As a result, a systemic infection settles as bacterial cells spread through the lymphatics, then into the bloodstream, liver and spleen. The *Salmonella* virulence plasmid (pSLT) enables bacterial cell proliferation within these organs (Kelly *et al.,* 2004; Ó Cróinín and Dorman, 2007). Chronic infection ocurrs as bacterial cells establish in the gall bladder (Menendez *et al.,* 2009).

1.2 Fis

Fis (FIS) stands for Factor for Inversion Stimulation because it was originally identified 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). It is a homodimeric, nucleoid-associated, 98 amino acid protein that consists of a 11.2-kDa subunit that binds to specific DNA sites (Ninneman *et al.,* 1992; Osuna *et al.,* 1995; Martínez-Antonio *et al.,* 2012). Fis is regarded as a member of the group of histone-like proteins because of its ability to alter DNA topology (Thompson and Landy, 1988; Gille *et al.,* 1991). Fis is also the most abundant known nucleoid-associated-protein (NAP) in bacteria entering exponential growth (Grainger *et al.*, 2008; Martínez-Antonio *et al.,* 2012). The Fis protein can bend DNA at angles of between 50° and 90° (Ball *et al.,* 1992; Hengel *et al.,* 1997; Martínez-Antonio *et al.,* 2012), and even through it binds DNA in a site-specific manner, the proposed core consensus sequence for Fis-binding sites 5'-KNNYRNNWNNYRNNM-3' is highly degenerate (Finkel and Johnson 1992; Hubner and Arber, 1989).

The gene that encodes Fis is located within an operon that has an open reading frame (ORF) called *yhdG*, that encodes a tRNA-dihydrouridine synthase, upstream of the *fis* gene (Fig. 1.2), (Morett and Bork, 1988; Ball *et al.,* 1992; Ninneman *et al.,* 1992; Osuna *et al.,* 1995; Bishop *et al.*, 2002). The *fis* gene is located second within a bicistronic operon present in *Enterobacteriaceae* family members of the *Gammaproteobacteria* (Beach and Osuna, 1998).

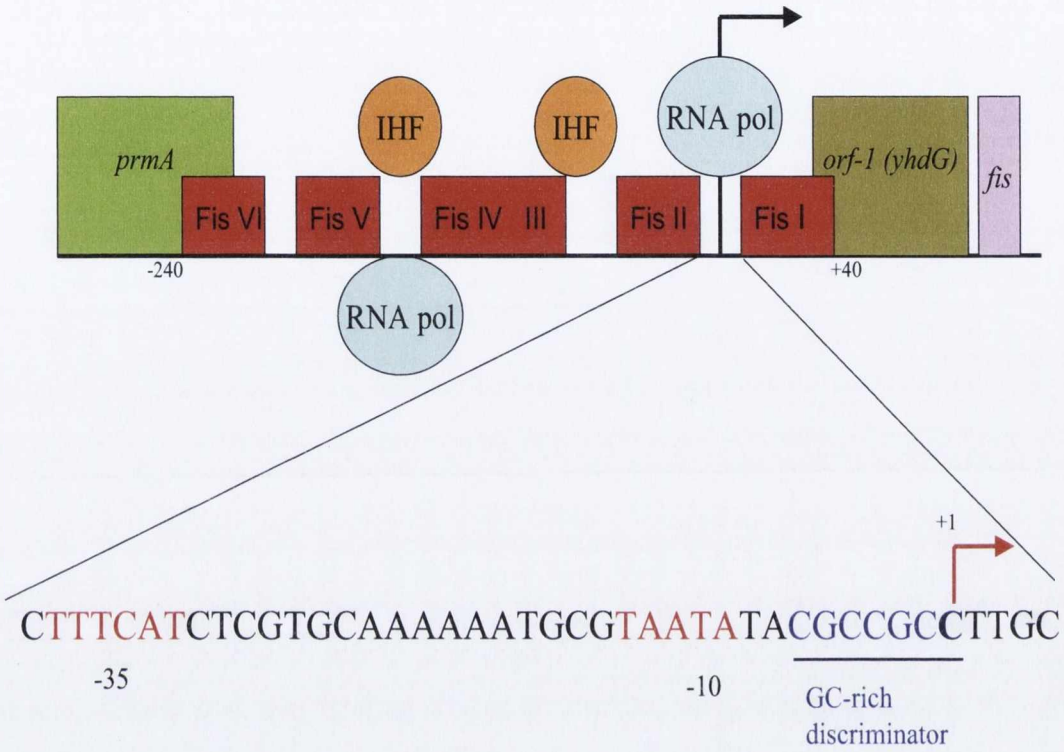


Fig. 1.2: The *fis* operon. The *fis* gene is in an operon with *yhdG*, located upstream of the *fis* gene. There is a single promoter and this region contains six binding sites (I-VI), a few of which overlap the RNA polymerase binding sites, implying Fis mediated repression could involve exclusion of RNA polymerase from the promoter. IHF is a transcriptional activator to the *fis* promoter that binds to two sites in the *fis* operon regulatory region. Fis and IHF overlap at their binding sites resulting in antagonistic functions. The sequences TTTCAT and TAATAT are located at the -35 and -10 regions, respectively. The main transcription initiation nucleotide lies at +1 (a C residue). A GC-rich motif known as a 'discriminator' is found downstream of the -10 region. This feature is important for the control of the *fis* promoter by the stringent response (section 1.4). *E. coli* and *S.* Typhimurium share the same overall *fis* operon organization, as well as Fis protein amino acid sequence. Although, there is *fis* promoter regions are conserved from -49 to +94, relative to the *E. coli* +1 position.

The Fis protein has been shown to repress its own promoter (*fis* P), as maximum *fis* mRNA levels are ten fold higher in the absence of Fis (Ball *et al.,* 1992; Ninneman *et al.,* 1992; Osuna *et al.,* 1995; Pratt *et al.,* 1997). The *fis* P is also dependent on a crucial level of a 49% nucleotide sequence divergence at positions -50 to -252 between the two species, their negative DNA supercoiling (section 1.7) to function optimally (Schneider *et al.,* 2000). The promoter region for the *fis* gene contains six binding sites (I-VI) for the Fis protein (Fig. 1.2), two of which overlap RNA polymerase binding sites, implying that Fis-mediated repression could involve keeping RNA polymerase from binding to the promoter (Ball *et al.,* 1992).

These multiple binding sites suggest that a highly wrapped nucleoprotein complex is responsible for *fis* repression (Ball *et al.,* 1992). IHF is a transcriptional activator of the *fis* promoter that binds to two sites in the *fis* operon regulatory region (Fig. 1.2), resulting in IHF and Fis having antagonistic functions there (Nasser *et al.,* 2002).

Fis is also known to be a global regulator for both housekeeping and virulence genes in *Salmonella* (Kelly *et al.,* 2004; Osuna *et al.,* 1995; Wilson *et al.,* 2001). In *E. coli* (Azam *et al.,* 1999; Ball *et al.,* 1992; Ninneman *et al.,* 1992; Mallik *et al.,* 2004) as in *Salmonella* (Osuna *et al.,* 1995), but in contrast to other global regulators like histone-like nucleoid-structuring protein (H-NS), the expression of Fis peaks in early exponential phase, but then decreases as exponential growth proceeds until it is almost undetectable by the onset of stationary phase for what it is also considered to have an important role in managing growth phase transitions (Schneider et al., 1997). Specifically, Fis protein levels rapidly increase upon subculturing cells in rich medium, reaching a peak of 25,000 to 40,000 dimers per cell prior to logarithmic cell division, about 75 to 90 minutes after subculturing, to become undetectable, fewer than 500 dimers per cell, at the onset of stationary phase (Appleman *et al.,* 1998; Ball *et al.,* 1992; Osuna *et al.,* 1995; Nilsson *et al.,* 1992). This occurs because Fis content per cell decreases due to the dilution of the protein population by cell division in the absence of *de novo* Fis synthesis (Appleman *et al.,* 1998). Also, Fis turnover by proteolysis has not been reported. On the other hand, a sudden increase in Fis protein concentration occurs when there is a rapid but transient increase in *fis* mRNA as a response to nutrient upshift (Ball *et al.,* 1992). One study also found that *fis* mRNA expression pattern is not regulated as *fis* mRNA decays (Pratt *et al.,* 1997). While Fis negative autoregulation controls

the level of *fis* mRNA, the classical *fis* expression pattern is still observed in the absence of Fis protein (Ball *et al.,* 1992; Osuna *et al.,* 1995). The level of *fis* expression is dictated by the nutritional quality of the medium, as higher mRNA levels are found in richer medium (Ball *et al.,* 1992; Nilsson *et al.,* 1992).

Moreover, if the predominant transcription initiation nucleotide is changed, high levels of *fis* mRNA are also detected in late exponential and early stationary phases (Walker *et al.,* 1999). However, this critical growth phase regulation of the *fis* P is throught to be due mainly to the availability of transcription initiation nucleotide CTP, and an inefficient transcription initiation process as promoter sequences deviate from the -10 to -35 consensus sequences (Walker *et al.,* 1999 and 2004). It has also been found that Fis can be readily detected in stationary phase for another important reason. Fis expression in *S.* Typhimurium was found to be sustained during the stationary phase of growth (Ó Cróinín and Dorman, 2007), where the protein was expected to be absent (Osuna *et al.,* 1995; Keane & Dorman, 2003). Specifically, this was found in *S.* Typhimurium cultures grown in non-aerated/microaerobic conditions, while cultures grown with standard aeration showed the classic Fis expression pattern (Ó Cróinín and Dorman, 2007). Furthermore, because these prolonged Fis expression levels in non-aerated cultures were also detected in some *E. coli* strains, this unexpected Fis expression pattern was found to be independent of sequence differences in the *fis* promoter regions of *Salmonella* and *E. coli* (Ó Cróinín and Dorman, 2007). Given the nature of these discoveries, there is an increasing number of findings on the relationships among Fis, virulence and aeration regimes.

1.3 Fis, virulence and aeration regimes

A critical virulence phenotype of *Salmonella* is the ability to invade mammalian cells (Jones, 2005). The Fis protein was found to be a key regulator of the *Salmonella* virulent phenotype (Kelly *et al.,* 2004; Ó Cróinín *et al.,* 2006; Ó Cróinín and Dorman, 2007; Wilson *et al.,* 2001; Yoon *et al.,* 2003). Fis, together with OmpR, is a transcriptional activator of virulence genes in SPI-1 (section 1.3.1) as the bacteria prepare to invade intestinal epithelial cells (Wilson *et*

al., 2001; Ohl and Miller, 2001; Waterman and Holden, 2003; Cameron and Dorman, 2012), and genes in SPI-2 (section 1.3.2), which are required for the intracellular survival of the bacteria (Waterman and Holden, 2003; Kelly *et al.,* 2004; Ó Cróinín *et al.,* 2006).

Fis fully induces the expression of *hilA* (section 1.4.2), which belongs to the SPI-1 invasion locus *hil* (hyperinvasion locus). Interestingly, *hil* was found while identifying and characterizing hyperinvasive mutants, which could enter epithelial cells even when grown aerobically (Lee *et al.,* 1992). This was based on the understanding that *Salmonella* invasiveness is regulated by oxygen, where wild type bacteria grown aerobically are less invasive than bacteria grown under low-oxygen conditions (Lee and Falkow, 1990; Schiemann and Shope, 1991). Similarly, studies done in pathogenic bacteria *S. enteriditis* and *Shigella flexneri* have shown that virulence relies on oxygen availability in localized regions in the intestine (Marteyn *et al.,* 2010). It has been suggested that aerobic zones dictate intestinal microbial interactions because they act as immune barriers to protect the mucosal surface from anaerobic bacteria, and are as well recognized as a signal to promote invasion by such pathogens (Marteyn *et al.,* 2010). Furthermore, it is not surprising that in various serovars of *S. enteritidis* like in *S. flexneri,* FNR (fumarate and nitrate reduction regulatory protein), like other regulators (such as the ArcBA system), plays different roles from mediating invasion to displaying inflammatory responses to infection according to oxygen levels, as FNR boxes are present upstream of genes required for T3SS function (Marteyn *et al.,* 2010).

During the infection process, it is clear that the intracellular environment of the macrophage has an impact on the expression of the virulence and housekeeping genes of *Salmonella.* Fis not only affects the expression of genes relevant to the intracellular growth of the pathogen *S.* Typhimurium, it also affects DNA topology (section 1.5). Fis has shown to modulate both the level of DNA supercoiling and SPI-2 *ssrA* and *ssrB* gene transcription (Ó Cróinín *et al.,* 2006; Cameron and Dorman, 2012).

It is not surprising to observe that the microaerobic conditions used to maintain *fis* transcription beyond its shut-off point in standard laboratory growth conditions are similar to those that induce maximum virulence in *S.* Typhimurium (Ó Cróinín and Dorman, 2007). Fis

is not only important in the virulence of *Salmonella* but also regulates virulence gene expression in other pathogens like *S. flexneri* (Falconi *et al.,* 2001; Prosseda *et al.,* 2004), pathogenic strains of *E. coli* (Goldberg *et al.,* 2001; Sheikh *et al.,* 2001), and even in the plant pathogens *Erwinia* (Lautier and Nasser, 2007).

It has been suggested that low aeration or low oxygen conditions of the intestinal lumen induce SPI-1 virulence (Lee and Falkow, 1990), as well as priming SPI-2 gene expression prior to entry to the intracellular environment (Osbourne and Coombes, 2011) in *S.* Typhimurium. Furthermore, virulence in Δ*fis* mutants is attenuated in mice, partly due to a reduced expression of SPI-1 encoded invasion genes, and poor SPI-2 priming for intracellular macrophage survival (Wilson et al. 2001; Ó Cróinín *et al.,* 2006; Yoon et al., 2009; Osbourne and Coombes, 2011). The ability of *Salmonella* to invade host mammalian cells is regulated by the concentration of oxygen in the growth medium, as high oxygen represses and low oxygen induces invasiveness (Ernst *et al.,* 1990; Schieman and Shope, 1991). Other studies have shown that oxygen limitation might be an important environmental cue that triggers SPI-1 gene expression, as *Salmonella* prepares to invade host cells within the intestinal lumen and other tissues (Jones and Falkow, 1994; Lee et al., 2000; Waterman and Holden, 2003). Such findings inspired more investigations to understand the effect of growth aeration regime on virulence and Fis.

Virulence in *S.* Typhimurium has also been linked to factors thought to be transcribed exclusively in the anaerobic growth regime. The virulence plasmid of *S. enterica* (pSLT), an F-like conjugative plasmid, transfers at high rates in the mammalian intestinal lumen, a microaerobic environment, but it was found that the anaerobic transcription factor ArcA activates the conjugation of the plasmid (Serna *et al.,* 2010).

As mentioned earlier (section 1.2), Fis expression in *S.* Typhimurium was found to be sustained during the stationary phase of growth (Ó Cróinín and Dorman, 2007), where the protein was expected to be absent (Osuna *et al.,* 1995; Keane & Dorman, 2003). This finding is significant because Fis is known to be a global regulator (section 1.6) of many *Salmonella* virulence genes and bacterial growth in non-aerated conditions is used routinely to maximize the infectivity of *Salmonella* during *in vitro* infection studies, as they would resemble *in vivo*

gut lumen conditions (Ó Cróinín and Dorman, 2007). In fact, the aims and objectives of this research investigation were designed (section 1.7) to reveal the underlying molecular mechanism responsible for this unusual pattern of *fis* gene expression.

1.3.1 *Salmonella* Pathogenicity Island 1 (SPI-1)

The *Salmonella* genes that were identified as essential for bacterial invasiveness in cell culture and complete oral virulence were later shown to be part of the horizontally-acquired SPI-1 (Blanc-Potard *et al.,* 1999). As previously mentioned (section 1.1.2), a major function of the SPI-1 genes is to enable bacterial invasion of epithelial cells (Coburn *et al.,* 1997; Ohl and Miller 2001; Waterman and Holden, 2003). SPI-1 uses T3SS effector proteins to penetrate epithelial cells (Lee *et al.,* 2000; Zhou *et al.,* 2001), inducing the cytoskeleton to rearrange, the microvilli to alter and the membrane to ruffle, allowing bacterial uptake (Zhou *et al.,* 2001) (Fig 1.1). The T3SS of SPI-1 in *S.* Typhimurium encodes at least eight effector proteins that control several processes, including host cell invasion, an apoptotic-like (programmed cell death) effect in macrophages, and trans-epithelial migration of neutrophils (Hersh *et al.,* 1999; Zhou *et al.*, 2001).

S. enterica serovars encode the T3SS within the SPI-1 at centisome 63 of their chromosome (Galán, 1996). A centisome is 1% of a chromosome length (Park and Riley, 2009). This system regulates the translocation of a series of bacterial effector proteins into host cells which can inhibit or trigger host cellular functions (Galán, 1998; Eichelberg and Galán, 1999) (Fig. 1.3). These effector proteins coordinate host cell actin cytoskeleton rearrangements and nuclear responses that eventually lead to bacterial internalization and production of pro-inflammatory cytokines (Hobbie *et al.,* 1997; Chen *et al.,* 1996a). Also, T3SS in *Salmonella* is involved in the initiation of programmed cell death in macrophages (Chen *et al.,* 1996b, Monack *et al.,* 1996), the activation of neutrophil migration across the intestinal epithelium (McCormick *et al.,* 1993), and fluid accumulation in ligated intestinal loops and the generation of diarrhoea (Eckmann *et al.,* 1997; Galyov *et al.,* 1997). A series of environmental cues are known to affect T3SS-associated gene expression in SPI-1 (Bajaj *et al.,* 1995; Bajaj *et al.,* 1996; Ernst *et al.,* 1990; Galán and Curtiss III, 1990; Lee and

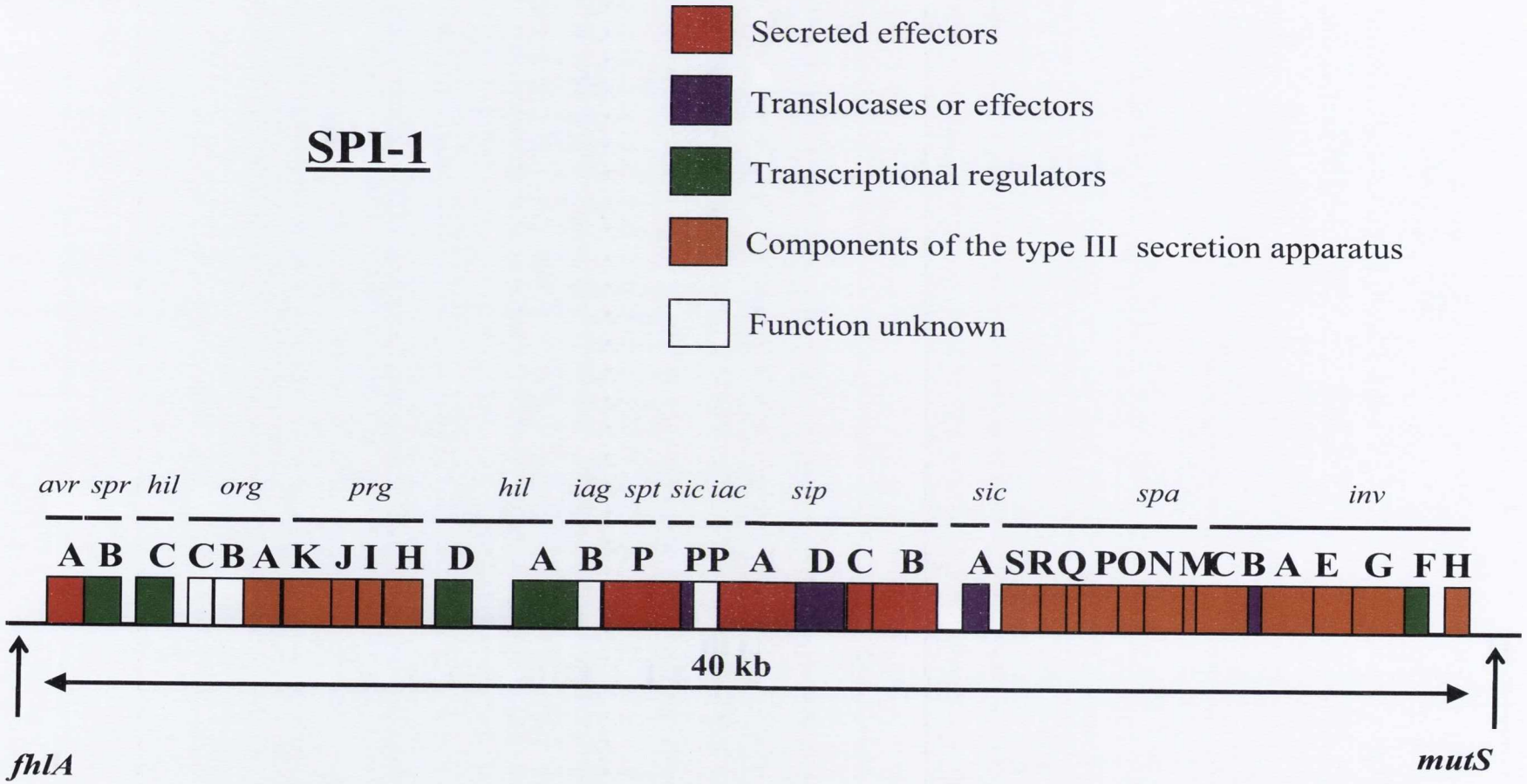
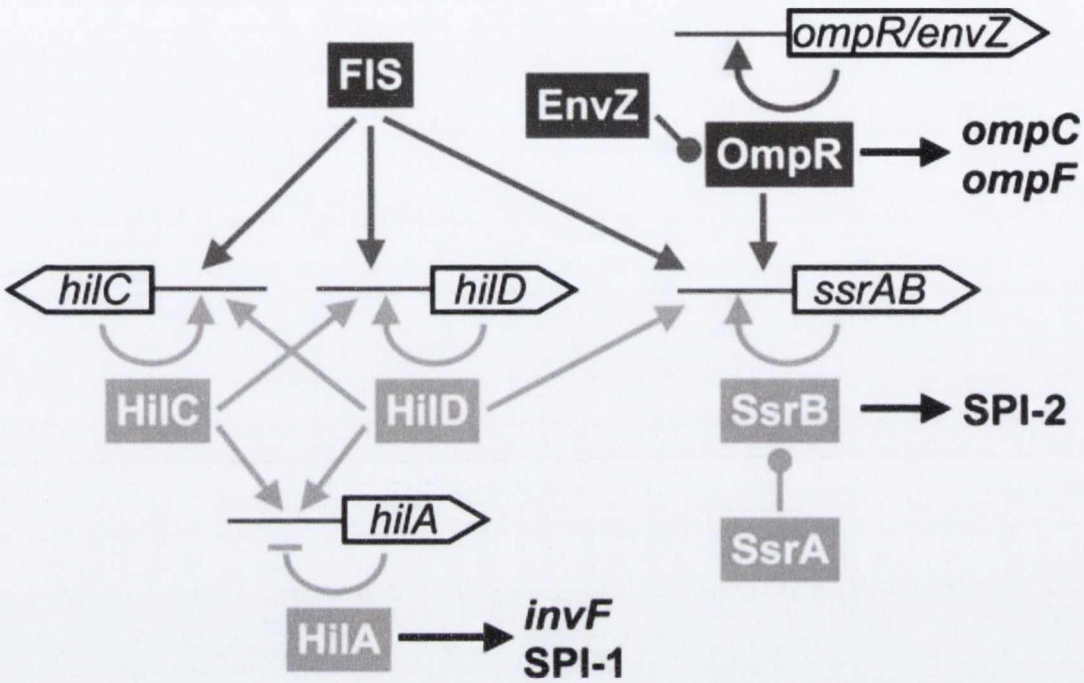


Fig. 1.3: Genetic framework of *Salmonella* Pathogenicity Island 1 (SPI-1). SPI-1 is approximately 40 kb in size and is found at 63 centisomes on the *S.* Typhimurium chromosome. SPI-1 is flanked by genes *fhlA* and *mutS*. SPI-1 genes that make up the type III secretion system/apparatus or T3SS; transcriptional regulators, secreted effectors and translocase/effectors are indicated. Length of each gene is shown approximately to scale.

Falkow, 1990; Schiemann and Shope, 1991; Tartera and Metcalf, 1993). Therefore, the expression of components and substrates of this T3SS is subject to complex regulatory mechanisms (Hueck, 1998). Bacterial growth under high-osmolarity and/or microaerobic conditions activates expression of T3SS associated proteins, generating increased levels of bacterial internalization within host cells (Lee and Falkow, 1990; Ó Cróinín and Dorman, 2007). In fact, the SPI-1 T3SS is most efficiently expressed when growth conditions reflect those of the lumen in the small intestine, including low-oxygen, high-osmolarity and mild alkalinity (pH 8) (Bajaj *et al.,* 1996). Furthermore, SPI-1 was induced in bacteria grown in low-aeration or microaerobiosis during stationary phase prior to infection of epithelial cells (Ó Cróinín and Dorman, 2007). The expression of the invasion-associated T3SS is influenced by several global regulatory networks. SPI-1 encodes four AraC-like transcription factors: Hil A, HilC, HilD, InvF (Kaniga *et al.,* 1994; Bajaj *et al.,* 1995; Klein et al., 2000; Cameron and Dorman, 2012). The transcription of *hilA* is activated through a complex feedback and feedforward mechanism, where HilC and HilD mutually control their own gene transcription (Fig. 1.4) (Cameron and Dorman, 2012). Nevertheless, Fis is required to induce expression of gene *hilA*, as it is the encoded activator of SPI-1 genes (Eichelberg and Galán, 1999; Wilson *et al.,* 2001), for which, as mentioned before, microaerobic growth conditions play a key role (Waterman and Holden, 2003; Ó Cróinín and Dorman, 2007). HilA together with InvF influence the expression of the invasion phenotype (Lee and Falkow, 1990; Kaniga *et al.,* 1994; Bajaj *et al.,* 1995; Klein *et al.,* 2000). Although OmpR-EnvZ was initially proposed to govern *hilC* expression (Lucas and Lee, 2001), OmpR was later shown to act post-transcriptionally through HilD protein function (Fig. 1.4) (Ellermeier and Slauch, 2007).

Relaxed DNA supercoiling (section 1.5) drives OmpR to bind directly to *hilC* and *hilD* promoters, as an activator and a repressor, respectively (Fig. 1.4) (Cameron and Dorman, 2012).

Another SPI-1 gene activated by low-oxygen conditions is the *orgA* gene (Jones and Falkow, 1994; Klein *et al.,* 2000). Because the microaerobic environment found in the small intestine induces the ability of *Salmonella* to enter mammalian cells (Ernst *et al.,* 1990; Jones and Falkow, 1994; Lee and Falkow, 1990; Lee *et al.,* 1992) (Fig. 1.1), OrgA also induces expression of the invasive phenotype and is essential for the entry process and virulence of *S.* Typhimurium (Jones and Falkow, 1994). Interestingly, the 200-bp region upstream of *orgA*



(Based on Cameron and Dorman, 2012)

Fig. 1.4: A regulatory network proposed for SPI-1 and SPI-2 gene expression. Regulatory connections at the gene promoters described earlier. Global regulators (dark grey), and local SPI-encoded regulators (light grey) are highlighted. Arrows indicate a positive regulatory effect while the perpendicular bars indicate a repressive regulatory effect, and rounded bars indicate activation by phosphorylation.

was found to have two direct repeats (TTGGATTNNNTTGATT) similar to those identified for *fnr*- (*oxrA*-) dependent promoters (Jones and Falkow, 1994). Since *fnr* is influenced also by aeration growth regime, it has been suggested that it may play a role in the oxygen regulation of *orgA* (Fink *et al.,* 2007). Furthermore, immediately downstream of *orgA* is *prgH,* another gene involved in bacterial entry. They are both expressed continuously in the same strain background (Klein *et al.,* 2000), indicating they are both regulated by the *hil* locus (Behlau and Miller, 1993). During *in vitro* studies it appears that the ability of *Salmonella* to invade tissue culture cells correlates with its abilities to invade and destroy M cells *in vivo* and to cause a systemic infection following oral infection (Fig. 1.1), nevertheless, it seems that the function of the *orgA* gene is required for later stages when the bacteria pass through the intestinal epithelium and the Peyer's patches (Fig. 1.1) (Jones and Falkow, 1994).

The growth of *S.* Typhimurium in a low-oxygen environment induces the ability of these bacteria to invade and enter mammalian cells. The oxygen regulation of bacterial entry has shown to be an important clue to determine individual components of the SPI-1 gene invasion arsenal (Klein *et al.,* 2000).

1.3.2 *Salmonella* Pathogenicity Island 2 (SPI-2)

During the early stages of invasion, SPI-1 genes in *S.* Typhimurium are expressed while in the intestinal lumen, however, expression decreases once across the epithelial cell as SPI-2 gene expression increases until it onsets, so to enable the bacteria to survive inside the macrophage vacuole (Hautefort *et al.,* 2008). The principal role of the *Salmonella* Pathogenicity Island 2 (SPI-2) genes is to facilitate the replication of intracellular bacteria in macrophages (Waterman and Holden, 2003). SPI-2 is situated at 30' (centisomes) on the *S.* Typhimurium chromosome, and it is composed of at least 15 genes that code for the second T3SS and for a two-component regulatory system (Ochman *et al.,* 1996). These set of genes are required for systemic growth of the *Salmonella* pathogen in its host (Hensel *et al.,* 1995; Ochman *et al.,* 1996; Shea *et al.,* 1996). The SPI-2 secretion system genes were categorized as follows: *ssa* for genes encoding the secretion system structure, *ssr* for genes encoding secretion system regulators, *ssc* for genes encoding secretion system chaperones and *sse* for genes encoding secretion system effectors (Fig 1.5) (Hensel *et al.,* 1997). The putative, two-component

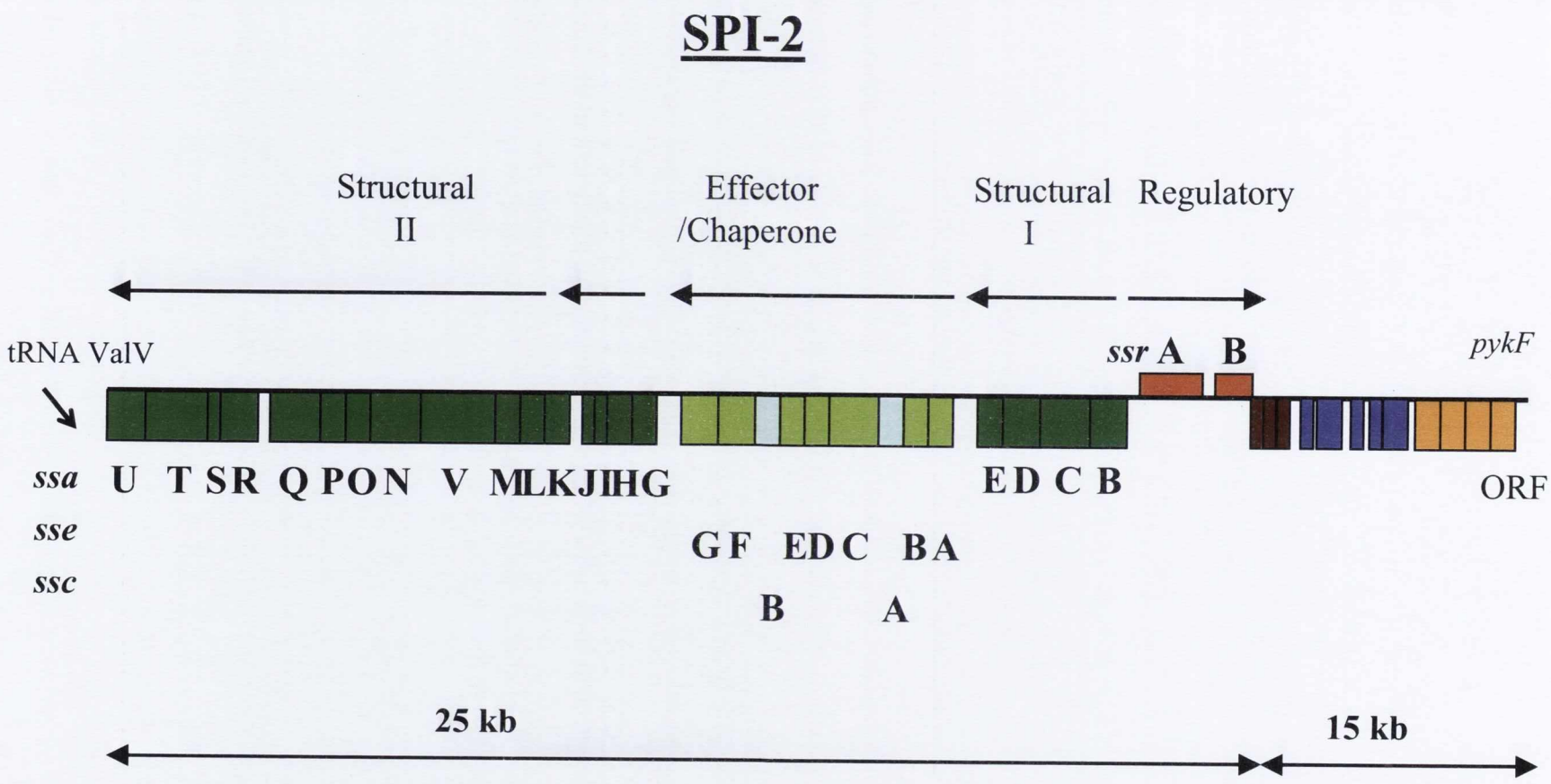


Fig. 1.5: Genetic framework of *Salmonella* Pathogenicity Island 2 (SPI-2). SPI-2 is approximately 40 kb in size and is found at 30' on the *S.* Typhimurium chromosome. SPI-2 genes that make up the structural, regulatory or effector/chaperone regions are indicated. Length of each gene is shown approximately to scale.

regulatory system, SsrA-SsrB, regulates the SPI-2 T3SS needed for replication inside macrophages and systemic infection in mice (Cirillo *et al.*, 1998). Although expression of *ssrA* and *ssrB* is autoregulated, it is dependent on a series of transcription factors including SlyA and Fis, and the two-component systems PhoP-PhoQ, OmpR-EnvZ (Fig. 1.4); while SPI-2 altogether is negatively regulated by H-NS, Hha and YdgT (Stapleton *et al.*, 2002; Feng *et al.*, 2003 & 2004; Bijlsma *et al.*, 2005; Coombes *et al.*, 2005; Silphaduang *et al.*, 2007). The OmpR protein binds directly to the *ssrA* promoter region, to activate the expression of *ssrA,* which together with *ssrB* co-transcribe with structural gene *ssaH* upon bacterial entry into host cells (Fig. 1.4) (Lee *et al.*, 2000; Cameron and Dorman, 2012). This is consistent with the finding that Δ*ompR* mutants have reduced virulence (Dorman *et al.*, 1989). Optimal transcription of the SPI-2 genes is controlled by DNA supercoiling, acting principally at the regulatory locus *ssrBA* (Cameron and Dorman, 2012).

Recently and quite interestingly, both OmpR and Fis were found to steer low levels of SPI-2 transcription in the intestinal lumen, where only SPI-1 gene transcription was throught to exist (Osborne and Coombes, 2011). There is increasing evidence that SPI-2 T3SS expression contributes to intestinal colonization (Miao *et al.*, 2002; Coombes *et al.*, 2004; Bustamante *et al.*, 2008). Because the SPI-2 phenotype required for enteric infection is not observed until after several days after infection, it is suggested that the early transcriptional activity measured for SPI-2 is independent to its functionality (Osborne and Coombes, 2011). For this reason, it was proposed that the quick activation of SPI-2, upon entry into the gut lumen in the host, is rather due to transcriptional priming needed for intracellular survival (Osborne and Coombes, 2011).

1.4 Fis and the Stringent Response

A lack of aeration at the epithelial interface in the intestinal lumen has been shown to sustain Fis expression at later stages of growth in batch culture (Ó Cróinín and Dorman, 2007). This is consistent with the previous finding that the Fis protein is a key regulator of the *Salmonella* virulent phenotype because the growth conditions that sustain expression of the *fis* gene are equivalent to those that induce the expression of the genes in the SPI-1 pathogenicity island (Kelly *et al.*, 2004; Thompson *et al.*, 2006; Wilson *et al.*, 2001). However, in order to

understand the molecular mechanism responsible for sustained expression of Fis during the stationary phase of growth, other avenues need to be studied. An important one is the examination of the genetic regulation mechanism already known to affect *fis* expression: the stringent response (Chang et al., 2002; Mallik *et al.,* 2006). During the stringent response, the polymerase-interacting DnaK suppressor regulatory factor protein DksA and the signal/alarmone molecule guanosine tetraphosphate (ppGpp) are known to play an important role in the repression of *fis* transcription in *E. coli* (Chatterji & Kumar-Ohja, 2001; Mallik *et al.,* 2006). In *S.* Typhimurium, *fis* displays elevated expression levels in a ppGpp deficient (ppGpp°) mutant strain under low-oxygen growth conditions (Thompson *et al.,* 2006).

Moreover, studies in *E. coli* explained how the *fis* promoter (*fis* P) is regulated by the stringent response (Mallik *et al.,* 2004 & 2006), through a mechanism in which the protein DksA (Blankschien *et al.,* 2009; Paul *et al.,* 2004; Rutherford et al., 2009) and signal molecules guanosine tetraphosphate (ppGpp) and pentaphosphate (pppGpp), regulated by *relA* and *spoT*, act jointly (Rutherford et al., 2009). Fis influences the transcription of several genes that are under the control of the stringent response (Paul *et al.,* 2004; Webb *et al.,* 1999). The *Salmonella fis* operon organization was shown to be the same as *E. coli*'s (Osuna *et al.,* 1995), suggesting that regulatory insights gained in studies of *fis* expression in *E. coli* might also apply to the same gene in *Salmonella*. The stringent response consists of a cascade of events initiated by the onset of stationary phase, amino acid starvation, or environmental stress, such as microaerobic conditions (Mallik *et al.,* 2006; Webb *et al.,* 1999; Ó Cróinín and Dorman, 2007) (Fig. 1.6). Bacteria detect these conditions as uncharged tRNAs begin to bind to ribosomes resulting in the accumulation of alarmones or signal molecules SpoT I (ppGpp), and SpoT II (pppGpp). RelA catalyzes the reaction from SpoT II to Spot I (Mallik *et al.,* 2006; Rutherford *et al.,* 2009). In *E. coli* as in *S.* Typhimurium, RelA and SpoT have opposing functions. RelA has a synthetic function as it produces pppGpp (or ppGpp) from ATP and GTP (or GDP), while SpoT degrades ppGpp, however, SpoT is a bifunctional enzyme and can also act as a ppGpp synthetase (Wendrich and Marahiel, 1997; Mittenhuber, 2001). These molecules are a signal to the cell to reduce the activity of its translation machinery as the demand for new protein synthesis diminishes. For this reason, the genes involved in the production of ribosomes, tRNA and other components of the translation apparatus are switched off (Mallik *et al.,* 2006; Chandrangsu *et al.,* 2011). As the levels of ppGpp increase, the ppGpp molecule binds to RNA polymerase (RNAP) in association with the transcription

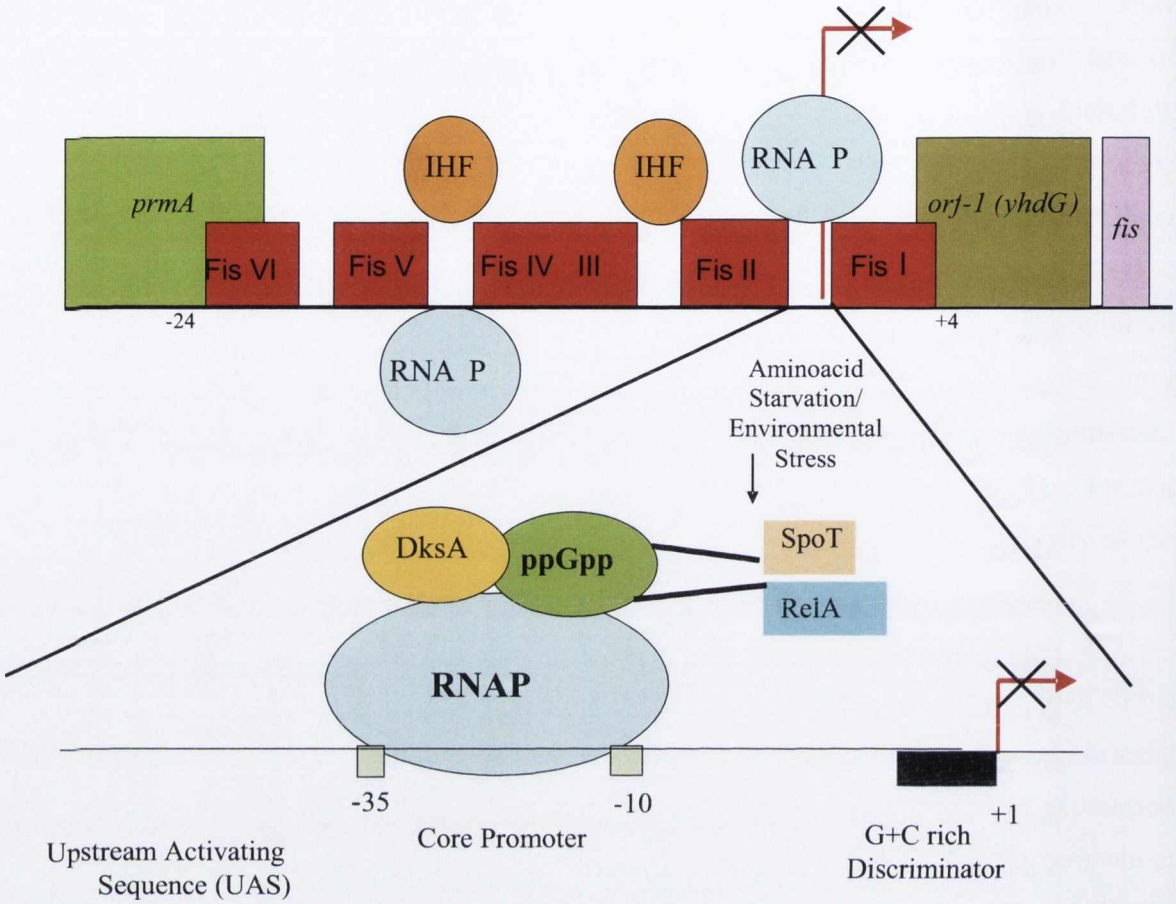
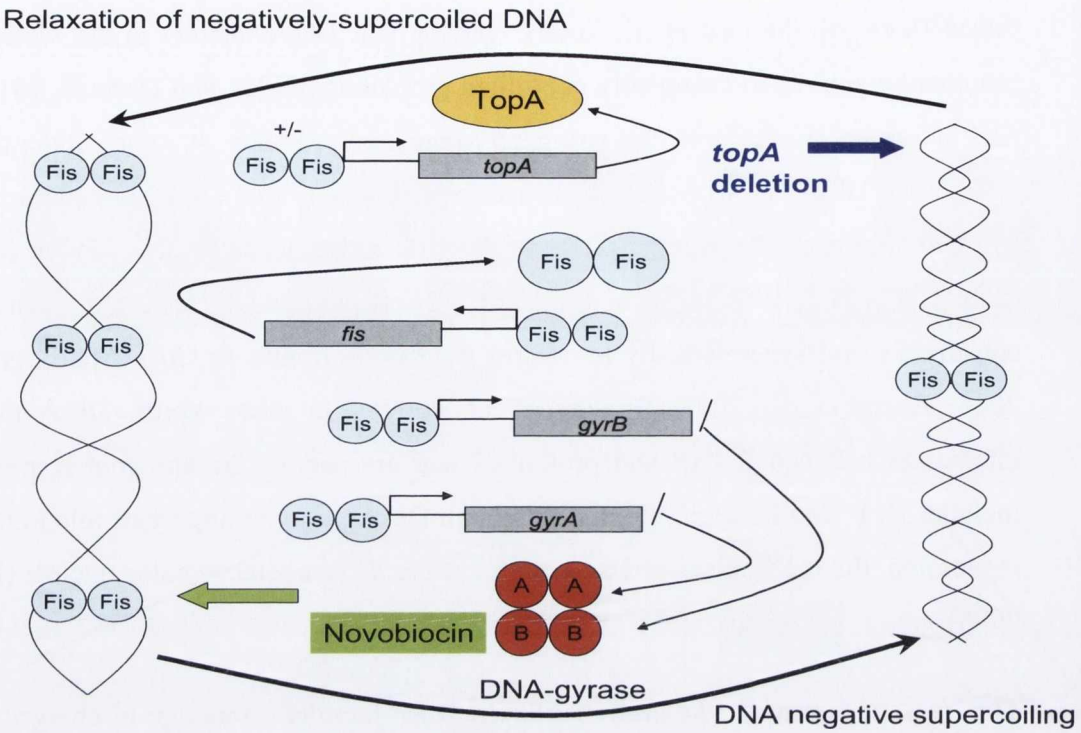


Fig. 1.6: Fis regulation by the Stringent Response. The stringent response is a mechanism initiated at and by the onset of stationary phase, amino acid starvation, or an environmental stressor. These conditions trigger uncharged tRNAs to bind to ribosomes. Signal protein molecules (alarmones) SpoT I (precursor to ppGpp), and SpoT II (precursor to pppGpp) accumulate, which is catalyzed by protein RelA. As ppGpp molecules accumulate in the cell, they prompt reduction in translation machinery activity as no new protein synthesis is needed. Genes involved in the production of ribosomes, tRNA and other components of the translation apparatus are shut down. Increased levels of ppGpp trigger binding to RNA polymerase (RNAP) by associating with the transcription factor DksA. *fis* P is a stringently-regulated promoter, being under the control of ppGpp and DksA. DksA and ppGpp inhibit *fis* P promoter function during amino acid starvation/environmental stress. These factors act in conjunction to negatively control *fis* P transcription.

factor DksA (Rutherford *et al.,* 2009). ppGpp and DksA interact in the weaking of RNA polymerase activity at stringently controlled promoters (Dillon and Dorman, 2010). Because *fis* P possesses the features of a stringently-regulated promoter, it comes under the control of ppGpp and DksA. It has been established experimentally that DksA and ppGpp are required for inhibition of *fis* P promoter activity following amino acid starvation and/or environmental stress (Ball *et al.*, 1992; Nilsson *et al.*, 1992; Ninnemann *et al.*, 1992), and that these factors act directly and synergistically to control *fis* P transcription *in vitro* (Mallik *et al.,* 2004 & 2006; Åberg *et al.,* 2009; Bradley *et a.l,* 2007). In other words, DksA modulates the interaction between RNAP and promoters that are part of the stringent response, and this includes *fis* P (Mallik *et al.,* 2006). Although DksA plays an important role in regulating Fis expression, the mechanism of DksA action at the *fis* promoter remains unclear (Mallik *et al.,* 2006).

The transcription of the *fis* gene is reduced when there is a shortage of charged tRNA when the bacterium experiences amino acid starvation and thus, the protein synthetic machinery of the cell should be down regulated. Stringently-regulated promoters like the *fis* P usually contain a GC-rich discriminator sequence upstream of the transcription start site (Schneider *et al.*, 2000) (Fig. 1.6). This discriminator sequence has been reported to be an energetic barrier to transcription initiation because it is hard to melt in relaxed DNA, the form of DNA that predominates in bacteria with a low energy charge (Dorman, 2009). Moreover, the discriminator sequence connects *fis* transcription to the physiological state of the cell through its sensitivity to the topological state of DNA (Fig. 1.7). This connection is interesting and becomes important when understanding that the Fis protein also regulates the transcription of the genes that encode the proteins responsible for introducing negative supercoiling (DNA gyrase) and removing it (DNA topoisomerase I) (section 1.5), (Keane and Dorman, 2003; Schneider *et al.*, 1999; Weinstein-Fischer and Altuvia, 2007). Furthermore, it should be noted that ppGpp has also been shown to regulate negatively another important global regulator, Fur (Thompson *et al.,* 2006), which is responsible for iron uptake (Tsolis *et al.,* 1995) (section 4.1). Fur, like Fis, is sensitive to environmental signals like non-aerated or low oxygen conditions (Lee & Falkow *et al.,* 1990; Ó Cróinín and Dorman, 2007) that activate important SPI-1 structural genes (Ellermeier & Slauch, 2008). Fur, therefore, also plays a substantial role in the invasion of intestinal epithelial cells by *S. enterica* serovar Typhimurium as it is part of the regulatory mechanism targeting SPI-1 type III secretion system (T3SS) genes



(Based on Dorman, 2009)

Fig. 1.7: Fis and DNA topology. The dimeric Fis protein represses the promoter of its own gene, *fis*, and genes encoding GyrA (*gyrA*) and GyrB (*gyrB*) subunits of the A_2B_2 heterotetrameric DNA gyrase. Fis has a double interaction with the gene coding for the monomeric DNA topoisomerase I (*topA*). That is, the Fis protein is an activator when present at low concentrations but becomes a repressor at high concentrations. This is indicated by the +/- symbol next to the *topA* promoter. (The angled arrows upstream of each gene represent transcription start sites). Moreover, the Fis protein binds to the chromosomal DNA where it can affect its availability as a substrate for DNA gyrase and DNA topoisomerase I. It is so that Fis can act as a homeostatic control of global DNA supercoiling by modulating the expression of the genes coding for the main topoisomerases and by regulating their function on the DNA substrate (Dorman, 2009). Furthermore, a deletion in the *topA* gene results in function impairment and as a result, DNA becomes more negatively supercoiled as it is unable to relax. Conversely, addition of novobiocin results in the relaxation of DNA, as impairment of the DNA gyrase subunit B prevents the introduction of negative supercoils into the DNA helix.

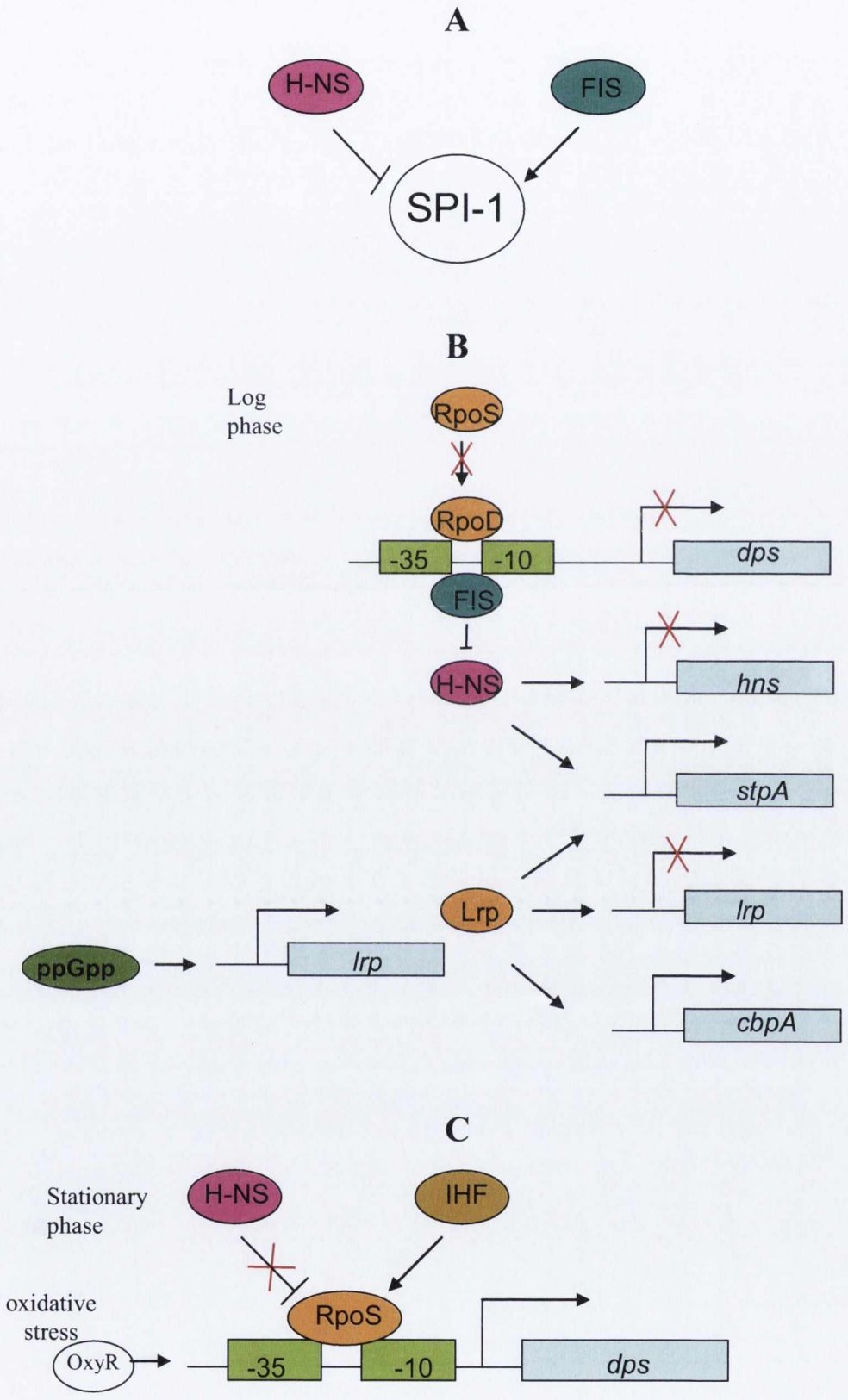
(Ellermeier & Slauch, 2008). This may prove to be important as it shows ppGpp to be a repressor not only of *fis* but also of other genes involved in epithelial cell invasion.

The *rpoS* gene encodes the alternative stress response sigma factor RpoS that regulates genes expressed during stationary phase in response to nutrient deprivation (Fang *et al.,* 1992; Loewen *et al.,* 1994; O'Neal *et al.,* 1994). The sigma factor RpoS was shown to play a role in repressing Fis expression during stationary phase (Ó Cróinín and Dorman, 2007), whereas during exponential phase, the opposite happens: Fis was shown to repress *rpoS* transcription (Hirsch and Elliott, 2005). Thereby, induction of Fis in the absence of aeration was facilitated by a reduction of RpoS levels under such conditions. During stationary phase, RpoS levels elevate to direct RNAP to induce transcription of stationary phase genes but, increased RpoS levels also correlate with decreased Fis expression (Ó Cróinín and Dorman, 2007). The RpoS interaction with Fis extends to a collaboration that the latter has with other global regulators/NAPs (section 1.7) like H-NS and IHF (Fig. 1.8C). IHF is a transcriptional activator to the *fis* promoter as it binds to two sites in the *fis* operon regulatory region (Fig. 1.3), resulting in IHF and Fis having antagonistic functions there (Nasser *et al.,* 2002). Conversely, the *dps* promoter is down regulated by the FIS (Fis NAP) and H-NS proteins, as H-NS represses transcription by RNA polymerase containing the RpoD housekeeping sigma factor but not the RpoS stationary phase and stress response sigma factor (Fig. 1.8B) (Grainger *et al.,* 2008).

1.5 Fis and DNA topology

Fis is a nucleoid-associated protein (section 1.2) that influences transcription by reshaping local chromatin structure (Muskhelishvili and Travers, 2003), to the point of indirectly and directly affecting RNA P (RNA polymerase) activity (Hengen *et al.*, 1997; McLeod *et al.*, 2002; Dorman and Deighan, 2003; Kelly *et al.*, 2004; Opel *et al.*, 2004). Since Fis is able to regulate the level of DNA supercoiling in the cell, and because DNA supercoiling also modulates this RNA P activity, it all results in global influences on transcription (Dorman, 1991; Cheung *et al.*, 2003; Travers and Muskhelishvili, 2005). Moreover, the Fis protein is a

Fig. 1.8: Regulatory networks of transcription by multiple nucleoid associated proteins (NAPs). The NAPs presented here are DNA protection from starvation protein (Dps), factor for inversion stimulation (FIS), histone-like nucleoid-structuring protein (H-NS), integration host factor (IHF), Leucine-responsive regulatory protein (Lrp), Salt tolerance protein A (StpA), and Curved-DNA-binding protein (CbpA). A) H-NS represses while FIS activates SPI-1 genes. B) Regulatory inputs at promoter *dps*. During the log phase of growth, FIS is abundant and binds at the *dps* promoter. A repression complex is formed as the RNA polymerase containing the RNA polymerase σ-factor RpoD becomes trapped. H-NS plays an inhibitory role. RNA polymerase containing the σ-factor RpoS cannot bind to the *dps* promoter while the Fis–RpoD–promoter repression complex is bound. As FIS levels decrease, this complex becomes less stable throughout the log phase and becomes undetectable at the onset of stationary phase. While H-NS autorepresses its own gene, it activates together with Lrp the expression of StpA. Lrp, on the other hand, is positively regulated by alarmone ppGpp (section 1.4) and also activates *cbpA* promoter function. C) At this point, RNA polymerase containing RpoS is able to counteract the repression of H-NS as IHF positively regulates *dps* at the promoter. As a result, by the end of log phase/beginning of stationary phase, Dps levels increase (Dillon and Dorman, 2010).



(Based on Dillon and Dorman, 2010)

regulator of the *Salmonella* virulence phenotype (Kelly et al., 2004; Ó Cróinín *et al.,* 2006) and because the expression of the *fis* gene is sensitive to changes in DNA supercoiling (Schneider *et al.,* 2000; Ó Cróinín *et al.,* 2006; Cameron *et al.,* 2011; Cameron and Dorman 2012) it was important to study proteins that are involved in modulating DNA topology, such as DNA gyrase and topoisomerase I (TopA).

Fis has been referred to as the master regulator of DNA supercoiling (Cameron and Dorman, 2012). The Fis protein influences DNA supercoiling both directly and indirectly (Travers *et al.,* 2001; Dorman, 2009) (Fig. 1.7). As previously mentioned (section 1.2), the *fis* P, the promoter of its own gene is responsive to negative supercoiling (Schneider *et al.*, 2000). The protein is also known to repress transcription of both genes encoding the DNA gyrase protein, *gyrA* and *gyrB* (Cozzarelli, 1980; Schneider *et al.,* 1997 & 1999; Travers *et al.,* 2001; Keane and Dorman, 2003), and to activate *topA,* the gene that encodes DNA topoisomerase I (TopA) (Cozzarelli, 1980; Richardson *et al.,* 1984; Weinstein-Fischer and Altuvia, 2007; Cho *et al.*, 2008), under specific conditions (Weinstein-Fischer *et al.*, 2000). DNA gyrase introduces negative supercoiling into DNA, whereas TopA relaxes it (Cozzarelli, 1980; Champoux, 2001; Travers and Muskhelishvili, 2005). In *E. coli,* DNA gyrase is an ATP-dependent type II topoisomerase (Hsieh *et al.,* 1991; Schneider *et al.,* 1999 & 2000), while TopA is an ATP-independent type I topoisomerase that relaxes negatively supercoiled DNA using the energy stored in the DNA to drive the reaction (Cozzarelli, 1980; Hsieh *et al.,* 1991; Travers and Muskhelishvili, 2005). In fact, there is a direct connection between aerobic metabolism and the [ATP]/[ADP] ratio in the cell and between this ratio and DNA gyrase activity (Hsieh *et al.,* 1991; Reece *et al.,* 1991). Fis acts as a local topological homeostat (Rochman *et al.*, 2002) by governing the action of gyrase A/B and topoisomerase IA on DNA. Fis binds to and preserves intermediately supercoiled forms of DNA by protecting them from extreme shifts in superhelicity due to the action of the topoisomerases (Schneider *et al.*, 1997; Travers *et al.,* 2001; Travers and Muskhelishvili, 2005).

Novobiocin, as well as other amino-coumarin antibiotics, like coumermycin A1 (CA1), has been frequently used in DNA topology studies. Novobiocin acts as a competitive inhibitor of the ATPase reaction catalyzed by the GyrB subunit of DNA gyrase (Gellert *et al.,* 1976; Cozzarelli, 1980). The potency of novobiocin is considerably higher than that of the fluoro-quinolones that also target DNA gyrase, but at a different site on the enzyme as the GyrA

subunit is also involved in the DNA nicking and ligation activity (Gellert *et al.,* 1976; Cozzarelli, 1980). Previous and current research in this laboratory has indicated that novobiocin at the final concentration of 50 μg/ml begins to affect DNA gyrase activity, specifically at the GyrB subunit, and decreases *fis* expression as well as DNA negative supercoiling (Ó Cróinín *et al.,* 2006; Cameron *et al.,* 2011). CA1, like novobiocin, inhibits DNA gyrase at the B subunit by blocking ATPase activity (Gellert *et al.,* 1976; Jovanovich & Lebowitz, 1987).

Although the Fis protein is considered a global regulator of supercoiling in *E. coli*, it does not have the same supercoiling control in *S. enterica*, suggesting that these inter-species differences fine-tune gene promoters to endogenous supercoiling and Fis levels (Cameron *et al.,* 2011). DNA supercoiling and Fis have been studied for their concerted ability to modulate SPI-1 and SPI-2 gene expression. As mentioned before (section 1.3), SPI-1 enables the bacterium to invade cells at the epithelial surface of the gut where microaerobic conditions prevail, while SPI-2 gene expression enables *Salmonella* to thrive intracellularly inside the host macrophages. A reporter plasmid in *S.* Typhimurium becomes relaxed when the bacteria reside in J774A.1 macrophages (Ó Cróinín *et al.,* 2006; Marshall *et al.*, 2000) and the macrophage-induced SPI-2 virulence genes require the Fis protein for full activity (Kelly *et al.*, 2004). Apparently, changes in the topological state of DNA take place as required by the bacterium, so it can thrive and adapt to a given environment. DNA becomes relaxed when *Salmonella* grow in murine macrophages but not in epithelial cells (Ó Cróinín *et al.,* 2006). As it turns out, the ability of DNA relaxation to activate SPI-2 is channeled through the cognate SsrA/B two-component regulator (Fig. 1.5) (Cameron and Dorman, 2012). Surprisingly, when DNA is highly relaxed, the induction of the SPI-2 T3SS and effector gene promoter, *ssrA* P (section 1.3.3) does not require Fis in simulated vacuolar environment culture conditions (Cameron *et al.,* 2011; Osborne and Coombes, 2011), and when the *ssrAB* promoter (*ssrA* P) is induced by novobiocin, the DNA supercoiling activity of the DNA gyrase subunit B (GyrB) is inhibited (Cameron and Dorman, 2012).

1.6 Fis as a global regulator

Fis is considered a global regulator (Hengen *et al.*, 1997; McLeod *et al.*, 2002; Dorman and Deighan, 2003; Kelly *et al.*, 2004; Opel *et al.*, 2004) due to its multifunctional nature as a protein affecting numerous processes including transcription, replication and recombination (Finkel and Johnson, 1992; Keane and Dorman, 2003), and as a nucleoid-associated protein (NAP) (section 1.7) in numerous DNA-based cellular activities (Dorman, 2009). Like many bacteria, *S.* Typhimurium regulates transcription in a hierarchical manner (Martinez-Antonio and Collado-Vides, 2003; Rhen and Dorman, 2005; Martinez-Antonio *et al.*, 2009). Fis, being a global regulator, functions at a high level in this hierarchy where just a few DNA binding proteins are needed to affect transcription of many genes (Ó Cróinín *et al.*, 2006). Fis creates an interwound and branched structure once it binds to the DNA and thus, allows for regions of high transcriptional activity (Schneider *et al.*, 2001).

Fis is considered a transcriptional regulator because of its expression pattern along the growth cycle. As mentioned earlier (section 1.2), the Fis protein is maximally expressed when entering the exponential phase of growth, correlating with an increasingly demand for components of the translation apparatus to support rapid growth (Osuna *et al.*, 1995; Dorman, 2009). Fis is, therefore, key in stimulating and sustaining the activity of the promoters of any relevant genes for growth. This is probably also why the multi-regulatory function of Fis to activate, repress or sustain according to aeration growth regime. During aerated growth conditions, Fis protein concentration levels are almost undetectable in stationary phase (Osuna *et al.*, 1995). This decline in concentrations levels is largely due to the negative autoregulatory nature of Fis (section 1.2) as it needs to remove its inhibitory control over *rpoS*, the gene encoding RNA polymerase σ-factor RpoS (Dillon and Dorman, 2010). Mainly because RpoS reprogrammes RNA polymerase to continue transcribing genes required for adaptation as growth ends (Hirsch and Elliot, 2005).

Fis has an important role not only in gene regulation but also in genome organization. According to independent studies, Fis activity is widely observed in the transcriptomes of *E. coli* and *S.* Typhimurium (Kelly *et al.*, 2004; Bradley *et al.*, 2007). The versatile and multifunctional nature of Fis as a global regulator prompted part of this research investigation

to map, by using Chromatin Immunoprecipitation in a chip --Microarray Technology (ChIP-chip), those Fis protein binding sites throughout the *Salmonella* genome when the bacterium is grown in different aeration regimes and at a certain phase in the growth cycle. Studies done on Fis transcription regulation in *Salmonella* and *E. coli* have been instrumental in this endeavour, as they provide clues as where to look for these binding sites (Kelly *et al.,* 2004; Cho *et al.,* 2008). For instance, bioinformatic analyses have detected potential binding sites for Fis along the *E. coli* genome, and ChIP-chip data has confirmed the binding at those sites, in particular the ones lying outside the coding regions of genes (Grainger *et al.,* 2006; Cho *et al.,* 2008). Furthermore, Fis binding sites also identified by ChIP-chip data indicate that the most representative Fis binding motif in DNA is 17 bp long, A+T rich and has G/C residues at positions 2 and 16 (Cho *et al.,* 2008). Also, throughout the *E. coli* genome there are A+T-rich DNA sequences that are frequently associated with promoters (Pedersen *et al.,* 2000).

Fis regulates the transcription of a long list of bacterial genes (Grainger et al., 2006; Kelly et al., 2004), as it can repress or activate promoters. It mainly depends on the location where Fis binds relatively to RNA polymerase (Dillon and Dorman, 2010). Fis can repress transcription initiation by alienating RNA polymerase as Fis becomes a barrier at the target promoter or, by modulating the RNA polymerase-mediated isomerization of the closed transcription complex to an open complex (Dillon and Dorman, 2010). As a transcription activator, Fis can actually bind to RNA polymerase at sites distant from the promoter by several mechanisms that involve DNA topology (McLeod *et al.,* 2002; Auner *et al.*, 2003).

Undoubtedly, the influence of Fis throughout the *Salmonella* chromosome as a global regulator has enabled the bacterium to transcend aeration regimes and various other environments. Fis regulation of genes that encode the T3SS machinery and the effector proteins required by the bacterium for invasion of host epithelial cells and for survival in macrophages, its interaction with stringent response elements and promoters, and ability to affect DNA topology, have positioned Fis as one of the most essential virulence features a gastro-enteric microbial pathogen can possess.

1.7 Nucleoid Associated Proteins (NAPs)

Bacteria, like many other organisms, are challenged as how to compact their chromosomal DNA. In the course of evolution, bacteria have succeeded in "folding" DNA into a compacted structure called the nucleoid. The shape and size of the nucleoid have been determined by a number of factors. The main factors involved being DNA supercoiling, macromolecular organization and proteins associated with the nucleoid, namely nucleoid-associated-proteins (NAPs). Fis (FIS), HU, IHF, H-NS, Dps, CbpA, Lrp, and StpA are the most commonly found NAPs in Gram negative bacteria (Dillon and Dorman, 2010) and most relevant in this research investigation.

It is now that the compaction and organization of DNA have become clearer thanks to the development in biophysical techniques. Electron microscopy (EM) provided evidence of eukaryotic-type of DNA compaction in nucleosome-like structures called "compactsomes" (Griffith, 1976). Such structures were the result of binding members within a group of DNA proteins to be later identified in *E. coli* (Vasharvsky *et al.,* 1977). There is also evidence that the bacterial chromosome is organized in chromatin as supercoiled loops have been observed, in fact, DNA supercoiling effectively reduces the radius of gyration (Olins and Olins, 1974; Lohr and Van Holde, 1975), NAPs and other proteins that bind to the DNA that display gene-silencing and anti-silencing functions have been found to be important antagonistic regulators of the nucleoid structure and transcritption processes (Dillon and Dorman, 2010). Interestingly, H-NS (histone-like nucleoid-structuring protein) and FIS, are abundant NAPs that have a binding preference for A+T-rich DNA sequences (Dorman, 2013) which are commonly found in horizontally acquired genes in enteric bacteria (Lucchini *et al.,* 2006; Dorman, 2007; Navarre *et al.,* 2008). However, unlike H-NS, which is present throughout the growth cycle (Dorman, 2013), high expression levels of FIS are found during early exponential phase (Beach and Osuna, 1998) because it aids the cell with the high transcription and translation demand from stable RNA operons (Schneider *et al.,* 2003). Also, bacterial cells can protect their genome during the stationary phase by extensive binding of NAP Dps (DNA protection during starvation) along their DNA (Dillon and Dorman, 2010). Furthermore, the growth phase determines the level of expression of the nucleoid associated proteins as there are NAPs absent during exponential growth but present during stationary phase or vice versa (Talukder *et al.,* 1999). The vast majority of NAPs not only influence

transcription, but are also capable of changing the direction of the DNA molecule itself by bending, wrapping or bridging it through their DNA-binding activity (Dorman, 2013). Moreover, horizontally acquired genes such as those in SPIs are sensitive to DNA relaxation as they are activated by FIS or repressed by H-NS (Fig. 1.8A) (Dorman, 2013) .

The activity of FIS has a considered effect on the transcriptomes of *E. coli* and *S.* Typhimurium (Bradley *et al.,* 2007; Kelly *et al.,* 2004). While it binds as a homodimer to an AT rich consensus sequence, dimeric FIS bends the DNA at its binding site (Pan *et al.,* 1996) enabling DNA to bind to the helix–turn–helix motif in each monomer (Skoko *et al.,* 2006), a common feature among NAPs. Also, as mentioned earlier, FIS has a tendency to bind to many locations, particularly those along intergenic regions throughout the *E. coli* genome, as a chromatin immunoprecipitation (ChIP) study shows (Grainger *et al.,* 2006), and an *in silico* work also confirms FIS preferential binding to sequences belonging to transcription promoters (Pedersen *et al.,* 2000). In this research investigation, FIS was also observed and studied for its ability to bind along the *S.* Typhimurium genome according to growth aeration regime. FIS can repress or activate promoters according on the position of its binding site relative to that of RNA polymerase (section 1.6). FIS can also make physical contact with the RNA polymerase by, for example, displacing DNA twisting from an upstream site to the target promoter by FIS-mediated DNA bending (Opel *et al.,* 2004). FIS thus, has an overall direct and indirect role as a NAP. It can directly bind to sites upstream of the stable RNA gene promoter or, indirectly by FIS repressing the promoters of genes encoding the A and B subunits of DNA gyrase, which are responsible for DNA negative supercoiling (section 1.5), and activating or repressing, according on growth conditions, the transcription of *topA* (section 1.5). FIS is therefore similar to other NAPs because it influences the global transcription pattern in response to changes in growth phase and physiological state (Dorman, 2009).

H-NS is considered the genome guardian and universal repressor. It is capable together with StpA to constrain supercoils in DNA, and negative supercoiling of the DNA is likely to facilitate the formation of DNA–H-NS–DNA bridges, as H-NS can bind at many bacterial promoters, thus influencing both nucleoid structure and gene expression simultaneously (Dillon and Dorman, 2010). Furthermore, important cross-regulations among NAPs include H-NS, StpA (Salt tolerance protein A) and FIS. H-NS represses the promoter at *stpA*

(Deighan *et al.,* 2003), while *hns* is itself regulated by FIS, thus compensating the autorepressive nature of H-NS (Falconi *et al.,* 1996) (Fig. 1.8B).

Dps is a ferritin-like polypeptide capable of shutting-off transcription during conditions that may be harmful to DNA (Almiron *et al.*, 1992; Talukder *et al.*, 1999; Dillon and Dorman, 2010). This protein is considered a NAP despite the little evidence available for its direct effect in transcriptional regulation. In *E. coli* it has been found to possess DNA-binding activity (Almiron *et al.,* 1992). Dps is commonly expressed during the stationary phase, when DNA needs protection the most from damage. RpoS-containing and RpoD- (housekeeping sigma factor) containing RNAP are regulated at the *dps* promoter by the dual action of FIS and H-NS (Dorman, 2009) (Fig. 1.8B). Interestingly, *dps,* the gene that encodes Dps, is also expressed in late stationary phase (Fig. 1.8C) as there is more demand of Dps to perform a biocrystallization process of the nucleoid in the chromosome (Wolf *et al.*, 1999) in order to protect the DNA from damage during stressful conditions (Grant *et al.,* 1998). Dps is negatively regulated by FIS (Grainger *et al.* 2008), but Dps classical expression pattern is very similar to that displayed by FIS, whose expression peak is found at the earliest stages of exponential growth (Azam and Ishihama, 1999). Nevertheless, Dps expression is activated later by the OxyR regulatory protein during oxidative stress, and together with RpoS, the *dps* promoter is co-activated by IHF (Fig. 1.8C) (Altuvia *et al.,* 1994). During this phase, *dps* requires RpoS and IHF for transcription (Almiron *et al.,* 1992; Dillon and Dorman, 2010) and FIS and H-NS for inhibition (Gottesman, 2004; Dillon and Dorman, 2010) (Fig. 1.8B).

The Integration Host Factor (IHF) is responsible for introducing U-turns in DNA. Eventhough IHF and HU share to a certain extent the same amino acid sequence, their mode of interacting with DNA is very different. HU wraps DNA in a sequence-independent manner, while IHF binds to a conserved nucleotide sequence and introduces a U-turn into the DNA, generated at the binding site (Swinger and Rice, 2004). In both, *E. coli* (Arfin *et al.,* 2000) and *S.* Typhimurium (Mangan *et al.,* 2006) IHF is a NAP that acts as a conventional transcription factor as it is capable of recruiting the σ54-RNA polymerase to promoters (Macchi *et al.,* 2003), and bend DNA in order to bring regulatory proteins and RNA polymerase into contact (Santero *et al.,* 1992). Also, as mentioned earlier, IHF is a transcriptional activator to the *fis* promoter as it binds to two sites in the *fis* operon regulatory region (Fig. 1.3), resulting in IHF and Fis having antagonistic functions there (Nasser *et al.,*

2002). And like FIS, IHF posesses the ability to displace supercoiling-induced duplex destabilization (sIDD) at stable RNA promoters (Opel *et al.,* 2004). This occurs because as IHF reduces the energy from a DNA twist at the IHF-binding site, and become single stranded by transferring this torsional energy to the promoter of the targeted gene, a bubble may form (Benham, 1993). Interestingly, stable RNA gene promoters like the *tyrT,* a tRNA gene, uses a torsional transmission mechanism where FIS stabilizes DNA writhe by binding at three consecutive sites, and then convert it into DNA untwisting at the promoter (Maurer *et al.,* 2009; Muskhelishvili *et al.,* 1997). Moreover IHF, like FIS and HU affect chromosome replication initiation at the chromosomal origin, *oriC* (Dillon and Dorman, 2010).

The leucine-responsive regulatory protein (Lrp) affects the nucleoid structure and the transcription of approx. 10% of the genes in *E. coli,* whereby according to the gene region, the activity of lrp can be enhanced, suppressed or unchanged by leucine (Cho *et al.,* 2008). Lrp negatively autoregulates its own gene (McFarland and Dorman, 2008), and is an activator of *stpA* (Fig. 1.8B) (Free and Dorman, 1997). The Lrp regulon affects genes involved in nutrient uptake, amino acid metabolism, virulence, and regulation of phase-variable expression of pili (Wang and Calvo, 1993). Lrp, like H-NS, bends, wraps and bridges DNA and influencing RNA expression in *E.coli* by collaborating with H-NS to repress rRNA operon transcription (Pul *et al.,* 2007). Furthermore, as it will become revelant later on (section 4.2.5), the *lrp* gene is positively controlled by ppGpp (Fig. 1.8B) (Landgraf *et al.,* 1996; Traxler *et al.,* 2011).

CbpA stands for curved-DNA-binding protein as it is related to the chaperone protein DnaJ but, unlike DnaJ, it has DNA-binding activity (Dillon and Dorman, 2010). In *E. coli,* as in many other bacteria, CbpA assists in cell growth at low and high temperatures and it is needed for normal cell division. Moreover, the transcription of *cbpA* is regulated by Lrp and by Rpos (Chenoweth and Wickner, 2008) the expression of which is influenced by FIS (Fig. 1.8C).

The Heat-Unstable (HU) protein is considered a regulator of DNA flexibility. It interacts with topoisomerase I resulting in drastically altering DNA supercoiling (Broyles and Pettijohn, 1986), and thus affecting gene expression and nucleoid structure. It does also contribute to DNA flexibility by bending the duplex as it minimizes the effective stiffness of DNA over short distances at low protein concentrations, but it stiffens DNA at high concentrations (Luijsterburg *et al.,* 2008). Induced flexibility enables loop formation, which is crucial in

gene regulation and chromosome organization (Dillon and Dorman, 2010). Interestingly, HU facilitates DNA loop formation, while H-NS discourages it (Becker *et al.,* 2003).

MukB is known for maintaining chromosomal structure and, like FIS, it modulates DNA topology (Weitao *et al.,* 2000). MukB-deficient mutants fail to generate daughter chromosomes at cell division, and *topA* suppressing such mutation phenotype result in increased negative supercoiling (Sawitzke and Austin, 2000). MukB is known to be involved with DNA gyrase, as it enables DNA topological shifts in the *E. coli* chromosome (Hsu *et al.,* 2006).

1.8 Aims and Objectives

This project aims to unveil the regulatory mechanism responsible for the prolonged expression of the *fis* gene in *S. enterica* serovar Typhimurium, during the stationary phase of growth and under microaerobic conditions. I followed a research approach based on those factors that are already known to influence Fis expression. These factors are: growth aeration regimes, the stringent response transcription factor DksA and signal molecule ppGpp, DNA supercoiling, and the regulatory role of the Fis protein in *fis* transcription, specifically during the mid-exponential and late-stationary phases of growth. I have also examined the impact of extended expression of the *fis* gene in microaerobic conditions on the pattern of Fis binding to the *Salmonella* chromosome and its influence on gene expression.

Chapter 2

Materials and Methods

2.1 Chemicals and growth media

2.1.1 Chemicals and reagents

The supplier for each reagent or chemical used in this research work is indicated in parenthesis after the product. Custom automated sequencing was performed by GATC-Biotech, Germany. Furthermore, several molecular biology kits were used throughout this study. The basic principle of each kit is summarized in the sections below, without giving exhaustive details.

2.1.2 Growth media

The growth medium used was rich Luria-Bertani (LB) broth.

Ingredients for preparing this medium were obtained from Bacto^{TM} , Difco. Media were sterilized by autoclaving at 120°C for 20 min prior to use, or storage at room temperature. Alternatively, antibiotic solutions, which are not suitable for autoclaving, were sterilized by filtration through sterile 0.22 μm Millipore Filters. All quantities described below are for the preparation of 1 liter of medium in distilled, deionised water (ddH_2O). Media were supplemented with the appropriate antibiotics as required. Given the known effect of salt content and osmolarity to influence DNA supercoiling, LB broth devoid of salt was used for those experiments in which DNA topology was measured. Agar for use in solid media was allowed to cool at 50°C prior to antibiotic addition.

LB agar plates were used throughout this research investigation to culture bacterial strains from frozen stocks, and to select transformants and transductants. Bacterial strains were grown in LB broth unless otherwise stated. LB broth contained 10 g Bacto® tryptone, 5 g Bacto yeast extract, and 10 g NaCl. LB broth with no salts contained 10 g Bacto® tryptone, and 5 g Bacto® yeast extract. LB agar contained 10 g Bacto® tryptone, 5 g Bacto® yeast extract, 10 g NaCl, and 15 g agar. LB agar with no salts contained 10 g Bacto® tryptone, 5 g Bacto® yeast extract, and 15 g agar.

Motility agar was used to measure bacterial motility. Equal numbers of bacteria were inoculated centrally into these plates and incubated at 37°C for 8 hours. The rate of spreading of rings of bacteria on the plate was a measure of chemotaxis and motility (Pratt and Kolter, 1998; Kelly *et al.*, 2004). It contained 3 g agar, 10 g Bacto® tryptone and 5 g NaCl.

In order to obtain isolates of *S.* Typhimurium free of P22 phage pseudo-lysogens, green agar plates were used following bacteriophage P22-mediated generalized transduction. Phage free colonies appear light green whereas pseudo-lysogens appear dark green in these plates because bacterial lysis causes a drop in the agar pH resulting in a dark green color change in the pH-sensitive dye. It contained 8 g tryptone, 1 g yeast extract, 5 g NaCl, and 15 g agar, as well as 3.3 ml of 2% (w/v) aniline blue, 21 ml 40% (w/v) glucose, and 25 ml 2.5% (w/v) Alizarin yellow (freshly prepared) were added after autoclaving.

2.2 Bacterial strains and culture conditions

2.2.1 Bacterial strains

S. enterica serovar Typhimurium SL1344 derivatives were used throughout this research work. The *E. coli* strain XL1 Blue was used for routine cloning work. Details of these strains and plasmids are listed in Table 2.1. Bacterial strains were maintained as permanent stocks in 20% (v/v) glycerol in LB broth and stored at -80°C.

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

Strain	Relevant details	Reference/source
Salmonella Typhimurium		
SL1344	SL1344 Virulent wild type	Hoiseth and Stocker, (1981).
SG02	SL1344 *dksA::kan*	This study
SG03	SL1344 *topA::kan*	This study
SL1344 *fis::cat*	SL1344 *fis::cat*	Keane & Dorman, (2003).
KT2160	SL1344 *relA::kan, spoT::cat*	Thompson et al., (2006).
KT4514	SL1344 Δ*relA* Δ*spoT28* *hisG rpsL*	Arthur Thompson
SG07	SL1344 *fis::3XFLAG::kan*	This study
SG08	SL1344 fis^+. *fis* gene function restored with *fis::3XFLAG::kan*	This study
Escherichia coli		
XL1Blue	cloning strain	Stratagene

2.2.2 Bacterial culture conditions

Bacteria were grown in LB medium at 37°C with shaking at 200 r.p.m. in a shaking incubator, either under aerated or non-aerated conditions. Starting cultures were grown by inoculating single colonies into 5 ml of liquid medium containing the appropriate antibiotic. Cultures were inoculated 1:100 (v/v) into 10 ml of liquid medium in 250 ml conical flasks (aerated cultures), or in 15 ml test tubes (non-aerated cultures). Cultures containing the temperature sensitive plasmid pKD46 were incubated at 30°C. Also, bacteria were grown overnight (~16 h) at 37°C on L-agar plates supplemented with antibiotics as required. LB medium used for *topA* mutant cultures was made without NaCl. Antibiotic solutions were made up at the following concentrations: carbenicillin (Cb) 50 μg/ml, chloramphenicol (Cm) 25 μg/ml, coumermycin A1 (CA1) 10 μg/ml, kanamycin (Kan) 50 μg/ml, novobiocin 50 μg/ml, and tetracycline (Tet) 12.5 μg/ml.

Gas-tight tubes (Bellco Glass, USA) designed for obtaining and maintaining anaerobic conditions were used. Tubes were capped with butyl rubber septum-type stoppers and aluminum seals (Bellco Glass, USA). Growth medium was prepared, as described previously. Tubes filled with medium and partially closed with stoppers were flushed for 10 minutes through a stainless steel cannula with oxygen-free nitrogen gas. This gas was obtained by passing commercial nitrogen over custom-designed oxygen scrubbers filled with a fine copper mesh and maintained at 350°C. Inoculation, antibiotic addition and any further manipulations were carried out by using syringes flushed with the anaerobic gas described earlier. The oxygen concentration in the gas used in the entire anaerobic procedure was lower than 100 ppb. It has been demonstrated that these anaerobic tubes are an alternative to the anaerobic cabinet since they provide similar oxygen-free conditions (Kafkewitz and Togna, 1999). All anaerobic samples were grown at 37°C in a static incubator.

2.3 Plasmids, GFP, 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. Specific details of plasmid constructions will be provided in the appropriate results chapters.

2.3.1.1 Plasmid pZEP08

High-copy-number plasmid pZEP08 (Table 2.2 & Fig. 2.1) was used throughout this investigation as the platform vector to measure *fis* promoter activity in different aeration regimes. Plasmid pZEP08 carrying the promoterless *gfp* reporter gene and lacking the Cm cassette, was termed pZEC09 (Table 2.2). Once the promoterless *gfp* reporter gene in plasmid was shown to be transcriptionally silent (section 3.2.1), *fis* promoter activity in different aeration regimes was determined in pZep*fis-gfp* Kan ^{S} recombinant plasmid (Table 2.2) by flow cytometry (section 2.6.3).

2.3.1.2 Plasmid pUC18

The small, high-copy-number plasmid pUC18 (Table 2.2) was used to monitor DNA topology (DNA supercoiling) in *S. enterica* serovar Typhimurium SL1344 cells. Plasmid supercoiling has been well established as reflecting the average supercoiling of the chromosome, including the particular growth conditions used in this research investigation (Hsieh *et al.,* 1991). The supercoiling state of plasmid pUC18 was analysed by using one-dimensional agarose gels containing 2.5 μg/ml chloroquine (section 2.13), which intercalates with DNA and relaxes negative supercoils.

Table 2.2 Plasmids used throughout this study.

Plasmids	Relevant details	Reference/source
pKD46	λ-red recombination system plasmid. Genes *gam, bet* and *exo* under control of the arabinose inducible pBAD promoter, Cb^R	Datsenko and Wanner, (2000) & Karlinsey (2007)
pUC18	Cb^R	Ó Cróinín *et al.*, (2006)
pSUB11	Kan^R	Ó Cróinín and Dorman (2007)
pZEP08	pZep*gfp*$^+$ carrying Kan^R and Cm^R, promoterless.	Hautefort *et al.*, (2003)
pZep*fis-gfp*	pZEP08 with *fis-gfp* fusion cloned, Kan^S	Ó Cróinín and Dorman (2007)
pZep*rpsM-gfp*	pZEP08 with *rpsM-gfp* fusion cloned, Kan^S	Ó Cróinín and Dorman (2007)
pZep*lrp-gfp*	pZEP08 with *lrp-gfp* fusion cloned, Kan^S	McFarland and Dorman (2008)
pZEC09	Cm^R cassette removed from pZEP08, Kan^S promoterless.	Cameron *et al.*, (2011)

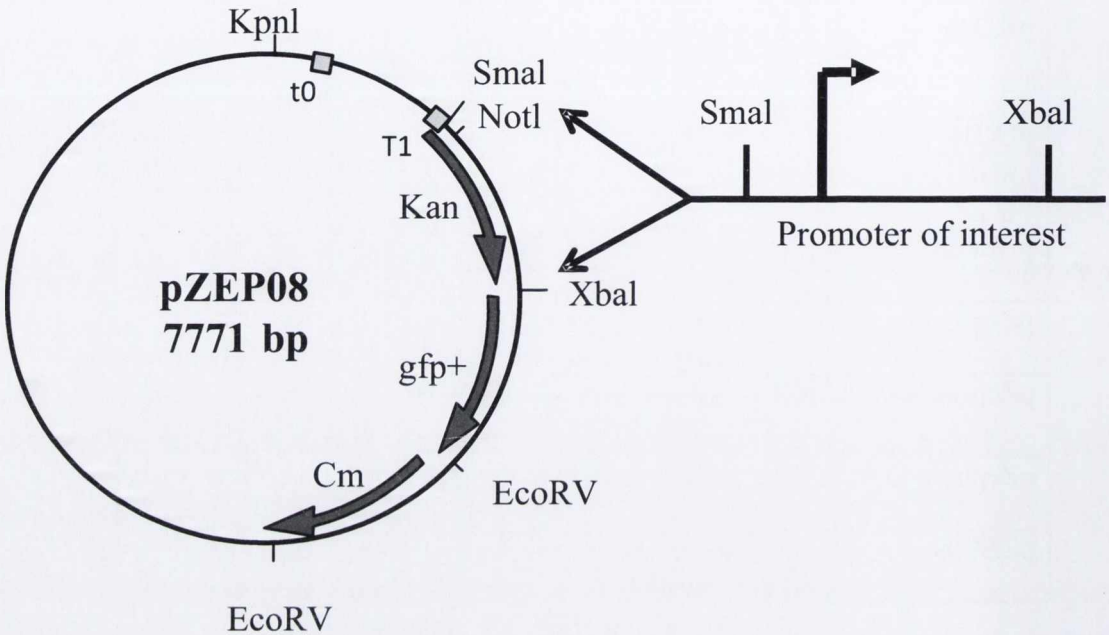


Fig. 2.1: Structure of plasmid pZEP08. Plasmid pZEP08 was used throughout this investigation as the platform vector to measure promoter activity in different aeration regimes (Hautefort *et al.,* 2003).

2.3.2 Green Fluorescent Protein (GFP)

The green fluorescent protein (GFP) that was used in this study comes from the jellyfish *Aequorea victoria* (Ghisla *et al.*, 1978). When calcium binds to the photoprotein aequorin, this monomeric and soluble protein displays visible fluorescent light at a peak wavelength of 508 nm when excited with ultraviolet light (Ward *et al.,* 1980) when expressed in prokaryotic or eukaryotic cells. The formation of the fluorophore requires covalent bonding with oxygen (Hansen et al., 2001). No exogenous substrates or co-factors are necessary for the fluorescence in the expression of GFP, thus rendering it as an ideal protein in the monitoring of gene expression and protein localization in living organisms (Chalfie *et al.,* 1994). GFP has a relatively long half-life: 4 hours in average (Morin *et al.,* 2001; Elowitz and Leibler, 2000). In this research investigation, once samples were taken they were immediately fixed and stored at 2-4°C to stop cell multiplication and slow down oxygen diffusion. Moreover, *fis-gfp* expression measurements were done in less than an hour from the moment the samples were taken at any given time point. Also, accurate assessments of *fis-gfp* expression in *Salmonella* growth cycle over a period of 24-30 hours was supported and further confirmed by Western Blotting examinations done in the present investigation (Fig. 3.4), and in previous studies (Ó Cróinín and Dorman, 2007). These confirmations guaranteed precise snapshots of *fis* expression in bacteria growing in any of the two aeration regimes. Equally important was to observe that the half-life of the GFP was similar to that of the wild-type protein (Morin *et al.,* 2001), in this case of Fis, for efficient assessment of fluorescence. Studies have shown that Fis degrades very slowly over time due to the tighter binding of Fis to DNA than to inorganic polyphosphate (polyP), which accumulates in response of aminoacid starvation, and resistance to mediated proteolysis (Kuroda *et al.,* 2006).

Furthermore, the *fis-gfp* transcriptional fusion used in this research investigation was created from putative *fis* transcription signals cloned into plasmid pZEP08 (Ó Cróinín and Dorman, 2007). This high-copy-number plasmid, pZEP08 *fis-gfp* (section 2.3.1.1.), allowed GFP to be used as a reporter of the *fis* promoter activity. Moreover, studies done by colleagues in the same laboratory where this investigation was performed showed that *fis-gfp* expression from pZEP08 was very similar to that generated directly from the chromosome (unpublished data).

2.3.3 Bacteriophages

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 chloroform.

2.3.4 Oligonucleotide primers

The sequences and nomenclature for all oligonucleotide primers used in this research work are listed in Table 2.3. Oligonucleotide primers were designed *in silico* (http://frodo.wi.mit.edu/), and purchased from IDT Integrated DNA Technologies, Belgium.

2.4 Transformation of bacterial cells with plasmid DNA

Transformation was done through electroporation, whereby recipient cells were made competent via high-voltage electroshock treatment for plasmid DNA uptake (Datsenko and Wanner, 2000; Karlinsey 2007).

2.4.1 Transformation via electroporation method

A volume of 350 µl of an overnight culture of the strain to be made electrocompetent for transformation was used to inoculate 25 ml of LB broth containing an appropriate antibiotic and grown to an OD_{600nm} of 0.5. Cells were incubated on ice for 1 hour and then pelleted by centrifugation (Eppendorf centrifuge 5415R) at 9,300 X G for 10 min. The bacterial pellet was resuspended in 250 µl of sterile, ice cold ddH_2O and incubated for another 20 min in ice. The cells were pelleted again by centrifugation (Eppendorf centrifuge 5415R) at 3,300 X G for 10 min and resuspended in 250 µl cold sterile ddH_2O. Aliquots of 50 µl of electrocompetent cells were used fresh for each transformation at a given time.

DNA to be electroporated was at a concentration of about 50 to 200 ng per µl, which was suspended in 5 µl of sterile ddH_2O. Aliquots of electrocompetent cells were added to 150 ng

Table 2.3. Oligonucleotide primers used in this study.

Primer Name	Sequence
dksA.KO.fwd	5'-CGA ACA TGG GGA TCG ATA GTG CGT GTT AAG GAG AAG CAA CGA CTA CAA AGA CCA TGA CGG-3'
dksA.KO.rev	5'-AAA CTC CCG CCT GTC ATA AAT AGG GTA GAA ACG AAC GGG ACA TAT GAA TAT CCT CCT TAG-3'
*dksA*_fwd	5'-TGC TAT CCG GAA AAG CAT CT-3'
*dksA*_rev	5'-GAA AAC TCC CGC CTG TCA TA-3'
*topAko*_rightfwd	5'-CTT ACG CGG CCC GCT TGA CTA TAG TGA CGA CAG GCA GGG GGA CTA CAA AGA CCA TGA CGG-3'
*topAko*_rightrev	5'-CGA CGC ATT CCT GGA AGA ATC AAC TTA GGT AAA GGT GAA TCA TAT GAA TAT CCT CCT TAG-3'
*topAko*_conf_fwd	5'-AGT AAT CCG TAG CCA ACA CAT AA-3'
FWD_*fis*_3xFlag	5'-TGG TAC GCT GCG TAA AAA ATT AAA AAA ATA CGG CAT GAA CGA CTA CAA AGA CCA TGA CGG-3'
REV_*fis*_3xFlag	5'-CGA GTA GCG CCT TTT TAA ACA AGC AGT TAG CTA ATC GAA ACA TAT GAA TAT CCT CCT TAG-3'
ST FLAG.*fis*.conf-For	5'- GCG TAA ACA CGT CTC CTG GT -3'
ST FLAG.*fis*.conf-Rev	5'- GTC GTC ATC TTT GTA GTC GAT ATC AT-3'
*fis*_prcorr_FWD	5'-TGC GTA AAC AGA AAT AAA GAG CTG-3'
*fis*_prcorr_REV	5'-AAC GGT AGA AAC GGT CAG TAC G-3'
*topA*_fwdorf	5'- TGT CTT CAC GCT CCA GTA CG-3'
*topA*_revorf	5'- GCT ACA GGT GAC GCA TCA GA-3'
*gyrA*_fwdprom	5'-TGT CCG AGA TCA GCG TAG TG-3'
*gyrA*_revprom	5'-AGC GTC GAG TTC TTC GTC AT-3'
*gyrB*_fwdprom	5'-CAC CGT CAG ATC TAC GAG CA-3'
*gyrB*_revprom	5'-GAC GTT GGT GAA GGT TTC GT-3'
*orgA*_fwdprom	5'-CTC GCA AAA TAC GCT TAG CC-3'
*orgA*_revprom	5'-GCC TGT TGA GGG GAT ACT GA-3'

Primer Name	Sequence
*relA*_fwd	5'-TTA CCC ACG CCT TCT ACC AC-3'
*relA*_rev	5'-AAT AAG CCG AGT GCA GAG GA-3'
*spoT*_fwd	5'-AGC CCA GGT AAA GGA CTG GT-3'
*spoT*_rev	5'-TTC CTG CTC CGT CTC TTT GT-3'
*fliA*_fwd	5'-CCG CAT TTA ATA ACC CGA TG-3'
*fliA*_rev	5'-CCG CTG AAG GTG TAA TGG AT-3'
*ssrA*_fwdprom	5'-GAC GTA ATG GGG TGT TTT CG-3'
*ssrA*_revprom	5'-CGC GCA GAG ATT TTA CTT CC-3'
nuoA _fwdprom	5'-GGG GGT CGG TTA CGA TCT-3'
nuoA _revprom	5'-ACC GCG AGG CAT TAA GAT G-3'
*guaC*_fwdprom	5'-GAG AAT TTC GCA GTC TTG TAC G-3'
*guaC*_revprom	5'-TGT ACG GAT TCC TGG GGT TA-3'

of DNA and incubated on ice for 1 min. DNA and cells were mixed together and transferred to an electroporation cuvette (Cell Technologies, 0.2-cm gap width). The cuvette was placed in the Gene Pulser chamber (Bio-Rad) for an electroshock. One ml of LB broth was added to the cuvette and transferred to a sterile tube and incubated at 37°C with aeration for 1 hour. Ten to 100 µl of the transformation mix were spread on selective plates, where cells containing the plasmid-borne antibiotic resistance marker formed colonies. Cells with no added DNA were treated identically, serving as a control for contamination. Transformants were single colony purified after overnight incubation at 37°C.

2.5 Transduction with bacteriophage P22

All P22 transductions were performed with P22 phage derivative HT105/1 *int*-201. Due to a DNA packaging defect that prevents the virus from recognising its own *pac* sequence, this phage incorporates bacterial rather than viral DNA about 50% of the time. For this reason it can transport bacterial DNA from one *S. enterica* SL1334 strain to another with high efficiency. DNA from strains with deletion mutations in the *dksA* or *topA* genes, or harbouring the C-terminal insertion 3XFLAG-*fis* in the *Salmonella* SL1344 donor strain was packed into the P22 phage heads and then transduced into a *Salmonella* SL1344, a recipient strain with wild type background. In the case of SL1344 *fis::cat*, it was *fis* restored using the *fis::3XFLAG* strain SG07. Here, SG07 was packed into the P22 heads and then transduced into recipient strain mutant *Salmonella* SL1344 *fis::cat*. The *int* mutation contained in each phage prevents the formation of stable lysogens; however, pseudolysogens can arise and these must be identified using green agar plates following the transductant selection step.

2.5.1 Preparation of a P22 lysate

The donor strain was grown overnight in 2 ml of LB broth with the appropriate antibiotic at 37°C in a shaking incubator. This culture was used to inoculate 10 ml of fresh LB broth at a 1:1000 dilution. The culture was incubated at 37°C with constant shaking until it reached an OD_{600nm} of 0.15. This is when 10 µl of P22 phage stock were added to a titre of approximately

10^{10} pfu/ml. Incubation continued for another 4 hours and then 500 µl of chloroform were added. The culture was mixed by vortexing and stored for 1 hour at 4°C. Cellular debris was removed by centrifugation in a bucket centrifuge (ALC PK131R Multispeed Refrigerated centrifuge) at 4,882 X G for 20 min. The lysate contained in the supernatant was transferred to a fresh tube and stored over chloroform.

2.5.2 P22 transduction

Insertions and deletion mutations were transferred using the general transducing properties of P22 lysates as described. The recipient strain was grown overnight at 37°C in 2 ml LB broth. Multiple transductions were set-up with the recipient strain in LB broth and the P22 phage lysate at different concentrations: 1:1, 1:10, 1:100, 1:0 and 0:1 dilutions, respectively. The mixtures were then incubated for 1 hour at 37°C without shaking. Transduced cells were selected by plating onto LB agar plates with the appropriate antibiotic. The plates were incubated at 37°C overnight. Pseudolysogens were distinguished from phage-cured transductants by streaking on green agar plates. Phage-cured colonies are light green, while pseudolysogens are dark blue green. *Salmonella* ferments the excess glucose contained in the green agar producing large amounts of acid. When pseudolysogen colony cells lyse, the pH indicator in the plate causes the agar to turn dark blue-green. Three repeated single colony purifications on green agar plates selected suitable transductants for further experimentation.

2.6 Assays based on spectrophotometry

2.6.1 Monitoring bacterial growth

Optical density measurements were performed at a wavelength of 600 nm (OD_{600nm}) to monitor growth of bacterial cultures. A culture volume ranging from 0.1 to 1 ml was transferred into a plastic disposable cuvette (Sarstedt), 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. Values obtained were linear in the range of 0.1-0.8 and, whenever necessary, each one was multiplied by the dilution factor.

2.6.2 Determination of nucleic acid concentration

Concentration of DNA and RNA samples was determined spectrophotometrically using Nanodrop ND 1000 V3.5.2 (Coleman Technologies, Inc. USA). Absorbance was measured at 260 nm. The sample volume tested was 2 µl and the final concentration was given in nanograms per microliter.

Purity assessments for DNA or RNA were done by measuring the A_{280nm}. For pure DNA, that is free of proteins or residual phenol, the ratio of A_{260nm} to A_{280nm} , is 1.8, whereas for pure RNA, the ratio of A_{260nm} to A_{280nm} is 2 (Sambrook *et al.*, 1989; Han *et al.*, 2010).

2.6.3 Flow cytometry

Expression of the Green Fluorescent Protein (GFP) was measured by flow cytometry. Flow cytometry is an assay that measures certain physical and chemical properties of cells or particles as they travel in suspension one by one as they pass through a sensing point. Light is emitted from a laser at a specific wavelength (488 nm). This emitted light is scattered and then collected by two lenses, positioned at the front and the other at a right angle. This enables the detection of cell size, shape, complexity and any other characteristic, such as mean fluorescence from the GFP by a fluorescent probe.

After incubating for a given number of hours, bacterial cells from all three growth conditions: aerated, non-aerated and anaerobic, were fixed immediately in 2% (v/v) formaldehyde and phosphate buffered saline (PBS). Samples were subsequently stored at 2-4°C to stop cell multiplication and slow down oxygen diffusion. Fis expression levels were analyzed using a Beckman Coulter Epics XL Flow Cytometer (Beckman & Coulter, USA), as previously described (Ó Cróinín & Dorman, 2007). In total, 10 000 bacteria were assayed for each

sample and results were displayed as the mean fluorescence. Analyses were done using the in-built Expo32 ADC software.

2.7 Preparation of plasmid DNA, and chromosomal DNA and RNA

2.7.1 Small-scale isolation of plasmid DNA

The RBC Plasmid Mini prep Kit (Bioscience) was used to extract plasmid DNA from 1.5 ml cultures according to the instructions provided. Cells were harvested at different ODs (0.05 to 2.0) depending on the growth condition and treatment. Bacteria are lysed via a modified alkaline method and proteins inhibited with protease inhibitors. RNA is degraded by addition of RNase, and denaturation of chromosomal and plasmid DNA was achieved by the addition of NaOH. The lysis mixture is neutralized with salts, which precipitates protein and chromosomal DNA. Re-annealed DNA and debris is pelleted by centrifugation. Plasmid DNA is finally eluted as the supernatant is washed and desalted through a mini-column with 100 µl of ddH_2O or elution buffer.

2.7.2 Purification of chromosomal DNA

Chromosomal DNA was purified for PCR using the PUREGENE Genomic DNA Purification kit (QIAGEN, Gentra Systems). A volume of 500 µl of an overnight culture was used for DNA extraction. The cell wall of the bacteria is removed by incubation in a Tris-buffered solution containing lysozyme which cleaves the peptidoglycan component, sucrose to cause osmotic stress, and EDTA to chelate divalent metal ions necessary for protease activity and destabilise the outer membrane by removing divalent cations from the lipopolysaccharide layer. Heating at 80°C in the presence of Sodium Dodecyl Sulphate (SDS) and NaCl denaturates and gives an osmotic shock to the cells, respectively. Also, the mix is supplemented with RNase to complete the lysis by breaking down RNA. Proteins are

denatured by SDS and together with cellular debris are removed by salt precipitation. Finally, the genomic DNA is recovered by precipitation with alcohol (isopropanol) and dissolved in a buffered solution containing a DNA stabilizer. The DNA was safely stored at –20°C.

2.8 Phenol extraction and ethanol precipitation of DNA

After each of the IP test and Input samples (section 2.14) was subjected to cross link reversal (section 2.14.6), DNA was extracted from each one using phenol (SIGMA) and chloroform, and concentrated by ethanol precipitation. A volume of 2 µl of yeast tRNA (5 mg/ml, Invitrogen) was added to each sample (except Input DNA) just before adding 250 µl of phenol (SIGMA) and 250 µl of chloroform. Samples were vortexed and centrifuged (ALC PK131R Multispeed Refrigerated centrifuge) at 15,800 X G for 5 min at room temperature. The aqueous layer (top layer) was carefully removed and placed in a fresh tube. A volume of 500 µl of chloroform was added to each sample. The samples were vortexed and centrifuged centrifuged (ALC PK131R Multispeed Refrigerated centrifuge) at 15,800 X G for 5 min at room temperature. The aqueous layer was again transferred to a fresh microfuge tube. At this point, 5 µg of glycogen (5 mg/ml, Roche), 1 µl of yeast tRNA (5 mg/ml, Invitrogen) (except Input DNA) and 50 µl (20 µl for Input IP) of 3M sodium acetate (pH 5.2) was added to each sample and mixed well. The DNA was precipitated with 1375 µl (550 µl for Input DNA) of 100% ethanol and incubated at -70ºC from 30 min to 1 to 2 hours maximum (or -20°C overnight). The samples were centrifuged (ALC PK131R Multispeed Refrigerated centrifuge) at 15,800 X G for 20 min at 4ºC. The DNA pellets were washed with 500 µl of ice-cold 70% ethanol and air-dried for 10-15 minutes. The DNA pellets of the IP test samples were resuspended in 50 µl of sterile distilled water, and 100 µl for the Input DNA sample. A volume of 5 µl from each sample was run on a 1% agarose 1X TBE (Tris Borate EDTA) gel and visualised with ethidium bromide to check DNA size. Samples were stored at -20°C.

2.9 Polymerase chain reaction

The polymerase chain reaction (PCR) was used to amplify DNA fragments to perform chromosomal mutations, or to carry out real-time quantitative PCR (qPCR) (section 2.9.2) reactions to determine Fis-enriched gene regions in DNA extracted from the IP test samples (section 2.8).

The basis of PCR is a thermostable DNA polymerase capable of amplifying DNA, primed from oligonucleotides annealed to denatured single-stranded templates (Bartlett & Stirling, 2003). The DNA template is denaturated at high temperatures, thereby enabling specific oligonucleotides to hybridize to complementary sequences on opposite strands of the DNA as they flank the sequence to be amplified. DNA polymerase uses the dNTPs provided to anneal the primers from their 3' ends and extend across the region of the original DNA template. As the amplification proceeds, each new strand is complementary to one of the primers, which can be used as a template in the annealing and extension cycles to come. These three steps: denaturation, annealing and extension, are repeated in that order for 25-35 cycles as they amplify exponentially the DNA target region.

2.9.1 Amplification of DNA

Phusion Polymerase ™ (FINNZYMES) was used in this study. Phusion™ DNA polymerase is comprised of a *Pyrococcus*-like enzyme and a processivity enhancing domain. The polymerase generates long templates with high accuracy and speed. The error rate of Phusion™ DNA polymerase in Phusion™ HF Buffer is determined to be 4.4×10^{-7} (as is, FINNZYMES Phusion product information sheet), which is approximately 50-fold lower than that of *Thermus aquaticus* DNA polymerase, and 6-fold lower than that of *Pyrococcus furiosus* DNA polymerase (Frey & Suppmann, 1995). Phusion DNA Polymerase possesses exonuclease (proof-reading) 5′-3′ and 3′-5′ DNA polymerase activity, which resulted in a reduced error rate of nucleotide misincorporation (FINNZYMES Phusion product information sheet).

PCR reactions were performed by mixing 10 µl of 5X High Fidelity (HF) Buffer, 0.2 mM of each dNTP, 10 µM of each oligonucleotide, 1 U of Phusion™ polymerase, 10-100 ng template DNA and sterile ddH_2O to a final volume of 50 µl in a sterile 500 µl PCR tube (Sarstedt). PCR reactions using Phusion™ polymerase were carried out as previously described. Reaction mixes were performed and always kept on ice. Immediately after, they were placed into the Peltier Thermal Cycler. A negative control was set-up by adding no template to one of the reaction tubes. A commonly used reaction cycle was as follows: 1) Initial denaturation was at 98°C for 3 min, 2) denaturation was at 98°C for 10 s, 3) annealing was at 63°C for 30 s, 4) extension was at 72°C for 1 min; 5) steps 2-4 above repeated for 34 cycles; and 6) final extension was at 72°C for 10 min.

Particularly, the annealing temperature (step 3) was often set one degree below the theoretical melting temperature (Tm) of the oligonucleotide being used. Tm's were calculated using the Phusion Primer Biomath calculator. According to Sambrook and Russell (2001), this calculation is based on the formula Tm= 2 x (A+T) + 4 x (G+C) -2, where A, T, G and C refer to the base nucleotide. Extension time (step 4) depended on the length expected for the PCR product (about 1 min per kilobase).

DNA sequences were either amplified from purified chromosomal or plasmid DNA (section 2.7). Occasionally, template DNA to be PCR amplified also came from a cell lysate, where a single colony was taken with a sterile plastic tip and resuspended in 50 µl of sterile ddH_2O and boiled for 3 min. A volume of 2 µl was used for each PCR reaction.

2.9.2 Real-Time quantitative PCR (qPCR)

To further support data generated from the ChIP-chip experiments (section 2.14), Fis enrichment levels were also determined by real-time quantitative PCR (qPCR).

DNA extracted from IP test samples ChIP DNA and control IgG DNA (section 2.8), was used for qPCR to measure the relative Fis enrichment level on promoter and ORF regions of known and potential Fis-binding sites at the *fis,topA, gyrA, gyrB, dksA, relA, spoT, nuoA, fliA, ssrA, orgA* and *guaC* genes. The gene regions used as controls were those belonging to the *topA*

and *fis* genes for positive controls, and to the *guaC* gene for the negative control. This selection was based on preliminary results (section 6.2.3 for *topA* and *fis* genes) and published work on Fis protein binding regions (Cho *et al.,* 2008). Briefly, DNA extracted (section 2.8) from each reverse-crosslinked (section 2.14.6) ChIP DNA and IgG DNA samples and which originated from the Fis FLAG-tagged bacterial strain SG07 grown at each of the four previously described conditions (section 2.2.2), was used separately as a template each (5 µl sample), and were included in a 20 µl reaction volume containing each 10 mM primers, nuclease free H_2O, and SYBR Go Taq® Master Mix (Promega). Amplifications were conducted in a Real Time-qPCR Standard mode 7500, and consisted of an initial denaturation step cycle of 2 min at 50°C, followed by 1 cycle of 10 min at 95°C, then 40 cycles of 15 s at 95°C, and a final extension period of 1 min at 60°C.

Fis enrichments were originally measured as mean quantities. These quantities reflect the amount of enrichment for Fis binding sites for the indicated promoter or ORF gene regions. Mean quantity of Fis enrichment was calculated based on a dilution series using SL1344 (SL1344) wild type genomic DNA as a template with the specific primer for the gene promoter in question. For simplification purposes, only for figure 6.4, Fis enrichment levels were indicated as mean quantities. Mean quantities for all subsequent figures were converted into $\log_2$ ratios using Microsoft Excel®, where IP test sample ChIP DNA $\log_2$ ratio was normalized to the internal control: IP test sample IgG DNA $\log_2$ ratio, in order to subtract background DNA 'noise'. Thus, Fis occupancy in 12 different gene regions was indicated as relative Fis enrichment values. All qPCR reactions were done in duplicates, and two independent biological replicates were prepared for each set of aeration conditions.

2.10 Chromosomal gene mutations by homologous recombination

Chromosomal mutant strains were constructed by homologous recombination using the lambda-red recombination system using plasmid pKD46 (Table 2.2), as previously described (Karlinsey, 2007; Datsenko and Wanner 2000). The λ-red recombination system enables the integration of PCR products to the chromosome. These PCR products must contain 40 bp of DNA sequence homology at both 5' and 3' ends to the region of the chromosome where the insertion is to be made.

2.10.1 Construction of mutant strains

dksA mutant

Oligonucleotides dksA.KO.fwd and dksA.KO.rev (Table 2.3) were designed by pairing pSUB11 epitope-side primers of 20 bp next to 40 bp sequences from the *dksA* ORF. These primers were subsequently used to PCR amplify the *kan* gene from plasmid pSUB11 and then integrated to the SL1344 *S.* Typhimurium chromosome to replace the *dksA* ORF. Strains and clones were confirmed by PCR (section 2.9.1) using primers dksA_fwd and dksA_rev; and by custom DNA sequencing (section 2.1.1).

topA mutant

Oligonucleotides topAko_rightfwd and topAko_rightrev (Table 2.3) were designed by pairing pSUB11 epitope-side primers of 20 bp next to 40 bp sequences from the *topA* open reading frame (ORF). These primers were subsequently used to PCR amplify the *kan* gene from plasmid pSUB11 and then integrated to the SL1344 *S.* Typhimurium chromosome to replace the *topA* ORF. Strains and clones were confirmed by PCR (section 2.9.1) using primers topAko_conf_fwd and topAko_rightrev (Table 2.3), and by custom DNA sequencing (section 2.1.1).

fis::3XFLAG mutant

C-terminal oligonucleotides FWD_Fis_3xFLAG and REV_Fis_3xFLAG (Table 2.3) were designed by pairing pSUB11 epitope-side primers of 20 bp next to the 40-bp. sequences before and after *fis* stop codon TAA. These primers were used to PCR amplify the *fis::3XFLAG::kan* gene from plasmid pSUB11 and then integrated to the SL1344 *S.* Typhimurium SL1344 chromosome to FLAG-tag the *fis* C-terminus end. The strain was FLAG-tagged in order to be detected by the respective antibody in Western Blot and ChIP-chip analyses. Strains and clones were confirmed by PCR (section 2.9.1) using primers ST.Fis.conf-For and FLAG.Fis.conf-Rev (Table 2.3), and by custom DNA sequencing (section 2.1.1). The 3XFLAG® system (SIGMA) fuses 3 tandem FLAG® epitopes for a total of 22 amino acids. Fusion proteins containing 3XFLAG® are better detected, up to 200 times more than any other system. The 3XFLAG® is hydrophilic, contains an enterokinase cleavage site, and is relatively small.

2.11 Gel Electrophoresis

2.11.1 Agarose gel electrophoresis

DNA was analyzed using 1% (w/v) agarose gels, which were prepared as follows: 1 g agarose added to 100 ml of TAE (40 mM Tris, 1 mM EDTA, 0.114% (v/v) glacial acetic acid, pH 3) was heated to 100°C. A final concentration of 1 µg/ml of ethidium bromide was added to the gel mixture once it cooled for a few minutes. Ethidium bromide is added as it intercalates DNA, strongly fluorescing in UV light. A volume of 1 µl loading dye Blue/Orange 6X (Promega) was added to each 5-µl sample. Also, 4 µl of a 1-kb DNA ladder (Promega) was run in a separate well and along every set of samples for size reference. Electrophoresis proceeded at 100V in TAE buffer for 45 min. DNA bands from separated samples were visualized under UV light.

2.11.2 SDS-PAGE

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) is a technique used to separate proteins according to their electrophoretic mobility, dictated by the length of the polypeptide chain or molecular weight (Laemmli, 1970). As proteins migrate fast along large pores in the stacking gel (5% acrylamide) they concentrate into narrow bands. The narrow pores of the separating or running gel (12% acrylamide) enable proteins to migrate according to their mass. Migration is facilitated by buffers of different pH in the stacking and running gels. SDS is added to 0.1% (w/v) in both gels. This is important as proteins bind to SDS according to length, giving almost identical charge densities for denatured proteins. Thus, migration occurs according to mass (Sambrook et al., 1989).

Running gels were prepared as previously described (Sambrook et al., 1989). Once these gels were poured into the electrophoretic chamber, 1 ml of isopropanol was overlaid onto the gel which was then allowed to polymerize for 1 h. The isopropanol was then drained and the stacking gel loaded. The 5% stacking gel was made by mixing 2.812 ml ddH_2O, 1.25 ml 0.5 M Tris-HCl (pH A.1), 50 µl 10% w/v SDS, 0.833 ml Protogel® (National Diagnostics)

acrylamide, 5 µl Tetramethylethylenediamine (Temed), and 50 µl 2% w/v ammonium persulphate (APS). Running buffer (25 mM Tris-HCl, 250 mM glycine (pH 8.3), 0.1% (w/v) SDS) was used as the gel was electrophoresed. Protein pellets were boiled in 2X Laemmli buffer (Laemmli, 1970) for 5 min before loading. The loaded gel was run at 100 V for 1.5 h.

2.12 Western immunoblot analysis

2.12.1 Preparation of total cellular extracts

A fraction of sample from every time point and aeration regime was saved for western blot analysis. Overnight samples were used in place of the zero-hour (0 h), as cells could not be harvest to sufficient numbers for western blot analysis at this 0-h time point. Protein pellets were boiled in 2X Laemmli buffer for 5 min and separated by SDS-PAGE (section 2.11.2) according to the method of Laemmli (Laemmli, 1970).

2.12.2 Transfer of proteins to nitrocellulose membrane

Separated protein was electrophoretically transferred to a 0.2-µm Millipore nitrocelloluse membrane using a BIO-RAD Mini-Protean Electrophoresis transfer cell system. This transfer cell was filled with transfer buffer (25 mM Tris, 192 mM glycine, 0.02% (w/v) SDS, 20% (v/v) methanol), at 90 V for 1 hour at 4°C.

2.12.3 Detection of bound antigens

Membranes were subsequently washed in 1X PBS (phosphate buffered saline), and incubated in blocking buffer (1X PBS plus 4% w/v Marvel® dried skim milk) with the monoclonal immune serum Anti-Green Fluorescent Protein (Anti-GFPuv, R&D, UK) or, Anti-3XFLAG Protein (Anti-3XFLAG, SIGMA, UK), and Anti-DnaK (BioLabs, USA) as a loading control (both diluted to 1/1000 in blocking buffer). Finally, the membranes were incubated with goat

anti-mouse immunoglobulin G conjugated to horse radish peroxidase (Chemicon, UK) (diluted to 1/10 000 in blocking buffer) and subsequently saturated in Luminol/Enhancer and stable peroxide solutions (Pierce, USA). Membranes were briefly exposed to Kodak film then developed, fixed and dried for later observation, sometimes using the ImageJ® package (National Institute of Health, USA) densitometry analysis where indicated.

2.13 Chloroquine gel electrophoresis

The effect of DNA supercoiling on *fis* gene expression was observed by using DNA gyrase-inhibiting antibiotics or aminocoumarins: novobiocin or coumermycin (CA1) at certain concentrations. Previous and current research in this laboratory have indicated that N at the final concentration of 50 μg/ml begins to affect DNA gyrase activity, specifically at the GyrB subunit, and decreases Fis expression as well as DNA supercoiling (Ó Cróinín et al., 2006). CA1 was used to corroborate the effect of DNA gyrase inhibition by N on Fis expression. Like novobiocin, CA1 inhibits DNA gyrase at the B subunit by blocking ATPase activity (Jovanovich & Lebowitz, 1987). The effects of these antibiotics caused topological shifts in reporter plasmid DNA. The resulting plasmid topoisomer distribution was assessed by the chloroquine gel electrophoresis method (Higgins et al., 1988).

The plasmid pUC18 was used as a reporter of DNA supercoiling in choloroquine agarose gel electrophoresis (section 2.3.1.2). pUC18 topoisomers were extracted from treated and non-treated samples at exponential and stationary phases. Plasmid was extracted using an RBC plasmid prep kit (section 2.7.1). Cells were harvested at different ODs (0.05 to 2.0) depending on the growth condition and treatment. Resuspension of cells was done with a RNase-containing buffer as previously described (RBC® mini prep kit information sheet; Birnboim and Doly, 1979) prior to cell lysis. Cells were subsequently neutralized, their DNA bound to special-filter columns, then washed and finally eluted in elution buffer. Sample cultures were grown under aerated or non-aerated (i.e. SPI-1 inducing) conditions. Topoisomers of the extracted plasmid DNA were then separated on a 1% agarose gels containing 2.5 μg/ml chloroquine. This is because at that concentration, an equal chloroquine amount is taken by each DNA molecule. Individual topoisomers are intercalated by the drug, resulting in negatively supercoiled molecules staying supercoiled while relaxed molecules

remain more relaxed. Under these conditions, the more negatively supercoiled topoisomers ran furthest into the gel. Samples were electrophoresed for 16 hours at 100V in 2X TBE which contained the same concentration of chloroquine as the gel. Staining of the gel was done with ethidium bromide at a concentration of 5 μg/ml and visualization of separated topoisomers by UV light. ImageJ® package (National Institute of Health, USA) densitometry analysis was used to determine accurately the location of the dominant topoisomer band in each lane in the gel.

2.14 Chromatin immunoprecipitation and microarray technology (ChIP-chip)

Constructed strain SG07 *fis::3XFLAG::kan* (section 2.10.1) was used for this set of experiments to identify peaks of Fis-binding on gene regions that are occupied by Fis. The chromatin immunoprecipitation on a chip (ChIP-chip) microarray technology was used to assess this portion of the research investigation.

Bacterial cells were grown under aerated and non-aerated conditions, and harvested from both exponential/mid-log (2 h) and late-stationary phases (24 h). All culture samples were normalized to a total OD A_{600} of 10, as culture samples replicates from each growth phase and condition were combined to result in that total OD. Subsequently, cells were fixed, lysed and sonicated (sections 2.14.3., 2.14.2 and 2.14.3, respectively). At this point, sheared chromatin (Ch) DNA from all culture samples was immunoprecipitated (IP), in which case it was either added IgG antibody or 3XFLAG antibody. DNA carrying 3XFLAG antibody was named ChIP DNA, while DNA carrying IgG antibody was named IgG DNA and in some instances they were also called IP test samples. The DNA without either antibody, but subsequently frozen at -80°C, was used later for the chip microarray portion as an internal DNA control, and which was labeled input or mock IP DNA. DNA from IP test samples was eluted (section 2.14.5), cross-link reversed (2.14.6) like the input or mock IP DNA, and all IP DNA samples were then each subsequently extracted (section 2.8), in preparation for either qPCR (section 2.9.2) (only IP test samples) or labelling for array hybridization (section 2.14.7) (except IgG DNA), for the chip microarray experiments.

2.14.1 Fixation

Cells were collected by centrifuging (ALC PK131R Multispeed Refrigerated centrifuge) at 4,882 X G for 8 min at room temperature and re-suspended in 50 ml of pre-warmed PBS (37°C) in a 250-ml glass flask. DNA-protein and protein-protein interactions were cross-linked by adding formaldehyde (37%). A volume of 1355 µl formaldehyde was added drop-wise to a final concentration of 1%. The cross-linking was carried out at room temperature with constant but gentle stirring for 30 min. A volume of 3.425 ml ice-cold 2M glycine was added to a final concentration of 0.125M with constant but gentle stirring for 5 min at room temperature to stop the cross-linking reaction. Cells were transferred to 50-ml Falcon® conical tubes and kept on ice whenever possible. The cells were pelleted by centrifuging (ALC PK131R Multispeed Refrigerated centrifuge) at 4,882 X G for 8 min at 4°C.

2.14.2 Cell lysis

After removing the supernatant, the cells were re-suspended in 0.6 ml of lysis buffer (50 mM Tris-HCl (pH 8.1), 10 mM EDTA, 1% w/v SDS, and protease inhibitor tablet® (SIGMA) and incubating on ice for 10 minutes.

2.14.3 Sonication

A volume of 1.4 ml of IP dilution buffer (IPDB) (20 mM Tris-HCl [pH 8.1], 150 mM NaCl, 2 mM EDTA, 1% v/v Triton X-100, 0.01% w/v SDS and protease inhibitor tablet® was added and the samples were transferred to 5-ml glass Falcon® tubes. The chromatin was sonicated to reduce the DNA length to an average size of approximately 500-bp using the Sanyo/MES Soniprep sonicator. The tip of the probe was dipped to reach approximately halfway down the total level of the liquid sample and the tube was kept constantly on ice (conditions for sonication like number of bursts, length of bursts and power setting depend on the sonicator tip used). The settings used for the sonicator were for amplitude, 10 microns; for number of bursts, 8; for length of bursts, 30 seconds.

The samples were allowed to cool on ice for 1 minute between each pulse (5 µl of the sheared chromatin was run on an agarose gel to check sonication). The sonicated chromatin was transferred to 2-ml microfuge tubes and spun down (Eppendorf centrifuge 5415R) at 15,682 X G for 10 min at 4°C.

2.14.4 Chromatin Immuno-Precipitation (ChIP)

The supernatant was transferred to a 15-ml Falcon® tube and 1.0 ml of IPDB was added (LB:IPDB ratio is 1:4). The chromatin was pre-cleared by adding 50 µl of 1 mg/mL normal rabbit IgG (Millipore). The samples were incubated for 1 hour at 4°C on a rotating wheel. A volume of 100 µl of homogeneous protein G-agarose suspension (Roche) was added to the precleared chromatin and the samples were incubated for 3-5 hours at 4°C on a rotating wheel. The samples were centrifuged (Eppendorf centrifuge 5415R) at 5,219 X G for 2 min at 4°C to pellet the protein G-agarose beads and the supernatant was used to set up various immunoprecipitation (IP) conditions in 2-ml microfuge tubes. A sample of 200 µl of chromatin was stored at -20°C to be used as input sample for array hybridisations. Experimental and control ChIP conditions were set up as follows: IgG control: 1350 µl of chromatin + 10 µg mouse specific IgG antibody (Millipore); while for ChIP conditions: 1350 µl chromatin + 10 µg of FLAG-tag mouse monoclonal antibody (SIGMA).

The samples were incubated at 4°C overnight on a rotating wheel. The samples were centrifuged (Eppendorf centrifuge 5415R) at 15,682 X G for 5 min at 4°C and the samples were transferred to fresh 2 ml microfuge tubes. A volume of 50 µl of homogeneous protein G-agarose suspension was added to each sample and the samples were incubated at 4°C for at least 3 hours on a rotating wheel. The samples were centrifuged (Eppendorf centrifuge 5415R) at 5,219 X G for 2 min at 4°C to pellet the protein G-agarose beads. The supernatant was removed and the protein G-agarose beads were carefully washed. For each wash, the wash buffer was added, the samples were vortexed briefly, were centrifuged (Eppendorf centrifuge 5415R) at 5,219 X G for 2 min at 4°C and left to stand on ice for 1 min before removing the supernatant. Washes were performed as follows: the beads were washed twice with 750 µl of cold IP wash buffer 1 (20 mM Tris-HCl (pH 8.1), 50 mM NaCl, 2 mM EDTA, 1% v/v Triton

X-100 and 1% w/v SDS) and then transferred to a 1.5 ml microfuge tube after the first wash. Second, the beads were washed once with 750 µl of cold IP wash buffer 2 (20 mM Tris-HCl (pH 8.1), 250 mM LiCl, 1 mM EDTA, 1% v/v NP-40 and 1% v/v deoxycholic acid). And third, the beads were washed twice with 750 µl of cold TE (10 mM Tris Base (pH 8.0) and 1 mM EDTA).

2.14.5 Elution of DNA

DNA-protein-antibody complexes were eluted from the protein G-agarose beads by adding 225 µl of IP elution buffer (IPEB) (100 mM $NaHCO_3$, and 1% w/v SDS). The bead pellets were resuspended in IPEB, briefly vortexed and centrifuged (Eppendorf centrifuge 5415R) at 5,219 X G for 2 min at room temperature. The supernatant was collected in fresh 1.5-ml microfuge tubes. The bead pellets in the original tubes were resuspended in 225 µl of IPEB again, briefly vortexed and centrifuged (Eppendorf centrifuge 5415R) at 5,219 X G for 2 min.

2.14.6 Reversal of cross-links

The reversal of cross-links step was carried out on the Input sample which was stored at -20°C previously. Then, 1 µg/µl of RNase A (Promega) and 0.3 M NaCl was added to the Input DNA sample. Similarly, 1 µg/µl of RNase A (Promega) and 0.3 M NaCl were added to each of the IP test samples. All three IP samples, including the Input DNA sample were incubated at 65°C for 6 hours to reverse the cross-links. A volume of 9 µl of Proteinase K (10 mg/ml, Promega) was added to each sample and incubated at 45°C overnight.

At this point, the DNA from the Input DNA and from IP test samples DNA (IP test sample that was originally either complexed with Anti-FLAG antibody, named ChIP DNA hereafter, or IgG antibody, named IgG DNA hereafter), has been cross-link reversed and extracted (section 2.8). This extracted DNA from each IP sample was either used for qPCR (section 2.9.2) (except Input DNA) or, labelled in preparation for array hybridizations for the chip microarray experiments (section 2.14.7) (except IgG DNA).

2.14.7 Labelling by random priming of DNA samples

The DNA was amplified and labelled using BioPrime Random Labelling Kit (Invitrogen) as described below.

2.14.7.1 Labelling method used for array hybridization

The reagents, 2.5 X random primer solution, ChIP DNA, or Input DNA, and sterile H_2O were mixed on ice in a microfuge tube. The DNA amount labelled was different for Input and ChIP DNA samples. 40 µl of unamplified ChIP DNA and approximately 200 ng of Input DNA were labelled. This mixture was heated at 100°C for 10 min to denature the DNA and then chilled briefly on ice. The following reagents were added to the tubes on ice: 10 X dNTP mix, 1 mM Cy3/Cy5 labelled dCTP (1 mM Cy3-dCTP, 1 mM Cy5-dCTP, GE Healthcare), (Input IP mock DNA samples were labelled with Cy5 (blue) dCTP, and ChIP DNA samples were labelled with Cy3 (red) dCTP), and 40 U/µl of Klenow fragment. The final volume per labelling reaction was 150 µl. The reagents were mixed gently but thoroughly and incubated at 37°C overnight. 5 µl of stop buffer (10 mM EDTA) was added to the reaction mix to terminate the reaction.

2.14.7.2 Purification of labelled DNA samples

Labelled DNA samples were purified as follows. Micro-spin G50 columns (GE Healthcare) were used to remove the unlabelled nucleotides from the labelled DNA samples. Three columns were used for each of the labelling reactions. The resin was resuspended in the columns by vortexing gently. The caps were loosened and the bottom of the tubes snapped off. The columns were placed in 2 ml microfuge tubes and centrifuged (Eppendorf centrifuge 5415R) at 1,500 X G for 1 min. A volume of 50 µl of sterile filtered HPLC water was applied to the resin-bed and the columns were centrifuged (Eppendorf centrifuge 5415R) at 1,500 X G for 1 min. The columns were placed in fresh 1-ml microfuge tubes and the labelled DNA samples were carefully applied to the resin-bed. The columns were then centrifuged

(Eppendorf centrifuge 5415R) at 15,682 X G for 1 min. The purified DNA samples were collected in the 1.5-ml microfuge tubes and the samples (Input + ChIP) from the same labelled reaction were pooled together. The final volume for the labelled DNA samples was approximately 180 µl. A volume of 5 µl of each labelled DNA was analyzed on a 1% agarose 1 X TBE gel and stained with ethidium bromide for visualization of smeared DNA. One tenth of the sample volume of 3M sodium acetate was added to precipitate DNA. Two and a half volumes of 100% EtOH were added to each sample. Samples were subsequently incubated at –80°C in the dark for 1-3 h or, at –20°C overnight. DNA was pelleted after spinning (Eppendorf centrifuge 5415R) at 15,682 X G in the dark for 20 min and at RT. Alcohol was drained and pellet resuspended in 80% EtOH. The tubes were allowed to air-dry for 15-30 min in a dark place. Pellet was resuspended in 100 µl hybridization buffer (1 M Sodium chloride, 50 mM MES (sodium salt SIGMA) (pH 6.5), 20 mM EDTA, 20% w/v formamide (SIGMA), and 1% v/v Triton X100). Labelled DNA was subsequently heated at 70°C for 10 min and denatured at 100°C for another 10 min. It was briefly placed on ice prior to hybridization.

2.14.8 Microarray Hybridizations

The entire volume of each labelled DNA sample was carefully pipetted over each of the four gaskets in a microarray glass slide. Fis-binding regions were identified with whole-genome microarray glass slide (Oxford Gene Technologies) that contained 21460 60-mer oligonucleotide probes which tiled the SL1344 chromosome and pSLT plasmid at an average spacing of 210 bp. This provided us with a genome-wide map of Fis interactions with the host chromosome. The order of samples, in each glass slide and from top to bottom, was as follows: aerated mid-log, aerated late-stationary phase, non-aerated mid-log, and non-aerated late-stationary phase. For orientation reference, the slide barcode was always at bottom. Subsequently, the microarray slide was placed carefully on top of the loaded gaskets slide, avoiding any bubble formation and with its reference barcode in the same orientation. Both slides were kept against each other for adequate hybridization in a tight metallic holder. Slides were incubated in the hybridization oven (Shel Lab, Hybridizer) at 55°C overnight (24 h).

2.14.9 Genome-wide chip microarray scanning (chip) and data handling

The hybridized microarray slide was separated from the gasket slide and washed twice in buffer 1 (10X Tween, 10% v/v Tween and sterile ddH_2O), and twice again in buffer 2 (20X PE (SIGMA), 100% Polyethylene Glycol (PEG) (Fluka) and sterile ddH_2O). The microarray slide was blot dried from the corners with a regular paper towel prior to scan reading. The slide was inserted side up into a microarray scanner (Axon) array. In order to view the microarray, each oligonucleotide on an OGT services array is assigned a unique feature number. The identity of the feature number is given by a CD-ROM with GenePix Array List (GAL) files. These files enable to get chromosome coordinates for the microarray data. The Chromatin ImmunoPrecipitation On Tiled arrays (ChIPOTle) peak-finding algorithm (Buck et al., 2005) was used to identify sites of significant Fis enrichment in the microarray data (P value < 0.00001, corrected P value < 0.05). The peak height of the identified Fis-binding peak was indicated and calculated as the $\log_2$ Fis enrichment ratio from Cy3 (ChIP DNA) over Cy5 (Input DNA) signal intensity of the probe corresponding to the identified peak. Occupied gene regions targeted by ChIPOTle were searched in the SL1344 genome browser from the Bioinformatics and Genetics website of Trinity College (http://bioinf.gen.tcd.ie/jbrowse/SL1344/) to translate SL1344 chromosome coordinates into STM coded genes. The function of STM coded genes were identified in the link to the LT-2 sequence gene function (http://www.ncbi.nlm.nih.gov/nuccore/16763390). Data obtained was uploaded and submitted to the Gene Expression Omnibus website. The accession number is GSE38661.

Chapter 3

Patterns of *fis* gene expression in cultures of *Salmonella* Typhimurium grown under different aeration regimes

3.1 Introduction

The goal of this part of the work was to examine *fis* gene expression patterns in *S.* Typhimurium wild type growing under aerated and non-aerated conditions, and under completely anaerobic growth conditions.

In one study, an epithelial cell component was proposed to provide the environmental cue that induced bacterial attachment and entry into the host cell (Finlay et al., 1989). This led another investigation to show that the *S.* Typhimurium *oxrA* gene that is required for the anaerobic induction of many proteins is not involved in the regulation of *Salmonella* invasiveness, but instead, it is oxygen limitation or non-aerated conditions that might be an environmental cue that triggers the expression of *Salmonella* invasiveness within the intestinal lumen and other tissues (Jones & Falkow, 1990). In fact, that same research group later reported that growth of *S.* Typhimurium in a low-oxygen environment induces the ability of these bacteria to enter mammalian cells (Jones & Falkow, 1994). Similarly, work on *Shigella flexneri* has shown that there is a zone of low aeration adjacent to the intestinal mucosa generated by diffusion from the capillary network at the tips of villi, therefore reversing the anaerobic mediated repression of the FNR (fumarate and nitrate reduction) regulator on the type III secretion system responsible for invasion and virulence (Marteyn *et al.,* 2010).

These findings point out that the microaerobic environment reigning in the brush border of the gut villi has played a crucial role in creating a niche for *Salmonella* transition from invasiveness to living intracellularly inside macrophages (Jones & Falkow, 1996; Rychlik & Barrow, 2005). It has been suggested that microaerobic conditions induce *S.* Typhimurium virulence (Lee & Falkow, 1990; Ó Cróinín *et al.*, 2007). Other factors like osmolarity have been shown also to affect virulence (Lee & Falkow, 1990). In this chapter, I show the extent to which aeration regime affects the transcription of the global regulator Fis as it has been found to be a key regulator of the virulent phenotype in *S.* Typhimurium (Adkins *et al.,* 2006; Kelly *et al.*, 2004; Ó Cróinín *et al.*, 2007; Nagy *et al.,* 2006). In chapter 5, I will report and discuss the influence of osmolarity on *fis* gene expression. Culture samples cited in that chapter were grown in low salt medium and under the same series of aeration regimes.

As mentioned earlier (section 1.2), *fis* gene expression peaks during early exponential phase and then decreases in early stationary phase (Osuna *et al.*, 1995; Keane & Dorman, 2003), as well as previous research done in this laboratory showed that in the absence of aeration the expression of the Fis protein in *S.* Typhimurium is sustained during the stationary phase of growth (Ó Cróinín *et al.*, 2007), where the protein might have been expected to be absent (Osuna *et al.*, 1995; Keane & Dorman, 2003). This has been underlined particularly important because Fis is involved in controlling the transcription of genes in both the *Salmonella* Pathogenicity Island 1 (SPI-1) and Pathogenicity Island 2 (SPI-2) invasion gene expression (Cameron *et al.,* 2011; Kelly *et al.,* 2004; Wilson *et al.,* 2001; Waterman and Holden, 2003). SPI-1 genes are induced in bacteria grown in low aeration into stationary phase prior to infection of epithelial cells (Ó Cróinín and Dorman, 2007). A major function of the SPI-1 genes is to enable bacterial invasion of epithelial cells and the principal role of SPI-2 genes is to facilitate the replication of intracellular bacteria (Waterman & Holden, 2003). Interestingly enough, the non-aerated conditions used to maintain *fis* transcription beyond its shut-off point are similar to those that induce maximum virulence in *S.* Typhimurium (Ó Cróinín and Dorman, 2007).

In this chapter, I report how changes in aeration regimes influence *fis* gene expression patterns.

3.2 Results

3.2.1 The promoterless *gfp* reporter gene in plasmid pZEP08 is transcriptionally silent

Throughout this investigation, *fis* promoter activity in the pZep*fis-gfp* recombinant plasmid was measured by flow cytometry (section 2.6.3) where the amount of GFP fluorescence that the plasmid expressed at a given time point was measured. Before embarking on work with the *fis-gfp* fusion, the level of background fluorescence from the pZEP08 vector was monitored. In order to verify absence of background fluorescence, a set of experiments were performed with a strain carrying promoterless plasmid pZEP08, in both aerated and non-aerated growth conditions. This promoterless plasmid was termed pZEC09 (Table 2.2). *S.*

Typhimurium wild type strain SL1344 carrying pZEC09 displayed no *gfp* expression levels throughout the growth cycle whether cultures were aerated or not (Fig. 3.1A & 3.1B).

These results showed that the promoterless vector/reporter plasmid pZEC09 does not emit background fluorescence, increasing confidence that fluorescence from the derivative containing the *fis* promoter (section 3.2.2) was reporting exclusively levels of *fis* transcription.

3.2.2 Non-Aerated growth conditions induce sustained expression of the *fis* gene during the stationary phase of growth

Published results from this laboratory indicated that *S.* Typhimurium SL1344 pZep*fis-gfp* grown under non-aerated conditions (SPI-1 inducing) displayed a sustained expression of the *fis-gfp* transcriptional fusion during the stationary phase of growth (Ó Cróinín & Dorman, 2007), where the *fis* gene had previously been throught to be silent (Keane & Dorman, 2003; Osuna *et al.*, 1995). These experiments were repeated to ensure consistency of methodology and familiarity with *S.* Typhimurium handling techniques. The strain SL1344 carrying reporter plasmid pZep*fis-gfp* (Table 2.2) was grown under aerated and non-aerated conditions, and *fis-gfp* expression levels were monitored.

Results (Fig. 3.2A & 3.2B) confirmed the published data (Ó Cróinín & Dorman, 2007). Expression levels of the *fis-gfp* fusion peaked during log phase for both growth conditions, whereas during the stationary phase of growth levels decreased gradually while aerated, but remained sustained under non-aerated conditions (Fig. 3.2A & 3.2B). These results corroborated earlier findings that non-aerated growth conditions induce sustained expression of Fis during the stationary phase of growth.

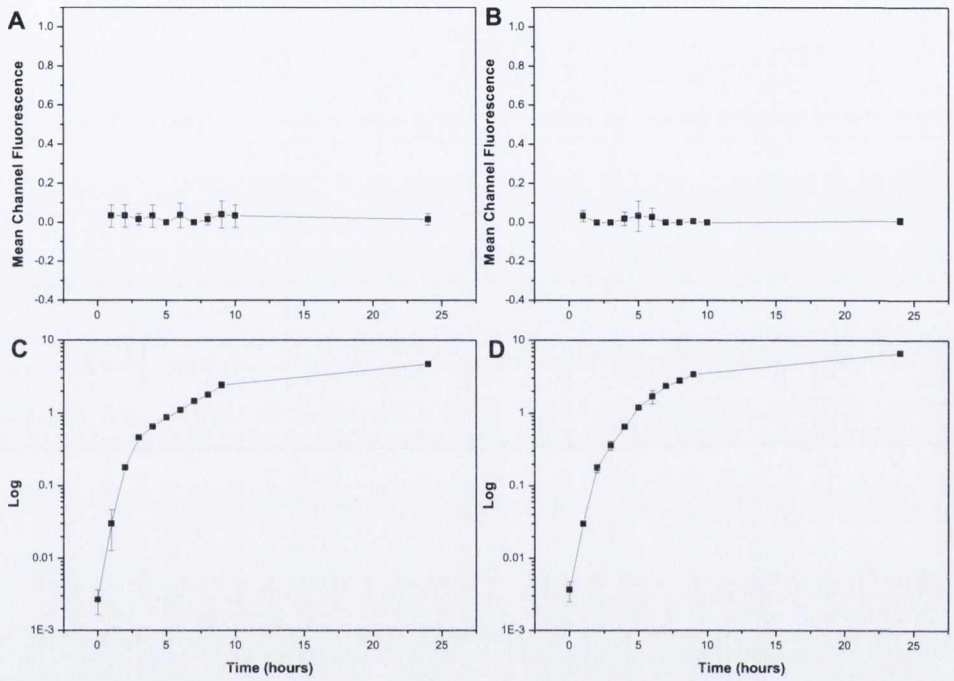


Fig. 3.1: Levels of *gfp* expression and growth curves of wild type strain SL1344 carrying pZEC09. A) *gfp* expression in sample cultures grown under non-aerated conditions; B) *gfp* expression in sample cultures grown under aerated conditions; C) growth curve of sample cultures grown under non-aerated conditions; and D) growth curve of sample cultures grown under aerated conditions. Promoterless plasmid pZEC09 did not display background fluorescence in either condition. Error bars represent standard deviations (n=3).

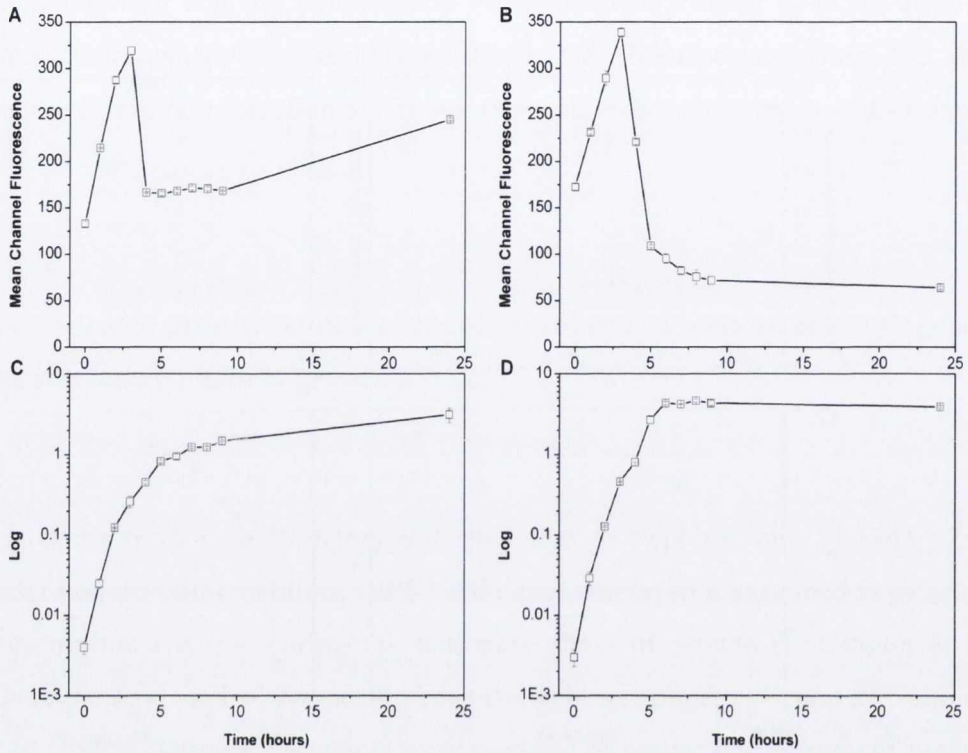


Fig. 3.2: Levels of *fis-gfp* expression and growth curves of wild type SL1344 pZep*fis-gfp*. Cultures were incubated under different conditions. A) *fis-gfp* expression of sample cultures grown under non-aerated conditions; B) *fis-gfp* expression of sample cultures grown under aerated conditions; C) growth curve of sample cultures grown under non-aerated conditions; D) growth curve of sample cultures grown under aerated conditions. For both panels, *fis-gfp* expression levels peaked during log phase in both growth conditions, whereas during the stationary phase of growth levels *fis-gfp* transcription decreased gradually while aerated, but remained sustained under non-aerated conditions. Error bars represent standard deviations (n=3).

3.2.3 Anaerobiosis does not result in the sustained transcription of *fis* during stationary phase

S. Typhimurium strain SL1344 was also cultured under fully anaerobic conditions in order to examine the possibility that a total absence of aeration might play a role in the sustained expression of the *fis* gene (Fig. 3.3). Since non-aerated growth conditions (or "SPI-1 inducing" conditions) have been shown to sustain *fis-gfp* expression (Fig. 3.2B), it was possible that strict anaerobic conditions could increase/promote sustained *fis* expression. SL1344 carrying pZep*fis-gfp* (Table 2.2) was grown under anaerobic, aerated and non-aerated conditions. Also, SL1344 carrying pZep*rpsM-gfp* (Table 2.2) was used as a heterologous gene control only under anaerobic conditions. The *rpsM* gene had been employed as a heterologous gene control in an earlier study of variations in *fis* transcription as a function of aeration (Ó Cróinín & Dorman, 2007). The product of the *rpsM* gene is a ribosomal protein produced continuosly in *Salmonella* throughout its growth cycle (Bylund *et al.,* 1997). The transcription of the *rpsM* gene was monitored in the SL1344 background and was found to be unaffected in aerated or non-aerated conditions during stationary phase (Ó Cróinín & Dorman, 2007).

Both strains SL1344 pZep*fis-gfp* (Fig. 3.3B) and SL1344 pZep*rpsM-gfp* (Fig. 3.3A) grown in anaerobic conditions showed a decrease in expression levels in both *fis* and *rpsM*, respectively, upon entry into stationary phase. These results differed from those showing the *fis-gfp* expression patterns in non-aerated and aerated conditions (Fig. 3.2), where *fis-gfp* expression levels peaked during log phase and were sustained during the stationary phase of growth while non-aerated (Fig. 3.2 A) but, decreased gradually while aerated (Fig. 3.2B).

As mentioned earlier (section 2.3.2), GFP requires oxygen for its fluorophore to fluoresce (Reid & Flynn, 1997). This makes monitoring of GFP activity problematic under conditions of anaerobic growth. It is possible to recover GFP activity by re-exposing the culture to atmospheric oxygen for about 1 hour following the anaerobic growth regime. This technique has been termed aerobic fluorescence recovery (AFR) (Zhang & Xing 2008). However, AFR was not applicable to our anaerobic study, since *fis-gfp* expression levels had to be directly measured from samples grown in strict anaerobic conditions. AFR would have interfered with

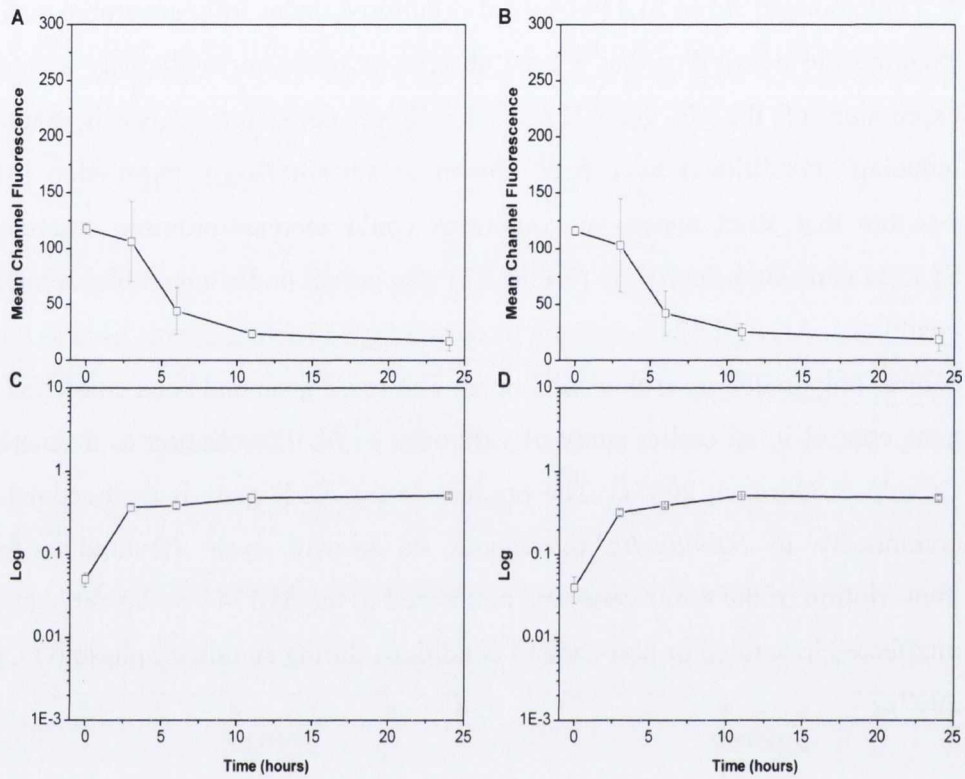


Fig. 3.3: Levels of *fis* expression as compared to *rpsM* expression. Flow-cytometry measurements of *fis-gfp* or *rpsM-gfp* expression levels and growth curves of strain SL1344 carrying pZep*fis-gfp* or pZep*rpsM-gfp* culture samples incubated under strict anaerobic growth conditions. A) *rpsM-gfp* expression; B) *fis-gfp* expression; C) growth curve of SL1344 pZep*rpsM-gfp* ; D) growth curve of SL1344 pZep*fis-gfp*. Both *fis-gfp* and *rpsM-gfp* expression in anaerobic conditions indicate a decrease of *gfp* fluorescence upon entry into stationary phase. Error bars represent standard deviations (n=2).

the very phenomenon that was being examined. Instead, western blot analysis was used to determine if the amount of GFP expression (from the *fis-gfp* fusion) present in the anaerobic samples (Fig. 3.4) corresponded to the *fis-gfp* expression levels detected by the flow cytometry measurements (Fig. 3.2 & 3.3)

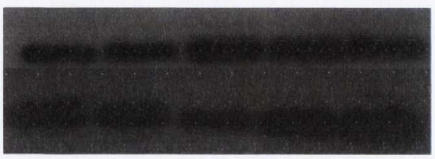
GFP expression levels were detected by western blotting and densitometry analyses. Strain SL1344 carrying pZep*fis-gfp* was incubated under aerated, non-aerated or anaerobic growth conditions and a fraction of the sample was analyzed by western blotting. Resulting protein bands indicated GFP protein expression from the *fis-gfp* fusion from culture samples grown in each aeration regime. Constitutive protein DnaK protein was used as a loading control for each western blot performed from every condition. Western blot bands from the GFP and DnaK proteins are shown for all three growth conditions (Fig. 3.4A-3.4C), and densitometry analyses (ImageJ®) of each protein band intensities allowed the GFP/DnaK ratios to be calculated. These ratios were then plotted as a function of time (Fig. 3.4D-3.4F).

Results showed *fis-gfp* fluorescence to decrease upon entry into stationary phase for cultures grown under aerated (Fig. 3.2B) and anaerobic regimes (Figs. 3.3A, 3.4C & 3.4F). Culture samples grown under non-aerated conditions clearly showed a sustained expression of the *fis-gfp* fusion during the stationary phase (Fig. 3.2A). Furthermore, western blot data showed that GFP expression levels behaved as expected under non-aerated and aerated conditions (Figs. 3.4A & B, respectively) because their corresponding band intensity ratio patterns (Figs. 3.4D & 3.4E, respectively) were very similar to *fis-gfp* expression levels measured by flow-cytometry (Fig. 3.2A & 3.2B, respectively). Samples grown in anaerobic conditions displayed GFP expression patterns in western blots with a virtually unchanged band intensity pattern for GFP expression for every time point along the growth curve (Fig. 3.4C & F). This may suggest sustained expression of the *fis* gene. The anaerobic GFP ratio expression pattern (Fig. 3.4F) was similar to that of aerated cultures (Fig. 3.4E), but it also remained well above the aerated samples all along the stationary phase of growth.

The GFP ratio expression for the last time point (27 h) in anaerobic conditions (Fig. 3.4F), was not lower than the first time point (0 h or o/n) (Fig. 3.4F), as occurred in the aerated culture samples graph (Fig. 3.4E). Western blots from the non-aerated and aerated (Figs. 3.4A & 3.4B) together with their corresponding GFP band expression area ratio (Figs. 3.4D &

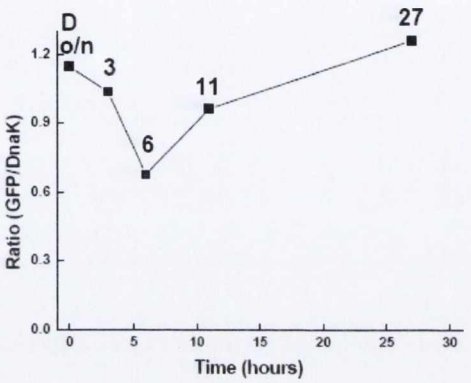
Fig. 3.4: GFP expression levels as detected by western blotting and densitometry analyses. SL1344 carrying pZep*fis-gfp* was incubated under different growth conditions, as indicated, and a fraction of the sample was analyzed by western blotting. Resulting protein bands came from: A) GFP protein expression from *fis-gfp* under non-aerated conditions; B) GFP protein expression from *fis-gfp* under aerated conditions; and C) GFP protein expression from *fis-gfp* under anaerobic conditions. The protein band profile of the o/n sample in panel C) was moved from the right to the left side to ensure consistency with the densitometry analysis ratio graph in panel F). The DnaK protein was used as a loading control. Densitometry of protein band intensities allowed the GFP/DnaK ratios to be calculated. These ratios were then plotted as a function of time: D) ratio of expression under non-aerated conditions; E) ratio of expression under aerated conditions; and F) ratio of expression under anaerobic conditions. Western blots and corresponding densitometry analysis ratios were performed in two separate occasions and typical data are shown.

A Non-Aerated



o/n 3 6 11 27

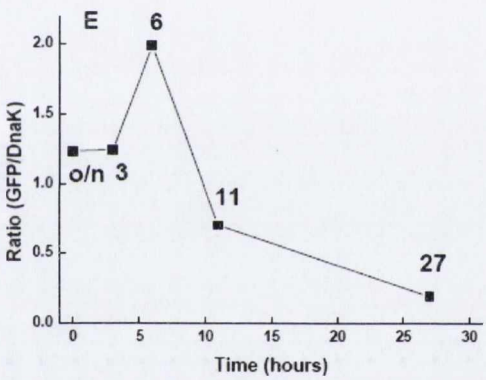
Time (hours)



B Aerated

o/n 3 6 11 27

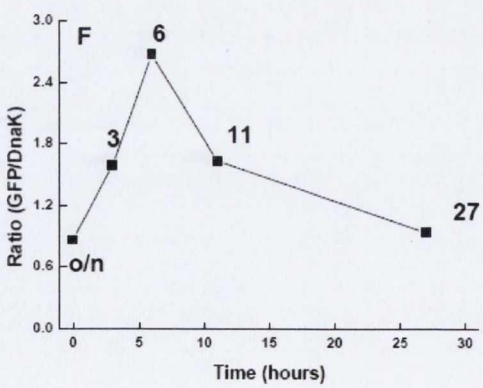
Time (hours)



C Anaerobic

3 6 11 27 o/n

Time (hours)



3.4E) correlated approximately with *fis-gfp* data obtained using flow-cytometry (Fig.3.4C & 3.4D). This GFP protein expression pattern corresponded to *fis-gfp* expression levels as they peaked during log phase for both conditions and during the stationary phase of growth, and the overall GFP protein band intensity pattern level decreased gradually while aerated (Fig. 3.4E), but remained sustained under non-aerated conditions (Fig. 3.4D).

3.3 Discussion

Previous findings showed that microaerobic conditions were found to induce virulence in *S.* Typhimurium (Lee & Falkow, 1990). Published and recent unpublished results from this laboratory have suggested that under non-aerated conditions there is sustained expression of the Fis protein during the stationary phase of growth (Ó Cróinín & Dorman, 2007). I have reproduced the observation that *fis-gfp* transcription is sustained in non-aerated cultures (Fig. 3.2). I have also shown that pZEC09 has no basal *gfp* gene expression that might confound these results (Fig. 3.1). GFP expression levels displayed by culture samples grown under different culture aeration regimes as detected by western blot analysis, corresponded to their respective *fis-gfp* expression level from flow-cytometry measurements. Furthermore, complete anaerobic growth (Figs. 3.3 and 3.4C & F) is not equivalent to non-aerated growth in terms of *fis* transcription. This may reflect the fact that niches that have proved to be optimal for *fis* promoter activity may be important for *Salmonella*, like the brush border of the gut epithelium which is microaerobic and not strictly anaerobic. This is consistent with the infection process of *Salmonella* in its initial invasive stages, where microaerobic conditions are much more advantageous than anaerobic ones. As *Salmonella* passages through the small intestine, oxygen availability decreases until it becomes anaerobic in the large intestine, where the bacterium is instead required to gradually switch from aerobic to a predominantly anaerobic metabolism, rather as a natural response to a gradually changing environment than as a stress response (Rychlik & Barrow, 2005).

Moreover, previous work showed that induction of Fis expression in the absence of aeration is facilitated by a reduction in the levels of RpoS, the alternative stationary phase stress response sigma factor (Ó Cróinín & Dorman, 2007). While it is true that RpoS levels increase at the onset of stationary phase, its expression is enhanced even further under stressful conditions,

such as microaerobicity, by Hfq (Vassilieva & Garber, 2002). Hfq, an important RNA chaperone involved in the posttranscriptional control of many genes, is in part positively regulated by the DksA protein (Sharma & Payne, 2006; Wilson *et al.,* 2007). In chapter 4, I will examine the role of DksA in the regulation of *fis* expression and provide data that may partly explain why *fis* is expressed without the repression of RpoS under non-aerated conditions (Ó Cróinín & Dorman, 2007). This might be possible if Hfq is not up-regulated to enhance RpoS levels (Vassilieva & Garber, 2002; Wilson *et al.,* 2007), but only when DksA levels are negligible (Sharma & Payne, 2006), as it could be the case in microaerobic conditions.

According to data shown in the present chapter, it is not anaerobiosis, but microaerobiosis that induces sustained expression of Fis during stationary phase growth. These data are also consistent with the fact that SPI-1 induction takes place in bacteria growing under very low oxygen conditions and into stationary phase prior to infection of epithelial cells (Lee & Falkow, 1990). It is also possible that the *fis* promoter is modulated by the availability of aeration/oxygen in the surrounding environment via changes in DNA supercoiling (see Chapter 5). Published data from this laboratory show that *fis* in *Salmonella* is sensitive to changes in DNA supercoiling (Ó Cróinín *et al.,* 2006), and that its expression depends on the level of aeration where ATP availability is maximal (Ó Cróinín & Dorman, 2007).

Chapter 4

A regulatory role for stringent response factors DksA and ppGpp in *fis* gene expression in *Salmonella* Typhimurium.

4.1 Introduction

The goal of this part of the work was to examine the regulatory role of stringent response factors DksA and ppGpp in *fis* gene expression in *S.* Typhimurium cultures grown under aerated and non-aerated conditions.

Environmental signals, specifically a lack of aeration, have been shown to sustain Fis expression at late stages of growth in batch culture (Ó Cróinín and Dorman, 2007). This is consistent with previous findings showing that the Fis protein is a key regulator of the *Salmonella* virulent phenotype because the growth conditions that sustain expression of the *fis* gene are equivalent to those that induce the expression of the genes in the SPI-1 pathogenicity island (Kelly *et al.,* 2004; Thompson *et al.,* 2006; Wilson *et al.,* 2001). However, the molecular mechanism responsible for sustained expression of Fis during stationary phase was still unclear. A first step involved an examination of the genetic regulation mechanism already known to affect *fis* expression: the stringent response (Chang et al., 2002; Mallik *et al.,* 2006). During the stringent response (section 1.4), regulatory factor DksA and signal molecule guanosine tetraphosphate (ppGpp) are known to play an important role in the repression of Fis transcription in *E. coli* (Chatterji & Kumar-Ohja, 2001; Mallik *et al.,* 2006). In *S.* Typhimurium SL1344, the gene encoding Fis displays elevated expression levels in a ppGpp deficient (ppGpp°) mutant strain under low oxygen growth conditions (Thompson *et al.,* 2006). Given this information, I wanted to understand if DksA played a role in the repression of the *fis* gene and if so, how. Furthermore, as it was not known if in *Salmonella* there was a partial interaction between DksA and ppGpp in *fis* gene regulation, experiments were conducted to investigate this possibility.

Also, as mentioned earlier (section 1.4), studies done in *E. coli* explained how the *fis* promoter (*fis* P) is regulated by the stringent response (Mallik *et al.,* 2006), through a mechanism in which the protein DksA (Blankschien *et al.,* 2009; Paul *et al.,* 2004; Rutherford et al., 2009) and the alarmones guanosine tetraphosphate (ppGpp) and pentaphosphate (pppGpp), regulated by the protein product of *relA* and *spoT*, act in conjunction (Rutherford et al., 2009). Fis influences the transcription of several genes that are under the control of the stringent response (Paul *et al.,* 2004; Webb *et al.,* 1999). The *Salmonella fis* operon organization was shown to be the same as *E. coli* (Osuna *et al.,* 1995), suggesting that regulatory insights

gained in studies of *fis* expression in *E. coli* might also apply to the same gene in *Salmonella*. Although DksA plays an important role in regulating Fis expression, the mechanism of DksA action at the *fis* promoter remains unclear (Mallik *et al.,* 2006).

As part of the investigation of the possible role of ppGpp in sustaining *fis* transcription, it was important to verify the integrity of the ppGpp° mutant used in the study. This strain was supposedly deficient in both *relA* and *spoT*, so experiments were performed to observe if the loss of ppGpp production had the expected effects on an unrelated member of the stringent response. Stringent response regulon member *lrp,* the gene coding for the Leucine responsive regulatory protein, Lrp, is positively regulated by ppGpp (Landgraf *et al.,* 1996; Traxler *et al.*, 2011). A reduction in the transcription of the *lrp* gene in the *relA spoT* double knockout strain would be consistent with the absence of ppGpp from that strain. However, no chemical or mass spectrophotometry methods were done to further demonstrate the absence of ppGpp.

In this chapter, I describe a role for DksA and ppGpp in the sustained expression of the *fis* gene. I performed experiments on a *S.* Typhimurium SL1344 wild type strain, a *dksA* mutant and a ppGpp° mutant, and analyzed their *fis* expression levels during all phases of growth and under aerated and non-aerated conditions. The results obtained corroborated published results and also identified DksA and ppGpp as negative regulators for Fis expression under aerated and non-aerated growth conditions for *S.* Typhimurium.

4.2 Results

4.2.1 Confirmation of a *dksA* mutation in strain SG02

A *dksA* knock-out mutant was constructed using a homologous recombination technique (section 2.10). The *dksA::kan* lesion was later transferred into a fresh SL1344 background by the bacteriophage P22 generalized transduction to create strain SG02 (section 2.5.2) (Table 2.1).

It was deduced by DNA sequence analysis that the 456-b.p. *dksA* gene had undergone a deletion of 151-b.p., and that a kanamycin resistance gene (*kan*) had been inserted in place of the 151-b.p. of deleted *dksA* DNA. The custom automated sequencing was performed at GATC-Biotech, Germany, using the primers dksA.KO.fwd and dksA.KO.rev (Table 2.3).

The presence of the *dksA*::*kan* mutation in *S.* Typhimurium was also confirmed by PCR amplification (Fig. 4.1) using the primer pair dksA_fwd and dksA_rev (Table 2.3) and genomic DNA prepared from SL1344 wild type, and SG02 (*dksA::kan).* The primer dksA_fwd binds the DNA directly downstream of the ATG translational start codon in the *dksA* ORF, and the dksA_rev binds 310-b.p. downstream of the translational stop site in the *dksA* ORF. Where SL1344 wild type SL1344 genomic DNA was used as a template for the PCR reaction, the primers generated an amplicon of 500-b.p. in size (Fig. 4.1). The primer pair produced an amplicon of approximately 2-k.b. in size from SL1344 SG02 *dksA::kan* genomic DNA, consistent with the disruption of the *dksA* gene (Fig. 4.1).

4.2.2 Levels of *fis* expression in SG02 *dksA* mutant strain are higher than those in SL1344 wild type strain

S. Typhimurium SL1344 wild type and SG02 *dksA::kan* mutant strains (Table 2.1) carrying pZep*fis-gfp* (Table 2.2) were used for this part of the work. All strains were grown under aerated and non-aerated conditions.

GFP fluorescence levels expressed from plasmid pZep*fis-gfp* in the SG02 *dksA*::*kan* strain, were higher than the control and displayed an increased expression at the onset and during the stationary phase of growth (Fig. 4.2A & 4.2B), in both aerated and non-aerated conditions. The *dksA* mutant and the wild type showed different growth profiles (Fig. 4.2C & 4.2D). However, given the extent of the increased *fis* expression in the *dksA* mutant when compared to the wild type it is possible that differences in the growth cycle were not a factor in the increased level of expression seen in the absence of DksA (Fig. 4.2C & 4.2D).

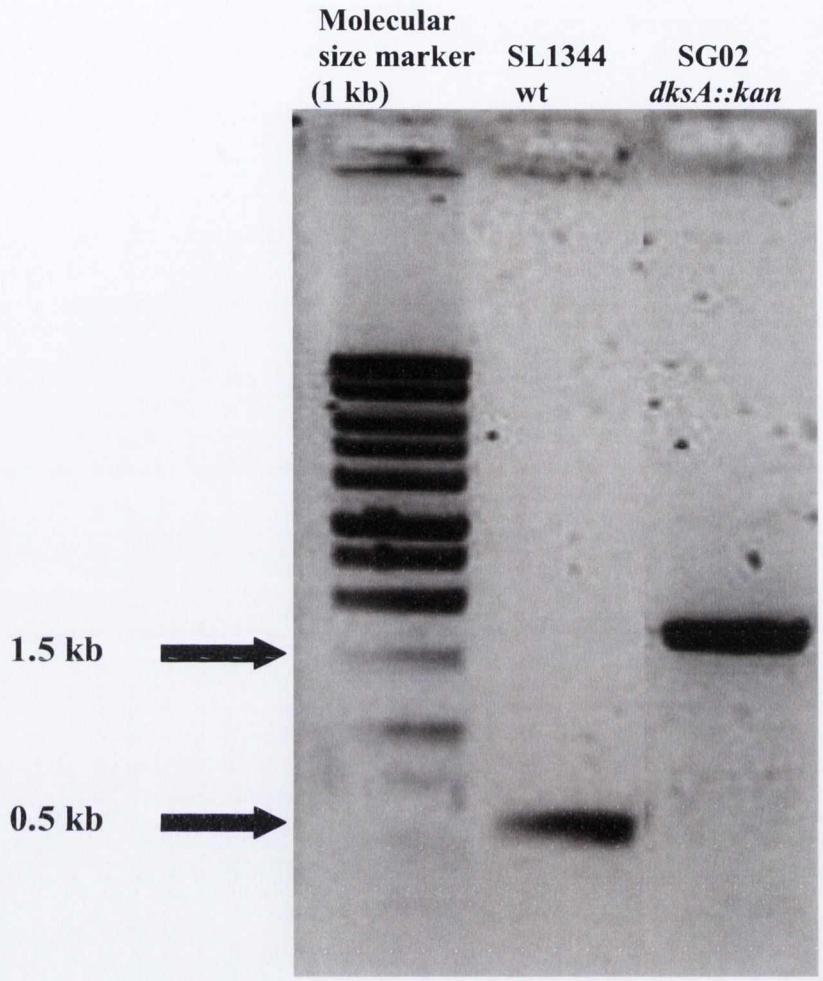


Fig. 4.1: Confirmation of the *dksA* mutation. PCR analysis confirmed the disruption of the *dksA* gene with the insertion of the kanamycin resistance gene cassette in SG02. The *dksA* gene was amplified by PCR using genomic DNA from SL1344 and SG02. An amplicon of 500-b.p. in size was generated in the wild type background compared to an amplicon of approximately 2-k.b. in size in the *dksA* mutant background. These results verify the insertion of the *kan* cassette within the *dksA* gene of SG02. A molecular size marker (DNA ladder) of 1 kb was used for size reference (section 2.11.1).

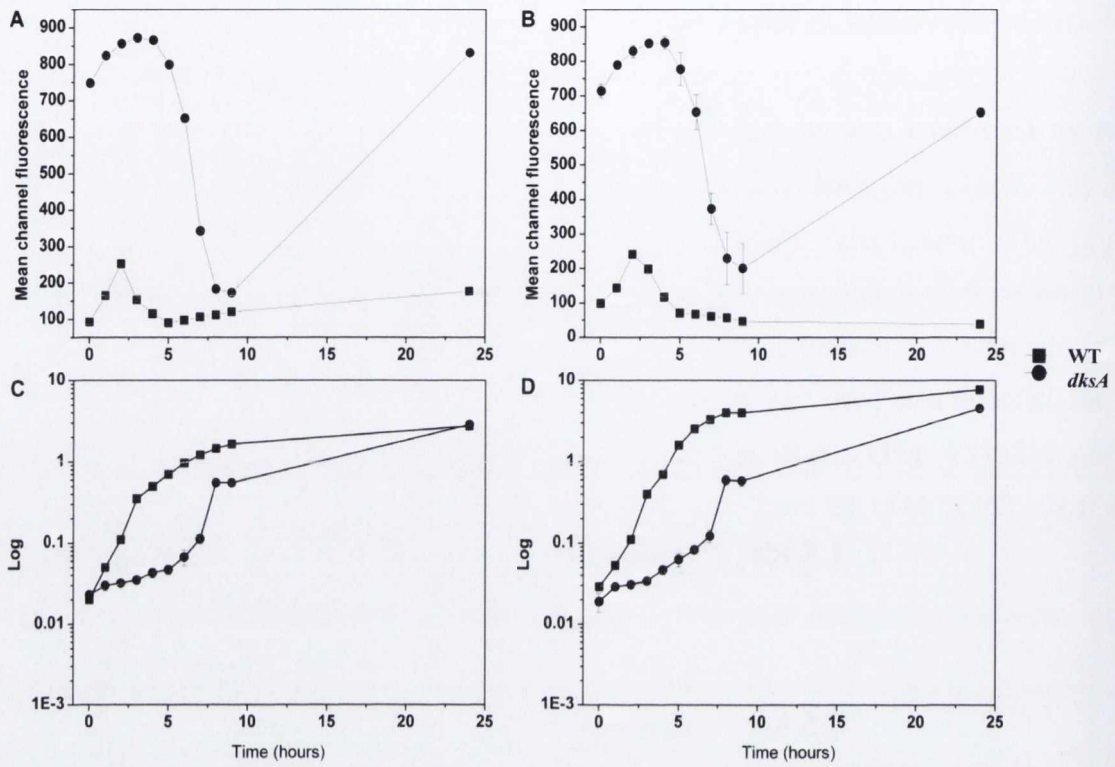


Fig. 4.2: Levels of *fis* expression and growth curves of wild type SL1344 and mutant SG02 strain cultures. Strains wild type SL1344 (filled squares) and mutant SG02 *dksA*::*kan* (filled circles), both carrying reporter plasmid pZep*fis-gfp* and grown under A) non-aerated or B) aerated conditions. SG02 expressed higher levels of *fis* than the SL1344 wild type in both conditions. Growth curves from each strain grown under C) non-aerated and D) aerated conditions. The *dksA* mutation affected the growth pattern of the strain as compared to the wild type. Error bars indicate standard deviations (n=3).

4.2.3 Levels of *fis* expression in the KT2160 ppGpp° mutant are similar to those in SL1344 wild type strain

In order to study the role of DksA in *fis* gene expression under the conditions of high and low aeration and in the context of the stringent response, additional experiments were performed to see if the signal molecule ppGpp made a contribution to the process.

S. Typhimurium SL1344 wild type and KT2160 ppGpp° *relA::kan, spoT::cat* mutant strain (Table 2.1) carrying pZep*fis-gfp* (Table 2.2) were used for this set of experiments. Both strains were grown under aerated and non-aerated conditions and their corresponding *fis* expression levels were measured (Fig. 4.3).

The striking similarity between the levels of *fis* expression between the wild type and the KT2160 ppGpp° mutant strain suggested, contrary to what occurs in *E. coli* (Mallik et al., 2006), that ppGpp plays no role in the regulation of *fis* transcription in *Salmonella*. This raised doubts about the integrity of the KT2160 ppGpp° mutant strain used. Therefore, knockout mutant strain was obtained: KT4514 ppGpp° Δ*relA* Δ*spoT28*, *hisG rpsL* (Table 2.1). This was transformed with plasmid pZep*fis-gfp* (Table 2.2) and used to re-evaluate the effect of ppGpp on *fis* expression in the presence or absence of the DksA regulatory protein (section 4.2.4).

4.2.4 The SG02 *dksA* mutant displays higher *fis* expression levels than KT4514 ppGpp° knockout mutant strain

S. Typhimurium SL1344 wild type, KT4514 ppGpp° Δ*relA* Δ*spoT28*, *hisG rpsL* knockout mutant, and SG02 *dksA*::*kan* strains (Table 2.1) carrying plasmid pZep*fis-gfp* (Table 2.2) were grown under aerated and non-aerated conditions and the corresponding *fis* expression levels were measured (Fig. 4.4).

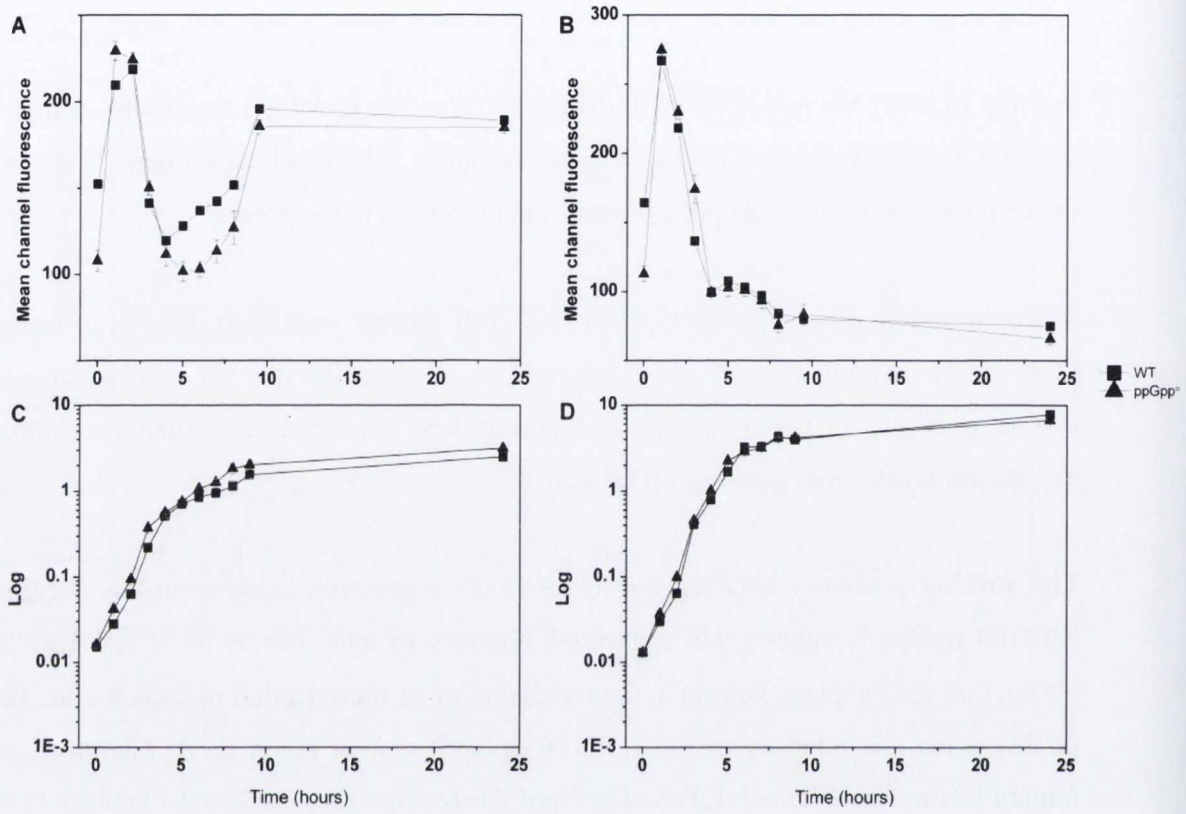


Fig. 4.3: Levels of *fis* expression and growth curves of wild type SL1344 and mutant KT2160 strain cultures. SL1344 wild type (filled square) and mutant KT2160 ppGpp° *relA::kan, spoT::cat* (filled triangle) strain cultures, both carrying reporter plasmid pZep*fis-gfp* and grown under A) non-aerated or B) aerated conditions. Growth curves from each strain grown under C) non-aerated and D) aerated conditions. Levels of *fis* expression from mutant cultures in both conditions were not higher than those from the wild type. The *relA spoT* mutations did not affect the growth of the bacteria across late-stationary phase when compared to the wild type. Error bars represent standard deviations (n=3).

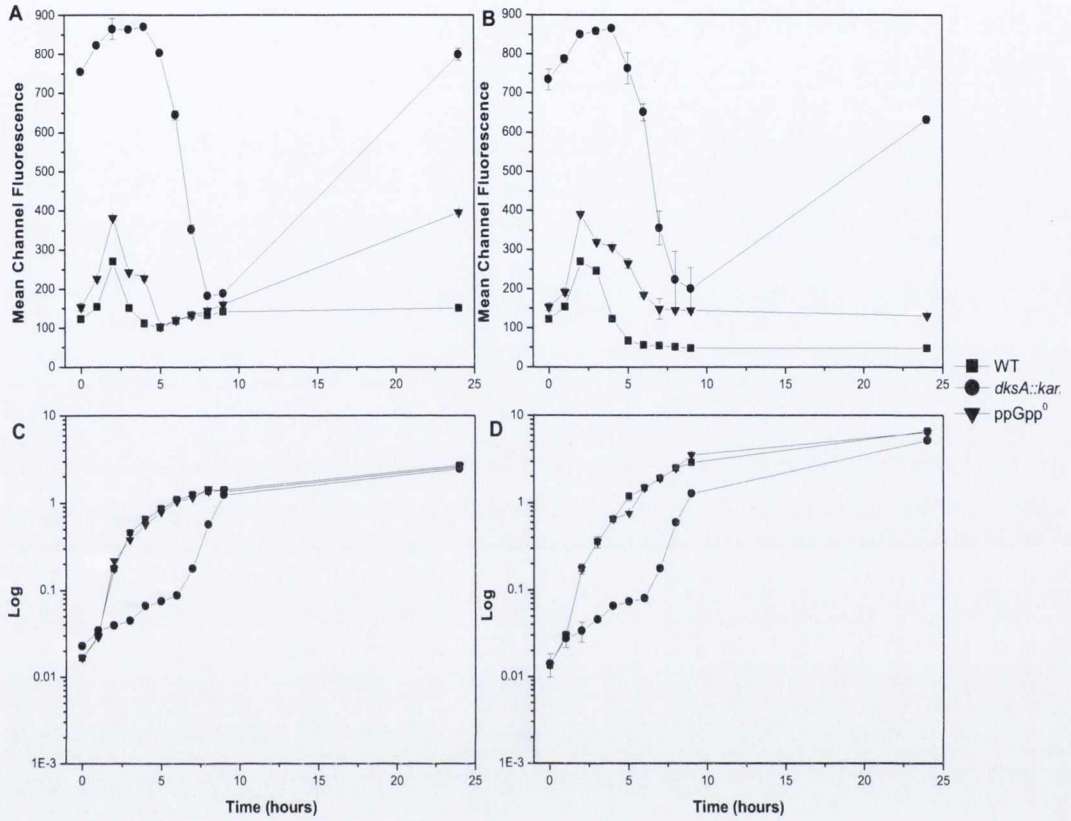


Fig. 4.4: Level of *fis* expression and growth curves of wild type SL1344 and mutants SG02 and KT4514. SL1344 wild type (filled squares), and mutants SG02 *dksA*::*kan* (filled circles) and KT4514 ppGpp° Δ*relA* Δ*spoT28, hisG rpsL* (filled inverted triangles) strains, all carrying reporter plasmid pZep*fis-gfp* and grown under A) non-aerated or B) aerated conditions. Data show increased expression levels of *fis* in the *dksA* mutant strain when compared to the ppGpp° or wild type strains. Comparably, although to a lesser extent, did the level of *fis* expression differ between the ppGpp° and wild type strain, and most remarkably during aerated conditions throughout the growth cycle. The *dksA* mutation seemed to have affected the growth cycle whereas, the *relA spoT* mutation produced almost no changes to the growth cycle when compared to the wild type. Error bars represent standard deviations (n=3).

Expression levels of *fis* are higher in the KT4514 mutant strain than in the SL1344 wild type in aerated growth conditions and, to a lesser extent, in non-aerated growth conditions (Fig. 4.4A & 4.4B). Nevertheless, expression levels of *fis* diplayed by the KT4514 mutant strain, in both growth conditions, were much higher than those displayed earlier by the KT2160 ppGpp° mutant strain (Fig. 4.3) when compared to the wild type strain. As for the SG02 *dksA*::*kan* mutant, its *fis* gene again showed higher expression levels than in the SL1344 wild type strain or, the KT4514 ppGpp° Δ*relA* Δ*spoT28*, *hisG rpsL* knockout mutant (Fig. 4.4A & 4.4B). The *relA spoT* mutations did not affect the growth of the bacteria in either aeration regime when compared to the wild type (Fig. 4.4C & 4.4D).

Transcription factor DksA and alarmone ppGpp have both been documented to target RNA polymerase in *E. coli* (Rutherford et al., 2009). However, the data from the present study suggest that ppGpp may interact *partially* with DksA in order to regulate *fis* transcription negatively in *Salmonella*. While the DksA mutation changed the pattern of *fis* transcription, the removal of ppGpp did not affect *fis* transcription to the same extent (Fig. 4.3 & 4.4).

Because of this apparent *partial* interaction between ppGpp and DksA in the regulation of *fis* transcription, and the not-yet-tested integrity of the KT4514 strain, further experiments were done to examine the connection between the stringent response and *fis* in the absence of DksA.

4.2.5 The KT4514 ppGpp° knockout mutant strain displays decreased *lrp* expression

Experiments were next performed to see if the loss of ppGpp production in the knockout mutant KT4514 (Table 2.1) had the expected effects on an unrelated element of the stringent response. Reporter plasmid pZep*lrp-gfp* was first transformed into *S.* Typhimurium SL1344 wild type, and into the SG02 *dksA*::*kan* and the KT4514 ppGpp° Δ*relA* Δ*spoT28, hisG rpsL* mutant strains as described earlier (section 2.4). Transformed strains were grown under both non-aerated and aerated conditions and their levels of *fis* gene expression were measured (Fig. 4.5 A & B). The *dksA* and the *relA spoT* mutations affected bacterial growth only during early log phase but not across late-stationary phase when compared to the wild type (Fig. 4.5 C & D).

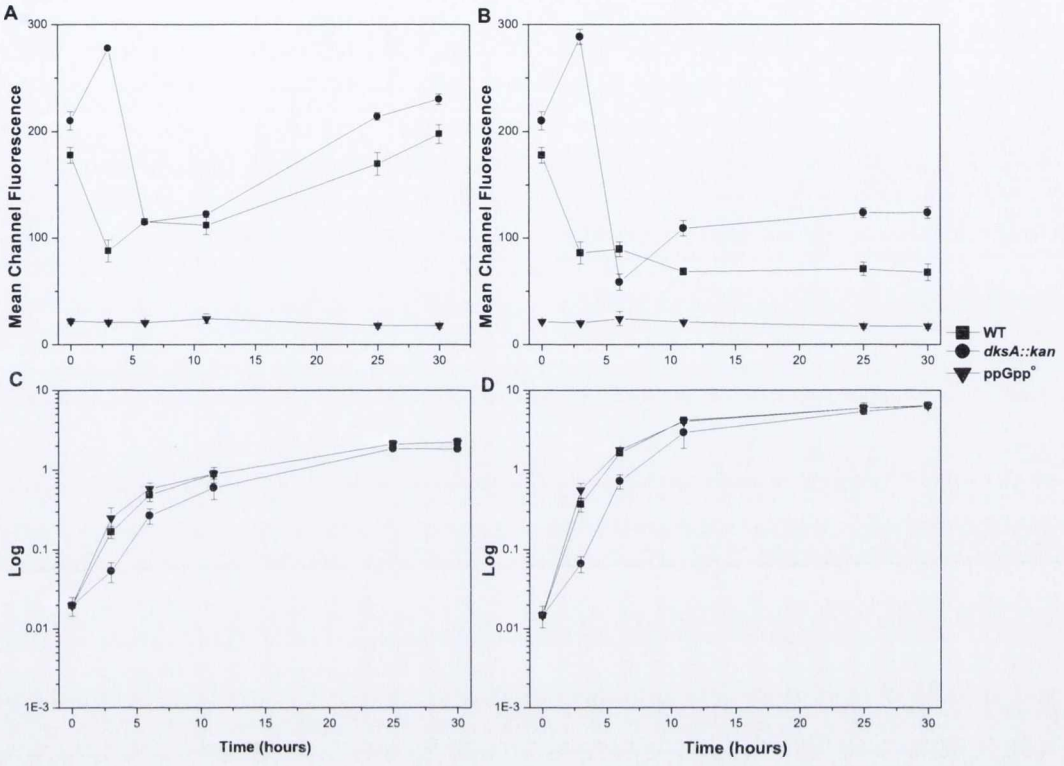


Fig. 4.5: Expression and growth curves of an *lrp-gfp* transcriptional fusion. Levels of *lrp* expression in SL1344 wild type (filled squares), and mutants SG02 *dksA*::*kan* (filled circles) and KT4514 ppGpp° (filled inverted triangles), all carrying reporter plasmid pZep*lrp-gfp* and grown under A) *lrp* expression in non-aerated or B) *lrp* expression in aerated conditions., C) and D) their respective growth curves. Patterns of *lrp* expression in panels A and B showed higher levels in the *dksA*::*kan* mutant strain than for the ppGpp° mutant and wild type strains like in the different strain variants of SL1344 with *fis-gfp* transcriptional fusion. *lrp* levels from the ppGpp° strain were lower than the wild type for both conditions thus confirming ppGpp deficiency in the strain. In panels C and D, the *dksA* nor the *relA spoT* mutations affected bacterial growth only during early log phase but not across late-stationary phase when compared to the wild type. Error bars represent standard deviations (n=3).

As stated earlier, ppGpp is required for activation of the *lrp* gene expression (Landgraf *et al.,* 1996; Traxler *et al.,* 2011). Data showed a reduction in transcription of the *lrp* gene in the KT4514 double knockout mutant strain as compared to the SL1344 wild type for both conditions (Fig. 4.5A & 4.5B). This set of data was consistent with the absence of ppGpp in the KT4514 strain.

Interestingly, the *dksA* mutant strain displayed higher levels of *lrp* expression than the control, in both growth conditions (Fig. 4.5A & 4.5B), almost comparable to the *fis* expression levels in the wild type strain also for both conditions (Fig. 4.3A & 4.3B). These results suggest that even in the absence of DksA, ppGpp is fully functional as it up-regulates *lrp* expression.

4.3 Discussion

Results obtained indicate that the transcription factor DksA and the alarmone ppGpp have negative roles in controlling *fis* expression. In *E. coli,* the *fis* gene is a member of the stringent response regulon and it responds to DksA (Mallik *et al.,* 2006). During the stringent response process, both DksA and ppGpp have been documented to target RNA polymerase (Rutherford et al., 2009), but data presented here show that each has an independent effect on *fis* transcription in *Salmonella*. The considerably higher *fis* expression levels in the *dksA* mutant when compared to the wild type, suggests that this factor has a repressive role. Other research findings show that DksA relieves chromosome replication arrest when an environmental stressor is encountered (e.g. microaerobiosis); in the absence of DksA, cell replication arrest occurs, but transcription persists (Tehanchi et al., 2010; Blankschien et al., 2009). According to those reports, even after cell replication arrest occurs, transcription still continues. Such finding could possibly explain why *fis* transcription is seen at its highest levels in the *dksA*::*kan* mutant strain. Data obtained in this study show that ppGpp is also a negative regulator of *fis* (Fig. 4.4 & 4.5). Since elimination of ppGpp production also has a positive effect on *fis* transcription at the stationary phase of growth (Fig. 4.4), these data suggest that DksA and ppGpp have parallel effects on *fis* transcription but affect its transcription to different degrees. It is possible that DksA regulates *fis* both directly and indirectly, allowing it to have a larger impact than ppGpp, which might only regulate directly.

Data from studies on *lrp* expression (Fig. 4.5) were consistent with the fact that KT4514 ppGpp^{o} Δ*relA* Δ*spoT28 hisG rpsL* mutant strain is, in fact, deficient in ppGpp. It was important to establish this fact given the ambiguity surrounding the ppGpp status of strain KT2160, which had been supplied to me as a strain deficient in this alarmone. However, there is no direct evidence because ppGpp levels were not measured either by chomatography or by mass spectrometry in either strain.

Furthermore, previous and present data suggest that ppGpp negatively influences global regulators that induce SPI-1 gene expression. This is underlined by the fact that *fis* levels were higher in the ppGpp-deficient strain that was non-aerated than they were in the ppGpp-deficient strain that was aerated (Fig. 4.4). Previous findings show that genes encoding the global regulators *fis* and *fur* have elevated expression levels in the Δ*relA* Δ*spoT* strain grown under low oxygen conditions (Thompson *et al.,* 2006). This is interesting because, as mentioned earlier (section 4.1), just like Fis, Fur is sensitive to SPI-1 inducing (i.e. non-aerated) conditions that activate important T3SS structural genes (Ellermeier & Slauch, 2008). This phenomenon shows that ppGpp is likely to have an important role in the invasion of epithelial cells.

Data presented in this study showed that the effects of a *dksA* knockout mutation and the abolition of ppGpp production on *fis* transcription were not completely equivalent. Instead, the results obtained here showed that ppGpp may interact *partially* with DksA in order to negatively regulate *fis* transcription in *Salmonella*.

Chapter 5

The contribution of DNA gyrase activity to the sustained expression of the *fis* gene

5.1 Introduction

The goal of this work was to examine the interaction between DNA supercoiling and *fis* expression levels, by inhibiting the activity of DNA gyrase and impairing the function of topoisomerase I in *S.* Typhimurium.

Previous results indicated that DksA plays a negative role in the regulation of *fis* expression and that ppGpp cannot act alone for the same purpose (Chapter 4). The next step was to discover the mechanism that prolonged *fis* expression. Fis can influence transcription, and other DNA transactions, through its ability to affect the level of DNA supercoiling in the cell (Muskhelishvili and Travers, 2003) (section 1.5). For this reason, both DNA supercoiling and Fis have been studied for their concerted ability to modulate SPI-1 gene expression. As mentioned before (section 1.3), SPI-1 enables the bacterium to invade cells at the epithelial surface of the gut where microaerobic conditions prevail. Since the Fis protein is an important regulator of the *Salmonella* virulence phenotype (Kelly et al., 2004; Ó Cróinín *et al.,* 2006) and because the expression of the *fis* gene is sensitive to changes in DNA supercoiling (Ó Cróinín *et al.,* 2006; Cameron *et al.,* 2011; Cameron and Dorman 2012) it was important to study proteins that are involved in modulating DNA topology such as DNA gyrase and topoisomerase I. Fis is known to repress transcription of both genes encoding the DNA gyrase protein, *gyrA* and *gyrB,* which are responsible for introducing negative supercoiling into DNA, (Cozzarelli, 1980; Schneider *et al.,* 1997; Travers *et al.,* 2001; Keane and Dorman, 2003), and to activate *topA,* the gene that encodes DNA topoisomerase I (TopA) which is responsible for relaxing DNA (Cozzarelli, 1980; Richardson *et al.,* 1984; Weinstein-Fischer and Altuvia, 2007; Cho *et al.*, 2008) under specific conditions (Ó Cróinín *et al.,* 2006; Ó Cróinín and Dorman, 2007).

Novobiocin as well as other amino-coumarin antibiotics like Coumermycin A1 (CA1), have been frequently used in DNA topology studies and their effects have been previously described (section 1.5).

In addition to the gyrase-inhibiting drugs, a genetic approach was taken to assess the impact of DNA topoisomerase I, in order to have a deeper understanding of the role of DNA gyrase in *fis* transcription as a function of growth phase.

5.2 Results

5.2.1 Confirmation of a *topA* mutation

A *topA* deletion mutant, SG03 (Table 2.1), was constructed using a homologous recombination technique (section 2.10). The *topA::kan* lesion was later transferred into a fresh SL1344 wild type background by bacteriophage P22 generalized transduction (section 2.5.2).

It was deduced by DNA sequence analysis that the 2601-b.p. *topA* gene had undergone a deletion of 750-b.p., and that a kanamycin resistance gene (*kan*) had been inserted in place of the 750-b.p. of deleted *topA* DNA. The custom automated sequencing was performed at GATC-Biotech, Germany, using the primers topAko_rightfwd and topAko_rightrev (Table 2.3).

The presence of the *topA::kan* mutation in *S.* Typhimurium was also confirmed by PCR amplification (Fig. 5.1) using the primer pair topAko_conf_fwd and topAko_rightrev (Table 2.3) and genomic DNA prepared from SL1344 SL1344 wild type, and SG03 SL1344 *topA::kan.* The primer topAko_conf_fwd binds the DNA directly downstream of the ATG translational start codon in the *topA* ORF and topAko_rightrev binds 1851-b.p. downstream of the translational stop site in the *topA* ORF. Where wild type SL1344 genomic DNA was used as a template for the PCR reaction, the primers generated an amplicon of 0.5-k.b. in size (Fig. 5.1). The primer pair produced an amplicon of approximately 2-k.b. in size from SL1344 *topA::kan* genomic DNA, consistent with the disruption of the *topA* gene (Fig. 5.1).

5.2.2 The concerted actions of DNA gyrase and topoisomerase I modulate *fis* expression levels

Experiments were performed to understand the role of DNA gyrase and DNA topoisomerase I as key enzymes in the supercoiling process of DNA in *fis* expression. SL1344 wild type and

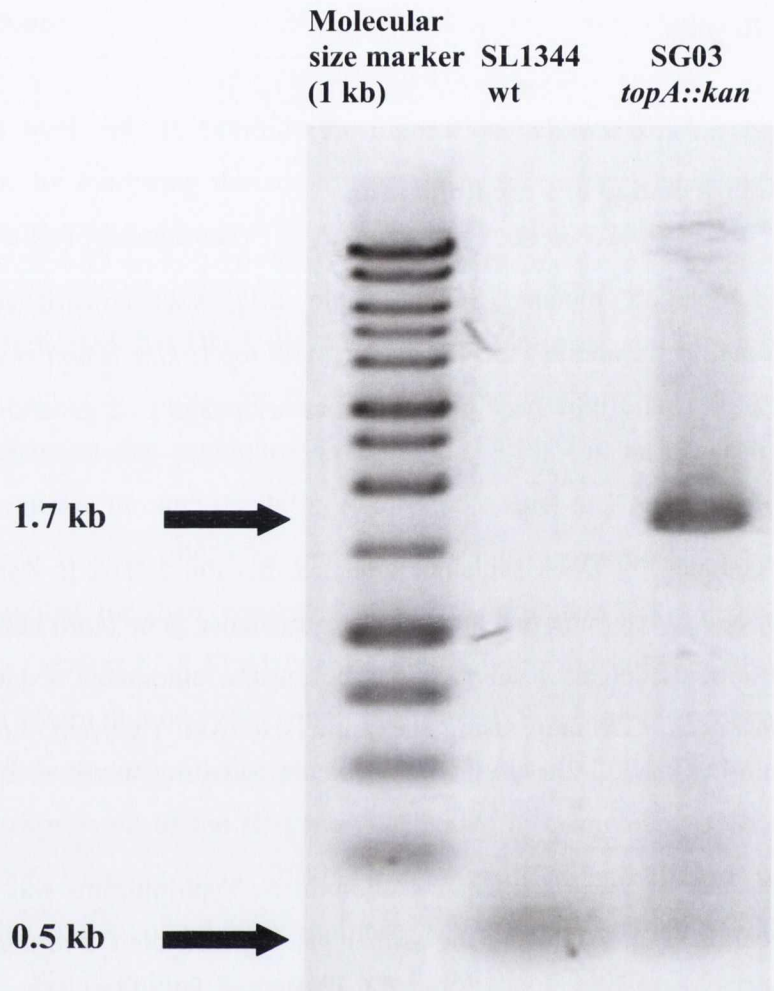


Fig. 5.1: Confirmation of the *topA* mutation. PCR analysis confirmed the disruption of the *topA* gene with the insertion of the kanamycin resistance gene cassette in SG03. The *topA* gene was amplified by PCR using genomic DNA from SL1344 and SG03. An amplicon of 0.5-k.b. in size was generated in the wild type background compared to an amplicon of approximately 2-k.b. in size in the *topA* mutant background. These results verify the insertion of the *kan* cassette within the *topA* gene of SG03. A molecular size marker (DNA ladder) of 1 kb was used for size reference (section 2.11.1).

SG03 *topA:kan* mutant strains (Table 2.1), both carrying reporter plasmid pZep*fis-gfp,* were assessed for their *fis* expression levels after inhibition of DNA gyrase activity. Both strains were grown under aerated and non-aerated conditions and treated with novobiocin or coumermycin A1 (CA1) as described (section 2.2.2).

Both drugs were administered 2 hours after the initial inoculum for the first set of experiments (Fig. 5.2), whereas only novobiocin was added and upon initial inoculum (0 h) for the second set of experiments (Fig. 5.3). Drug administration was done at different times because it was observed that CA1 addition after 2 hours from initial inocula was not lethal and, in order to make it comparable, novobiocin had to also be added 2 hours after initial inocula. Addition of novobiocin upon inoculation at 0 h was observed to be not lethal. The corresponding *fis* expression levels were measured as previously described (section 2.6.3), and compared to a non-treated control for the wild type (Fig. 5.2 & 5.3, red), and SG03 strain (Fig. 5.3, black).

Expression of the *fis-gfp* transcriptional fusion in the SL1344 wild type during stationary phase decreased in aerated cultures and increased in the non-aerated ones (Fig. 5.2A & B, red), as previously published. However, *fis* expression levels in novobiocin-treated samples were higher than the control but then decreased by the 25 h time point in both conditions (Fig. 5.2A & B, green), thus not showing sustained expression of *fis*. CA1-treated samples displayed a gradual decrease of *fis* expression levels all along the growth cycle in aerated and non-aerated conditions (Fig. 5.2A & B, blue). Novobiocin and CA1 treated samples grew at a slower rate than the control (Fig. 5.2C & D). These data suggest that when DNA gyrase is not fully functional *fis* expression levels drop even in cultures grown under non-aerated conditions during late-stationary phase. These data are also indicative of a possible correspondence between less supercoiled DNA and lower *fis* expression levels. The results confirm that gyrase activity is important for maintaining the *fis* transcription pattern but that after the maximum level of *fis* transcription that occurs in early exponential growth, gyrase activity normally suppresses *fis* expression. When gyrase is inhibited, the transcription of *fis* goes up until very late in stationary phase.

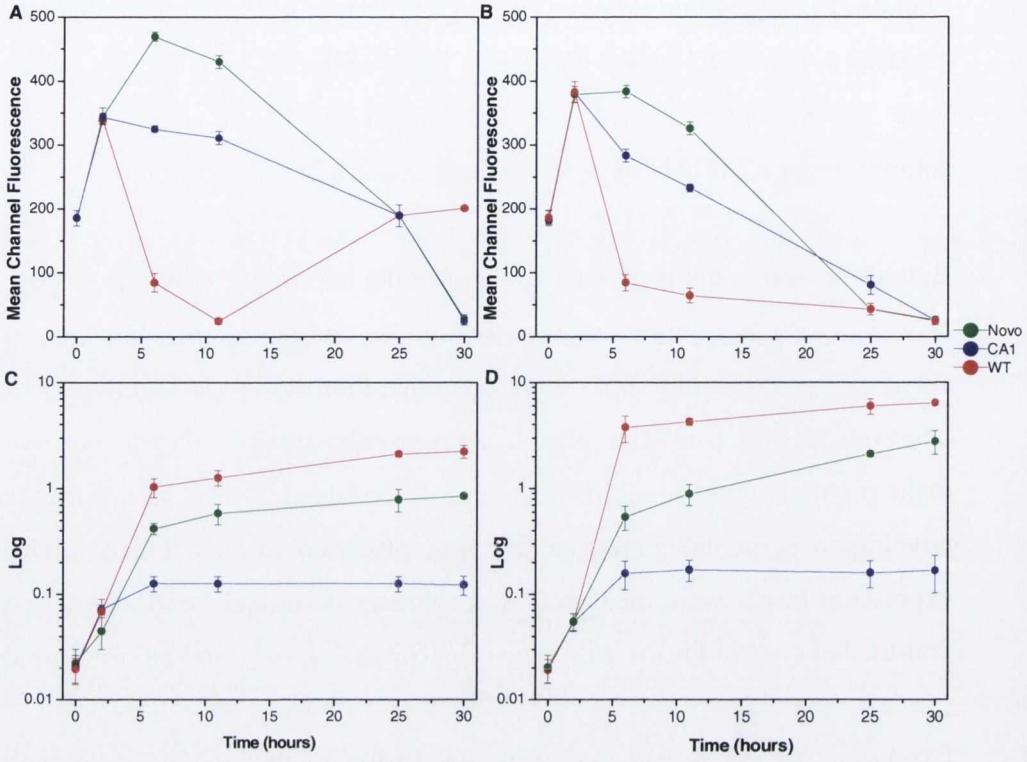


Fig. 5.2: Expression levels of the *fis* gene and growth curves of SL1344 pZep*fis-gfp* treated with novobiocin or CA1. SL1344 pZep*fis-gfp* without (red) or with (green) novobiocin (50 μg/ml), or with (blue) CA1 (10 μg/ml), was incubated under aerated or non-aerated conditions. Novobiocin or CA1 was added 2 hours after the initial inoculum. A) *fis* expression under non-aerated conditions, and C) the corresponding growth curve; and B) *fis* expression under aerated conditions, and D) the corresponding growth curve. Both treatments affected the growth pattern of the strain as compared to the wild type. However, novobiocin had a lower effect than CA1 on growth. *fis* expression levels in novobiocin treated samples were higher than the control except at the 25-h time point. CA1-treated samples showed higher *fis* expression levels than the control throughout growth under aerated and non-aerated conditions, but eventually decreased to the same levels by the 25-h time point in both aerated and non-aerated conditions. Error bars represent standard deviations (n=3).

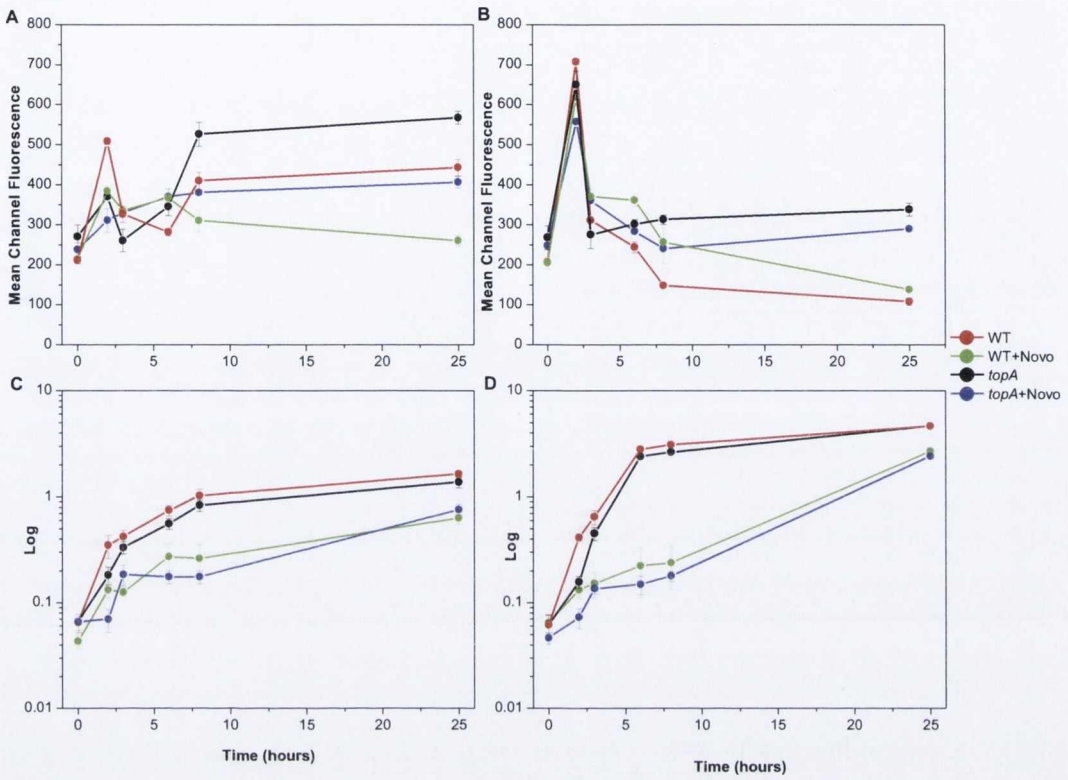


Fig. 5.3: Levels of *fis* expression and growth curves of SL1344 (wild type) and SG03 (*topA::kan)* treated with novobiocin. Levels of *fis* expression were measured and growth curves observed for SL1344 (wild type) (red), and SG03 (*topA::kan)* (black), both carrying pZep*fis-gfp*. Both strains were treated with novobiocin at 50 µg/ml: SL1344 (wild type) (green), SG03 (*topA::kan)* (blue), and incubated under aerated or non-aerated conditions. Novobiocin was added upon initial inoculum at 0 h. A) *fis* expression in non-aerated conditions, and C) the corresponding growth curve; B) *fis* expression in aerated conditions, and D) the corresponding growth curve. During stationary phase, *fis* expression was higher and sustained in the mutant SG03 (black) cultures grown in aerated conditions, and even increased in non-aerated growth conditions. Conversely, treated mutant samples (blue) expressed *fis* in a very similar pattern to that of the non-treated wild type (red) during stationary phase in both growth conditions. Treated wild type samples (green), displayed the least *fis* expression levels in non-aerated conditions during stationary phase. Both, treated mutant (blue) and treated wild type (green) strains showed difficulty growing as compared to the non-treated wild type control (red), but growth increased to similar levels in late-stationary phase. Error bars represent standard deviations (n=3).

Expression of the *fis-gfp* fusion in the SG03 *topA* mutant strain was predicted to be higher than in the wild type since its DNA would be more negatively supercoiled. It was anticipated that novobiocin-treated *topA* mutants would display *fis* expression levels similar to those expressed by the non-treated wild type because novobiocin treatment would be expected to convert any hyper-negatively-supercoiled DNA to a more relaxed state, approximating to DNA supercoiling levels in the wild-type strain. Figure 5.3 shows the levels of *fis* expression in SL1344 wild type pZep*fis-gfp* and SG03 *topA::kan* pZep*fis-gfp,* with or without novobiocin at a final concentration of 50 μg/ml, incubated under aerated or non-aerated conditions. Novobiocin had possibly much more of a negative effect in *fis* expression along the growth cycle in samples in Fig. 5.3 because the drug was administered at the time of inoculation at 0 h, as described. Conversely, for samples in Fig. 5.2, where drugs were administered after two hours upon inoculation, *fis* expression did not decreased but only by the time it reached the 25 h time point.

During stationary phase, *fis* expression was higher and sustained during stationary phase in the SG03 *topA* mutant strain cultures grown under aerated conditions (Fig. 5.3B, black), and even increased further under non-aerated conditions (Fig. 5.3A, black). Conversely, antibiotic-treated *topA* mutant samples (Fig. 5.3A & B, blue) expressed *fis* during the stationary phase in a similar pattern to that of the non-treated wild type under both growth conditions (Fig. 5.3A & B, red). The difference in *fis* expression levels between the antibiotic-treated *topA* mutant samples (Fig. 5.3A, blue) and the non-treated wild type under non-aerated conditions (Fig. 5.3A, red) may be due to *faster* impairment effect of novobiocin on DNA gyrase during non-aerated conditions and because the drug was administered at the time of inoculation (0-h), as it also occurred in the treated wild-type during non-aerated conditions (Fig. 5.3A, green).

It is important to note that in *topA* mutant and in wild type cells grown under microaerobic conditions, growth is slower than in aerated conditions (Fig. 5.3C, black vs. red). By the time the aerated wild type has reached stationary phase, the *topA* mutant or microaerobic cells are still coming out from logarithmic phase. Perhaps this allows DNA to be more negatively supercoiled during the stationary phase of growth under non-aerated conditions. In other words, *topA* mutants tend to be negatively hypersupercoiled thus allowing for an extended logarithmic phase as it is known that at the onset of stationary phase DNA starts relaxing (section 1.5).

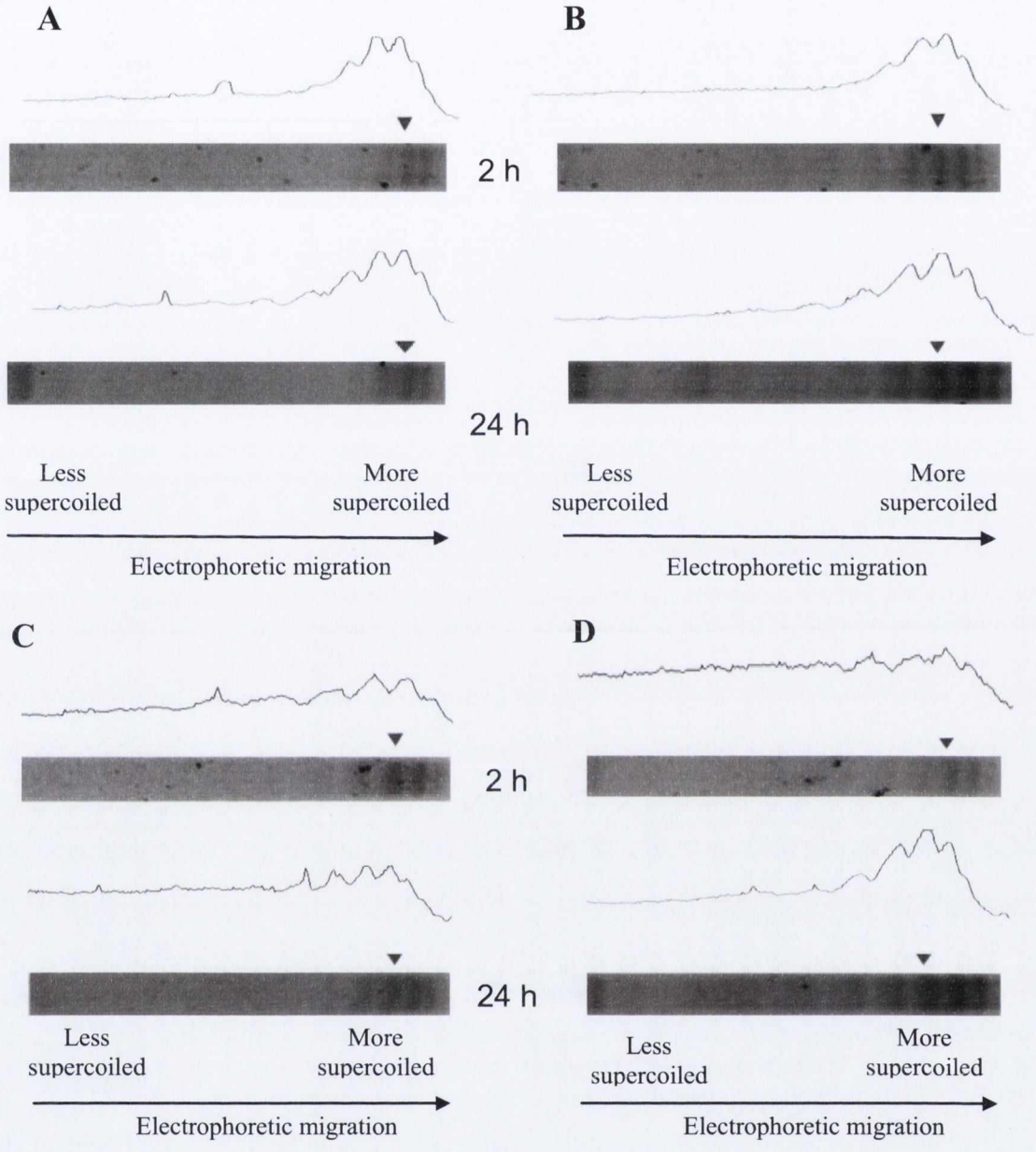
These results demonstrate that when the wild type strain carries a *topA* mutation, *fis* expression levels are sustained during late-stationary phase even under aerated growth, and increase under non-aerated conditions. The data are consistent with concerted action where DNA gyrase and TopA modulate *fis* transcription. Also, data suggest a correspondence between highly negatively supercoiled DNA and increased *fis* expression levels, which will be explored in the next section.

5.2.3 Changes in DNA topology correspond to changes in *fis* expression levels

Experiments in the previous section (5.2.2) had shown that the expression pattern of the *fis* gene was sensitive to mutations and drug treatments that altered the degree of DNA supercoiling in SL1344. The effects of these treatments on DNA supercoiling were monitored directly using a suitable reporter plasmid and a gel electrophoresis assay. The plasmid was isolated from the strains of interest (with or without drug treatment) and the populations of plasmid topoisomers were then analysed by electrophoresis in an agarose gel containing chloroquine. These experiments were done using SL1344 wild type and the *topA:kan* mutant SG03 (Table 2.1), both carrying the 2-k.b. reporter plasmid pUC18. Each strain was grown under aerated and non-aerated conditions. During the mid-log and late-stationary phase of growth, plasmid DNA was extracted and supercoiling was then assessed by the chloroquine gel electrophoresis method (section 2.13).

Plasmid DNA was isolated after 2 and 24 h and, where applicable, following inhibition of DNA gyrase by novobiocin. The pUC18 topoisomers were separated on agarose gels containing 2.5 μg/ml chloroquine. Under these conditions, topoisomers with higher superhelical density migrate farther (*x*-axis). Image J® densitometry analysis confirmed the location of the dominant topoisomer (highest peak indicated with an arrow) for each culture sample and each aeration regime, and designated it either more negatively supercoiled (i.e. further to the right) or less negatively supercoiled (i.e. further to the left) relative to the non-aerated or aerated control (Fig. 5.4A & C, respectively). Since the goal of these experiments was to examine the absence or presence of negatively supercoiled DNA topoisomers at certain time points, and due to the nature of the obtained results, no quantitative data analyses (i.e. statistical proof) were possible to perform.

Fig. 5.4: Effects of novobiocin on pUC18 DNA supercoiling in SL1344 wild type. The effects of novobiocin at a final concentration of 50 μg/ml on pUC18 reporter plasmid DNA supercoiling in SL1344 wild type cultures grown under aerated or non-aerated conditions were monitored by chloroquine gel electrophoresis. A) non-aerated; B) novobiocin-treated non-aerated; C) aerated; D) novobiocin-treated aerated. Plasmid DNA was isolated after 2 h (exponential), and 24 h (late-stationary) and, where applicable, following inhibition of DNA gyrase by novobiocin. The pUC18 topoisomers were separated on agarose gels containing 2.5 μg/ml chloroquine. Under these conditions, topoisomers with higher superhelical density migrate furthest (*x*-axis). Image J® densitometry analysis confirmed the location of the dominant topoisomer (highest peak indicated with an arrow) for each culture sample and each aeration regime, and designated it either more negative (i.e. further to the right) or less (i.e. further to the left) negatively supercoiled than the control (non-treated wild type). Topoisomers of plasmids isolated from cultures grown under aerated conditions (C) were less supercoiled for the 24 h time point, whereas those from non-aerated growth conditions were more supercoiled (A). Novobiocin treated samples (B & D) were less supercoiled than the non-treated (A & C). Experiments were performed on three separate occasions and typical data are shown.


A
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Less supercoiled
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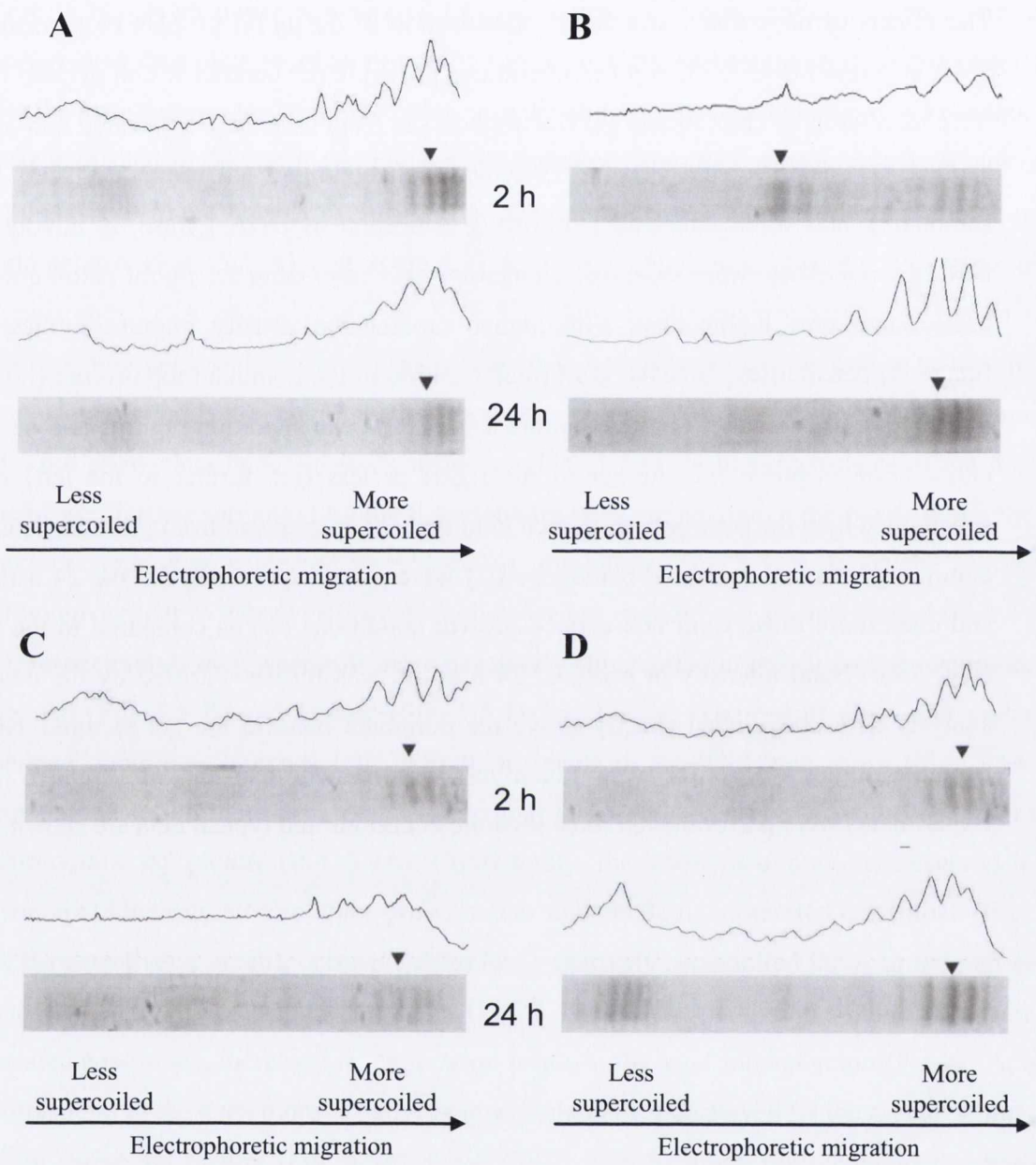
Electrophoretic migration

Topoisomers of pUC18 plasmids isolated from wild type SL1344 (Fig. 5.4), or mutant SG03 (Fig. 5.5), grown under non-aerated (A) and aerated (C) conditions, were more negatively supercoiled than their novobiocin-treated non-aerated (B) and aerated (D) sample counterparts for the stationary phase 24 h time point. As the ability of the *topA* gene product to relax DNA was abolished in the *topA*::*kan* mutant, topoisomers from the untreated SG03 mutant culture showed a different negatively supercoiled DNA profile at 2 h and 24 h time points, in both non-aerated (Fig. 5.5 A) and aerated (Fig. 5.5 C) conditions, than the wild type control topoisomer DNA profile (Fig. 5.4 A & C). Due to the electrophoretic migration of the chloroquine gels, and as mentioned before, it is understood that the farther to the right the topoisomer band is the more negatively supercoiled it is. Moreover, because chloroquine gels did not show clear bands in some instances (Fig. 5.5 C, 24 h) the presence of the dominant band was further supported by the densitometry peak shown above the band. When the *topA* mutant strain was subjected to novobiocin inhibition (Fig. 5.5 B & D) the topoisomers although similar to those in the treated wild type non-aerated and aerated control (Fig. 5.4 B & D, respectively), they were still more negatively supercoiled in the culture grown under non-aerated (Fig. 5.5 B) and aerated (Fig. 5.5 D) conditions. The results show that under non-aerated conditions, increased *fis* expression levels in the wild type strain (Fig. 5.3A, red) correspond to the more negatively supercoiled DNA displayed by the wild type strain in the chloroquine gel picture (Fig. 5.4A). Consistently, the lower *fis* expression observed in wild type novobiocin-treated samples grown under non-aerated and aerated conditions (Fig. 5.3A & B respectively, green), corresponds to less negatively supercoiled DNA in the non-aerated and aerated novobiocin-treated samples (Fig. 5.4B & D, respectively). Likewise, under non-aerated conditions, increased *fis* expression levels in the *topA* mutant strain (Fig. 5.3A, black) correspond to the even more negatively supercoiled DNA displayed by the mutant strain in the chloroquine gel picture (Fig. 5.5A, 3 h & 24 h). Furthermore, the increased *fis* expression observed at the 24 h time point in the non-drug-treated mutant samples grown under non-aerated conditions (Fig. 5.3A green), correspond to the more negatively supercoiled DNA in the non-treated mutant culture samples grown under non-aerated conditions at the 24 h time point (Fig. 5.5A).

These data show that inhibition of DNA gyrase results in more relaxed DNA and that the genetic elimination of TopA results in increased negative supercoiling. These changes in DNA could correspond to changes in levels of *fis* expression.

Fig. 5.5: Effects of novobiocin on pUC18 DNA supercoiling in mutant SG03 *topA::kan*. The effects of novobiocin at a final concentration of 50 μg/ml on pUC18 reporter plasmid DNA supercoiling in SG03 *topA::kan* cultures grown under aerated or non-aerated conditions were monitored by chloroquine gel electrophoresis. A) non-aerated; B) treated non-aerated; C) aerated; D) treated aerated. Plasmid DNA was isolated after 2 h (exponential), and 24 h (late-stationary), and where applicable following inhibition of DNA gyrase by novobiocin. The pUC18 topoisomers were separated on agarose gels containing 2.5 μg/ml chloroquine. Under these conditions, topoisomers with higher superhelical density migrate furthest (*x*-axis). Image J® densitometry analysis confirmed location of the dominant topoisomer (highest peak indicated with an arrow) for each culture sample and each aeration regime, and designated it either more negative (i.e. further to the right) or less (i.e. further to the left) negatively supercoiled than the control (non-treated wild type). Topoisomers from plasmids isolated from cultures grown under aerated conditions (C) were more supercoiled for the 24 h time point, and even more those from non-aerated growth conditions (A) as compared to the wild type (Fig. 5.4). Band intensity in panel C, 24 h point, was further clarified by the densitometry analysis arrow positioned exactly above the dominant band in the gel picture. Novobiocin treated samples (B & D) were much less supercoiled than the non-treated (A & C). Experiments were performed on three separate occasions and typical data are shown.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Electrophoretic migration

5.3 Discussion

In the previous chapters I showed the environmental conditions and a possible regulation strategy that favours a sustained expression of *fis* during the stationary phase of growth. However, the question still remained as to what maintains such sustained expression. To answer this, I decided to focus in DNA supercoiling, mainly because bacterial cells have a subset of genes whose expression corresponds with changes in DNA supercoiling brought about by microaerobic (Ó Cróinín *et al.,* 2006; Ó Cróinín and Dorman, 2007) and anaerobic growth (O'Byrne *et al.,* 1994). I wanted to investigate the possibility that *fis* was among them.

I investigated the role in *fis* expression of the two key proteins in supercoiling: DNA gyrase and topoisomerase I (TopA). Inhibition of DNA gyrase activity with the drugs novobiocin or coumermycin (CA1) increased *fis* transcription after early exponential phase (Figs. 5.2 & 5.3). This confirms a role for DNA negative supercoiling in optimal expression of the *fis* gene, and shows that gyrase activity determines the shape of the *fis* expression curve. Genetic removal of DNA topoisomerase I also changes the pattern of *fis* expression throughout growth (Fig. 5.3 black), confirming a role for DNA supercoiling in the regulation of the *fis* promoter. Moreover, the intact *topA* gene is needed for the decrease in *fis* expression that is seen in wild type cells in aerated cultures (Fig. 5.3 B red). All these findings are consistent with the known links between DNA supercoiling and the metabolic flux of the cell, and the role of Fis protein responding to changes in nutrient availability (Schneider *et al.*, 1997; Travers *et al.,* 2001; Travers and Muskhelishvili, 2005). The likelihood of a physiologically-significant link between microaerobic growth, DNA supercoiling and *fis* promoter activity seem particularly compelling. Furthermore, DNA supercoiling and Fis have been studied since for their concerted ability to modulate SPI-1 gene expression. It has been shown that this concerted action enables the bacterium to invade cells at the epithelial surface of the gut where microaerobic conditions prevail (Ó Cróinín *et al.,* 2006; Ó Cróinín and Dorman, 2007; Cameron *et al.,* 2011; Cameron *et al.,* 2012).

Data obtained in the present study showed that impaired function of DNA gyrase in the wild type strain enhance *fis* expression in cultures grown under aerated and non-aerated conditions (Fig. 5.2). Loss of TopA function seemed to maintain continuous expression of the *fis* gene. These results also may suggest that when DNA becomes less supercoiled in the wild type

during late-stationary phase (Fig. 5.4C, 24 h), *fis* expression levels cannot be maintained and become reduced as in aerated conditions (Fig. 5.3B red, 24 h). Therefore, if *fis* expression in the wild type is sustained during late-stationary phase and in non-aerated conditions (Fig. 5.3A red) then, it may be possible, although not conclusive, that DNA is more negatively supercoiled during this time (Fig. 5.4A, 24 h). This is in keeping with the observation that *fis* expression in the *topA* mutant is sustained in both aerated and non-aerated conditions (Fig. 5.3A & B, black), as happens in the wild type strain in non-aerated conditions (Fig. 5.3A red).

In both *E. coli* and *Salmonella, topA* mutants exhibit increased levels of negative DNA supercoiling, just as wild type bacteria do when exposed to high-osmolarity growth conditions, and this results in a supercoiling-dependent induction of the transcription of *proU*, an operon that only expresses in high-osmolarity conditions (Higgins *et al.,* 1988). Interestingly, a connection has been established between the dynamic nature of DNA supercoiling and changes in several environmental parameters that are relevant to infection of mammalian hosts, such as pH, temperature and oxygen availability (Higgins *et al.,* 1988; Dorman, 1991). When *Salmonella* grows at low oxygen concentrations, its DNA becomes more negatively supercoiled (Dorman *et al.,* 1988). Therefore, it is plausible that *Salmonella topA* mutants possess levels of negative supercoiling in their DNA that approximate to those seen in the wild type when growing under microaerobic conditions and that this leads to increased levels of *fis* expression.

Overall, the results presented here suggest that *fis* expression is sustained during the later part of the growth cycle as a result of a counter-action that sustains DNA gyrase activity but sequesters TopA function, simultaneously, as a response to low oxygen conditions (Fig. 5.3A, (wt) red and (*topA* + novo) blue). This is most likely to involve the maintenance into stationary phase of a ratio of [ATP] to [ADP] that is favorable for DNA gyrase activity with a concomitant repression of *topA* gene transcription by the Fis protein.

Chapter 6

Fis binding patterns in different aeration and growth regimes in *Salmonella enterica* serovar Typhimurium

6.1 Introduction

In this chapter, I will show the effect of aeration on the level of Fis occupancy along selected and non-selected gene regions of the chromosome in *S.* Typhimurium. Attention was given to 20 pre-selected gene regions containing genes known to be induced by low aeration or microaerobiosis, including those genes whose products influence DNA supercoiling; and also to other 20 non pre-selected gene regions displaying the highest Fis enrichments. This is important from the standpoint of bacterial cell growth and its relationship to Fis, in order to predict the ability of *Salmonella* to invade its host. The goal of this chapter is to provide information, by the application of chromatin immunoprecipitation-on-chip (ChIP-chip) (sections 1.16 & 2.14), on how Fis interacts with different genetic regions, known and unknown, along the *S.* Typhimurium chromosome depending on the growth aeration regime. ChIP-chip studies like this one have the potential to aid understanding of how Fis affects transcription and other DNA transactions globally (Finkel & Johnson, 1992) through its ability to affect the level of DNA supercoiling in the cell (Muskhelishvili & Travers, 2003). This chapter describes a novel Fis-binding pattern that may help elucidate the molecular mechanism responsible for the sustained expression of the *fis* gene under microaerobic conditions.

As previously described (section 1.2), expression of *fis* peaks during mid-log phase and then decreases to very low levels in late-stationary phase (Osuna *et al.,* 1995). However, as it also was shown in Chapter 3, that the nature of the growth aeration regime determines the level of *fis* expression at a given time-point in *Salmonella* Typhimurium. The finding that *fis* expression levels are sustained in late-stationary phase when bacterial cells are grown under non-aerated or microaerobic conditions (Ó Cróinín *et al.,* 2007), led me to investigate Fis protein occupancy at pre-selected and non pre-selected genetic regions. Pre-selected regions containing genes involved in the onset of stationary phase, respiration, stringent response, or DNA supercoiling, were studied because Fis plays an important role in these processes. The pre-selected gene regions were: *nuoA, guaC, fis, topA, gyrA, gyrB, dksA, relA, spoT, fliA, ssrA, orgA, hilA, tyrT, arcA, arcB, rpoS, nrfA, fur,* and *brnQ.*

In Chapter 3, I confirmed *fis* sustained expression during the late-stationary phase of growth under non-aerated or microaerobic (i.e. SPI-1-inducing) conditions. In this chapter 6, the

genes *ssrA, orgA, hilA, nrfA, fur, arcA*, and *arcB* were selected for further investigation of their interaction with Fis as these could be considered 'aeration response' genes (sections 1.3 and 6.3).

In Chapter 4, I studied a regulatory role for stringent response factors DksA and ppGpp in *fis* gene expression in *Salmonella* Typhimurium. Those results indicated that the transcription factor DksA and the alarmone ppGpp have negative roles in controlling *fis* expression. Genes *dksA, relA* and *spoT* (ppGpp precursors) were selected for inclusion in the Fis binding pattern analysis for this reason. Also, the stationary phase sigma factor gene *rpoS* was investigated for its Fis binding pattern, due to its concomitant interaction with *fis* and its role in the onset of the stationary phase and the stringent response. During the stringent response, bacterial multiplication stops, as nutrients become depleted, giving way to the stationary phase of growth. Studies have shown that entering and surviving in stationary phase involves complex physiological control in *Salmonella* and *E. coli* (Siegele & Kolter, 1992; Kolter *et al.*, 1993). Multiple factors including nutrient starvation brought about by limiting carbon, nitrogen or phosphate sources can stop growth (O'Neal *et al.*, 1994; Seymour *et al.*, 1996). In addition, electron acceptors, such as oxygen, are also important in bacterial metabolism. In anaerobic conditions, when oxygen is absent, the stationary phase of growth may occur not only because of depleted nutrients but also because of limiting electron acceptors. Also, *Salmonella* must adapt quickly to the environmental transitions experienced in its infection process. It has to face several challenges as it passages from the gastrointestinal tract to the intracellular milieu. These include fluctuations in the oxygen concentration and a series of host-immune defenses like the reactive oxygen species (ROS) and reactive nitrogen species (RNS) (Morgan et al., 2004; Galan et al., 2001; Wallis et al., 2000; Cirillo et al., 1998). For this reason, the *nrfA* locus, encoding nitrogen respiration factor A, was also investigated for Fis-binding patterns.

In Chapter 5, I showed how DNA gyrase contributes to the sustained expression of Fis. Much like H-NS, Fis is known to modify the topology of the bound DNA (Dillon and Dorman 2010; Dorman and Kane 2009). Interestingly, the Fis protein was found to be a global regulator of supercoiling in *E. coli*, but the protein had less influence over supercoiling control in *S. enterica* (Cameron et al., 2010). For this reason, genes known to affect DNA topology, such as *gyrA, gyrB, topA* and *nuoA* were studied for their Fis binding patterns in different aeration regimes.

In this chapter, the global regulatory role of Fis in different aeration regimes was studied to better understand the downstream consequences of the sustained expression of the *fis* gene during microaerobic conditions. In the first part, an SL1344 *fis::3X::FLAG* strain (SG07) was constructed and tested for Fis functionality by motility and western blot assays. In the second part, Fis enrichment in known and possible Fis-binding genomic regions in culture samples of this strain was investigated by real-time qPCR, as well as by genome-wide microarray analysis using ChIP-chip technology, where Fis binding peaks were indicated using the bio-informatic tool ChIPOTle. Additionally, an overall heatmap was produced based on the data obtained from the SL1344 genome-wide microarray of Fis binding regions, to observe 20 of the most Fis-enriched gene regions across all the four different aeration growth regimes (aerated exponential and stationary growth phases, and non-aerated exponential and stationary growth phases).

6.2 Results

6.2.1 Construction of *Salmonella* Typhimurium SL1344 *fis*-FLAG-tagged strain

A SL1344 *fis::3XFLAG::kan* tagged strain, SG07 (Table 2.1), was constructed using a homologous recombination technique (section 2.10). The *fis::3XFLAG::kan* gene construct was later transferred into a fresh SL1344 background by bacteriophage P22 generalized transduction (section 2.5.2).

It was deduced by DNA sequence analysis that a 40-bp C-terminal 3XFLAG epitope had been added to the 297-b.p. *fis* gene, together with a kanamycin resistance cassette. The custom automated sequencing was performed at GATC-Biotech, Germany, using the primers ST FLAG.Fis.conf-For and ST FLAG.Fis.conf-Rev (Table 2.3). The 3XFLAG epitope was added at the part of the *fis* gene encoding the C-terminal of the Fis protein by a PCR-based method using plasmid pSUB11 as template. Primers used for introducing the 3XFLAG epitope were FWD_Fis_3xFLAG and REV_Fis_3xFLAG (Table 2.3). These primers were designed by pairing pSUB11 epitope-side primers of 20-b.p. next to the 40-b.p. sequences

before and after the *fis* stop codon TAA. These PCR products contained 40-b.p. of DNA sequence homology at both the 5' and 3' ends of the region of the chromosome where thetagged gene was to be integrated. The tagged *fis* gene construct and its associated *kan* cassette were then introduced onto the chromosome of *S.* Typhimurium SL1344 using the λ-red recombination system. Where wild type SL1344 genomic DNA was used as template for the PCR reaction, the primers generated an amplicon of 500-b.p. in size (Fig. 6.1). And, where the SG07 *fis::3XFLAG::kan* genomic DNA was used as a template the primer pair produced amplicons of approximately 1.5-k.b. and 0.5-k.b. in size (Fig. 6.1).

6.2.2 Tests to examine functionality of Fis in a constructed strain

6.2.2.1 Motility tests

The Fis protein is known to be essential for motility in *S.* Typhimurium (Kelly *et al.,* 2004; Osuna *et al.,* 1995; Yoon *et al.,* 2003). Motility assays were performed as an indirect assay for the functionality of the FLAG-tagged Fis protein in strain SG07. Levels of motility were compared to those of the wild-type strain SL1344, a *fis::cat* mutant strain SG04, and a strain with restored *fis* function, *fis* $^{+}$, SG08 (section 2.5 and Table 2.1), (Fig. 6.2).

The SG07 *(fis::3XFLAG::kan)* tagged strain displayed a similar swarming pattern to SL1344 (wild type), while the SL1344 *(fis::cat)* mutant strain showed significantly reduced motility (Fig. 6.2A, B and C). Introduction of the *fis::3XFLAG::kan* construct into a *fis* knockout mutant restored full motility (Fig. 6.2D). The diameter of the swarming patterns varied according to the genetic construct of each strain. The swarming diameters from every strain were as follows: SL1344 (wild type) 7.2 cm.; SG07 *(fis::3XFLAG::kan),* 7.0 cm.; SL1344 *(fis::cat),* 3.5 cm.; and SG08 (*fis*$^{+}$), 6.9 cm. (Fig. 6.2).

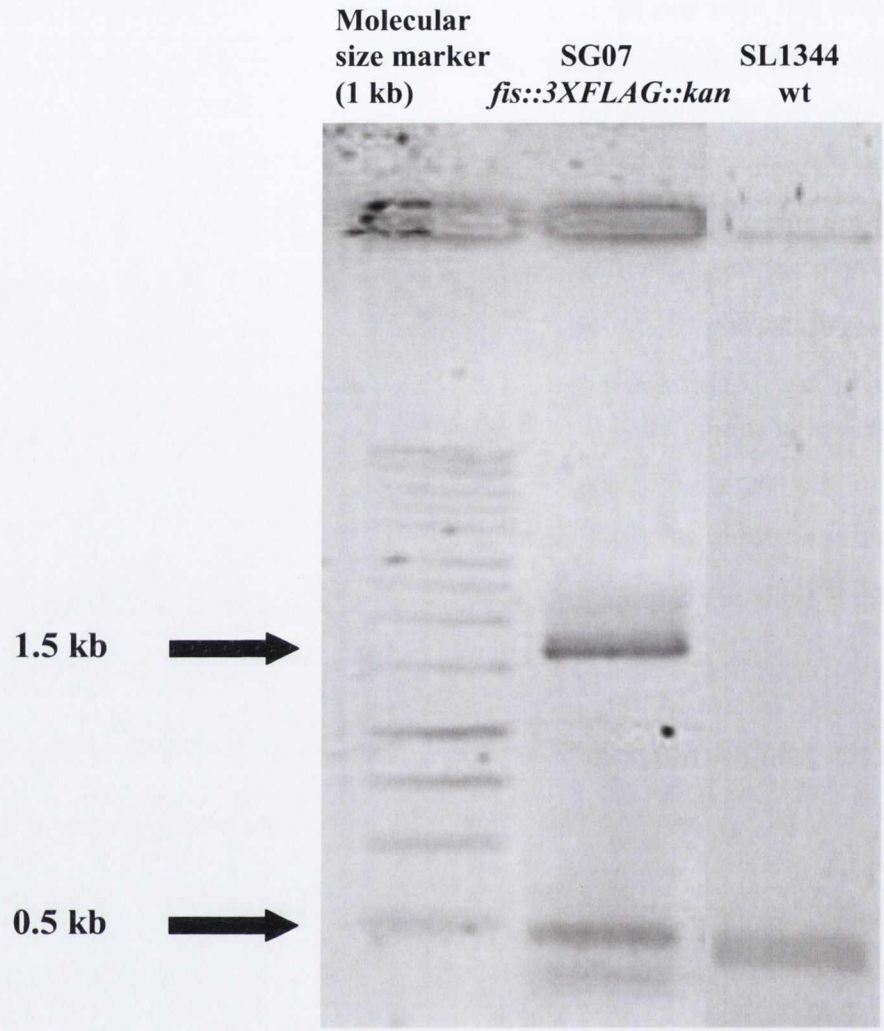


Fig. 6.1: Confirmation of a C-terminal 3XFLAG tag insertion in *fis*. A 3XFLAG epitope was added at the C-terminal of the Fis protein. The *fis::3XFLAG::kan* gene construct was amplified by PCR using genomic DNA from SG07 and the corresponding *fis* locus was amplified from wild-type strain SL1344. An amplicon of 0.5 kb in size was generated in the wild type (SL1344) background and amplicons of approximately 1.5 kb (*kan* cassette) and 0.5 kb (FLAG-tagged *fis*) in size in the FLAG-tagged (SG07) strain background. These results verified the insertion of the DNA sequence specifying the 3XFLAG tag at the 3' end of *fis* and the presence of the *kan* cassette in the chromosome downstream of the tagged *fis* gene. A molecular size marker (DNA ladder) of 1-k.b. was used for size reference (section 2.11.1).

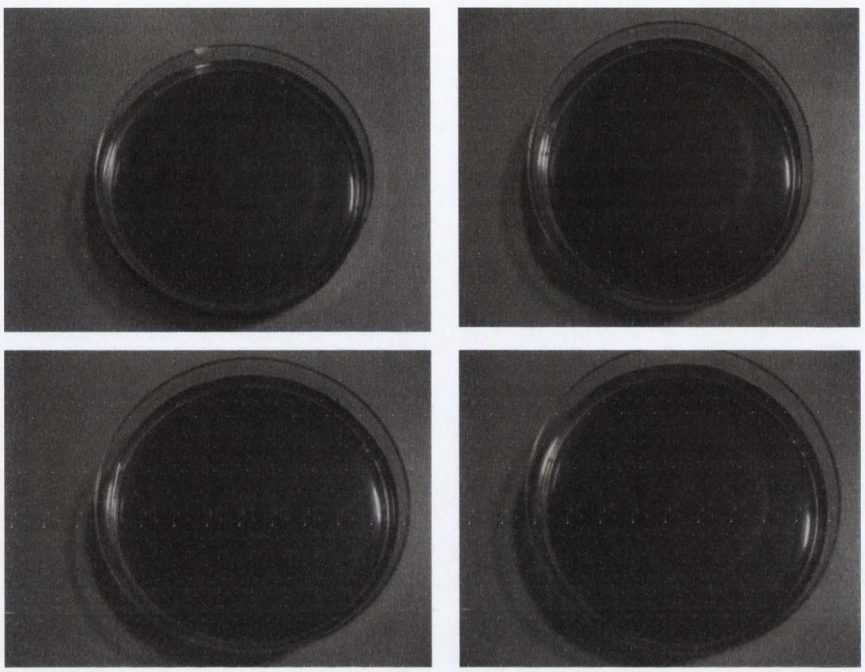


Fig. 6.2: Effect of *fis::3XFLAG* insertion on the motility of *S.* Typhimurium. A) SL1344 (wild type), B) SG07 (*fis::3XFLAG::kan*), C) SL1344 (*fis::cat)*, and D) SG08 (fis^+) were all compared for motility. Semi-solid swarming plates were inoculated centrally with equal numbers of bacteria and incubated at 37°C for 6 hours. The "swarming" or rate of bacterial ring diameter spreading on a swarm plate was indicative of chemotaxis and motility. The diameter of swarming patterns were as follows: A) SL1344 (wild type), 7.2 cm.; B) SG07 *(fis::3XFLAG::kan),* 7.0 cm.; C) SL1344 (*fis::cat),* 3.5 cm.; and D) SG08 (fis^+), 6.9 cm.

6.2.2.2 Immunodetection of the FLAG-tagged Fis protein in different aeration regimes

Western blots were performed with culture samples from each aeration regime to detect the presence of the Fis protein in the FLAG-tagged strain SG07 (*fis::3XFLAG::kan)* during mid-log (2 h) and late-stationary (24 h) growth phases (Fig. 6.3) using the antibody SIGMA Anti-3X FLAG (section 2.12.3). The SL1344 (wild type) strain was used as negative control to show absence of bands in those culture samples lacking the FLAG-tagged Fis protein. The aerated 2 h culture sample from SG07 (*fis::3XFLAG::kan)* served as an internal positive control to show Fis protein presence when compared to the bands from the non-aerated growing conditions. The DnaK protein was used as a loading control. Because evidence of presence and size of the FLAG-tag insertion to the C-terminus of the *fis* gene was given earlier (Fig. 6.1), no size marker was shown in this immunoblot. Fis protein was detected in SG07 (*fis::3XFLAG::kan)* cultures grown under aerated and non-aerated conditions, at the mid-log (2 h) and late-stationary phases (24 h) of growth, while the SL1344 (wild type) carrying no FLAG-tagged Fis protein did not show such protein bands.

The confirmation, motility, and immunoblot tests were considered enough evidence to suggest that the SG07 (*fis::3XFLAG::kan)* strain was functional and thus be effectively used for the next set of experiments.

6.2.3 Binding patterns of the Fis protein in different aeration regimes

S. Typhimurium SG07 (*fis::3XFLAG::kan*) was grown under aerated and non-aerated conditions, and harvested at both exponential (2 h) and late-stationary (24 h) phases of growth. To determine the genomic binding patterns of the Fis protein, samples were prepared for chromatin immunoprecipitation and microarray technology (ChIP-chip) (section 2.14) and real-time quantitative PCR (qPCR) (section 2.9.2).

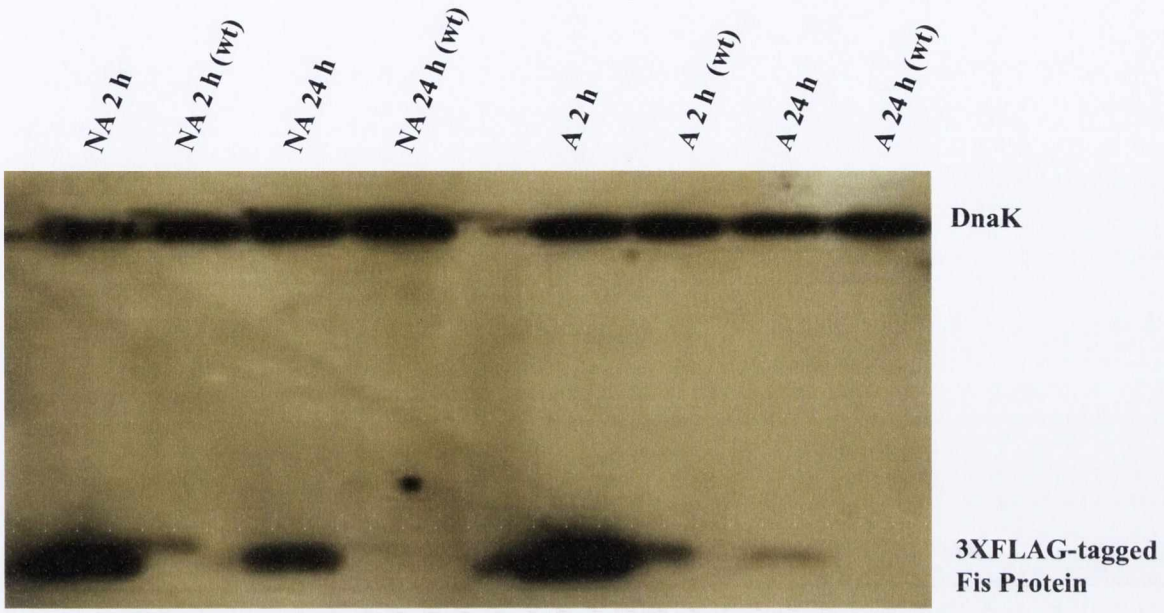


Fig. 6.3: Western blot showing FLAG-tagged Fis protein carrier strain SG07 versus comtrol non-carrier FLAG-tagged Fis protein strain SL1344 wild type (wt). FLAG-tagged Fis protein detected in SG07 (*fis::3XFLAG::kan*) cultures grown under aerated (A) and non-aerated (NA) conditions, and during mid-log (2 h) and late-stationary phases (2 h). DnaK protein was used as a loading control. The SG07 strain displayed clear FLAG-tagged Fis protein bands for culture samples grown under both aerated and non-aerated conditions, and during mid-log and late-stationary phases, as opposed to the SL1344 (wild type), negative control.

Bacterial culture samples from 2 h or 24 h time points were normalized to an OD at A_{600}=10. Subsequently, the cells were fixed, lysed and sonicated (section 2.14) and sheared chromatin DNA from all culture samples was immuno-precipitated. DNA treated with mouse specific IgG antibody was named IgG DNA, and that treated with FLAG-tag antibody was named ChIP DNA. IgG and ChIP DNA were designated the name of test samples. DNA that was not antibody-treated was frozen at -80°C for later use in the chip microarray experimental portion as an internal DNA control. This DNA control was designated the name of 'input' or 'mock IP' DNA. DNA from immunoprecipitated test DNA samples was eluted (section 2.14.5) and the cross-links reversed (2.14.6). DNA was subsequently extracted (section 2.8) in preparation for either qPCR (section 2.9.2) (except input DNA), or labelling for microarray hybridization (section 2.14.7) (except IgG DNA). Prior to microarray hybridization, qPCR reactions were peformed to determine the quality of the immunoprecipitated DNA from the strain harbouring the FLAG-tagged Fis protein, by confirming Fis occupancy levels along a few genomic regions known to contain Fis-binding sites. Extracted DNA (section 2.8) from either ChIP DNA or IgG DNA samples was used as a template for all qPCR experiments in sections 6.2.3.1 and 6.2.3.2.

ChIP-chip mean quantity values of Fis enrichment were calculated based on a dilution series using SL1344 (wild type) genomic DNA as a template with the specific primer for the gene region in question. Mean quantities were converted into $\log_2$ values using the Microsoft Excel® programme function. ChIP DNA $\log_2$ values were normalized over the control IgG DNA $\log_2$ values to obtain the $\log_2$ Fis enrichment ratio and thus make them comparable to the ChIP-on-chip data. This $\log_2$ Fis enrichment ratio along the Y-axis in all panels B) (qPCR panels) ranges from 0.1 to 10, where 1 is considered the threshold cut-off value between high (above 1), or low (below 1) Fis enrichment, based on the positive *topA* (section 6.2.3.1), and negative *guaC* (sections 6.2.3.1 & 6.2.3.2) control $\log_2$ enrichment ratio values, and based also in the threshold cut-off value of 1, in the ChIP-chip $\log_2$ Fis enrichment ratio values. All ChIP-chip experiments were performed in duplicate, and three independent biological replicates were prepared for each set of conditions. Results represent the average $\log_2$ Fis enrichment ratio to a binding gene region from each set of triplicates in every of the four conditions from the ChIP-chip experiments. The ChIPOTle peak-finding algorithm (Buck *et al.*, 2005) was used to identify and average the sites of significant Fis enrichment from in all replicates (P value < 0.00001, corrected P value < 0.05). The peak height of the identified Fis-

binding peaks was calculated as a $\log_2$ enrichment ratio by dividing the Cy3 (ChIP DNA) over the Cy5 (input DNA) signal intensities of the gene probe corresponding to the identified peak, and are indicated with solid rectangles below the peaks. These gene regions were selected based on chip microarray data in *E. coli* as a function of the aeration regime, as culture samples were harvested from aerobic and anaerobic conditions and in known Fis regulation studies over these gene regions (Cho *et al.*, 2008). For example, the *nuoA* gene region which displayed high levels of Fis enrichment along its promoter site in *E. coli* during aerobic mid-log conditions (Cho *et al.*, 2008) showed similar high enrichment levels in *S.* Typhimurium under the same conditions (Appendix fig. A.18, panel A1 & panel B1). Similarly, the *guaC* gene region that did not show Fis occupancy in *E. coli* during aerobic mid-log conditions (Cho *et al.*, 2008) did not display Fis enrichment either in *S.* Typhimurium during the same conditions (sections 6.2.3.1 and 6.2.3.2). Furthermore, a dozen genes from the 20 pre-selected gene regions were also assessed for Fis enrichment by qPCR (sections 6.2.3.1 & 6.2.3.2 and appendix figures A.4B; A.6B; A.11B; A.14B; A.15B; and A.17B to A.19B). For these qPCR experiments, Fis enrichments were originally measured also as mean quantities. These quantities reflect the amount of enrichment for Fis binding sites for the indicated promoter or ORF. All real-time qPCR reactions were performed in duplicate; and two independent biological replicates were prepared for each set of conditions. The $\log_2$ Fis enrichment ratio present in the qPCR panels is the average from each set of duplicates in every of the four conditions from the qPCR reactions.

6.2.3.1 Confirmation of Fis occupancy in known binding regions

Based on data from studies in *E. coli,* a high level of Fis occupancy was predicted at the *fis* promoter and the *topA* ORF (Cho *et al.,* 2008; Kahamanoglou *et al.,* 2011). For this reason, the *fis* and *topA* gene regions were chosen as the positive control genomic regions to determine high Fis enrichment levels.

ChIP and IgG DNA from the *fis::3XFLAG::kan* strain SG07, grown with aeration to exponential phase (2 h), was used to determine Fis occupancy at the *fis* promoter and the *topA*

open reading frame (ORF). Specific primers for the *fis* promoter and *topA* ORF were used to assess Fis enrichment at those regions using qPCR. Results indicated that Fis protein was highly enriched in the ChIP DNA compared to the IgG DNA control in the aerated 2 h cultures, at both the *fis* and the *topA* genomic regions (Fig. 6.4). These results concur with known Fis occupancy at these sites. The mean quantity of protein reflects the extent of Fis occupancy at either genomic region. This quantity was calculated based on a dilution series using SL1344 (wild type) genomic DNA as a template with *fis* or *topA* primers for the *fis* gene promoter or *topA* ORF region, respectively. These qPCR reactions were performed in duplicate, and two independent biological replicates were prepared.

Next, a negative control genomic region for Fis enrichment was determined. The *guaC* gene, encoding GMP reductase, was used as the negative control site since Fis has been reported not to associate with that gene region in *E. coli* (Cho *et al.*, 2008). ChIP and IgG DNA from the SG07 (*fis::3XFLAG::kan*) strain grown to exponential phase (2 h) and late-stationary phase (24 h) in aerated or non-aerated conditions was used. Primers for the *guaC* promoter region were used to confirm absence or minimal Fis enrichment at that region.

Results indicated that in all four growth regimes Fis occupancy at the *guaC* gene promoter region for the ChIP DNA was even lower than the IgG DNA control (Fig. 6.5). These data were consistent with the low Fis occupancy level previously reported at the *guaC* gene in *E. coli* (Cho *et al.*, 2008).

6.2.3.2 Fis enrichment levels at pre-selected and non pre-selected genomic regions

Once Fis occupancy was confirmed in known relevant genomic regions, further experiments were done using genome-wide ChIP-chip microarray technology (section 2.14) to determine Fis enrichment levels at pre-selected and non pre-selected (not selected prior to ChIP-chip experiments) genomic regions. Forty overall chromosomal regions in the SL1344 genome-wide microarray were studied for their Fis enrichment levels in response to aeration regime.

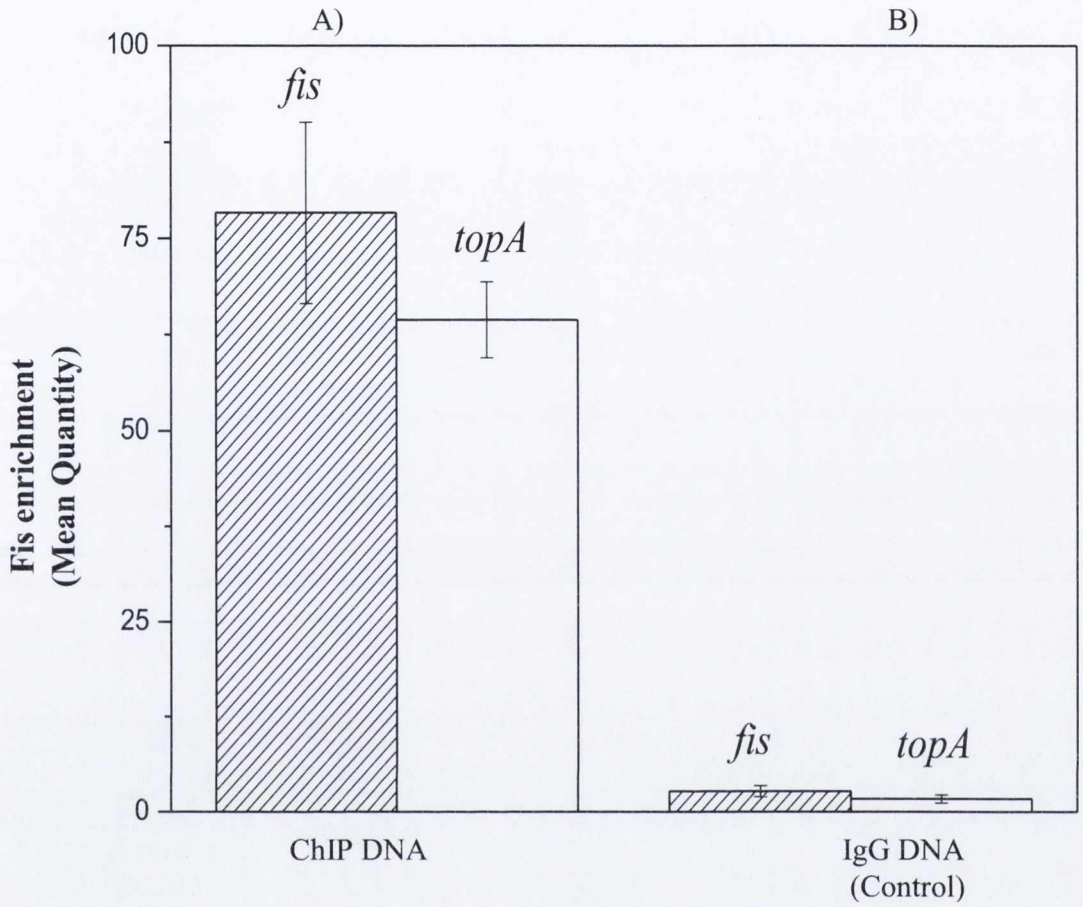


Fig. 6.4: Fis enrichment at the *fis* and *topA* gene regions. The association of the Fis protein with the *fis* promoter (line-crossed bar) and the *topA* ORF (white bar), from SG07 culture samples in mid-log phase (2 h), grown under aerated conditions. Mean quantity reflects the amount of Fis occupancy at either the *fis* promoter or the *topA* ORF using qPCR. This quantity was calculated based on a dilution series using SL1344 wild-type genomic DNA as a template with *fis* or *topA* primers for the *fis* gene promoter or the *topA* ORF region, respectively. Fis enrichment along the *fis* promoter and *topA* ORF in A) ChIP DNA or, B) control IgG DNA samples. A) During mid-log (2 h) growth phase, in ChIP DNA samples, Fis enrichment levels are similar in both gene regions; B) during mid-log (2 h) growth phase, both gene regions in the respective control IgG DNA samples show much lower Fis enrichment levels. Error bars represent standard deviations (n=2).

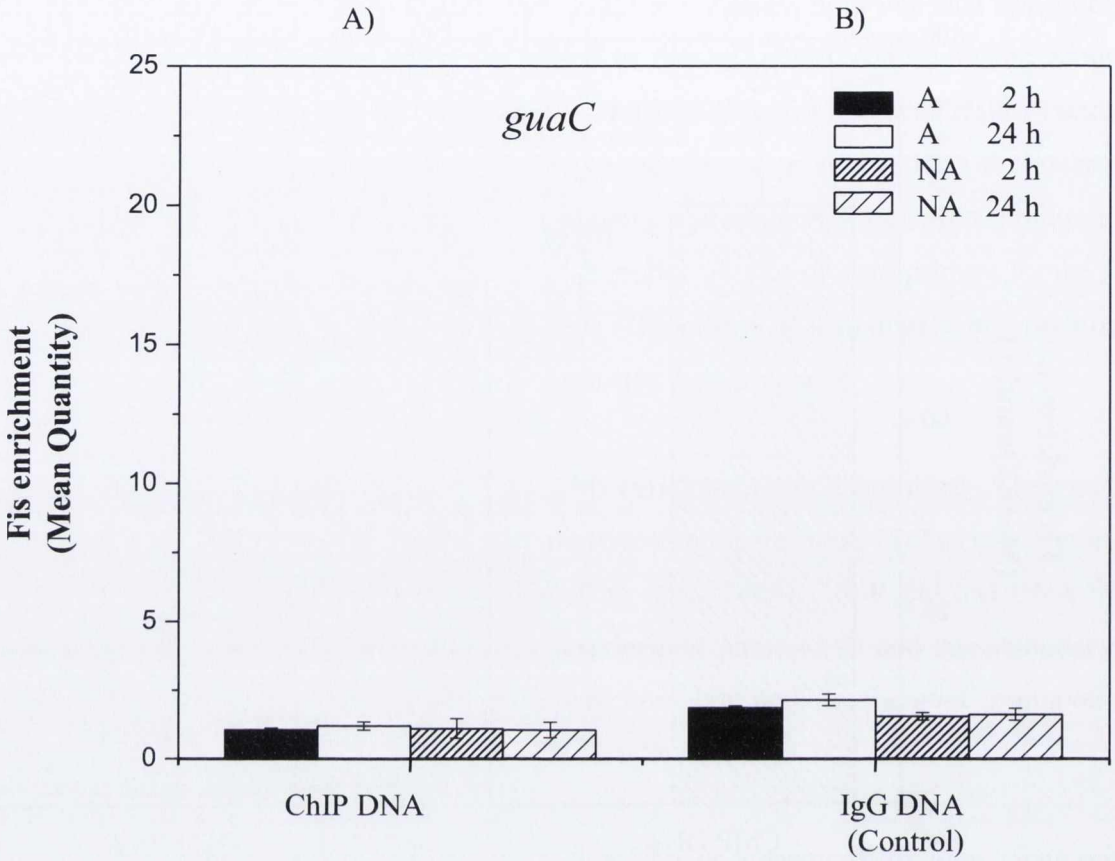


Fig. 6.5: Fis enrichment at *guaC* gene region. Fis association with the promoter region of *guaC* in SG07 culture samples in mid-log (2 h) or late-stationary (24 h) growth phase, grown under aerated or non-aerated growth conditions. Mean quantity on the Y-axis represents the amount of Fis enrichment at the *guaC* gene site in A) ChIP DNA or B) IgG DNA, using qPCR. Mean quantity was calculated based on a dilution series using SL1344 wild-type genomic DNA as a template with *guaC* promoter region primers. In all four growth conditions, ChIP DNA showed little Fis enrichment at the promoter region of *guaC* and was lower than its IgG DNA control. Error bars represent standard deviation between biological replicates (n=2).

Twenty gene regions of these forty were pre-selected prior to ChIP-chip experiments (Fig. 6.7A and appendix figures A.1-A.19), while the other twenty were not pre- selected but, were identified and studied for having the overall highest levels of Fis-enriched regions (Fig. 6.8B).

The twenty pre-selected gene regions were analyzed for Fis enrichment levels by ChIP-chip microarray technology (2.14.8) and for Fis-binding peaks by using the ChIPOTle software (sections 2.14.9 and 6.2.3) as a function of aeration regime growth condition. Measuring and unit value criteria were previously described (section 6.3.2). Additional real-time quantitative PCR (qPCR) experiments were done in a dozen of these pre-selected gene regions to further validate the ChIP-chip data, (Figs. 6.7B, and appendix figures A.1 to A.19, panel B). Mid-log (2 h) and late-stationary phase (24 h) culture samples were used for both the ChIP-chip experiments and qPCR reactions.

Fis enrichment levels from these pre-selected gene regions were revealed during exponential phase and late-stationary phase, in aerated and non-aerated growth conditions (Figs. 6.6 & 6.7A, and appendix figures A.1 to A.19). The $\log_2$ enrichment ratio on the *Y*-axis was calculated from Cy3 (ChIP DNA) and Cy5 (input DNA) signal intensities of each probe and plotted against each gene location along the 4878-kbp *S.* Typhimurium chromosome on the *X*-axis. Probe signal intensity, reflecting levels of Fis enrichment, is most marked in the non-aerated, 24 h growth regime as compared to the other conditions. Specifically, analyses revealed a total of 2214 Fis binding regions, where 450 were observed in the aerated, 2 h condition; 651 in the aerated, 24 h condition; 142 in the non-aerated, 2 h condition; and 971 in the non-aerated, 24 h condition. These results identified regions of the genome that are Fis-enriched in the ChIP DNA samples, thereby allowing construction of a genome-wide picture of interactions between Fis and the *S.* Typhimurium genome during specific growth aeration regimes (Fig. 6.6).

There are 20 figures showing each of the pre-selected gene regions and their Fis enrichment levels by ChIP-chip (Fig. 6.7A and appendix A.1-A.19), and twelve of which were also analyzed by qPCR (Figs. 6.7B; A.1 to A.4B; A.6B; A.11B; A.14B; A.15B; and A.17B to A.19B). Gene regions were ordered by their level of Fis enrichment as revealed by ChIP-chip analyses. Among all twenty regions, the negative control *guaC* gene region showed the least

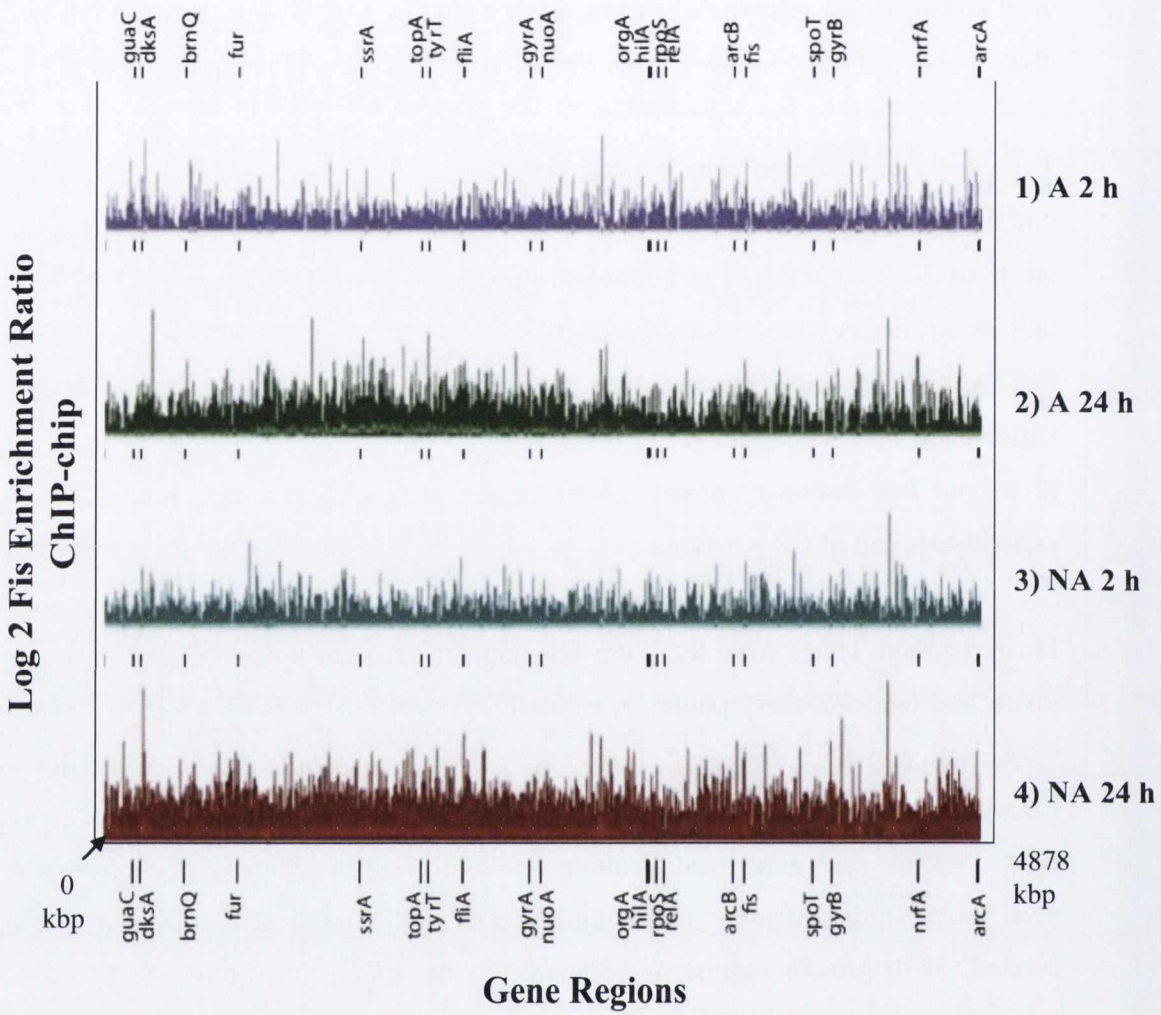
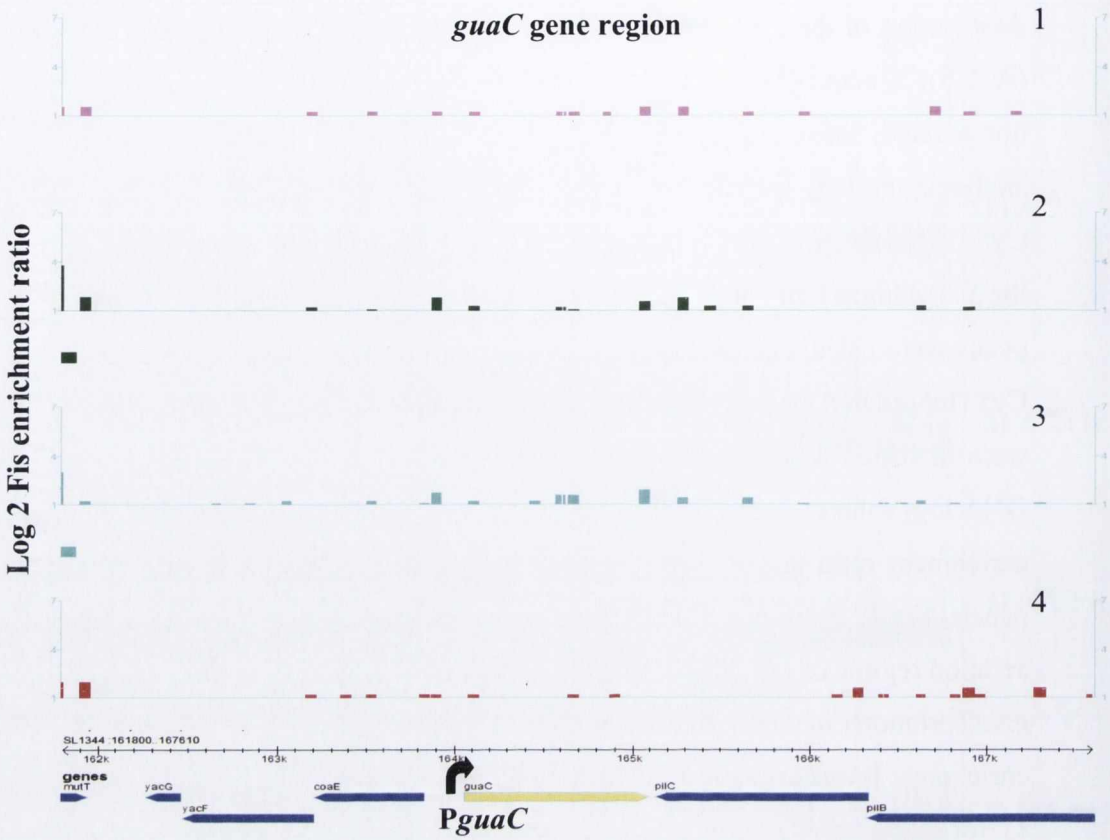


Fig. 6.6: Genome wide microarray analysis of Fis-binding regions in *S.* Typhimurium in different aeration regimes. The indicated twenty known or potentially Fis-regulated genes reveal their corresponding Fis enrichment levels during 1) aerated, mid-log (A, 2 h); 2) aerated, late-stationary phase (A, 24 h); 3) non-aerated, mid-log (NA, 2 h); and 4) non-aerated, late-stationary phase 24 h (NA, 24 h), growth conditions. Log_2 enrichment ratio is indicated on the *Y*-axis, representing Cy3 (ChIP DNA) over Cy5 (Input DNA) signal intensities for each probe and plotted against each gene location along the 4878-kbp *S.* Typhimurium chromosome on the *X*-axis. Probe signal intensity indicates highest levels for Fis enrichment in the NA 24 h growth regime (red) as compared to the other 3 conditions.

Fig. 6.7: Fis binding at the promoter region of *guaC* in different aeration regimes. Association of the Fis protein with the promoter region of *guaC*, during 1) aerated, mid-log (A, 2 h); 2) aerated, late-stationary phase (A, 24 h); 3) non-aerated, mid-log (NA, 2 h); and 4) non-aerated, late-stationary phase (NA, 24 h), growth conditions. A) For the ChIP-chip analyses, the $\log_2$ enrichment ratio on the *Y*-axis was calculated from Cy3 (ChIP DNA) and Cy5 (Input DNA) signal intensity of each probe and plotted against each gene location along the *S.* Typhimurium chromosome on the *X*-axis. The peak heights of the identified Fis-binding peaks were calculated as a $\log_2$ enrichment ratio by dividing the Cy3 (ChIP DNA) over the Cy5 (Input DNA) signal intensities of the gene probe corresponding to the identified peak, and were indicated with solid rectangles below such peak(s). B) For the qPCR analyses, ChIP DNA $\log_2$ values were normalized over the control IgG DNA $\log_2$ values to obtain the $\log_2$ Fis enrichment ratio and thus make them comparable to the ChIP-chip data. Both analyses, panels A) and B), indicate similar low levels of Fis binding at the *guaC* promoter regardless of aeration regime or growth phase. In panel A), no Fis enrichment peaks are identified along the *guaC* promoter in any of the aeration/growth phase regimes 1-4. Consistently, in panel B), Fis enrichment levels are non-existent for aeration/growth regimes 1, and 4, and very low (below 1) for 2, and 3. All qPCR reactions were performed in duplicate, and two independent biological replicates were prepared for each set of conditions.

A) ChIP-chip



B) qPCR

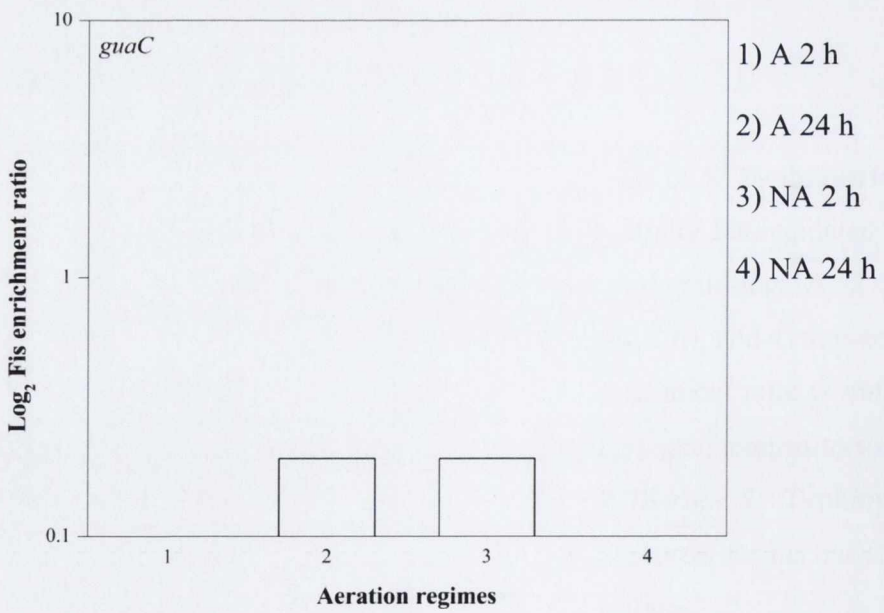
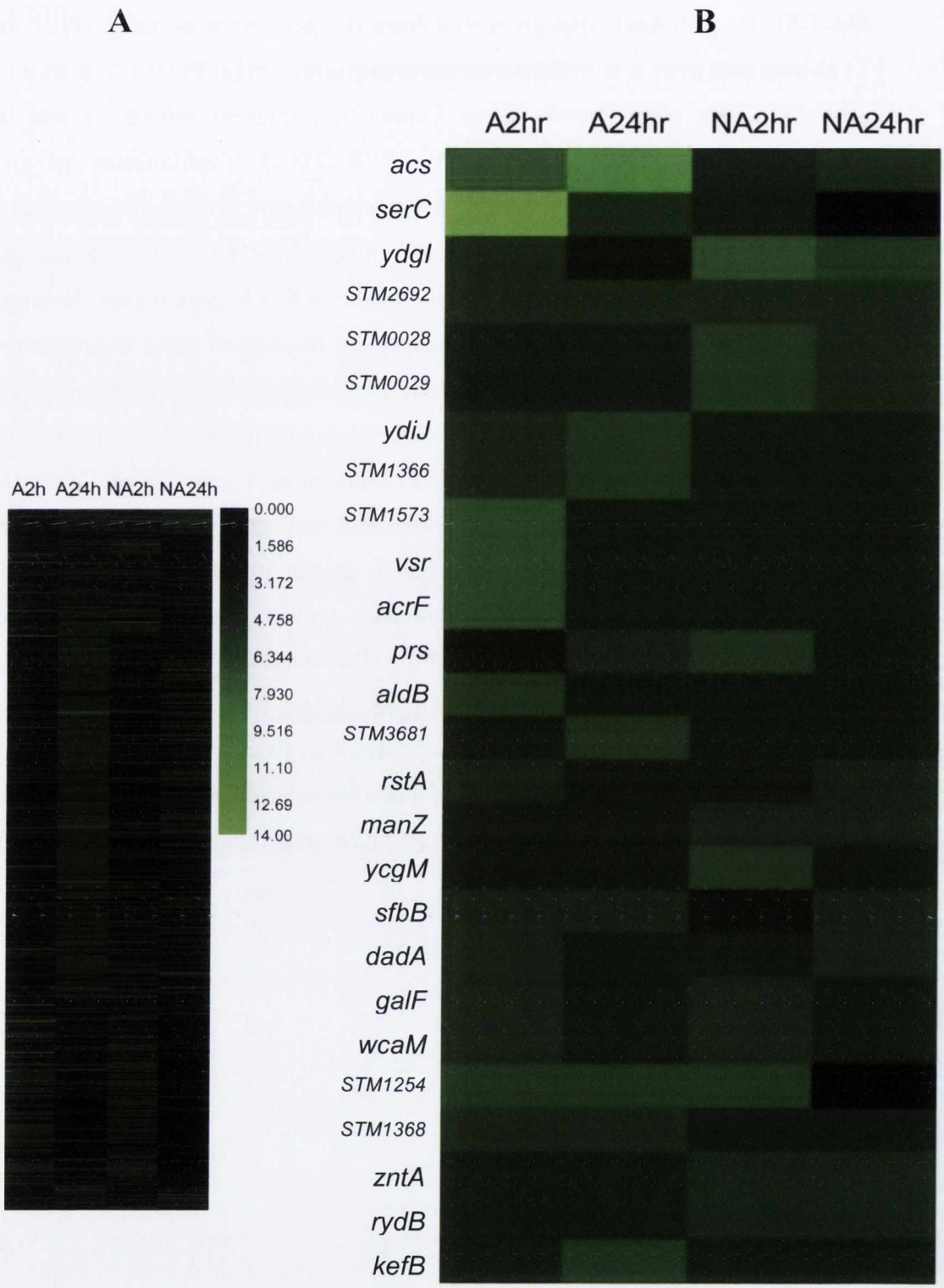


Fig. 6.8: Overall heat map generated from the genome wide microarray data set of *S.* Typhimurium grown in different aeration regimes. Origin Pro 8.5 ® software was used to generate a heat map where a green fluorescence gradient reflects several levels of Fis enrichment along diverse gene regions of SL1344 *S.* Typhimurium grown in aerated, exponential/log phase (A 2 h); aerated, stationary phase (A 24 h); non-aerated, exponential/log phase (NA 2 h); and non-aerated, stationary phase (NA 24 h). A) Genome-wide heat map displaying SL1344 Fis-binding gene regulation according to gene region (Y-axis), and growth aeration regime (X-axis). Gene regions displaying the highest green fluorescence possess the highest Fis-enrichment. B) Magnified heat map image of the 26 top most Fis-enriched gene regions. The fluorescence gradient table in panel A provides a quantitative reference between different levels of green fluorescence and Fis-enrichment levels, where 0 is the lowest and 14 the highest $\log_2$ Fis-enrichment ratio value. The *serC* gene region displayed the most Fis-enrichment and during aerated, exponential growth phase, but did not show any Fis-enrichment during the non-aerated, stationary growth phase. Similarly, gene *STM1254,* appears to be regulated by Fis-binding during all aeration growth regimes except, non-aerated, stationary growth phase. The highest Fis enrichment $\log_2$ ratio values that resulted from adding all four $\log_2$ ratio enrichment values from all four growth aeration regimes were the determinant basis for identifying the top 26 non pre-selected gene regions. Proteins of unclear known function were omitted from that set of 26 gene regions resulting in a final set of 20 gene regions.


A
B
A2h A24h NA2h NA24h
0.000
1.586
3.172
4.758
6.344
7.930
9.516
11.10
12.69
14.00
A2hr
A24hr
NA2hr
NA24hr
acs
serC
ydgI
STM2692
STM0028
STM0029
ydiJ
STM1366
STM1573
vsr
acrF
prs
aldB
STM3681
rstA
manZ
ycgM
sfbB
dadA
galF
wcaM
STM1254
STM1368
zntA
rydB
kefB

overall Fis enrichment levels as depicted by Fis peak absence (Fig. 6.7A, (1-4)), and as noted by qPCR Fis levels below 1 (Fig. 6.7B, (1-4)); whereas the positive control *topA* gene region displayed the highest Fis enrichment levels as depicted by Fis peak presence (Fig. A.19A, (1-4)), and as noted by qPCR Fis levels above 1 (Fig. A.19B, (1-4)). The gene regions *spoT*, *dksA*, *ssrA, gyrB* and *arcA* for both ChIP-chip and qPCR (except *gyrB*) assessments, showed the least overall association with the Fis protein (Figs. A.1A & B; A.2A & B; A.4A & B; and A.5, respectively). The qPCR Fis occupancy levels remained below 1 at all these sites, where applicable, and in all four growth aeration conditions (1-4) levels were similar to the negative control *guaC* (Fig. 6.7B, (1-4)), consistent with the absence of Fis peaks in their respective ChIP-chip results (Figs. A.1A; A.2A; and A.4A). Conversely, the *gyrB* gene region ChIP-chip data (Fig. A.3A) differ from the qPCR data (Fig. A.3B) in all four growth conditions, 1-4. Along the *gyrB* gene region, Fis occupancy is almost absent as there were no Fis peaks identified in either of all four growth conditions in the ChIP-Chip panel (Fig. A.3A), whereas Fis enrichment levels are above 1 in all four conditions in the qPCR panel (Fig. A.3B). The *arcA* gene region showed no Fis peaks that could be identified by the ChIPOTle software in either of the four growth conditions (Fig. A.5, (1-4)). In the ChIP-chip analysis, the genes *arcB*, *relA*, *rpoS, nrfA, fur, fliA, tyrT* and *hilA* showed partial association with the Fis protein as Fis peaks were identified in only one (Figs. A.6; A.7A; A.8, A.9, A.10; A.11A and A.12, respectively) or two aeration regimes in *hilA* (Fig. A.13). The *arcB* and *rpoS* gene regions showed Fis peaks only in the non-aerated, late-stationary (24 h) growth condition (Figs. A.6 (4) & A.8 (4), respectively). The *relA* and *nrfA* gene regions displayed Fis peaks only in the non-aerated, mid-log (2 h), growth condition (Figs. A.7A (3) & A.9 (3), respectively). The *tyrT* gene region displayed Fis peaks at only the late-stationary phase, (24 h), but in both conditions, in aerated (Fig. A.12 (2)), and to a lesser extent in non-aerated conditions (Fig. A.12 (4)).

And the *fur*, *fliA,* and *hilA* gene regions indicate a Fis peak only in the aerated, late-stationary (24 h), growth condition (Figs. A.10 (2), A.11A (2), and A.13 (2), respectively). Consistently, the qPCR assessment done for *relA* and *fliA* gene regions show Fis enrichment levels above 1 for the same growth condition where Fis peaks were identified: non-aerated, 2 h for *relA* (Fig. A.7B (3)), and aerated, 24 h for *fliA* (Fig. A.11B (2)).

The genetic loci *orgA, fis,* and *nuoA* showed Fis binding in three different aeration growth regimes according to ChIP-chip analysis (Figs. A.14A, A.15A and A.16A, respectively). The *orgA* and *fis* gene regions displayed Fis peaks in the aerated, mid-log (2 h), aerated, late-stationary (24 h), and non-aerated, mid-log (2 h) growth conditions (Figs. A.14A (1-3) & A.15A (1-3), respectively). No Fis peaks were detected in either the *orgA* or *fis* gene regions in the non-aerated, late-stationary (24 h) growth condition (Figs. A.14A (4) & A.15A (4), respectively). The *nuoA* gene region showed Fis peaks in all aeration growth regimes except in the aerated, mid-log (2 h) growth condition (Figs. A.16A (4)). Consistently, the qPCR assessment for the *orgA, fis,* and *nuoA* gene regions showed Fis enrichment levels above 1 for the same growth conditions where Fis peaks were identified: aerated, mid-log (A, 2 h); aerated, late-stationary (A, 24 h); and non-aerated, mid-log (NA, 2 h), growth conditions for *orgA* and *fis* (Figs. A.14B (1-3) & A.15B (1-3), respectively), and for *nuoA* in all aeration growth regimes except in the aerated, mid-log (A, 2 h) growth condition (Figs. A.16B (4)). Furthermore, low Fis enrichment levels (below 1) were detected in the *orgA* and *fis* gene regions in the non-aerated, late-stationary (24 h), growth condition (Figs. A.14B (4) & A.15B (4), respectively). On the other hand, *brnQ, gyrA,* and the positive control *topA* gene regions showed high Fis enrichment levels in all four aeration growth regimes according to ChIP-chip analysis (Figs. A.17, A.18A and A.19A, respectively). All four genetic regions displayed Fis peaks in the aerated, mid-log (A, 2 h); aerated, late-stationary (A, 24 h); non-aerated, mid-log (NA, 2 h); and non-aerated, late-stationary (NA, 24 h) growth conditions (Figs. A.17 (1-4), A.18A (1-4), and A.19A (1-4), respectively). Similarly, the qPCR assessment done for the *gyrA* and *topA* gene regions showed Fis enrichment levels well above 1 in all four growth conditions where Fis peaks were identified in both *gyrA* and *topA* gene regions (Figs. A.18B (1-4) & A.19B (1-4), respectively). Moreover, both the *gyrA* and *topA* gene regions displayed the highest qPCR Fis enrichment levels in the aerated, late-stationary, (24 h), growth condition (Figs. A.18B (2) & A.19B (2), respectively), which also corresponded to the most Fis peaks identified among all four conditions in the ChIP-chip assessment for both *gyrA* and *topA* gene regions (Figs. A.18A (2) & A.19A (2), respectively).

Furthermore, an overall heat map was generated by using Origin Pro 8.5 ® software with the previously obtained genome-wide microarray ChIPOTle (section 2.14.9) data set from *S.* Typhimurium grown in different aeration regimes. The heatmap provides an overall picture of the different Fis-enrichment levels across all four aeration growth regimes (Fig. 6.8A). A

sub-set heatmap of twenty-six gene regions, known as non pre-selected, was also produced based on the highest Fis-enrichment levels across all four growth aeration regimes (Fig. 6.8B). The green fluorescence gradient shows several levels of Fis enrichment along diverse gene regions in SL1344 *S.* Typhimurium grown in aerated, exponential/log phase (A 2 h); aerated, stationary phase (A 24 h); non-aerated, exponential/log phase (NA 2 h); and non-aerated, stationary phase (NA 24 h). The highest Fis enrichment $\log_2$ ratio values that resulted from adding all four $\log_2$ ratio enrichment values from all four growth aeration regimes were the determinant basis for identifying the top 26 non pre-selected gene regions. All 26 gene regions were placed together in a sub-heatmap and magnified (Fig. 6.8B) for clear viewing. Proteins of unclear known function were omitted from that set resulting in a final set 20 gene regions. As previously mentioned, these genomic sites were identified because of their high Fis-enrichment levels (Fig. 6.8B), according to the color gradient table (Fig. 6.8A). This table was based on the $\log_2$ ratio enrichment levels from the ChIP-chip data. The observed twenty non pre-selected most Fis-enriched gene regions were: *acs, serC, ydgI, STM2692, ydiJ, STM1366, vsr, acrF, aldB, rstA, manZ, ycgM, sfbB, dadA, galF, wcaM, STM1254, STM1368, zntA, and kefB*.

The most fluorescence was displayed in aerated, exponential growth phase conditions by *serC*, the gene encoding for phosphoserine aminotransferase, which, interestingly, did not show any fluorescence in the non-aerated, stationary growth phase condition (Fig. 6.8B, A 2 h vs. NA 24 h). The other was *acs*, the gene encoding for acetyl-coenzyme A synthetase, which showed most Fis-binding in the aerated, stationary growth phase (Fig.6.8B, A 24 h). Intriguingly, the *ydgI*, the gene encoding for a nitroreductase protein, showed a high level of Fis occupancy in the non-aerated conditions, especially during the exponential phase (Fig.6.8B, NA 2 h & 24 h), whereas *STM2692,* the gene encoding for a secretion membrane fusion protein, showed a more evenly distribution of high Fis-enrichement across all four aeration growth regimes (Fig. 6.8B). Interestingly, *rstA* showed the most Fis occupancy in the aerated, exponential phase and non-aerated, stationary phase (Fig.6.8B, A 2 h & NA 24 h). The protein it encodes for is a DNA binding transcriptional regulator that responds to changes in the environment. The *ydiJ*, the gene encodes for an iron oxidoreductase protein involved in energy conversion, displayed more Fis binding during aerated conditions rather that during non-aerated (Fig.6.8B, A vs. NA). AcrF, part of the multidrug efflux AcrA/B system, and a protein encoded by *acrF* a gene responsible for acriflavin resistance, showed Fis occupancy

mostly during the exponential growth phase in aerated conditions (Fig.6.8B, A 2 h). Along gene regions *STM1366*, which encodes for a thioesterase protein; *vsr,* which encodes for very short patch repair endonuclease protein; *aldB*, which encodes for aldehyde dehydrogenase B protein; and on *STM1368*, which encodes for sodium dicarboxylate symporter, high levels of Fis binding were detected but mostly during aerated conditions (Fig.6.8B, A). On the other hand, *manZ,* which encodes for a putative mannose specific permease protein; *ycgM,* which encodes for catechol catabolism protein; and *zntA*, which encodes for zinc-cadmium-mercury-lead transporting ATPase, showed abundant Fis occupancy but mainly during non-aerated conditions (Fig.6.8B, NA). During both aerated and non-aerated conditions, the following genes displayed high levels of Fis occupancy: *sfbB* (encodes for ABC transporter ATPase), *dadA* (encodes for aminoacid dehydrogenase small subunit), *galF* (encodes for UTP-glucose transferase), and *wcaM* (encodes for colonic acid biosynthesis protein) (Fig.6.8B, A & NA). *STM 1254*, which encodes for an outer-membrane lipoprotein displayed Fis binding during all aeration regimes except, during the non-aerated, stationary phase (Fig.6.8B, A & NA). And finally, *kefB*, which encodes the glutathione regulated potassium efflux synthetase protein, showed Fis enrichment mostly only during aerated, stationary growth phase (Fig.6.8B, A 24 h). All genes were identified using the National Center for Biotechnology Information website (NCBI Gene entrez, http://www.ncbi.nlm.nih.gov).

6.3 Discussion

Fis protein binding patterns change not only as a function of growth phase, but also as a function of the aeration regime. ChIP-chip assessments were performed to examine Fis protein binding sites along the *S.* Typhimurium genome during four different aeration growth regimes. The following pre-selected gene regions were studied: *guaC, relA, spoT, dksA, ssrA, fis, topA, gyrA, gyrB, nuoA, fliA, orgA, hilA, tyrT, arcA, arcB, rpoS, nrfA, fur,* and *brnQ*. Also, qPCR analyses were made in twelve of those gene regions, *guaC, relA, spoT, dksA, nuoA, ssrA, fis, topA, gyrA, gyrB, fliA,* and *orgA*.

Of the twenty loci that were examined, the gene regions *guaC* (negative control), *relA, spoT, dksA*, *gyrB, ssrA* and *arcA* displayed the least overall association with the Fis protein (Figs.

6.7A & B; A.7A; A.1A & B; A.2A & B; A.3A & B; A.4A & B and A.5, respectively) in all four growth-phase/aeration conditions (1-4). The *relA* gene region displayed a few Fis peaks only in the non-aerated, mid-log (NA, 2 h) growth condition (Fig. A.7A, 3), and in the qPCR assessment the gene region barely shows Fis enrichment above 1 in the NA 2 h growth condition (Fig. A.7B, 3). Consistently, in *E. coli,* similar low or non-existent Fis-binding occurs along gene regions of *guaC*, *relA*, *spoT, dksA* and *arcA* (Cho *et al.,* 2008). Interestingly, Although *dksA* (DksA protein), *relA* and *spoT* (ppGpp metabolism) appear to have a role in Fis expression (see chapter 4), this low level of Fis-binding along their gene regions suggests that the Fis protein does not feed back onto the expression of these regulators of *fis* gene transcription. The *ssrA* gene region, which belongs to the SPI-2 virulence arsenal (sections 1.3.2 & 1.5), is known to be regulated by Fis but, it is also dependent on the surrounding gut conditions and to the topological state of the DNA (section 1.5). Surprisingly, when DNA is highly relaxed, the induction of the SPI-2 T3SS and effector gene promoter, *ssrA* P (section 1.3.3) does not require Fis in simulated vacuolar environment culture conditions (Cameron *et al.,* 2011; Osborne and Coombes, 2011). This is consistent with the data obtained here as Fis binding is neither observed in late-stationary aerated conditions (where DNA is highly relaxed), nor in non-aerated mid-log (NA, 2 h) or late-stationary conditions (NA, 24 h), which may resemble the low-oxygen regime in the vacuolar environment. Similarly, when the *ssrAB* promoter (*ssrA* P) is induced by novobiocin, the DNA supercoiling activity of the DNA gyrase subunit B (GyrB) is inhibited (Cameron and Dorman, 2012). Novobiocin acts as a competitive inhibitor of the ATPase reaction catalyzed by the GyrB subunit of DNA gyrase (Gellert *et al.,* 1976; Cozzarelli, 1980), thus rendering DNA more relaxed, as it is found in aerated, late-stationary conditions. The Fis protein represses transcription of both genes encoding the DNA gyrase protein *gyrA* and *gyrB* by binding to their promoter region (Cozzarelli, 1980; Schneider *et al.,* 1997 & 1999; Travers *et al.,* 2001; Keane and Dorman, 2003), and it is also known that it does it in correspondence to the DNA supercoiling changes as required by the growth phase of the cell. In other words, the Fis protein influences DNA supercoiling both directly and indirectly (Travers *et al.,* 2001; Dorman, 2009) (Fig. 1.6). Furthermore, the Fis protein is also known to activate *topA,* the gene that encodes DNA topoisomerase I (TopA) (Cozzarelli, 1980; Richardson *et al.,* 1984; Weinstein-Fischer and Altuvia, 2007; Cho *et al.*, 2008), under specific conditions (Weinstein-Fischer *et al.*, 2000). In fact, even through Fis occupancy in the ChIP-Chip panel is strangely absent along the *gyrB* gene region, as Fis peaks were not identified in any of the four

conditions (Fig. A.3A, 1-4), there are, in the qPCR panel, Fis enrichment levels above 1 in all four conditions (Fig. A.3B, 1-4), being the highest during aerated, late-stationary (A, 24 h), followed by aerated, mid-log (A, 2 h), non-aerated, mid-log (NA, 2 h), and non-aerated, late-stationary (NA, 24 h) conditions, in that order. Interestingly, the *topA* gene region is also Fis-enriched mostly during A, 24 h, followed by A, 2 h, NA, 24 h, and NA, 2 h conditions. As a matter of fact, Fis appears to be most enriched along all three *gyrA, gyrB* and *topA* gene regions during the A, 24 h aeration regime, and less enriched in the A, 2 h; NA, 2 h; and NA, 24 h aeration/growth regimes. Taking into account the role of each of these genes in DNA supercoiling, this clearly suggests Fis involvement by orchestrating the reduction of DNA negative supercoiling in the cell during the A, 24 h condition, and an increase in DNA negative supercoiling during the A, 2 h, NA, 2 h, and NA, 24 h aeration/growth regimes. Fis involvement in DNA topology shifts is further supported by the Fis binding patterns along *tyrT*. The *tyrT*, the gene encoding for a species of tyrosine tRNA, displayed Fis enrichment along its gene region not during exponential phase but during the late-stationary phase, (24 h), in both conditions, in aerated (Figs. A.12, 2), and to a lesser extent in non-aerated conditions (Figs. A.12, 4). This is in agreement with the literature stating that in *E. coli*, Fis is neither necessary nor responsible for the stimulation of transcription from the wild-type promoter for the *tyrT* operon that occurs upon resumption of exponential growth (Lazarus and Travers, 1993). The Fis enrichment at the *tyrT* gene region increases DNA relaxation via TopA in a concentration dependent manner, as highly negatively supercoiled DNA species gradually disappear (Schneider *et al.,* 1997). Consistently, during the aerated, late-stationary phase (A, 24 h) where extensive Fis binding occurs in the *tyrT* gene region (Fig. A.12, 2) DNA is most relaxed (chapter 5). Therefore, it is deduced that DNA becomes more supercoiled or less relaxed as less Fis binding occurs along the *tyrT* region. In fact, during the non-aerated, late-stationary (NA, 24 h) condition, the *tyrT* region shows less Fis enrichment (Fig. A.12, 4) than during the A, 24 h condition. This concurs with the data obtained in chapter 5, where the NA, 24 h condition displays less relaxed DNA than the A, 24 h condition. Altogether, these results suggest that the Fis protein acts as a modulator of DNA topology to enable *S.* Typhimurium to adapt to sudden changes in the growth aeration regime, like when transiting into the microaerobic environment of the gut lumen.

The gene site *fliA* showed Fis enrichment during the A, 24 h condition (Fig. A.11A & B, 2), and the *nuoA* region during the A, 24 h, NA, 2 h, and NA, 24 h, conditions (A.16A & B, 2, 3

and 4, respectively). The *brnQ,* like *gyrA,* and positive control *topA* gene regions showed high Fis enrichment levels in all four aeration growth regimes (Figs. A.17, A.18A & B and A.19A & B, respectively). The *brnQ* gene, which encodes for the branched chain amino acid transporter, was an arbitrarily-selected housekeeping gene chosen merely to understand if there are any given similarities between *E. coli* and *S.* Typhimurium Fis-binding patterns, and which are also independent from aeration regime. Indeed, as in *E. coli*, Fis binds also along the *brnQ* gene promoter region in *S.* Typhimurium, and not only during aerobic, mid-log conditions, but also during non-aerated conditions. The *fliA* gene, which encodes alternative sigma factor 28, is a middle flagellar gene involved in bacterial motility. Previous studies demonstrated a role for Fis in *Salmonella* motility (Osuna et al., 1995; Yoon et al., 2003). The Fis binding to the *fliA* gene region that is only observed during the aerated, late-stationary (A, 24 h) condition may act as a repressor, as motility is no longer useful as it would be during exponential phase and/or microaerobic conditions (Fig. A.11A & B, 1, 2, and 4) when epithelial cell invasion occurs. On the other hand, the *nuoA* (NADH dehydrogenase subunit A) gene region Fis enrichment levels observed in the A, 24 h NA, 2 h and NA, 24 h, aeration regimes (Fig. A.16A & B, 2, 3 and 4, respectively) are consistent with the interaction in the literature between Fis dimers and specific binding sites in the *nuo* promoter, and the influence of the dimers with the compaction of the DNA (Zhang *et al.,* 2004). Furthermore, it is also possible that the Fis enrichment observed mostly in aeration regimes A, 24 h and NA, 2 h, downstream of *nuoA*, where gene region *lrhA* (NADH dehydrogenase transcriptional repressor) is, activates the repressing function of the *lrhA* gene and thus modulates the level of compaction of DNA.

Intriguingly, the *arcB* gene region, like *rpoS*, but unlike *arcA*, reveals Fis enrichment in the non-aerated, late-stationary (NA, 24 h) growth condition (Fig. A.6, 4), where sustained expression of *fis* has been observed. The *arcB* gene encodes for the transmembrane histidine kinase sensor, and together with the *arcA* gene, they belong to the ArcA regulon that is part of a two-component global regulatory system, ArcA/ArcB (Evans *et al.,* 2011). The Arc system provides aerobic respiratory control, as it helps the cells to sense and respond to the presence of dioxygen. As it turns out, *fis* is one of the gene regions most highly repressed by ArcA (Evans *et al.,* 2011), which is consistent with the notion that perhaps the *arcB* gene needs to be repressed by Fis for the cell to thrive not in an anaerobic, but in a microaerobic environment. The gene sites *arcB*, *rpoS*, *nrfA,* and *fliA* showed limited association with the Fis

protein as Fis peaks were identified in only one aeration regime (Figs. A.6; A.8; A.9, and A.11A), or two aeration regimes in the case of the *hilA* gene region (Fig. A.13). Interestingly, the *rpoS* gene region revealed Fis enrichment in the non-aerated, late-stationary (NA, 24 h) growth condition (Figs. A.8, 4), where sustained expression of *fis* has been observed. The *rpoS* gene encodes for the alternative stress response sigma factor RpoS that regulates genes expressed during stationary phase in response to nutrient deprivation (Fang *et al.,* 1992; Loewen *et al.,* 1994; O'Neal *et al.,* 1994). The sigma factor RpoS was shown to play a role in repressing Fis expression during stationary phase (Ó Cróinín and Dorman, 2007), whereas during exponential phase, the opposite happens: Fis was shown to repress *rpoS* transcription (Hirsch and Elliott, 2005). However, and quite surprisingly, Fis enrichment along the *rpoS* gene region occurred neither during the aerated nor the non-aerated exponential phase conditions but during the non-aerated late-stationary (NA, 24 h) growth regime. Thereby, induction of Fis in the absence of aeration was facilitated by a reduction of RpoS levels under such conditions. During stationary phase, RpoS levels elevate to direct RNAP to induce transcription of stationary phase genes but, increased RpoS levels also correlate with decreased Fis expression (Ó Cróinín and Dorman, 2007). Nonetheless, during non-aerated, stationary phase (NA, 24 h), it appears that *rpoS* transcription needs to be brought to a halt as increased Fis binding occurs along the *rpoS* gene region, and as result Fis expression is not decreased but rather sustained. On the other hand, the *nrfA* gene, encoding a nitrite reductase periplasmic cytochome c(552) (Evans *et al.,* 2011), displayed Fis enrichment only in the NA, 2 h growth condition (Figs. A.9, 3). The *nrfA* gene region is part of the operon promoter *nrf* P, likewise the *acs* and *ogt* promoter regions, where Fis displaces the essential activator by acting as a common repressor, as the role of Fis is to repress the promoter in response to nutrient richness (Browning *et al.*, 2005). The function of this operon is crucial for *S.* Typhimurium when faced with multiple host-immune defenses that include nitrogen species (RNS) (Cirillo *et al.,* 1998; Galán *et al.,* 2001; Evans *et al.,* 2011). Since the *nrfA* gene is part of the *nrf* operon promoter that is involved in nitrogen respiration orchestration, it is possible that the actual *nrf* operon promoter is repressed when conditions are aerobic or microaerobic. This may explain why Fis binding occurs along the *nrf* operon promoter in all four aeration regimes (Fig. A.9, 1-4).

The *fur* and *hilA* gene regions displayed Fis enrichment only in the aerated, late-stationary (A, 24 h), growth condition (Figs. A.10, 2, and A.13, 2, respectively), while the *orgA* and *fis* gene

regions revealed Fis enrichment in three aeration regimes, the aerated, mid-log (A, 2 h), late-stationary (A, 24 h), and non-aerated, mid-log (NA, 2 h) growth conditions (Figs. A.14, 1-3, and A.15, 1-3, respectively). The Fis-binding patterns along these gene regions suggest an interesting interconnection that may further reveal the purpose behind the sustained expression of the *fis* gene. As previously mentioned in Chapter 3, during the non-aerated (NA, 24 h) growth conditions, which resemble the microaerobic conditions of the intestinal lumen, *fis* expression was shown and confirmed to be sustained. Consistently, in this chapter, the *fis* promoter gene region displays Fis enrichment levels in the NA, 24 h condition far lower than in any of the other three aeration regimes, and such levels also reside below one (Fig. A.15A & B). Considering Fis is a negative autoregulator of its own promoter, low Fis enrichment along its promoter would mean not increased but continuous *fis* transcription. It is possible too, that the Fis binding patterns along the other three gene regions: *fur, orgA* and *hilA*, may have an influence in this prolonged *fis* transcription. The *fur*, ferric uptake regulator, encodes for global regulator Fur (Thompson *et al.,* 2006; Lee and Helmann, 2007). Fur is responsible for the global regulation of iron metabolism, including iron uptake via siderophore-dependent transport systems (Tsolis *et al.,* 1995). This sensing capability is important because the availability of free iron could assist pathogenic bacteria in sensing their host environment (Rhen and Dorman, 2005). Although Fur is commonly throught of as a metal-dependent repressor, Fur also activates the expression of many genes by either indirect or direct mechanisms (Lee and Helmann, 2007). Interestingly, iron availability correlates with the induction of several virulence factors, including those encoded on SPI-1 (Deiwick *et al.,* 1999; Janakiraman and Slauch, 2000). In fact, Fur, like Fis, is sensitive to environmental signals like non-aerated or low oxygen conditions (Lee & Falkow *et al.,* 1990; Ó Cróinín *et al.,* 2007) that activate important SPI-1 structural genes (Ellermeier & Slauch, 2008). Fur therefore also plays a substantial role in the invasion of host intestinal epithelial cells by *S.* Typhimurium as it is part of the regulatory mechanism targeting SPI-1 type III secretion system (T3SS) genes (Ellermeier & Slauch, 2008). This may prove to be important as it shows ppGpp to be a repressor not only of *fis*, but also of other genes involved in epithelial cell invasion. This is in keeping with the Fis-binding pattern observed at the *fur* gene, which is similar to the pattern observed along it own *fis* promoter (*fis* P). The *fis* promoter gene region was found to display Fis enrichment levels in the NA, 24 h condition far lower than in any of the other three aeration regimes (Fig. A.15A & B). This is in agreement with the fact that Fis is a negative regulator of its own promoter, as lower enrichment along its promoter would mean not

increased but continuous *fis* transcription. This prolonged expression may be also the result of Fis interaction with other genes, in particular the *hilA/B* genes, which are hyperinvasion locus genes (Teixido *et al.,* 2011), and the *orgA* gene, which is an oxygen-regulated gene (Jones & Falkow, 1994). As mentioned in the previous chapter, Fur binds to the *hilD* promoter and activates it, which in turn activates *hilA* via HilD together with HilC, thus enabling *S.* Typhimurium to invade epithelial cells through the use of SPI-1 (Teixido *et al.,* 2011). Furthermore, HilA directly activates the expression of *prgH*, which lies within the *orgA* promoter, and together with *invF* encode the components of the T3SS apparatus (Lostroh *et al.,* 2000; Lostroh & Lee, 2001) necessary for epithelial cell invasion during microaerobic conditions (SPI-1 inducing). Also here, similar to the *fur* and *hilA* (A.13, (4)) gene regions, there is no Fis-enrichment along the *orgA* gene region in the NA, 24 h condition (A.14A & B, (4)), while enrichment levels are higher during the other conditions. Fis may act here also as a repressor on this gene region, much like it does on its own *fis* promoter (*fis* P).

Moreover, a comprehensive heatmap was produced from the ChIP-chip microarray data set and it revealed 20 non pre-selected gene regions with the highest Fis enrichment levels (Fig. 6.8B). These gene regions were *acs, serC, ydgI, STM2692, ydiJ, STM1366, vsr, acrF, aldB, rstA, manZ, ycgM, sfbB, dadA, galF, wcaM, STM1368, zntA,* and *kefB*. However, not much is known about them interacting with Fis in response to microaerobic conditions.

Nevertheless, *rstA* is known to be transcribed in response to changes in the environment (Cabeza *et al.,* 2007). RstA expression promotes RpoS degradation, affects RpoS-modulated genes and is involved in modulating *Salmonella* biofilm formation. This places RstA as a powerful contributor to *Salmonella* pathogenic traits (Cabeza *et al.,* 2007). Furthermore, it is important to mention that deletion in the *rpoS* gene, on the other hand, has resulted in non-restricted, increased anaerobic gene *arcA* expression in stationary-phase cultures under microaerobic conditions (Sevcik *et al.,* 2001).

Also, high levels of Fis enrichment along the *ydgI* gene region were present mostly during non-aerated conditions. This is interesting given the fact that the protein it encodes for, nitroreductase (Prosser *et al.,* 2013), is important during low-oxygen/microaerobic conditions when the bacteria needs to switch to a nitrogen reducing mode.

Another interesting gene showing abundant Fis binding is the gene encoding iron-sulfur oxido-reductase, *ydiJ.* As previously mentioned, iron availability correlates with the induction of several virulence factors, including those encoded on SPI-1 (Deiwick *et al.,* 1999; Janakiraman and Slauch, 2000). Furthermore, it is possible that this gene is also regulated by Fur, which as mentioned before, controls the intracellular concentration of Fe.

Therefore, it is possible that *fis* expression is concurrent to *fur, hilA*, *orgA, rstA, ydiJ,* and *ydgI* expressions during microaerobic conditions. This is not surprising given the sensitivity of these genes to microaerobic conditions.

Chapter 7

General Discussion

Several studies have established a correlation between microaerobicity and virulence in *S.* Typhimurium (Lee & Falkow, 1990; Ó Cróinín *et al.*, 2007; Cameron *et al.,* 2011; Teixido *et al.,* 2011; Cameron *et al.,* 2012). They show that oxygen limitation is an environmental cue that primes the expression of *Salmonella* invasiveness within the intestinal lumen and other tissues. Also, published and unpublished work from this laboratory has shown that under non-aerated, microaerobic conditions, exponential-growth-phase expression of the Fis protein is sustained into the stationary phase (Ó Cróinín & Dorman, 2007). I have reproduced this observation, confirming that *fis-gfp* transcription is maintained into the stationary phase of growth in non-aerated cultures (Fig. 3.2). This observation is important because the Fis protein is a key regulator of the *Salmonella* virulent phenotype (Kelly *et al.,* 2004; Ó Cróinín *et al.,* 2006; Ó Cróinín and Dorman, 2007; Wilson *et al.,* 2001; Yoon *et al.,* 2003). Consistently, in chapter 6, I have shown that the *fis* promoter gene region reveals Fis enrichment levels in the NA, 24 h condition far lower than in any of the other three aeration regimes (Fig. A.15A & B). Considering Fis is a negative autoregulator of its own promoter, low Fis enrichment levels along its promoter in non-aerated conditions and during stationary growth phase, would probably suggest not increased but the continuous/sustained transcription of *fis*.

I also discovered that growth under completely anaerobic conditions (Figs. 3.3 and 3.4C & F) is not equivalent to non-aerated growth in terms of *fis* transcription. DNA associated with the *arcB* gene is bound by Fis to high levels in the non-aerated, late-stationary (NA, 24 h) growth condition (Fig. A.6, 4). The *arcB* gene is part of the ArcA/ArcB aerobic respiratory control system and the Fis binding pattern at *arcB* is suggestive of a physiologically-significant link between aeration levels and *fis* gene expression. It is important to recall that *fis* is one of the genetic loci that is most highly repressed by ArcA (Evans *et al.,* 2011), which is consistent with a reciprocal relationship between *fis* and *arcAB* that leads to mutual modulation in a microaerobic environment. It is reasonable to propose that *fis* promoter activity must be modulated in *Salmonella* as part of the process of adaptation to environmental niches, such as the brush border of the gut epithelium which is microaerobic and not strictly anaerobic. This adaptation is likely to be important during the infection process when *Salmonella* is being primed for epithelial cell invasion, an event that must take place in the microaerobic environment of the epithelial boundary layer (Rychlik & Barrow, 2005). Thus, it is not

anaerobiosis, but microaerobiosis that induces sustained expression of Fis during stationary phase growth. This scenario is completely consistent with data from *in vitro* work showing that induction of invasiveness takes place in *S.* Typhimurium growing under very low oxygen conditions and into stationary phase prior to infection of epithelial cells (Lee & Falkow, 1990).

The *fis* gene is part of the stringent response and its repression during this response depends in part on the DNA supercoiling sensitivity of the *fis* promoter. Important components of the stringent response are the transcription factor DksA and the alarmone ppGpp. Thus, in addition to assessing the role of DNA supercoiling in the maintainance of *fis* transcription into stationary phase in microaerobic cultures, my work has also investigated the contributions of elements of the stringent response, specifically DksA and ppGpp (Chapter 4). The findings obtained point to a collaborative effect between DksA and ppGpp in repressing *fis* transcription. The effects of eliminating ppGpp production and *dksA* expression were additive and extended into the stationary phase of growth (Fig. 4.4). These data suggest that DksA and ppGpp have parallel, independent effects on *fis* transcription and that they affect *fis* transcription to different degrees. It is possible that DksA regulates *fis* both directly and indirectly, allowing it to have a larger impact than ppGpp, which might only regulate directly via modulation of RNA polymerase activity.

Furthermore, it seems likely that changes in DNA supercoiling form part of the mechanism by which the *fis* promoter adjusts to a microaerobic environment (see Chapter 5). Published data from this laboratory show that *fis* transcription in *Salmonella* is sensitive to changes in DNA supercoiling (Ó Cróinín *et al.,* 2006), and that its expression depends on the level of aeration where the ratio of ATP to ADP is maximal (Ó Cróinín & Dorman, 2007). Fis and DNA supercoiling operate collaboratively to set the expression of numerous genes in *Salmonella,* including the major virulence genes in SPI-1 and SPI-2 (Kelly *et al.,* 2004). When DNA supercoiling is relaxed as a result of gyrase inhibition by novobiocin treatment in vitro, the promoter of the SPI-2 master regulator gene *ssrA*, which is normally Fis-dependent, is activated in a Fis-independent manner under simulated vacuolar environment culture conditions (Cameron *et al.,* 2011; Osborne and Coombes, 2011). This shows that the link between DNA supercoiling sensitivity and Fis dependence is conditional and can be broken by modulation of growth conditions. Furthermore, the recent demonstration that the ability of Fis

to bind to its DNA target is contingent on the superhelicity of that target provides a mechanism to disconnect the Fis protein concentration in the cell from the level of Fis binding at specific sites, such as the *ssrA* promoter (Cameron and Dorman, 2012).

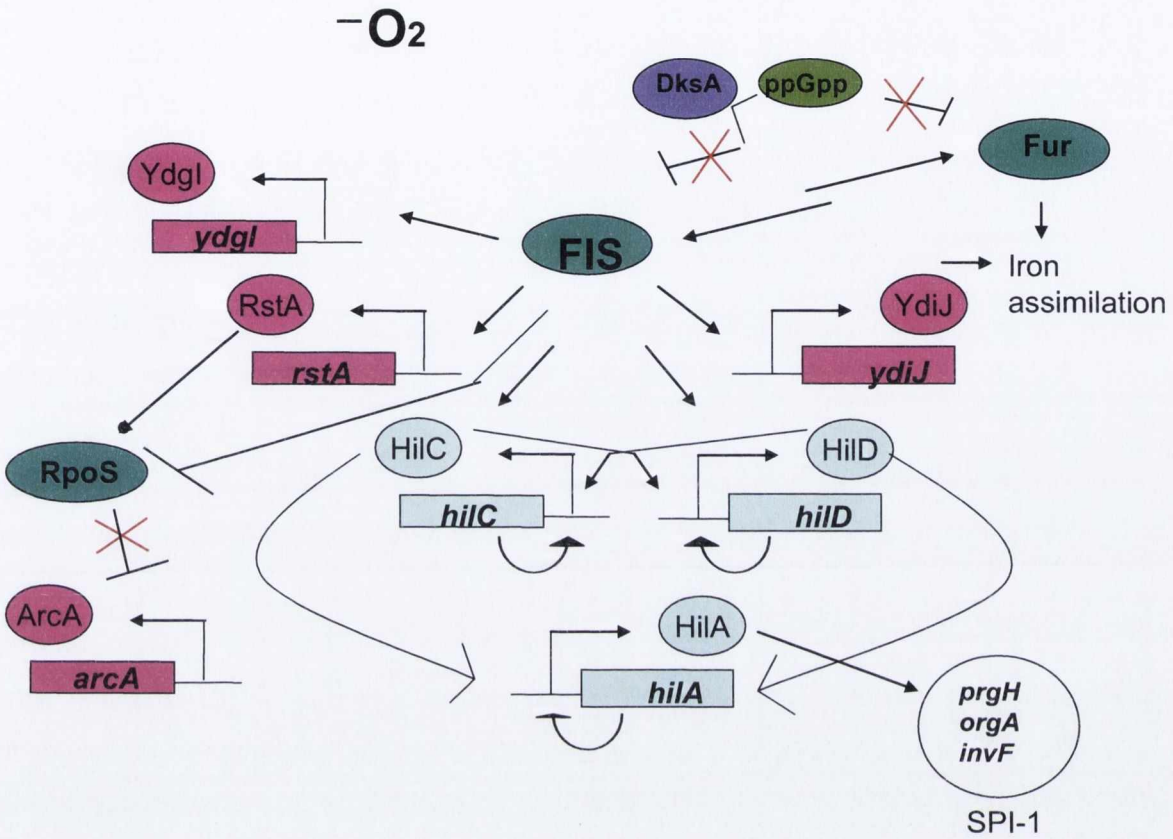
As mentioned before, the Fis protein represses transcription of both the *gyrA* and *gyrB* genes encoding the DNA gyrase by binding to their promoter regions (Cozzarelli, 1980; Schneider *et al.,* 1997 & 1999; Travers *et al.,* 2001; Keane and Dorman, 2003). It also influences DNA supercoiling both directly and indirectly (Travers *et al.,* 2001; Dorman, 2009) (Fig. 1.6), by activating *topA,* the gene that encodes DNA topoisomerase I (TopA) (Cozzarelli, 1980; Richardson *et al.,* 1984; Weinstein-Fischer and Altuvia, 2007; Cho *et al.*, 2008), under specific conditions (Weinstein-Fischer *et al.*, 2000). This is important because while DNA gyrase introduces negative supercoiling into DNA, *topA* relaxes it (Cozzarelli, 1980; Champoux, 2001; Travers and Muskhelishvili, 2005). As a matter of fact, in both *E. coli* and *Salmonella, topA* mutants exhibit increased levels of negative DNA supercoiling, just as wild-type bacteria do when exposed to high-osmolarity growth conditions, and this results in a supercoiling-dependent induction of the transcription of *proU*, an operon that only expresses in high-osmolarity conditions (Higgins *et al.,* 1988). When *Salmonella* grows at low oxygen concentrations, its DNA becomes more negatively supercoiled (Dorman *et al.,* 1988). Therefore, *Salmonella topA* mutants possess levels of negative supercoiling in their DNA that approximate to those seen in the wild type when growing under microaerobic conditions and that this leads to increased levels of *fis* expression. This is in agreement with the results reported in the previous chapter, where Fis protein binding appears to be most enriched along all three *gyrA, gyrB* and *topA* gene regions during the A, 24 h aeration regime. The same regions were much less Fis-enriched during the A, 2 h, and NA, 2 h in the *topA* mutant. The *gyrA* and *gyrB* gene regions showed the least Fis enrichment in the NA, 24 h aeration regime. These data are in keeping with the level of DNA negative supercoiling observed in each of those same conditions (Fig. 5.4 A & C) and with the known stimulatory effect of DNA negative supercoiling of *fis* promoter activity. The sustained expression of the *fis* gene observed during non-aerated (microaerobic), late-stationary (NA, 24 h) conditions arises because DNA is less relaxed than that in its aerated counterpart. Consequently, there is less binding of Fis at *gyrA* and *gyrB*, leading to more *gyr* gene transcription and a concomitant increase in gyrase activity.

The patterns of Fis protein binding to the *Salmonella* chromosome discovered in this study are in keeping with links between this protein and DNA supercoiling sensitivity at other loci, such as the tRNA gene *tyrT.* The Fis protein serves as a topological buffer at the *tyrT* promoter in *E. coli,* maintaining a micro-domain of negative superhelicity there as DNA relaxes elsewhere in the genome (Schneider *et al.,* 1997). I have found that during aerated growth, in late-stationary phase (A, 24 h) extensive Fis binding occurs in the *tyrT* gene region (Fig. A.12, 2), just as the DNA is becoming most relaxed (Fig. 5.4C, 24 h). Extrapolating from the situation in *E. coli*, this suggests that as the DNA becomes more supercoiled or less relaxed the amount of Fis binding to the *tyrT* region is diminished. In fact, during the non-aerated, late-stationary (NA, 24 h) condition, the *tyrT* region shows less Fis enrichment (Fig. A.12, 4) than during the A, 24 h condition. Once again, this concurs with previously obtained data, where the NA, 24 h condition displays less relaxed DNA than the A, 24 h condition (Fig. 5.4A & C). As previously mentioned, DNA becomes less negatively supercoiled in the wild type during aerated, late-stationary phase (Fig. 5.4C, 24 h), where *fis* expression levels cannot be maintained and decrease (Fig. 5.3B red, 24 h). Therefore, when *fis* expression in the wild type is sustained during late-stationary phase in non-aerated conditions (Fig. 5.3A red), DNA is more likely to be less relaxed during this time, and my chloroquine gel results corroborate this (Fig. 5.4A, 24 h). These findings are in keeping with the observation that *fis* expression in a *topA* mutant is sustained into stationary phase in both aerated and non-aerated conditions (Fig. 5.3A & B, black), a pattern that is seen in the wild type strain in non-aerated conditions (Fig. 5.3A red). Overall, my results suggest that *fis* expression is maintained during the later part of the growth cycle as a result of sustained DNA gyrase activity, linked to the colligative response to low oxygen conditions (Fig. 5.5B). This is most likely to involve the maintenance into stationary phase of a ratio of [ATP] to [ADP] that is favourable for DNA gyrase activity with a concomitant repression of *topA* gene transcription by the Fis protein.

Therefore, the Fis binding patterns along the *tyrT, gyrA, gyrB* and *topA* gene regions, and their related interaction with *fis* expression and DNA topology, establish the Fis protein as an important orchestrator between DNA supercoiling and *S.* Typhimurium virulence.

The comprehensive heatmap produced in Chapter 6 revealed possible important gene regions as they were identified for having the highest overall Fis enrichment levels (Fig. 6.8B). Although, their involvement in virulence and/or microaerobicity is not yet clear, heatmap observations suggested that *rstA*, which is known to be transcribed in response to changes in the environment, is highly enriched with Fis especially during non-aerated, stationary growth phase conditions. This is in keeping with the fact that RstA negatively affects RpoS and that it is a powerful contributor to *Salmonella* pathogenic traits (Cabeza *et al.,* 2007). As RstA induces the degradation of RpoS, the expression of anaerobic gene *arcA* is de-repressed in stationary-phase cultures under microaerobic conditions (Sevcik *et al.,* 2001). Also, high levels of Fis enrichment along the *ydgI* gene region during non-aerated conditions, provides evidence of Fis involvement in nitrogen reduction during those conditions.

The different interactions that Fis has shown to have with a number of other regulators and important virulent genes has drawn an interesting link to a possible regulatory network (Fig. 7.1) where *fis* sustained expression has entered the spotlight in the infection process of S. Typhimurium. In this proposed regulatory network, Fis abundantly binds along the *rstA* gene site to assist in its transcription in response to microaerobic conditions, while RpoS is degraded by the protein product of *rstA*, and thus *arcA* may proceed into transcription. By the same token, during non-aerated conditions, *ydgI* is activated by high levels of Fis occupancy along its gene region, resulting in the transcription of YdgI, enabling *S.* Typhimurim to function even in conditions where nitrogen is more abundant than oxygen. Moreover, Fis-binding along *ydiJ* suggest further contributions of Fis in the regulation of iron reduction and it also supports the finding that iron availability correlates with the induction of several virulence factors, including those encoded on SPI-1 (Deiwick *et al.,* 1999; Janakiraman and Slauch, 2000). Thus, the proposed regulatory network may interact with other known regulatory networks found already for Fis in the literature, such as the one for Fis and Fur (section 1.3). Fur interacts indirectly with Fis in regulating the intracellular concentration of iron, situation that has been linked to *S.* Typhimurium virulent phenotype (Ellermeier & Slauch, 2008). Also Fur, like Fis, is sensitive to environmental signals like non-aerated or low oxygen conditions (Lee & Falkow *et al.,* 1990; Ó Cróinín and Dorman, 2007) that activate important SPI-1



(Based on Cameron and Dorman, 2012)

Fig. 7.1: A regulatory network proposed for Fis during microaerobic conditions. Regulatory connections at the gene promoters described earlier. Global regulators (blue), local SPI-encoded regulators (light blue), other Fis regulated genes/proteins (pink), transcription factor (purple) and alarmone (green) are highlighted. Arrows indicate a positive regulatory effect while the perpendicular bars indicate a repressive regulatory effect, and rounded bars indicate degradation.

structural genes (Ellermeier & Slauch, 2008). Fur is negatively regulated by ppGpp (Thompson *et al.,* 2006), and the finding that DksA and ppGpp have a also negative effect on Fis, and that *dksA* and ppGpp deficient mutants display higher levels of Fis expression (chapter 4), leads to the notion that their negative influence is abolished during microaerobic conditions (Fig. 1.7). This is in agreement with the literature that when Fur is expressed, it binds to the *hilD* promoter and activates it, which in turn activates *hilA* via HilD together with HilC, thus enabling *S.* Typhimurium to invade epithelial cells through the use of SPI-1 (Teixido *et al.,* 2011). Furthermore, HilA directly activates the expression of *prgH*, which lies within the *orgA* promoter, and together with *invF* encode the components of the T3SS apparatus (Lostroh *et al.,* 2000; Lostroh & Lee, 2001) necessary for epithelial cell invasion during microaerobic conditions (SPI-1 inducing). Furthermore, this is in keeping with the findings that similar to the *fur* and *hilA* (A.13, (4)) gene regions, there is no Fis-enrichment along the *orgA* gene region in the NA, 24 h condition (A.14A & B, (4)), while enrichment levels are higher during the other conditions. Fis may act here also as a repressor on this gene region, much like it does on its own *fis* promoter (*fis* P).

As mentioned throughout this thesis, in the microaerobic conditions of the gut lumen, SPI-1 and SPI-2 genes are continually transcribed along the growth cycle as their protein products are required to invade epithelial cells and to survive inside macrophages, respectively (Kelly *et al.,* 2004; Ó Cróinín and Dorman, 2007). Fis constant interaction with a suite of genes in SPI-1 and SPI-2, and with *tyrT*, *topA* and *gyrA/B;* Fis role as a NAP influencing DNA supercoiling; and as a global regulator having high binding affinity along specific *Salmonella* gene regions involved in responding to changes in environmental conditions late in the growth cycle, suggest a possible explanation for the sustained expression of the Fis protein into the stationary phase of growth during non-aerated conditions.

In summary, in this research investigation, I have: 1) shown that earlier work on the classical pattern of *fis* gene expression, where *fis* expression peaks during the exponential phase of growth, is not all-inclusive as manipulation of the aeration regime can extend the period of *fis* transcription into stationary phase; 2) shown that ppGpp and DksA, known to be regulators of *fis* transcription, also modulate *fis* gene expression in microaerobic growth, but are not themselves responsible for the extended pattern of *fis* expression; 3) suggested that deferment of DNA relaxation is an important part of the mechanism by which *fis* transcription is

maintained for longer under microaerobic conditions; and 4) examined the pattern of Fis protein binding in cells grown under microaerobic conditions and compared that pattern with the one seen in cells grown aerobically, which is concomitant with the presence of more Fis protein in the bacteria that are grown microaerobically.

Bibliography

Ali Azam, T., Iwata, A., Nishimura, A., Ueda, S., and Ishihama, A. 1999. Growth phase-dependent variation in protein composition of the *Escherichia coli* nucleoid. J. Bacteriol. **181:** 6361–6370.

Almiron, M., Link, A. J., Furlong, D., and Kolter, R. 1992. A novel DNA-binding protein with regulatory and protective roles in starved *Escherichia coli*. Genes Dev. **6:** 2646–2654.

Altmeyer, R. M., McNern, J. K., Bossio, J. C., Rosenshine, I., Finlay, B. B, and Galán, J. E. 1993. Cloning and molecular characterization of a gene involved in *Salmonella* adherence and invasion of cultured epithelial cells. Mol. Microbiol. 7:89-98.

Altuvia, S., Almirón, M., Huisman, G., Kolter, R., and Storz, G. 1994. The *dps* promoter is activated by OxyR during growth and by IHF and sigma S in stationary phase. Mol. Microbiol. **13**:265–272.

Appleman, J. A., Ross, W., Salomon, J., and Gourse, R. L. 1998. Activation of *Escherichia coli* rRNA transcription by FIS during a growth cycle. J. Bacter. **180:** 1525-1532.

Arfin, S. M., Long, A. D., Ito, E. T., Tolleri, L., Riehle, M. M., Paegle, E. S. and Hatfield, G. W. 2000. Global gene expression profiling in *Escherichia coli* K12. The effects of integration host factors. J. Biol. Chem. **275:** 29672±29684.

Auner, H., Buckle, M., Deufel1, A., Kutateladze, T., Lazarus, L., Mavathur, R., Muskhelishvili, G., Pemberton, I., Schneider, R., and Travers, A. 2003. Mechanism of Transcriptional Activation by FIS: Role of Core Promoter Structure and DNA Topology. J. Mol. Biol. **331:** 331–344.

Azam, T. A., and Ishihama, A. 1999. Twelve species of the nucleoid-associated protein from *Escherichia coli*: Sequence recognition specificity and DNA binding affinity. J. Biol. Chem. **274**: 33105–33113.

Bajaj, V., Hwang, C., and Lee, C. A. 1995. *hilA* is a novel *ompR toxR* family member that activates the expression of *Salmonella typhimurium* expression genes. Mol. Microbiol. **18:**715–727.

Bajaj, V., Lucas, R. L., Hwang, C., and Lee, C. A. 1996. Co-ordinate regulation of *Salmonella typhimurium* invasion genes by environmental and regulatory factors is mediated by control of *hilA* expression. Mol. Microbiol. **22:**703–714.

Ball, C. A., Osuna, R., Ferguson, K. C., and Johnson, R. C. 1992. Dramatic changes in FIS levels upon nutrient upshift in *Escherichia coli*. J. Bacter. **174:** 8043-8056.

Barlett, J. M. S., and Stirling, D. A. 2003. A Short History of the Polymerase Chain Reaction. Methods Mol. Biol. **226:**3-6.

Baumler, A. J., Tsolis, R. M., Ficht, T. A., and Adams, L. G. 1998. Evolution of host adaptation in *Salmonella enterica*. Infect. Immun. **66:** 4579–4587.

Beach, M. B., and Osuna, R. 1998. Identification and characterization of the *fis* operon in enteric bacteria. J. Bacteriol. **180**:5932–5946.

Becker, N. A., Kahn, J. D. and Maher, L. M. III. 2007. Effects of nucleoid proteins on DNA repression loop formation in *Escherichia coli.* Nucleic Acids Res. **35:** 3988–4000.

Behlau, I., and Miller, S. I. 1993. A PhoP-repressed gene promotes Salmonella typhimurium invasion of epithelial cells. J. Bacteriol. **175**:4475-4484.

Benham, C. J. 1993. Sites of predicted stress-induced DNA duplex destabilization occur preferentially at regulatory loci. Proc. Natl Acad. Sci. USA **90:** 2999–3003.

Bijlsma, J. J., and Groisman, E. A. 2005. The PhoP/PhoQ system controls the intramacrophage type three secretion system of *Salmonella enterica*. Mol. Microbiol. **57**: 85-96.

Birnboim, H. C., and Doly, J. 1979. RNase based buffers. Nucleic Acids Res. **7:** 1513.

Bishop, A. C., Xu, J., Johnson, R. C., Schimmel, P., and de Crecy-Lagard, V. 2002. Identification of the tRNA-dihydrouridine synthase family. J. Biol. Chem. **277**:25090-25095.

Blanc-Potard, A. B., Solomon, F., Kayser, J., and Groisman, E. A. 1999. The SPI-3 pathogenicity island of *Salmonella enterica.* J. Bacteriol. **181**: 998-1004.

Blankschien, M. D., Lee, J., Grace, E. D., Lennon, C. W., Halliday, J. A., and Ross, W. 2009. Super DksAs: substitutions in DksA enhancing its effects on transcription initiation. EMBO J. **28:** 1720-1731.

Bradley, M. D., Beach, M. B., de Koning, A. P., Pratt, T. S., and Osuna, R. 2007. Effects of Fis on *Escherichia coli* gene expression during different growth stages. Microbiology **153**, 2922–2944 .

Bustamante, V. H., Martinez, L. C., Santana, F. J., Knodler, L. A., Steele-Mortimer, O., and Puente, J. L. 2008. HilD-mediated transcriptional cross-talk between SPI-1 and SPI-2. Proc. Natl. Acad. Sci. U.S.A. **105**: 14591–14596.

Browning, D., Grainger, D., Beatty, C., Wolfe, A., Cole, J., and Busby, S. 2005. Integration of three signals at the *Escherichia coli nrf* promoter: a role for Fis protein in catabolite repression. Mol. Microbiol. **57:** 496–510.

Broyles, S. S. and Pettijohn, D. E. 1986. Interaction of the *Escherichia coli* HU protein with DNA. 1986. Evidence for formation of nucleosome-like structures with altered DNA helical pitch. J. Mol. Biol. **187:** 47–60.

Bylund, G. O., Persson, B. C., Lundberg, L. A., Wikström, P.M. 1997. A novel ribosome-associated protein is important for efficient translation in Escherichia coli. J Bacteriol. **179**: (14) 4567–4574.

Cabeza, M.L., Aguirre, A., Soncini, F. C., and Garcia-Vescovi, E. 2007. Induction of RpoS degradation by the twocomponent system regulator RstA in Salmonella enterica. J. Bacteriol. **189:** 7335–7342.

Cameron, A. D. S., Stoebel, D. M., and Dorman, C. J. 2011. DNA supercoiling is differentially regulated by environmental factors and FIS in *Escherichia coli* and *Salmonella enterica.* Mol. Microbiol. **80**: 85-101.

Cameron, A. D. S., and Dorman, C. J. 2012. A fundamental regulatory mechanism operating through OmpR and DNA topology controls expression of *Salmonella* pathogenicity islands SPI-1 and SPI-2. PLoS Genet. **8**(3): e1002615.

Chakravortty, D., Hansen-Wester, I., and Hensel, M. 2002. *Salmonella* pathogenicity island 2 mediates protection of intracellular Salmonella from reactive nitrogen intermediates. J. Exp. Med. **195**: 1155–1166.

Chalfie,M., Tu, Y., Euskirchen, G.,Ward,W. and Prasher, D. 1994. Green fluorescent protein as a marker for gene expression. Science **263:** 802–805.

Champion, K., and Higgins, N. P. 2007. Growth rate toxicity phenotypes and homeostatic supercoil control differentiate *Escherichia coli* from *Salmonella enterica serovar* Typhimurium. J. Bacteriol. **189**: 5839-5849.

Champoux, J. J. 2001. DNA topoisomerases: Structure, function, and mechanism. Ann. Rev. Biochem. **70**: 369-413.

Chang, D. E., Smalley, D. J., and Conway, T. 2002. Gene expression profiling of *Escherichia coli* growth transitions: an expanded stringent response model. Mol. Microbiol. **45:**289–306.

Chatterji, D., and Ojha, A. K. 2001. Revisiting the stringent response, ppGpp and starvation signaling. Curr. Opin. Microbiol. **4**: 160–165.

aChen, L. M., Hobbie, S., and Galán, J. E. 1996. Requirement of CDC42 for *Salmonella*-induced cytoskeletal and nuclear responses. Science **274:** 2115–2118.

bChen, L. M., Kaniga, K., and Galán, J. E. 1996. *Salmonella* spp. are cytotoxic for cultured macrophages. Mol. Microbiol. **21:** 101–1115.

Chenoweth, M. R. and Wickner, S. 2008. Complex regulation of the DnaJ homolog CbpA by the global regulators σ^s and Lrp, by the specific inhibitor CbpM, and by the proteolytic degradation of CbpM. J. Bacteriol. **190***:* 5153–5161.

Cho, B. W., Knight, E. M., Barrett, C. L., and Palsson, B. Ø. 2008. Genome-wide analysis of Fis binding in *Escherichia coli* indicates a causative role for A-/AT-tracts. Genome Res. **18:** 900-910.

Cho, B. W., Barrett, C. L. Knight, E. M., Park, Y. S. and Palsson, B. Ø. 2008. Genome scale reconstruction of the Lrp regulatory network in *Escherichia coli.* Proc. Natl Acad. Sci. USA **105:** 19462–19467.

Cirillo, D. M., Valdivia, R. H., Monack, D. M. and Falkow, S. 1998. Macrophage-dependent induction of the Salmonella pathogenicity island 2 type III secretion system and its role in intracellular survival. Mol. Microbiol. **30**: 175–188.

Coburn, B., Grassl, G. A., and Finlay, B. B. 2007. *Salmonella*, the host and disease: a brief review. Immunol. and Cell. Biol. **85:**112–118.

Coombes, B. K., Brown, N. F., Valdez, Y., Brumell, J. H., and Finlay, B. B. 2004. Expression and secretion of *Salmonella* pathogenicity island-2 virulence genes in response to acidification exhibit differential requirements of a functional type III secretion apparatus and SsaL. J. Biol. Chem. **279**: 49804–49815.

Coombes, B. K., Wickham, M. E., Lowden, M. J., Brown, N. F., and Finlay, B. B. 2005. Negative regulation of *Salmonella* pathogenicity island 2 is required for contextual control of virulence during typhoid. Proc. Natl. Acad. Sci. USA **102**:17460–17465.

Cozzarelli, N. R. 1980. Dna gyrase and the supercoiling of DNA. Science **207:** 953-960.

Crump, J. A., Luby, S. P., and Mintz, E. D. 2004. Bull. The global burden of typhoid fever.World Health Organ. **82:** 346-353.

Dame, R. T., and Dorman, C. J. (eds.) 2010. Bacterial Chromatin. 3DOI 10.1007/978-90-481-3473-1_1. Springer Science Business Media B.V.

Datsenko, K. L., and Wanner, B. L. 2000. One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. Proc. Natl. Acad. Sci. USA **97:** 6640-6645.

Deighan, P., Beloin, C. and Dorman, C. J. 2003. Three-way interactions among the Sfh, StpA and H-NS nucleoid structuring proteins of *Shigella flexneri* 2a strain 2457T. Mol. Microbiol. **48:** 1401–1416.

Deiwick, J., Nikolaus, T., Erdogan, S., and Hensel, M. 1999. Environmental regulation of *Salmonella* pathogenicity island 2 gene expression. Mol. Microbiol. **31**: 1759–1773.

Detweiler, C. S., Monack, D. M., Brodsky I. E., Mathew, H. and Falkow, S. 2003. *virK*, *somA* and *rcsC* are important for systemic *Salmonella enterica* serovar Typhimurium infection and cationic peptide resistance. Mol. Microbiol. **48(2)**, 385–400.

Dillon, S. C., and Dorman, C. J. 2010. Bacterial nucleoid-associated proteins, nucleoid structure and gene expression. Nature Rev. Microbiol. **8**: 185-195

Dillon, S. C., Cameron, A. D. S., Hokamp, K., Lucchini, S., Hinton, J. C. D., and Dorman, C. J. 2010. Genome-wide analysis of the H-NS and Sfh regulatory networks in *Salmonella typhimurium* identifies a plasmid-encoded transcription silencing mechanism. Mol. Microb. **76**: 1250–1265.

Dorman, C. J., Chatfield, S., Higgins, C. F., Hayward, C., and Dougan, G. 1989. Characterization of porin and *ompR* mutants of a virulent strain of *Salmonella typhimurium*: *ompR* mutants are attenuated in vivo. Infect. Immun. **57:** 2136–2140.

Dorman, C. J. 1991 DNA supercoiling and environmental regulation of gene expression in pathogenic bacteria. Infect. Immun. **59:** 745–749.

Dorman, C. J., and Deighan, P. 2003. Regulation of gene expression by histone-like proteins in bacteria. Curr. Opin. Genet. Devel. **13:** 179–184.

Dorman, C.J. 2007. H-NS, the genome sentinel. Nat. Rev. Microbiol. **5:** 157–161

Dorman, C. J. 2009. Nucleoid-associated proteins and bacterial physiology. Adv. Appl. Microbiol. **67:** 47–64.

Dorman, C. J. 2013. Co-operative roles for DNA supercoiling and nucleoid-associated proteins in the regulation of bacterial transcription. Biochem. Soc.Transact. **41:** part 2.

Eckmann, L., Rudolf, M. T., Ptasznik, A., Schultz, C., Jiang, T., Wolfson, N., Tsien, R., Fierer, J., Shears, S. B., Kagnoff, M. F., and Traynor-Kaplan, A. E. 1997. D-*myo*-Inositol-1,4,5,6-tetrakisphosphate produced in human intestinal epithelial cells in response to *Salmonella* invasion inhibits phosphoinositide 3-kinase signaling pathways. Proc. Natl. Acad. Sci. U.S.A. **94:** 14456–14460.

Eichelberg, K., and Galán, J. E. 1999. Differential Regulation of Salmonella typhimurium Type III Secreted Proteins by Pathogenicity Island 1 (SPI-1)-Encoded Transcriptional Activators InvF and HilA. Infect. Immun. **67**: 4099-4105.

Eiglmeier, K., Honore, N., Luchi, S., Lin, E. C., and Cole, S. T. 1989. Molecular genetic analysis of FNR-dependent promoters. Mol. Microbiol. **3**:869-878.

Ellermeier, J. R. and Slauch, J. M. 2007. Adaptation to the host environment: regulation of the SPI1 type III secretion system in *Salmonella enterica* serovar Typhimurium. Curr. Opin. Microbiol. **10**: 24–29.

Elowitz, M. B. and Leibler, S. 2000. A synthetic oscillatory network of transcriptional regulators. Nature. **403:** 335±338.

Elsinghorst, E. A., Baron, L. S., and Kopecko, D. J. 1989. Penetration of human intestinal epithelial cells by *Salmonella*: molecular cloning and expression of *Salmonella typhi* invasion determinants in *Escherichia* coli. Proc. Natl. Acad. Sci. USA **86**:5173-5177.

Ernst, R. K., Domboski, D. M., and Merrick, J. M. 1990. Anaerobiosis, type 1 fimbriae, and growth phase are factors that affect invasion of HEp-2 cells by *Salmonella typhimurium*. Infect. Immun. **58:**2014–2016.

Evans, M. R., Fink, R. C., Vazquez-Torres, A., Porwollik, S., Jones-Carson, J., McClelland, M., Hassan, H. M. 2011. Analysis of the ArcA regulon in anaerobically grown *Salmonella enterica sv.* Typhimurium. BMC Microbiol. **11:**58.

Falconi, M., Brandi, A., La Teana, A., Gualerzi, C. O. and Pon, C. L. 1996. Antagonistic involvement of FIS and H-NS proteins in the transcriptional control of *hns* expression. Mol. Microbiol. **189:** 965–975.

Falconi, M., Prosseda, G., Giangrossi, M., Beghetto, E., and Colonna, B. 2001. Involvement of FIS in the H-NS-mediated regulation of virF gene of *Shigella* and enteroinvasive *Escherichia coli* . Mol. Microbiol. **42:** 439-452

Fang, F.C., Libby, S. J., Buchmeier, N. A., Loewen P. C., J. Switala, J. Harwood, and Guiney, D. G.. 1992. The alternative sigma factor *katF* (*rpoS*) regulates *Salmonella* virulence. Proc. Natl. Acad. Sci. U.S.A. **89:** 11978–11982.

Feng, X., Oropeza, R., and Kenney, L. J. 2003. Dual regulation by phospho-OmpR of *ssrA/B* gene expression in *Salmonella* pathogenicity island 2. Mol. Microbiol. **48**: 1131–1143.

Feng, X., Walthers, D., Oropeza, R., and Kenney, L. J. 2004. The response regulator SsrB activates transcription and binds to a region overlapping OmpR binding sites at *Salmonella* pathogenicity island 2. Mol. Microbiol. **54**: 823–835.

Fierer, J., and Guiney, D. G. 2001. Diverse virulence traits underlying different clinical outcomes of Salmonella infection. J. Clin. Invest. **107:** 775–780.

Fink, R. C., Evans, M. R., Porwollik, S., Vazquez-Torres, A., Jones-Carson, J., Troxell, B. Libby, S. J., McClelland, M., and Hassan, H. M. 2007. FNR is a Global Regulator of Virulence and Anaerobic Metabolism in *Salmonella enterica* Serovar Typhimurium (ATCC 14028s). J. Bacteriol. **189**: 2262–2273.

Finkel, S. E. and Jonhson, R. C. 1992. The Fis protein: it's not just for DNA inversion anymore. Mol. Microbiol. **6:** 3257–3265.

Finlay, B. B., and Brumell, J. H. 2000. *Salmonella* interactions with host cells: *in vitro* to *in vivo*. Phil. Trans. R. Soc. Lond. B **355**: 623-631.

Foster, J. W. 1991. *Salmonella* acid shock proteins are required for the adaptive acid tolerance response. J. Bacteriol. **173:** 6896–6902.

Free, A. and Dorman, C. J. 1997. The *Escherichia coli stpA* gene is transiently expressed during growth in rich medium and is induced in minimal medium and by stress conditions. J. Bacteriol. **179:** 909–918.

Galán, J. E., and Curtiss III, R. 1989. Cloning and molecular characterization of genes whose products allow *Salmonella typhimurium* to penetrate tissue culture cells. Proc. Natl. Acad. Sci. U.S.A. **86:**6383-6387.

Galán, J. E., and Curtiss III, R. 1990. Expression of *Salmonella typhimurium* genes required for invasion is regulated by changes in DNA supercoiling. Infect. Immun. **58:** 1879-1885.

Galán, J. E. 1996. Molecular bases of *Salmonella* entry into host cells. Mol. Microbiol. **20:** 263–271.

Galán, J. E. 1998. Interaction of *Salmonella* with host cells: encounters of the closest kind. Proc. Natl. Acad. Sci. USA **95:** 14006–14008.

Galán, J. E. 2001. *Salmonella* interactions with host cells: type III secretion at work. Annu. Rev. Cell Dev. Biol. **17**:53–86.

Galyov, E.E., Wood, M.W., Rosqvist, R., Mullan, P.B., Watson, P.R., Hedges, S., and Wallis, T.S. 1997. A secreted effector protein of *Salmonella dublin* is translocated into eukaryotic cells and mediates inflammation and fluid secretion in infected ileal mucosa. Mol. Microbiol. **25:**1903–1912.

Garner, C.D., Antonopoulos, D.A., Wagner, B., Duhamel G.E., Keresztes I., and Ross, D.A. 2009. Perturbation of the small intestine microbial ecology by streptomycin alters pathology in a *Salmonella enterica* serovar Typhimurium murine model of infection. Infect. Immun. 77:2691–2702.

Gellert, M., O'Dea, M. H., Mizuuchi, K. and Nash, H. 1976. DNA gyrase: an enzyme that introduces superhelical turns into DNA. Proc. Natl. Acad. Sci. USA **73:**3872–3876.

Ghisla, S., Hastings, J. W., Favaudon, V. and Lhoste, J.-M. 1978. Structure of the oxygen adduct intermediate in the bacterial luciferase reaction: 13C nuclear magnetic resonance determination. Proc. Natl Acad. Sci. USA **75:** 5860–5863.

Giannella, R. A., S. A. Broitman, and Zamcheck, N. 1972. Gastric acid barrier to ingested microorganisms in man: studies *in vivo* and *in vitro*. Gut **13:** 251-256.

Gille, H., Egan, J.B., Roth, A., and Messer, W. 1991. The Fis protein binds and bends the origin of chromosomal DNA replication, oriC, of Escherichia coli. Nucleic Acids Res. **19:** 4167–4172.

Ginocchio, C., Pace, J., and Galán, J. E. 1992. Identification and molecular characterization of a Salmonella typhimurium gene involved in triggering the internalization of salmonellae into cultured epithelial cells. Proc. Natl. Acad. Sci. U.S.A. **89:** 5976-5980.

Goldberg, M. D., Johnson, M., Hinton, J. C. D., and Williams, P. H. 2001. Role of the nucleoid-associated protein Fis in the regulation of virulence properties of enteropathogenic *Escherichia coli.* Mol. Microbiol. **41(3):** 549–559.

Gottesman, S. 2004. The small RNA regulators of *Escherichia coli*: roles and mechanisms. Annu. Rev. Microbiol. **58:** 303–328.

Ghosh, P. 2004. Process of protein transport by the type III secretion system. Microbiol. Mol. Biol. Rev. **68:** 771–795.

Grainger, D. C., Hurd, D., Goldberg, M. D. and Busby, S. W. J. 2006. Association of nucleoid proteins with coding and non-coding segments of the *Escherichia coli* genome. Nucleic Acids Res. **34:** 4642–4652.

Grainger, D. C., Goldberg, M. D., Lee, D. J. and Busby, S. J. W. 2008. Selective repression by Fis and H-NS at the *Escherichia coli dps* promoter. Mol. Microbiol. **68:** 1366–1377.

Grant, R. A., Filman, D. J., Finkel, S. E., Kolter, R. and Hogle, J. M. 1998. The crystal structure of Dps, a ferritin homolog that binds and protects DNA. Nat. Struct. Biol. **5:** 294-303.

Griffith, J. D. 1976. Visualization of prokaryotic DNA in aregularly condensed chromatin-like fiber. Proc. Natl. Acad. Sci. USA. **73:** 563-567.

Groisman, E. A., and Ochman, H. 1993. Cognate gene clusters govern invasion of host epithelial cells by *Salmonella typhimurium* and *Shigella flexner*i. EMBO J. **12:** 3779-3787.

Groisman, E. A., and Ochman, H. 1997. How *Salmonella* became a pathogen. Trends Microbiol. **5**:343–349.

Hacker, J., and Kaper, J. B. 1999. The concept of pathogenicity islands. In Pathogenicity Islands and Other Mobile Virulence Elements. pp, 1–11. Washington, DC: Am. Soc. Microbiol.

Hansen, M. C., Palmer, R. J. Jr, Udsen, C., White, D. C. and Molin, S. 2001. Assessment of GFP fluorescence in cells of *Streptococcus gordonii* under conditions of low pH and low oxygen concentration. Microbiol **147:** 1383–1391.

Hansen-Wester, I., and Hensel, M. 2001. *Salmonella* pathogenicity islands encoding type III secretion systems. Microbes Infect. **3:** 549–559.

Hardt, W. D., Chen, L. M., Schuebel, K. E., Bustelo, X. R., and Galán, J. E. 1998. *Salmonella typhimurium* encodes an activator of Rho GTPases that induces membrane ruffling and nuclear responses in host cells. Cell **93:**815–826.

Hautefort, I., Proenc, M. J., and Hinton, J. C. D. 2003. Single-copy green fluorescent protein gene fusions allow accurate measurement of *Salmonella* gene expression in vitro and during infection of mammalian cells. Appl. Environ. Microb. **69:** 7480-7491.

Hautefort, I., Thompson, A., Eriksson-Ygberg, S., Parker, M. L., Lucchini, S., Danino, V., Bongaerts, R. J., Ahmad, N., Rhen, M., and Hinton, J. C. D. 2008. During infection of epithelial cells *Salmonella enterica* serovar Typhimurium undergoes a time-dependent transcriptional adaptation that results in simultaneous expression of three type 3 secretion systems. Cell Microbiol. **10**: 958–984.

Hirsch, M., and Elliott., T. 2005. Stationary-phase regulation of RpoS translation in *Escherichia coli*. J. Bacteriol. **187:**7204–7213.

Heichman, K. A., and Johnson, R. C. 1990. The Hin invertasome: protein-mediated joining of distant recombination sites at the enhancer. Science **249**: 511-517.

Hengen, P. N., Bartram, S. L., Stewart, L. E., and Schneider, T. D. 1997. Information Analysis of Fis Binding Sites. Nucl. Acids Res. **25**: 4994-5002.

Hensel, M., Shea, J. E., Gleeson, C., Jones, M. D., Dalton, E., and Holden, D. W. 1995. Simultaneous identification of bacterial virulence genes by negative selection. Science **269:** 400–403.

Hensel, M., Shea, J. E., Raupach, B., Monack, D., Falkow, S., Gleeson, C., and D. W. Holden. 1997. Functional analysis of ssaJ and the *ssaK/U* operon, 13 genes encoding components of the type III secretion apparatus of *Salmonella* pathogenicity island 2. Mol. Microbiol. **24:** 155–167.

Hensel, M., Nikolaus, T., and Egelseer, C. 1999. Molecular and functional analysis indicates a mosaic structure of Salmonella Pathogenicity Island 2. Mol. Microbiol. **31**: 489-498.

Hensel, M. 2000. Salmonella Pathogenicity Island 2. Mol Microbiol **36**: 1015-1023.

Herrick, R. L., Buchberger, S. G., Clark, R. M., Kupferle, M., Murray, R. and Succop, P. 2011. A Markov model to estimate Salmonella morbidity, mortality, illness duration, and cost. Health Econ. **21**: 1169–1182. doi: 10.1002/hec.1779

Hersh, D., Monack, D. M., Smith, M. R., Ghori, N., Falkow, S. and Zychlinsky, A. 1999. The *Salmonella* invasin SipB induces macrophage apoptosis by binding to caspase-1. Proc Natl. Acad. Sci. U.S.A. **96**:2396–2401.

Higgins, C. F., Dorman, C. J., Stirling, D. A., Waddell, L., Booth, I. R., May, G., and Bremer, E. 1988. A physiological role for DNA supercoiling in the osmotic regulation of gene expression in *S. typhimurium* and *E. coli.* Cell **52:** 569-84.

Hobbie, S., Chen, L. M., Davis, R., and Galán, J. E. 1997. Involvement of the mitogen-activated protein kinase pathways in the nuclear responses and cytokine production induced by *Salmonella typhimurium* in cultured intestinalcells. J. Immunol. **159:**5550–5559.

Hsieh, L., Rouviere-Yaniv, J., and Drlica, K. 1991. Bacterial DNA supercoiling and ATP/ADP ratio: changes associated with salt shock. J. Bacteriol. **173**: 3914-3917.

Hsu, Y. H., Chung, M. W. and Li, T. K. 2006. Distribution of gyrase and topoisomerase IV on bacterial nucleoid: implications for nucleoid organization. Nucleic Acids Res. **34:** 3128–3138.

Huber, H. E., Iida, S. Arber, W., and Bickle, T. A. 1985. Site-specific DNA inversion is enhanced by a DNA sequence element in cis Proc. Nat. Acad. Sci. U.S.A. **82**: 3776-3780.

Hübner, P., and Arber, W. 1989. Mutational analysis of a prokaryotic recombinational enhancer element with two functions. EMBO J. **8**: 577–585.

Hueck, C. J. 1998. Type III protein secretion systems in bacterial pathogens of animals and plants. Microbiol. Rev. **62:** 379–433.

Janakiraman, A., and Slauch, J.M. 2000. The putative iron transport system SitABCD encoded on SPI-1 is required for full virulence of *Salmonella typhimurium.* Mol. Microbiol. **35:** 1146–1155.

Johnson, R. C. and Simon, M. I. 1985. Hin-mediated site-specific recombination requires two 26 by recombination sites and a 60 by recombinational enhancer. Cell **41:**781-791.

Jones, B. D., N. Ghori, and Falkow, S. 1994. *Salmonella typhimurium* initiates murine infection by penetrating and destroying the specialized epithelial M cells of the Peyer's patches. J. Exp. Med. **180:** 15–23.

Jones, B. D., and Falkow, S. 1994. Identification and Characterization of a Salmonella typhimurium Oxygen-Regulated Gene Required for Bacterial Internalization. Infect. Immun. **62(9),** 3745-3752.

Jones, B. D. 2005. Salmonella invasion gene regulation: a story of environmental awareness. J. Microbiol. **43:** 110–117.

Jovanovich, S. B. and Lebowitz, J. 1987. Estimation of the effect of coumermycin A1 on *Salmonella typhimurium* promoters by using random operon fusions. J. Bacteriol. **169:** 4431-4435.

Kafkewitz, D., and Togna, M. T. 1999. The methods of anaerobic microbiology with emphasis on environmental applications. Intern. J. Environ. Studies **56:** 141-170.

Kahmann, R. Rudt, F., Koch, C., and Mertens, G. 1985. G inversion in bacteriophage Mu DNA is stimulated by a site within the invertase gene and a host factor. Cell **41**: 771-780.

Kahamanoglou, C., Seshasayee, A. S. N., Prieto, A. I., Ibberson, D., Schmidt, S., Zimmermann, J., Benes, V., Fraser, G. M., and Luscombe, N. M. 2010. Direct and indirect effects of H-NS and Fis on global gene expression control in *Escherichia coli*. Nucleic Acids Res. **39(6):**2073–2091.

Kaniga, K., Bossio, J. C., and Galán, J. E. 1994. The *Salmonella typhimurium* invasion genes *invF* and *invG* encode homologues to the PulD and AraC family of proteins. Mol. Microbiol. **13:**555–568.

Karlinsey, J. E. 2007. λ-Red Genetic Engineering in *Salmonella enterica serovar* Typhimurium. Methods Enzymol. **421:** 199-209.

Karp, P. D., and Riley, M. 2009. Representations of Metabolic Knowledge. Proceed. First Internat. Conf. Intel. Syst. Mol. Biol. Morgan Kaufmann Publishers, pp207–215.

Keane, O. M., and Dorman, C. J. 2003. The *gyr* genes of *Salmonella enterica serovar* Typhimurium are repressed by the factor for inversion stimulation, Fis. Mol. Gen. Genomics. **270:** 56-65.

Kelly, A., Goldberg, M. D., Carroll, R. K., Danino, V., Hinton, J. C. D., 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.

Kingsley, R. A., and Bäumler, A. J. 2000. Host adaptation and the emergence of infectious disease: the *Salmonella* paradigm. Mol. Microbiol. **36(5):** 1006-1014.

Klein, J. R., Fahlen, T. F., and Jones, B. D. 2000. Transcriptional organization and function of invasion genes within *Salmonella enterica* serovar Typhimurium pathogenicity island 1, including the *prgH, prgI, prgJ, prgK, orgA, orgB,* and *orgC* genes. Infect. Immun. **68:** 3368-3376.

Kröger, C., Dillon, S. C., Cameron, A. D., Papenfort, K., Sivasankaran, S. K., Hokamp, K., Chao, Y., Sittka, A., Hebrard, M., Handler, K., and Hinton, J. 2012. The transcriptional landscape and small RNAs of *Salmonella enterica* serovar Typhimurium. Proc. Natl. Acad. Sci. USA. **109:** E1277–E1286.

Kuroda, A., Nomura, K., Kato, J., Takiguchi, N., and Ohtake H. 2006. Inorganic polyphosphate stimulates Lon-mediated proteolysis of nucleoid proteins in Escherichia coli. Cell Mol. Biol. **52:** 22-29.

Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of the bacteriophage T4. Nature **227:** 680-685.

Landgraf, J. R., Wu, J., and Calvo, J. M. 1996. Effects of nutrition and growth rate on Lrp levels in *Escherichia coli*. J. Bacter. **178:** 6930-6936.

Lautier, T., and Nasser, W. 2007. The DNA nucleoid-associated protein Fis co-ordinates the expression of the main virulence genes in the phytopathogenic bacterium Erwinia chysanthemi. Mol. Microbiol. **66**: 1474–1490.

Lazarus, L. R., and Travers, A. A. 1993. The *Escherichia coli* FIS protein is not required for the activation of *tyrR* transcription on entry into exponential growth. EMBO J. **12:** 2483–2494.

Lee, C. A., and Falkow, S. 1990. The ability of *Salmonella* to enter mammalian cells is affected by bacterial growth state. Proc. Natl. Acad. Sci. U.S.A. **87**: 4304–4308.

Lee, C.A., Jones, B.D., and Falkow, S. 1992. Identification of a *Salmonella typhimurium* invasion locus by selection for hyperinvasive mutants. Proc. Natl. Acad. Sci. U.S.A. **89:** 1847-1851.

Lee, A. K., Detweiler, C. S., and Falkow, S. 2000. OmpR regulates the two-component system SsrA-ssrB in *Salmonella* pathogenicity island 2. J. Bacteriol. **182:** 771–781.

Lee, J. W., and Helmann, J. D. 2007. Functional specialization within the Fur family of metalloregulators. Biometals. 20: 485–499

Loewen, P. C., and Hengge-Aronis, R. 1994. The role of the sigma factor sigma S (KatF) in bacterial global regulation. Annu. Rev. Microbiol. **48:**53–80.

Lohr, D., and Van Holde, K. E. 1975. Yeast subunit chromatin structure. Science. **188:** 165-168.

Lostroh, C. P., Bajaj V., and Lee, C. A. 2000. The cis requirements for transcriptional activation by HilA, a virulence determinant encoded on SPI-1. Mol. Microbiol. **37:** 300–315.

Lostroh, C. P., Lee, C. A. 2001. The Salmonella pathogenicity island-1 type III secretion system. Microbes Infect. **3:** 1281–1291.

Lucas, R. L. and Lee, C. A. 2001. Roles of *hilC* and *hilD* in regulation of *hilA* expression in *Salmonella enterica* serovar Typhimurium. J. Bacteriol. **183:** 2733–2745.

Lucchini, S., Rowley, G., Goldberg, M.D., Hurd, D., Harrison, M. and Hinton, J.C. 2006. H-NS mediates the silencing of laterally acquired genes in bacteria. PLoS Pathog. **2:** e81

Luijsterburg, M. S., White, M. F., van Driel, R. and Dame, R. T. 2008. The major architects of chromatin: architectural proteins in bacteria, archaea and eukaryotes. Crit. Rev. Biochem. Mol. Biol. **43:** 393–418.

Macchi, R., Montesissa, L., Murakami, K., Ishihama, A., De Lorenzo, V., and Bertoni, G. 2003. Recruitment of σ54-RNA polymerase to the *Pu* promoter of *Pseudomonas putida* through integration host factor-mediated positioning switch of α subunit carboxyl-terminal domain on an UP-like element. J. Biol. Chem. **278:** 27695–27702.

Mallik, P., Pratt, T. S., Beach, M. B., Bradley, M. D., Undamatla, J., and Osuna, R. 2004 Growth-phase dependent regulation and stringent control of *fis* are conserved processes in enteric bacteria and involve a single promoter (*fis* P) in *Escherichia coli*. J. Bacteriol. **186:** 122–135.

Mallik, P., Paul, B. J., Rutherford, S. T., Gourse, L. R. and Osuna, R. 2006. DksA is required for growth-phase dependent regulation, growth rate-dependent control, and stringent control of Fis expression in *Escherichia coli*. J. Bacter. **188:** 5775-5882.

Mangan, M. W., Lucchini, S., Danino, V., Ó Cróinín, T., Hinton, J. C. D., and Dorman, C. J. 2006. The integration host factor (IHF) integrates stationary-phase and virulence gene expression in *Salmonella enterica* serovar Typhimurium. Mol. Microbiol. **59:** 1831–1847.

Marcus, S. L., Brumell, J. H., Pfeifer, C. G., and Finlay, B. B. 2000. Salmonella pathogenicity islands: big virulence in small packages. Microb. Infec. **2**:145–156.

Martínez-Antonio, A. and Collado-Vides, J. 2003. Identifying global regulators in transcriptional regulatory networks in bacteria. Curr. Opin. Microbiol. **6**: 482–489.

Martínez-Antonio, A., Medina-Rivera, A., and Collado-Vides, J. 2009. Structural and functional map of a bacterial nucleoid. Genome Biol. **10**:247.

Martínez-Antonio, A., Lomnitz, J. G., Sandoval, S., Aldana, M., and Savageau, M. A. 2012. Regulatory Design Governing Progression of Population Growth Phases in Bacteria. PLoS ONE **7(2)**: e30654.

Marteyn, B., West, N. P., Browning, D. F., Cole, J. A., Shaw, J. G., Palm, F., Mounier, J., Prevost, M. C., Sansonetti, P., and Tang, C. M. 2010. Modulation of *Shigella* virulence in response to available oxygen in vivo. Nature **465:** DOI:10.1038/nature08970

Maurer, S., Fritz, J. and Muskhelishvili, G. 2009. A systematic *in vitro* study of nucleoprotein complexes formed by bacterial nucleoid-associated proteins revealing novel types of DNA organization. J. Mol. Biol. **387:** 1261–1276.

McClelland, M., Sanderson, K. E., Clifton, S. W., Latreille, P., Porwollik, S., Sabo, A., Meyer, R., Bieri, T., Ozersky, P., McLellan, M., Harkins, C. R., Wang, C., Nguyen, C., Berghoff, A., Elliott, G., Kohlberg, S., Strong, C., Du, F., Carter, J., Kremizki, C., Layman, D., Leonard, S., Sun, H., Fulton, L., Nash, W., Miner, T., Minx, P., Delehaunty, K., Fronick, C., Magrini, V., Nhan, M., Warren, W., Florea, L., Spieth, J., and Wilson, R. K. 2004. Comparison of genome degradation in Paratyphi A and Typhi, human-restricted serovars of *Salmonella enterica* that cause typhoid. Nat. Gen. **36(12):**1268-1274.

McLeod, S. M., Aiyar, S. E., Gourse, R. L., and Johnson, R. C. 2002. The C-terminal domains of the RNA polymerase alpha subunits: Contact site with Fis and localization during coactivation with CRP at the *Escherichia coli* proP P2 promoter. J. Mol. Biol. **316**: 517–529.

McCormick, B.A., Colgan, S. P., Delp-Archer, C., Miller, S. I., and Madara, J. L. 1993. *Salmonella typhimurium* attachment to human intestinal epithelial monolayers: transcellular signalling to subepithelial neutrophils. J. Cell Biol. **123:** 895–907.

McFarland, K. A., and Dorman, C. J. 2008. Autoregulated expression of the gene coding for the leucine-responsive protein, Lrp, a global regulator in *Salmonella enterica serovar* Typhimurium. Microbiol. **154:** 2008-2016.

Menendez, A., Arena, E. T., Guttman, J. A., Thorson, L., Vallance, B. A., Vogl, W., and Finlay, B. B. 2009. *Salmonella* Infection of Gallbladder Epithelial Cells Drives Local Inflammation and Injury in a Model of Acute Typhoid Fever. J. Infect. Diseases **200:** 1703–13.

Miao, E. A., Freeman, J. A., and Miller, S. I. 2002. Transcription of the SsrAB regulon is repressed by alkaline pH and is independent of PhoPQ and magnesium concentration. J. Bacteriol.**184**: 1493–1497.

Mittenhuber, G. 2001. Comparative genomics and evolution of genes encoding bacterial (p)ppGpp synthetases/hydrolases (the Rel, RelA and SpoT proteins). J. Mol. Microbiol. Biotechnol. **3(4):** 585-600.

Monack, D. M., Raupach, B., Homockyj, A. E., and Falkow, S. 1996. *Salmonella typhimurium* invasion induces apoptosis in infected macrophages. Proc. Natl. Acad. Sci. U.S.A. **93:** 9833–9838.

Morett, E., and Bork, P. 1998. Evolution of new protein function: recombinational enhancer Fis originated by horizontal gene transfer from the transcriptional regulator NtrC. FEBS Lett. **433:** 108-112.

Muskhelishvili, G., Buckle, M., Heumann, H., Kahmann, R. and Travers, A. A. 1997. FIS activates sequential steps during transcription initiation at a stable RNA promoter. *EMBO J.* **16**: 3655–3665.

Muskhelishvili, G., and Travers, A. 2003. Transcription factor as a topological homeostat. Front. Biosci. **8:** 279-285.

Navarre, W. W., Porwollik, S., Wang, Y., McClelland, M., Rosen, H., Libby, S. J., and Fang, F. C. 2006. Selective silencing of foreign DNA with low GC content by the H-NS protein in *Salmonella*. Science **313**: 236–238.

Nasser, W., Rochman, M., and Muskhelishvili, G. 2002. Transcriptional regulation of *Fis* operon involves a module of multiple coupled promoters. EMBO J. **21:** 715–724.

Nilsson, L., Verbeek, H., Vijgenboom, E., van Drunen, C., Vanet, A., and Bosch, L. 1992. Fis-dependent *trans* activation of stable RNA operons of *Escherichia coli* under various growth conditions. J. Bacteriol. **174:** 921–929.

Ninneman, O., Koch, C., and Kahmann, R. 1992. The *E. coli fis* promoter is subject to stringent control and autoregulation. EMBO J. **11:** 1075–1083.

O'Byrne, C. P., Ní Bhiain, N., and Dorman, C. J. 1992. The DNA supercoiling-sensitive expression of the *Salmonella typhimurium his* operon requires the *his* attenuator and is modulated by anaerobiosis and by osmolarity. Mol. Microbiol. **6:** 2647-2476.

Ó Cróinín, T., Carroll, R. K., Kelly, A., and Dorman, C. J. 2006. Roles for DNA supercoiling and the Fis protein in modulating expression of virulence genes during intracellular growth of *Salmonella enterica serovar* Typhimurium. Mol. Microbiol. **62:** 869-882.

Ó Cróinín, T., and Dorman, C. J. 2007. Expression of the Fis protein is sustained in late-exponential and stationary-phase cultures of *Salmonella enterica serovar* Typhimurium grown in the absence of aeration. Mol. Microbiol. **66:** 237-251.

Ochman, H., Soncini, F. C., Solomon, F., and Groisman, E. A. 1996. Identification of a pathogenicity island required for *Salmonella* survival in host cells. Proc. Natl. Acad. Sci. U.S.A. **93**: 7800–7804.

Ohl, M. E., and Miller, S. I. 2001. *Salmonella*: A Model for Bacterial Pathogenesis. Annu. Rev. Med. **52:** 259-274.

Olins, A. L., and Olins, D. E. 1974. Spheroid chromatin units (v bodies). Science. **183:** 330-332.

O'Neal, C. R., Gabriel, W. M., Turk, A. K., Libby, S. J., Fang, F. C., and Spector, M. P. 1994. RpoS is necessary for both the positive and negative regulation of starvation survival genes during phosphate, carbon, and nitrogen starvation in *Salmonella typhimurium*. J. Bacteriol. **176:** 4610–4616.

Opel, M. L, Aeling, K. A., Holmes, W. M., Johnson, R. C., Benham, C. J., and Hatfield, G. W. 2004. Activation of transcription initiation from a stable RNA promoter by a Fis protein-mediated DNA structural transmission mechanism. Mol. Microbiol. **53**: 665–674.

Osuna, R., Lienau, D., Hughes, K. T., and Johnson, R. C. 1995. Sequence, regulation, and functions of *fis* in *Salmonella typhimurium*. J. Bacter. **177:** 2021-2032.

Pan, C. Q. *et al.* 1996. Variable structures of Fis-DNA complexes determined by flanking DNA-protein contacts. J. Mol. Biol. **264:** 675–695.

Paul, B. J., Barker, M. M., Ross, W., Schneider, D. A., Webb, C., Foster, J. W., and Gourse, R. L. 2004. DksA: a critical component of the transcription machinery that potentiates the regulation of rRNA promoters by ppGpp and the initiating NTP. Cell **118:** 311-322.

Pedersen, A. G., Jensen, L. J., Brunak, S., Staerfeldt, H. H. and Ussery, D. W. 2000. A DNA structural atlas for *Escherichia coli.* J. Mol. Biol. **299:** 907–930.

Pratt, T. S., Steiner, T. Feldman, L. S., Walker, K. A., and Osuna, R. 1997. Deletion analysis of the *fis* promoter region in *Escherichia coli*: antagonistic effects of integration host factor and *fis*. J. Bacteriol. **179**: 6367–6377.

Pratt, L.A., and Kolter, R. 1998. Genetic analysis of *Escherichia coli* biofilm formation: defining the roles of FLAGella, motility, chemotaxis and type I pili. Mol Microbiol **30:** 285–293.

Prosseda, G., Falconi, M., Giangrossi, M., Gualerzi, C. O., Micheli, G. and Colonna, B. 2004. The virF promoter in *Shigella*: more than just a curved DNA stretch. Mol. Microbiol. 51: 523–537.

Prosser, G. A. *et al.* 2013. Creation and screening of a multi-family bacterial oxidoreductase library to discover novel nitroreductases that efficiently activate the bioreductive prodrugs CB1954 and PR-104A. Biochem. Pharmacol. **85:** 8, 1091–1103.

Pullinger, G. D., van Diemen, P. M., Dziva, F., and Stevens, M. P. 2010. Role of two-component sensory systems of *Salmonella enterica* serovar Dublin in the pathogenesis of systemic salmonellosis in cattle. Microbiol. **156:** 3108-3122.

Pul, U., Wurm, R. and Wagner, R. 2007. The role of LRP and H-NS in transcription regulation: involvement of synergism, allostery and macromolecular crowding. J. Mol. Biol. **366**: 900–915.

Reece, R. J. and Maxwell, A. 1991. DNA Gyrase: Structure and function. Crit. Rev. Biochem. Mol. Biol. **26**: 335-375.

Reid, B. G., and Flynn, G. C. 1997. Chomophore formation in green fluorescent protein. Biochemistry **36:** 6786-6791.

Rhen, M. and Dorman, C. J. 2005. Hierarchical gene regulators adapt *Salmonella enterica* to its host milieus. Int. J. Med. Microb. **294**: 487–502.

Richardson, S. M. H., Higgins, C. F., and Lilley, D. M. J. 1984. The genetic control of DNA supercoiling in *Salmonella typhimurium*. EMBO J **3:** 1745-1752.

Rubin, R. H., and Weinstein, L. 1977. Salmonellosis: microbiologic, pathogenic, and clinical features. Stratton Intercontinental Medical Book Corp., New York.

Rutherford, S. T., Villers, C. L., Lee, J. H., Ross, W., and Gourse, R. L. 2009. Allosteric control of *Escherichia coli* rRNA promoter complexes by DksA. Genes & Develop. **23:** 236-248.

Santero, E. *et al.* 1992. Role of integration host factor in stimulating transcription from the σ54-dependent *nifH* promoter. J. Mol. Biol. **227:** 602–620.

Sawitzke, J. A. and Austin, S. 2000. Suppression of chromosome segregation defects of *Escherichia coli muk* mutants by mutations in topoisomerase I. Proc. Natl Acad. Sci. USA **97:** 1671–1676 .

Schiemann, D. A., and Shope, S. R. 1991. Anaerobic growth of *Salmonella typhimurium* results in increased uptake by Henle 407 epithelial and mouse peritoneal cells in vitro and repression of a major outer membrane protein. Infect. Immun. **59:**437–440.

Schneider, R., Travers, A., and Muskhelishvili, G. 1997. FIS modulates growth phase-dependent topological transitions of DNA in Escherichia coli. Mol. Microbiol., **26**:519–530.

Schneider, R., Kutateladze, T., Travers, A.A., and Muskhelishvili, G. 1999. A DNA architectural protein couples cellular physiology and DNA supercoiling in *Escherichia coli*. Mol. Microbiol. **34**: 953-964.

Schneider, R., Travers, A. A., and Muskhelishvili, G. 2000. The expression of the *Escherichia coli* Fis gene is strongly dependent on the superhelical density of DNA. Mol. Microbiol. **38**: 167-175.

Schneider, R., Lurz, R., Lüder, G., Tolksdorf, C., Travers, A., and Muskhelishvili, G. 2001. An architectural role of the Escherichia coli chromatin protein FIS in organising DNA. Nucl. Ac. Res. **29**: 5107–5114.

Schneider, D.A., Ross, W., and Gourse, R. L. 2003. Control of rRNA expression in *Escherichia coli*. Curr. Opin. Microbiol. **6:** 151-156.

Serna, A., Espinosa, E., Camacho, E. M., and Casadesús, J. 2010. Regulation of Bacterial Conjugation in Microaerobiosis by Host-Encoded Functions ArcAB and SdhABCD. Genetics **184**: 947-958.

Sevcik, M., Sebkova´, A., Volf, J., and Rychlı´k, I. 2001. Transcription of arcA and rpoS during growth of *Salmonella typhimurium* under aerobic and microaerobic conditions. Microbiology **147:** 701–708.

Sharma, A. K., and Payne, S. M. 2006. Induction of expression of *hfq* by DksA is essential for *Shigella flexneri* virulence. Mol. Microbiol. **62:** 469-479.

Shea, J. E., Hensel, M., Gleeson, C., and Holden, D. W. 1996. Identification of a virulence locus encoding a second type III secretion system in *Salmonella typhimurium*. Proc. Natl. Acad. Sci. USA **93:** 2593–2597.

Sheikh, J., Hicks, S., Dall'Agnol, M., Phillips, A. D., and Nataro, J. P. 2001. Roles for Fis and YafK in biofilm formation by enteroaggregative *Escherichia coli*. Mol. Microbiol. **41:** 983–997.

Silphaduang, U., Mascarenhas, M., Karmali, M., and Coombes, B. K. 2007. Repression of intracellular virulence factors in *Salmonella* by the Hha and YdgT nucleoid-associated proteins. J. Bacteriol. **189:** 3669–3673.

Silva, C., and Wiesner, M. 2009. An introduction to systematics, natural history and population genetics of *Salmonella.* Molecular Biology and Molecular Epidemiology of *Salmonella* Infections. Kerala, India: Research Signpost. Chapter 1: 1-17.

Sittka, A., Pfeiffer, V., Tedin, K., and Vogel, J. 2007. The RNA chaperone Hfq is essential for the virulence of *Salmonella typhimurium*. Mol. Microbiol. **63:** 193-217.

Skoko, D. *et al.* 2006. Mechanism of chromosome compaction and looping by the *Escherichia coli* nucleoid protein Fis. J. Mol. Biol. **364:** 777–798.

Spiro, S., and Guest, J. R. 1990. FNR and its role in oxygen-regulated gene expression in *Escherichia coli.* FEMS Microbiol. Rev. **6:** 399-428.

Stapleton, M. R., Norte, V. A., Read, R. C., and Green, J. 2002. Interaction of the *Salmonella typhimurium* transcription and virulence factor SlyA with target DNA and identification of members of the SlyA regulon. J. Biol. Chem. **277**: 17630-17637.

Swinger, K. K. and Rice, P. A. 2004. IHF and HU: flexible architects of bent DNA. Curr. Opin. Struct. Biol. **14:** 28–35.

Talukder, A. A., Iwata, A., Nishimura, A., Ueda, S., and Ishihama, A. 1999. Growth phase-dependent variation in protein composition of the *Escherichia coli* nucleoid. J. Bacteriol. **181:** 6361–6370.

Tartera, C., and Metcalf, E. S. 1993. Osmolarity and growth phase overlap in regulation of *Salmonella typhi* adherence to and invasion of human intestinal cells. Infect. Immun. **61:** 3084–3089.

Tedin, K., and Norel, F. 2001. Comparison of ΔrelA strains of *Escherichia coli* and *Salmonella enterica serovar* Typhimurium suggests a role for ppGpp in attenuation regulation of branched-chain aminoacid biosynthesis. J. Bacter. **183:** 6184-6196.

Tehanchi, A. K., Blankschien, M. D., Zhang, Y., Halliday, J. A., Srivatsan, A., Peng, J., Herman, C., and Wang, J.D. 2010. The transcription factor DksA prevents conflicts between DNA replication and transcription machinery. Cell **141:** 595-605.

Teixido, L., Carrasco, B., Alonso J. C., Barbe, J., and Campoy, S. 2011. Fur Activates the Expression of *Salmonella enterica* Pathogenicity Island 1 by Directly Interacting with the *hilD* Operator *in Vivo* and *in Vitro*. PLoS ONE 6(5): e19711. doi:10.1371/journal.pone.0019711

Thompson, J. F., and Landy, A. 1988. Empirical estimation of protein-induced DNA bending angles: applications to lambda site-specific recombination complexes. Nucleic Acids Res 16: 9687–9705.

Thompson, A., Rolfe, M. D., Lucchini, S., Schwerk, P., Hinton, J. C. D., and Tedin, K. 2006. The bacterial signal molecule, ppGpp, mediates the environmental regulation of both the invasion and intracellular virulence gene programs of *Salmonella*. J. Biol. Chem. **281:** 30112-30121.

Thomson, N. R., Vernikos, G. S., and Parkhill, J. 2009. Fluidity in the *Salmonella* chromosome. Molecular Biology and Molecular Epidemiology of *Salmonella* Infections. Kerala, India: Research Signpost. Chapter 2: 19-46.

Tomljenovic-Berube, A. M., Mulder, D. T., Whiteside, M. D., Brinkman, F. S., and Coombes, B. K. 2010. Identification of the regulatory logic controlling *Salmonella* pathoadaptation by the SsrA-SsrB two-component system. PLoS Genet. **6**: e1000875.

Travers, A., Schneider, R., and Muskhelishvili, G. 2001. DNA supercoiling and transcription in *Escherichia coli*: the FIS connection. Biochimie **83:**213-217.

Travers, A., and Muskhelishvili, G. 2005. DNA supercoiling — a global transcriptional regulator for enterobacterial growth? Nat. Reviews Microbiol. **3**: 157-169.

Varshavsky, A. J., Nedospasov, S. A., Bakayev, V. V., Bakayeva, T. G. and Georgiev, G. 1977. Histone-like proteins in the purified Escherichia coli deoxyribonucleoprotein. Nucleic Acids. Res. **4:** 2725-2745.

Vassilieva, I. M., and Garber, M. B. 2002. The regulatory role of the Hfq protein in bacterial cells. Mol. Microbiol. **36:** 785-791.

Vazquez-Torres, A., Xu, Y., Jones-Carson, J., Holden, D. W., Lucia, S. M., Dinauer, M. C., Mastroeni, P., and Fang, F. C. 2000. *Salmonella* pathogenicity island 2-dependent evasion of the phagocyte NADPH oxidase. Science **287**: 1655-1658.

Walker, K. A., Atkins, C. L., and Osuna, R. 1999. Functional determinants of the *Escherichia coli fis* promoter: Roles of −35, −10, and transcription initiation regions in the response to stringent control and growth phase-dependent regulation. J. Bacteriol. **181**: 1269–1280.

Walker, K. A., Mallik, S. P., Pratt, T. S., and Osuna, R. 2004. The *Escherichia coli fis* promoter is regulated by changes in the levels of its transcription initiation nucleotide CTP. J. Biol. Chem. **279:** 50818–50828.

Wang, Q., and Calvo, J. M. 1993. Lrp, a major regulatory protein in *Escherichia coli*, bends DNA and can organize the assembly of a higher order nucleoprotein structure. EMBO J. **12:** 2495–2501.

Ward, W. W., Cody, C. W., Hart, R. C., & Cormier, M. J. 1980. SPECTROPHOTOMETRIC IDENTITY OF THE ENERGY TRANSFER CHROMOPHORES IN RENILLA AND

AEQUOREA GREEN - FLUORESCENT PROTEINS. Photochem. Photobiol. **31(6):** 611-615.

Waterman, S. R., and Holden, D. W. 2003. Functions and effectors of the *Salmonella* pathogenicity island 2 type III secretion system. Cell Microbiol. **5:** 501–511.

Webb, C., Moreno, M., Wilmes-Riesenberg, M., Curtiss III, R., and Foster, J. 1999. Effects of DksA and ClpP protease on sigma S production and virulence in *Salmonella typhimurium.* Mol. Microbiol. **34:** 112-123.

Weitao, T., Nordstrom, K. and Dasgupta, S. 2000. *Escherichia coli* cell cycle control genes affect chromosome superhelicity. EMBO Rep. **1**: 494–499 .

Weinstein-Fischer, D., Elgrably-Weiss, M., and Altuvia, S. 2000 *Escherichia coli* response to hydrogen peroxide: a role for DNA supercoiling, Topoisomerase I and Fis. **35(6):** 1413-1420.

Weinstein-Fischer, D., and Altuvia, S. 2007. Differential regulation of *Escherichia coli* topoisomerase I by Fis. Mol. Microbiol. 63: 1131-1144.

Wendrich, T. M., Marahiel, M. A. 1997. Cloning and characterization of a *relA/spoT* homologue from Bacillus subtilis. Mol Microbiol. **6(1):** 65-79.

Wilson, R. L., Libby, S. J., Freet, A. M., Boddicker, J. D., Falhen, T. F., and Jones, B. D. 2001. Fis, a DNA nucleoid-associated protein, is involved in *Salmonella typhimurium* SPI-1 invasion gene expression. Mol. Microbiol. **39:** 79-88.

Wilson, J. W., Ott, C. M., and Bentrup, K. H. Z. 2007. Space flight alters bacterial gene expression and virulence and reveals a role for global regulator Hfq. Proc. Natl. Acad. Sci. U.S.A. **104:** 16299-16304.

Wolf, S. G., Frenkiel, D., Arad, T., Finkel, S.E., Kolter, R., and Minsky, A. 1999. DNA protection by stress-induced biocrystallization. Nature **400:** 83–85.

Yoon, H., S. Lim, S. Heu, S. Choi, and Ryu, S. 2003. Proteome analysis of *Salmonella enterica* serovar Typhimurium *fis* mutant. FEMS Microbiol. Lett. **226:** 391-396.

Yoon, H., McDermott, J. E., Porwollik, S., McClelland, M., and Heffron, F. 2009. Coordinated regulation of virulence during systemic infection of *Salmonella enterica* serovar Typhimurium. PLoS Pathog. **5:** e1000306.

Yu, X., McGourty, K., Liu, M., Unsworth, K. E., and Holden, D. W. 2010. pH Sensing by Intracellular Salmonella Induces Effector Translocation. Science **328:** 1040-3.

Zhang, C., and Xing, X. H. 2008. Quantification of a specific bacterial strain in an anaerobic mixed culture for biohydrogen production by the aerobic fluorescence recovery (AFR) technique. Biochem. Eng. J. **39:** 581-585.

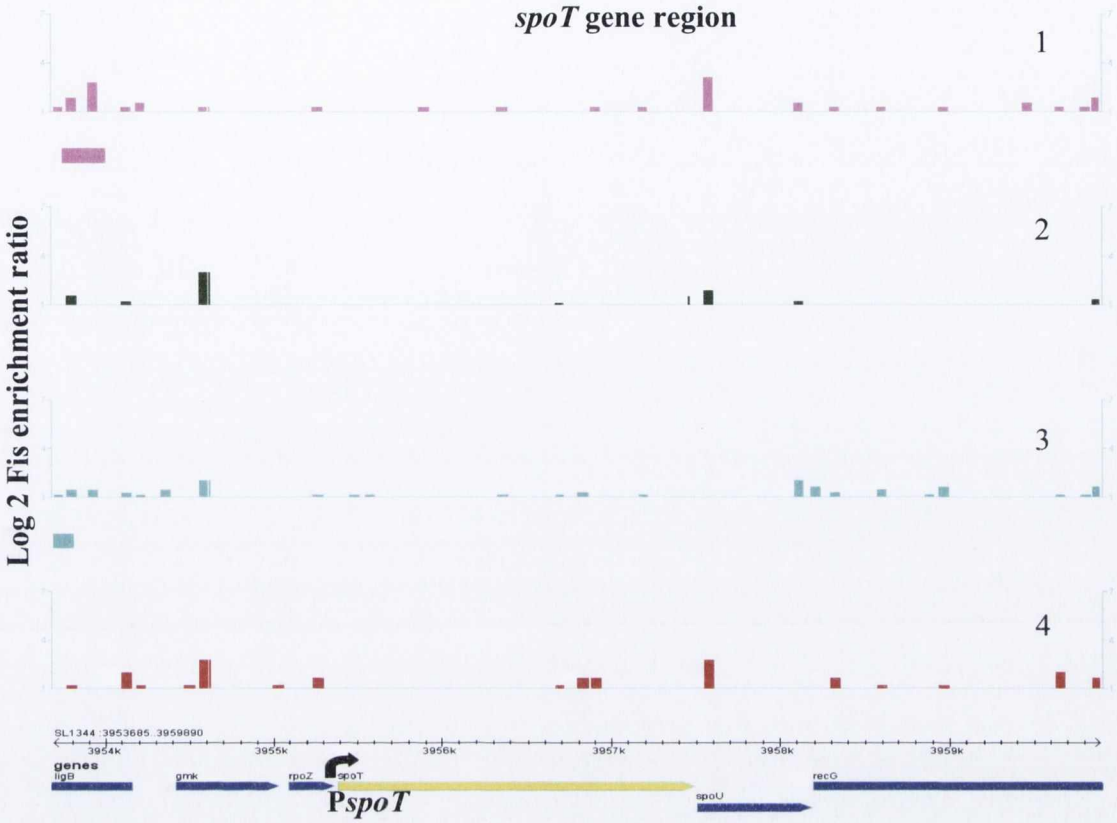
Zhang, J., Zeuner, Y., Kleefeld, A., Unden, G., and Janshoff, A. 2004. Multiple site-specific binding of Fis protein to *Escherichia coli nuoA-N* promoter DNA and its impact on DNA topology visualised by means of scanning force microscopy. Chembiochem. **5:** 1286–1289

Zhou, D., L. M., Chen, L. Hernandez, S. B. Shears, and Galán, J. E. 2001. A *Salmonella* inositol polyphosphatase acts in conjunction with other bacterial effectors to promote host cell actin cytoskeleton rearrangements and bacterial internalization. Mol. Microbiol. **39:** 248–259.

Appendix

Fig. A.1: Fis binding at the gene region of *spoT* in different aeration regimes. Association of the Fis protein with the ORF region of *spoT*. See legend to Fig. 6.8 for details. Both analyses, panels A) and B), indicate similar levels of Fis binding at the *spoT* ORF region and according to the growth phase. In panel A), no Fis enrichment peaks are identified along the *spoT* gene region in any of the aeration regimes 1-4. Consistently, in panel B), Fis enrichment levels are non-existent in aeration regimes 1, 2, and low (below 1) in 3 and 4. All qPCR reactions were performed in duplicate, and two independent biological replicates were prepared for each set of conditions.

A) ChIP-chip



B) qPCR

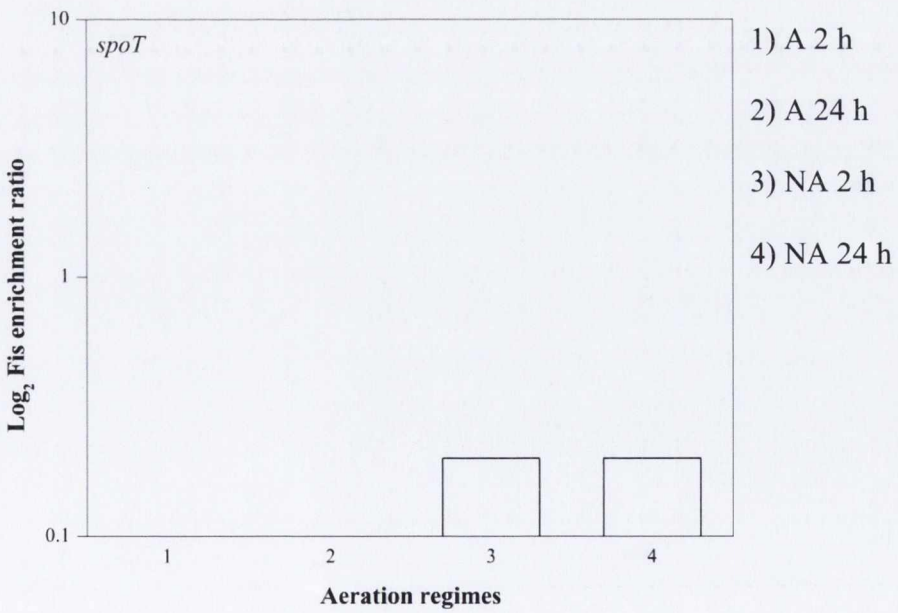
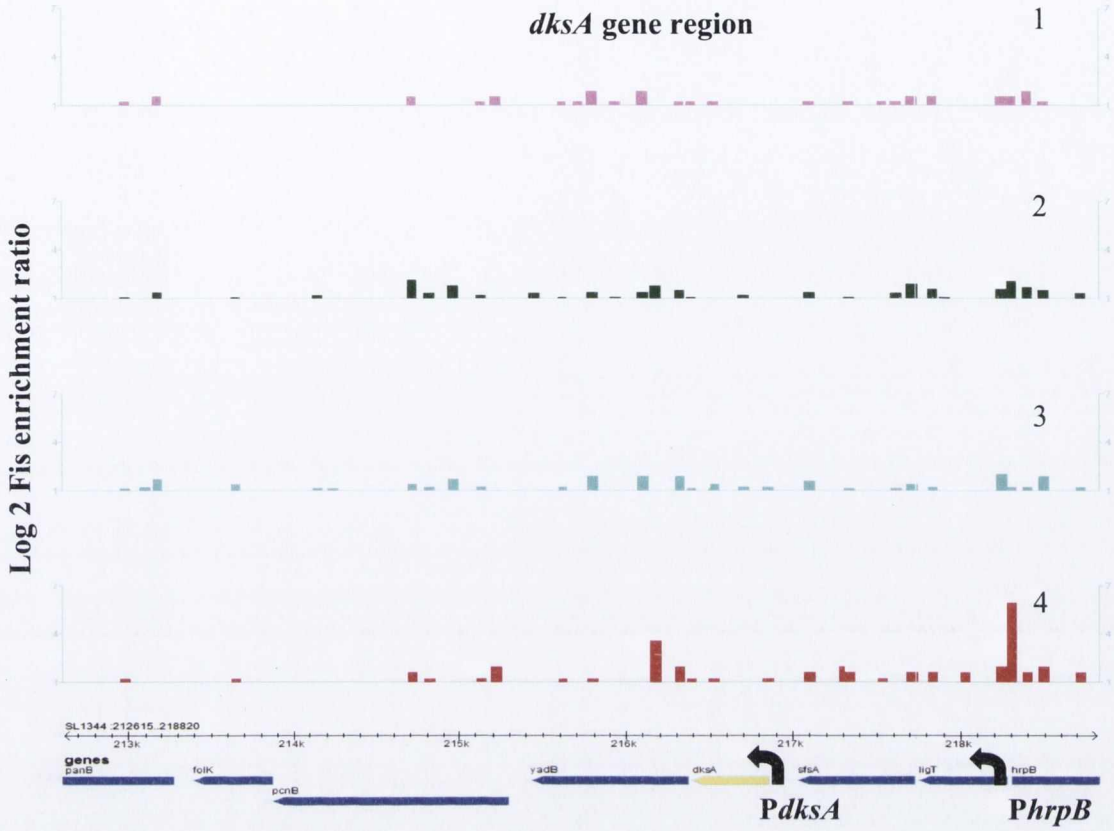


Fig. A.2: Fis binding at the promoter region of *dksA* in different aeration regimes. Association of the Fis protein with the ORF region of *dksA*. See legend in Fig. 6.8 for details. Both analyses, panels A) and B), indicate similar levels of Fis binding at the *dksA* ORF region in every growth phase. In panel A) No Fis enrichment peaks are identified along the *dksA* gene region in either aeration regime 1-4. Consistently, in panel B), Fis enrichment levels are low (below 1) in all four aeration regimes 1-4. All qPCR reactions were done in duplicate, and two independent biological replicates were prepared for each set of conditions.

A) ChIP-chip



B) qPCR

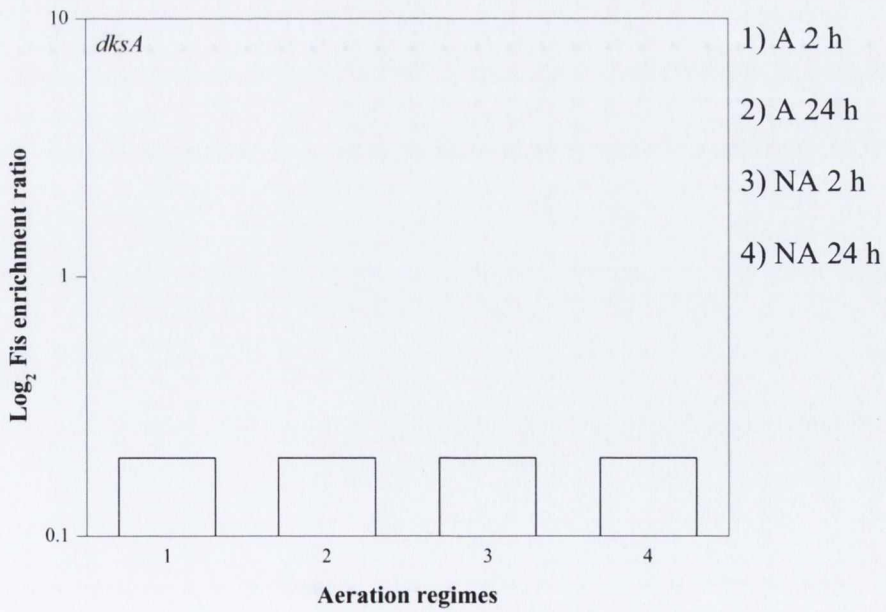
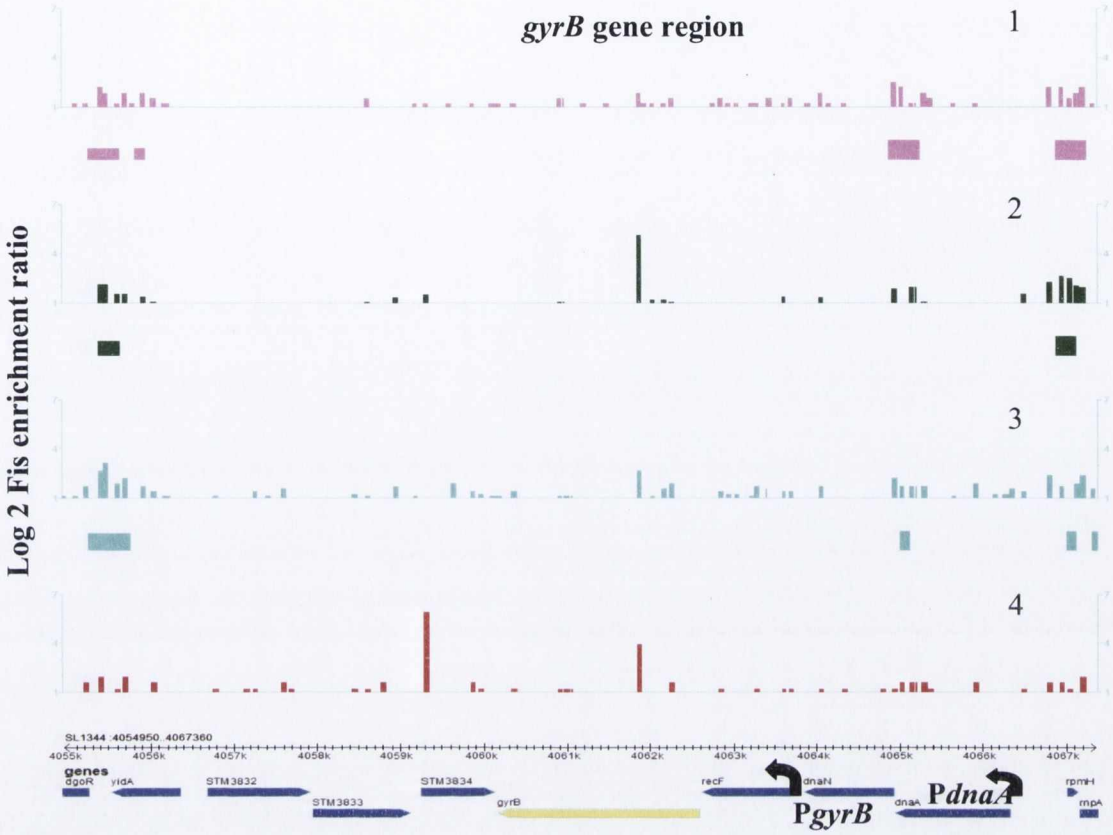


Fig. A.3: Fis binding at the promoter region of *gyrB* in different aeration regimes. Association of the Fis protein with the promoter region of *gyrB*. See legend in Fig. 6.8 for details. Panel A) data differs from panel B) data, as Fis binding reveals differently at the *gyrB* promoter in either growth phase, 1-4. In panel A), no Fis enrichment peaks are identified along the *gyrB* gene region in either aeration regime 1-4. However, in panel B), Fis enrichment levels are higher than the threshold (above 1) in all four aeration regimes 1-4. All qPCR reactions were done in duplicate, and two independent biological replicates were prepared for each set of conditions.

A) ChIP-chip



B) qPCR

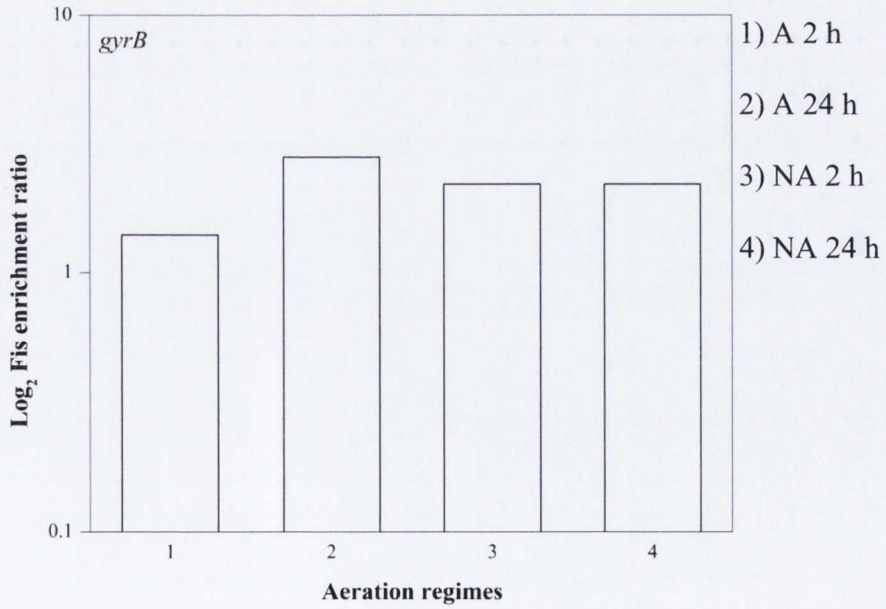
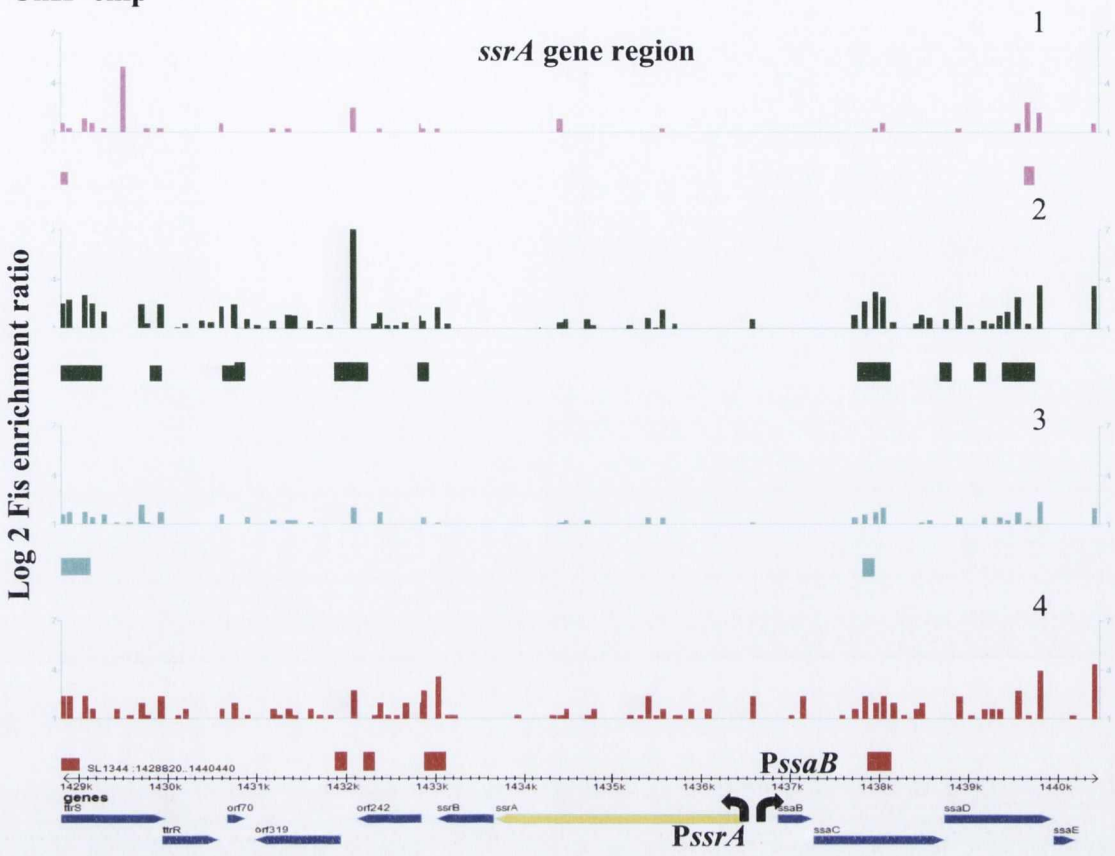
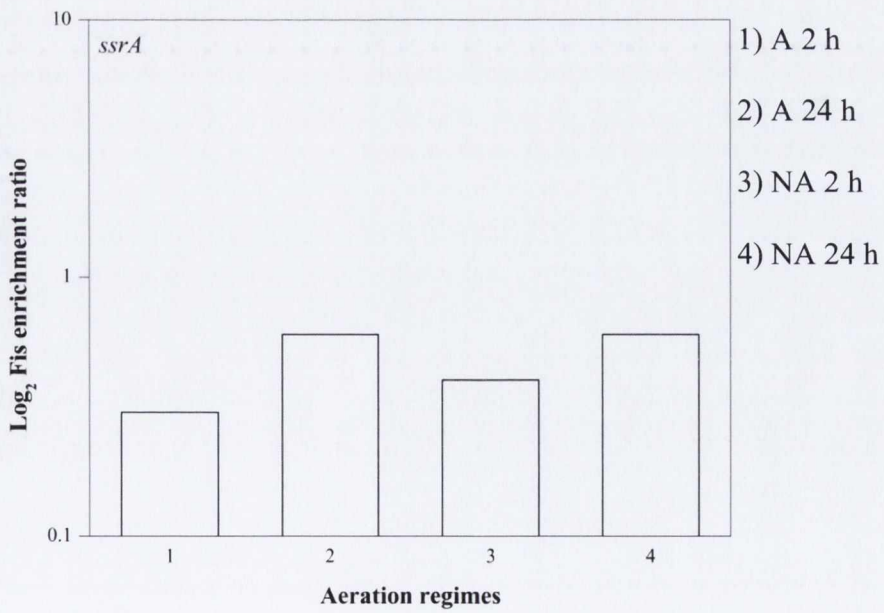


Fig. A.4: Fis binding at the promoter region of *ssrA* in different aeration regimes. Association of the Fis protein with the promoter region of *ssrA*. See legend in Fig. 6.8 for details. Both analyses, panels A) and B), indicate similar low levels of Fis binding at the *ssrA* promoter region in either growth phase. In panel A), no Fis enrichment peaks are identified along the *ssrA* gene region in any aeration regime 1-4. Consistently, in panel B), Fis enrichment levels are low (below 1) in all four aeration regimes 1-4. All qPCR reactions were performed in duplicate, and two independent biological replicates were prepared for each set of conditions.

A) ChIP-chip



B) qPCR



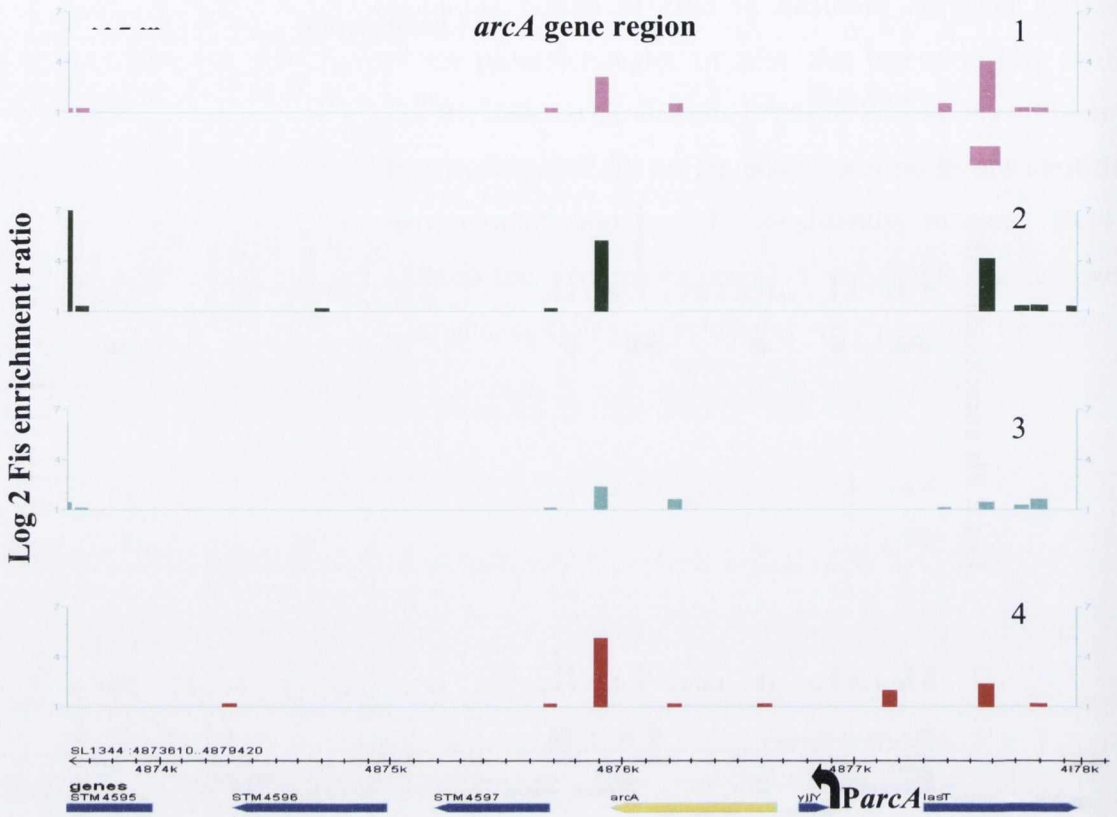


Fig. A.5: Fis binding at the gene region of *arcA* in different aeration regimes. Association of the Fis protein with the ORF region of *arcA*. during 1) aerated, mid-log 2 h (A, 2 h); 2) aerated, late-stationary phase 24 h (A, 24 h); 3) non-aerated, mid-log 2 h (NA, 2 h); and 4) non-aerated, late-stationary phase 24 h (NA, 24 h), growth conditions. The $\log_2$ enrichment ratio on the *Y*-axis was calculated from Cy3 (ChIP DNA) and Cy5 (Input DNA) signal intensity of each probe and plotted against each gene location along the *S.* Typhimurium chromosome on the *X*-axis. The peak height of the identified Fis-binding peaks was calculated in $\log_2$ enrichment ratio from dividing the Cy3 (ChIP DNA) over the Cy5 (Input DNA) signal intensity of the gene probe corresponding to the identified peak, and were indicated with solid rectangles below the peaks. No Fis enrichment peaks are identified along the *arcA* ORF region in either aeration regime, 1-4.

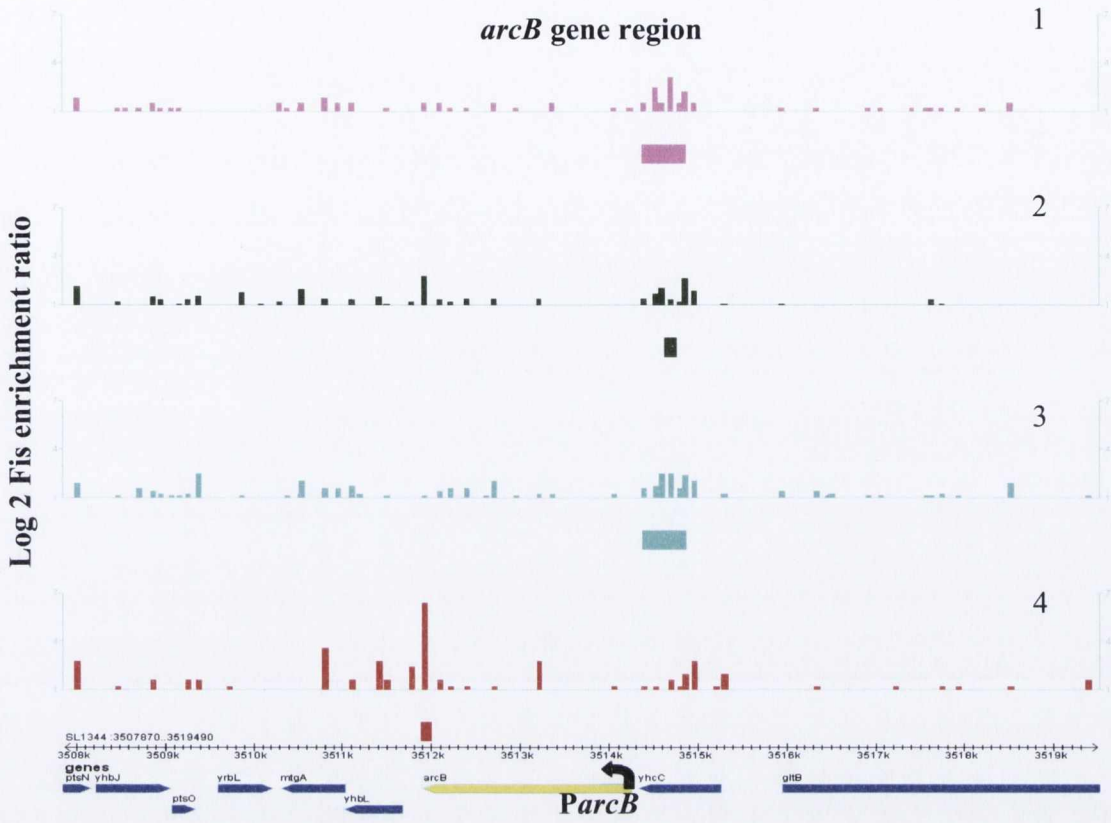
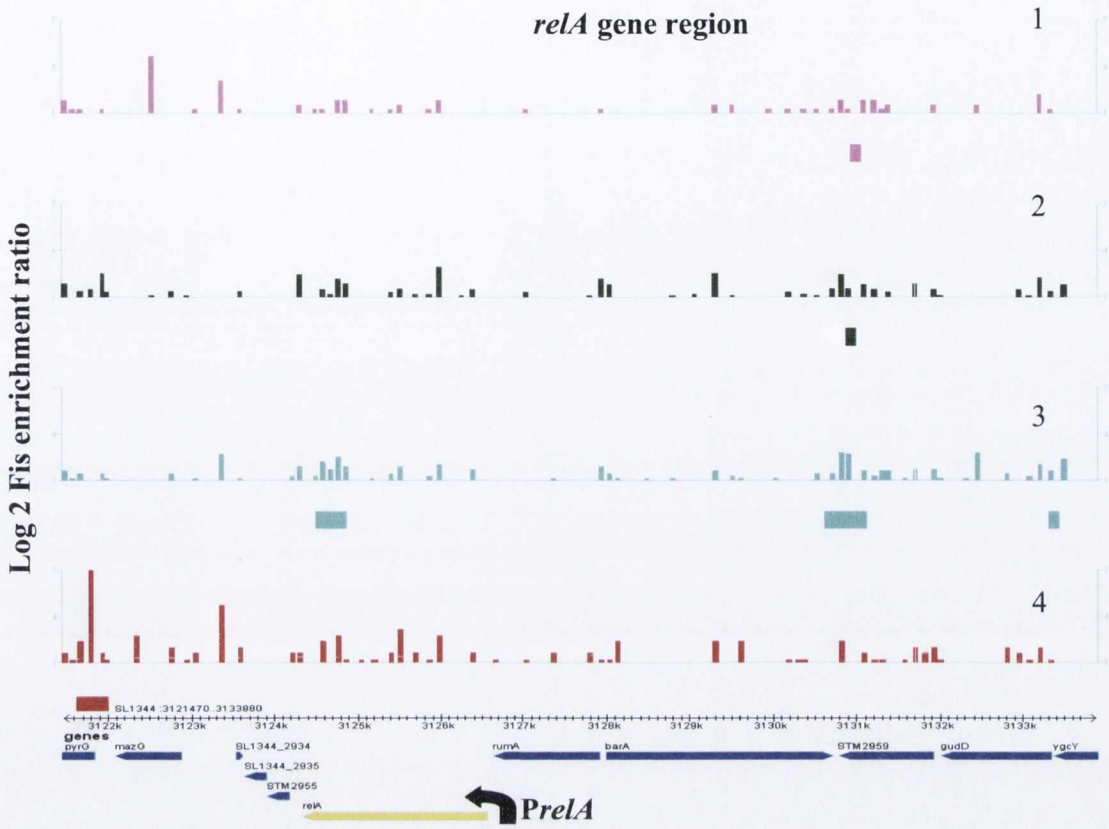


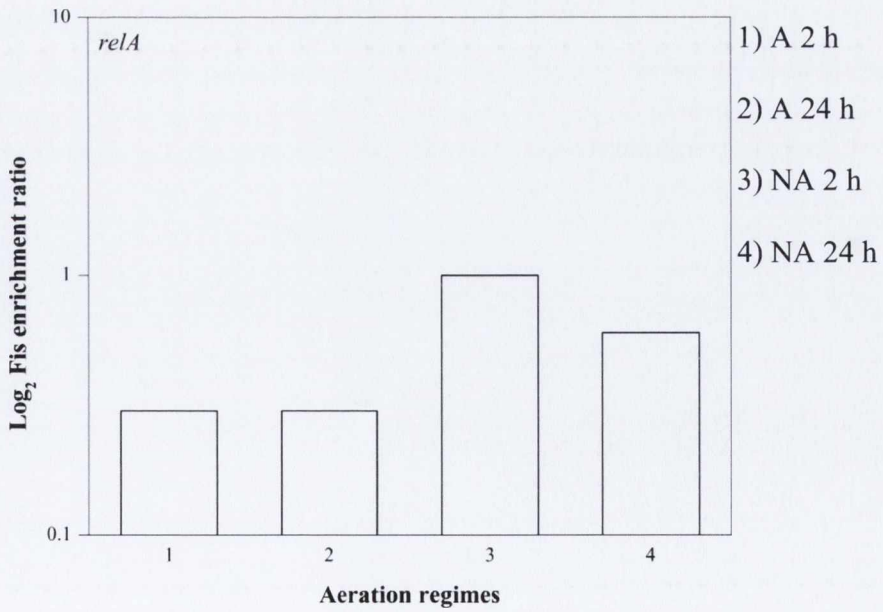
Fig. A.6: Fis binding at the gene region of *arcB* in different aeration regimes. Association of the Fis protein with the ORF region of *arcB*. See legend in Fig. A.5 for details. Fis enrichment peaks are identified along the *arcB* ORF gene region only in the aeration regime number 4.

Fig. A.7: Fis binding at the gene region of *relA* in different aeration regimes. Association of the Fis protein with the ORF region of *relA*. See legend in Fig. 6.8 for details. Both analyses, panels A) and B), indicate similar levels of Fis binding at the *relA* ORF gene region in all four growth phase conditions 1-4. In panel A), Fis enrichment peaks are identified along the *relA* gene region only in aeration regime number 3. Consistently, in panel B), Fis enrichment levels are high (above 1) in the same aeration regime number 3. All qPCR reactions were done in duplicate, and two independent biological replicates were prepared for each set of conditions.

A) ChIP-chip



B) qPCR



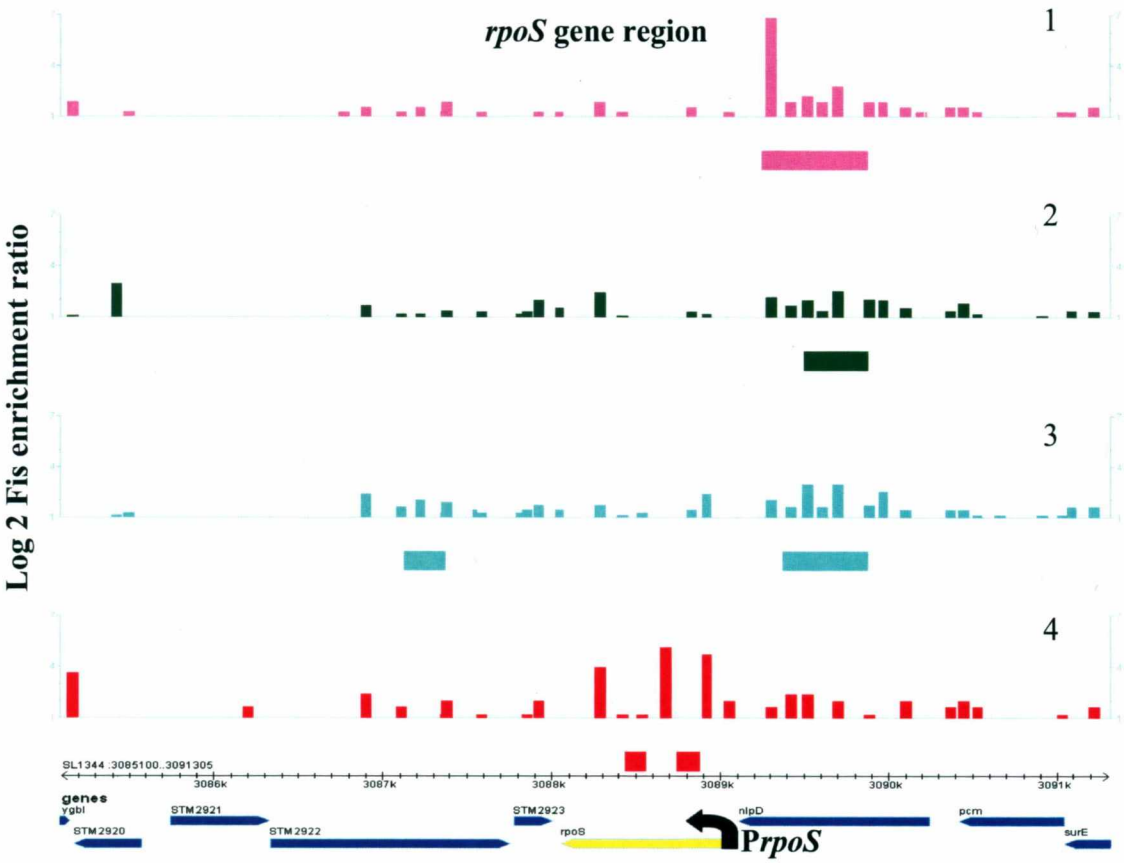


Fig. A.8: Fis binding at the gene region of *rpoS* in different aeration regimes. Association of the Fis protein with the ORF region of *rpoS*. See legend in Fig. A.5 for details. Fis enrichment peaks are identified along the *rpoS* ORF gene region only in aeration regime number 4.

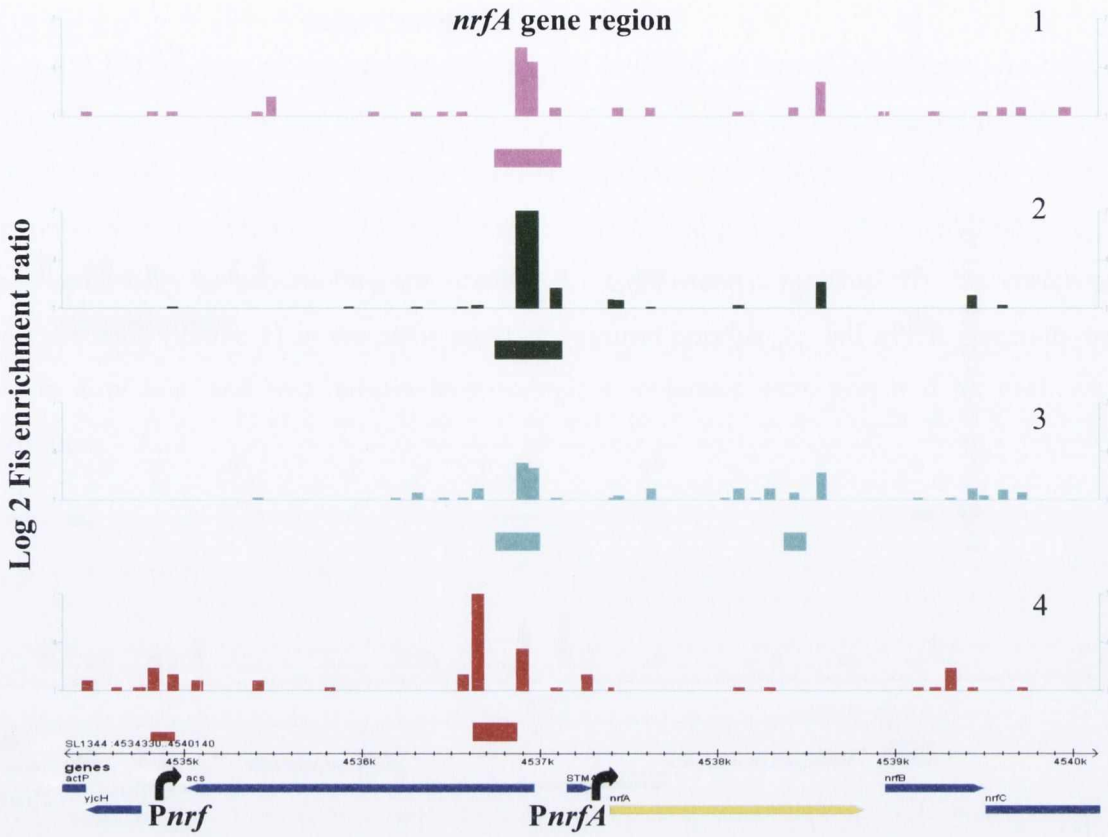


Fig. A.9: Fis binding at the gene region of *nrfA* in different aeration regimes. Association of the Fis protein with gene region of *nrfA*. See legend in Fig. A.5 for details. Fis enrichment peaks are identified along the *nrfA* gene region only in aeration regime number 3.

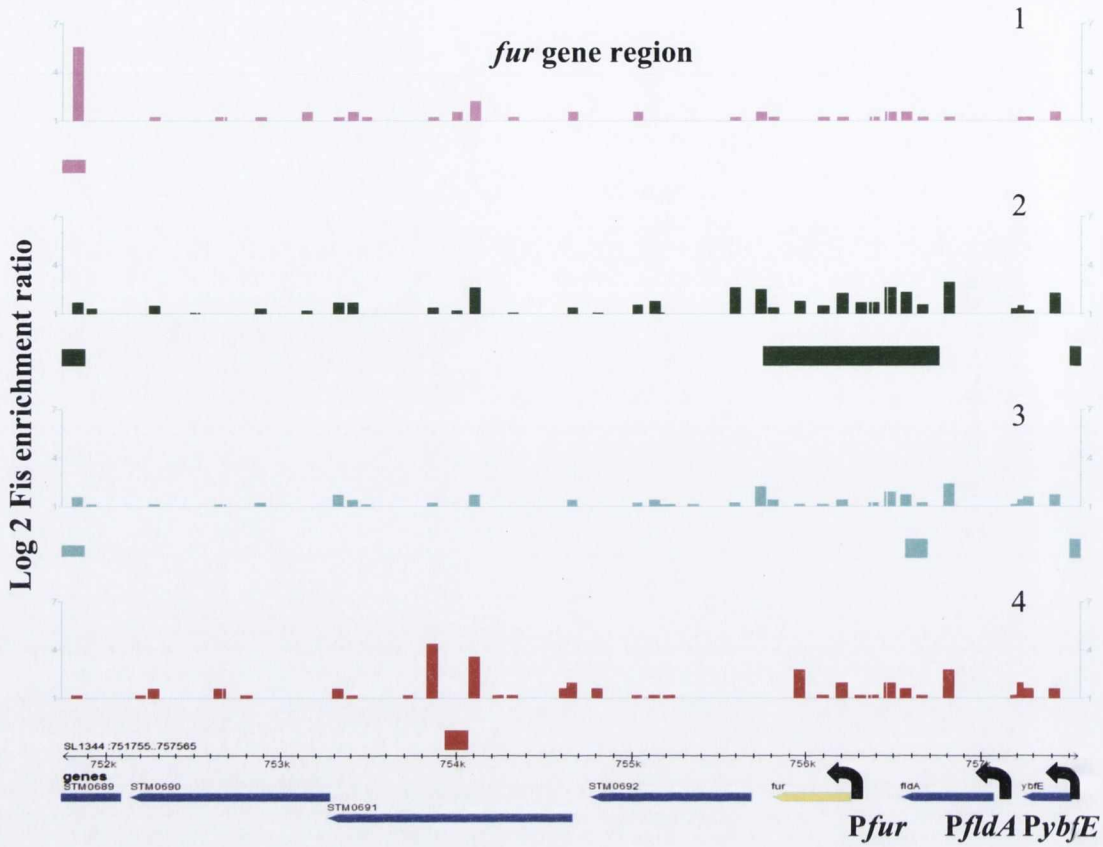
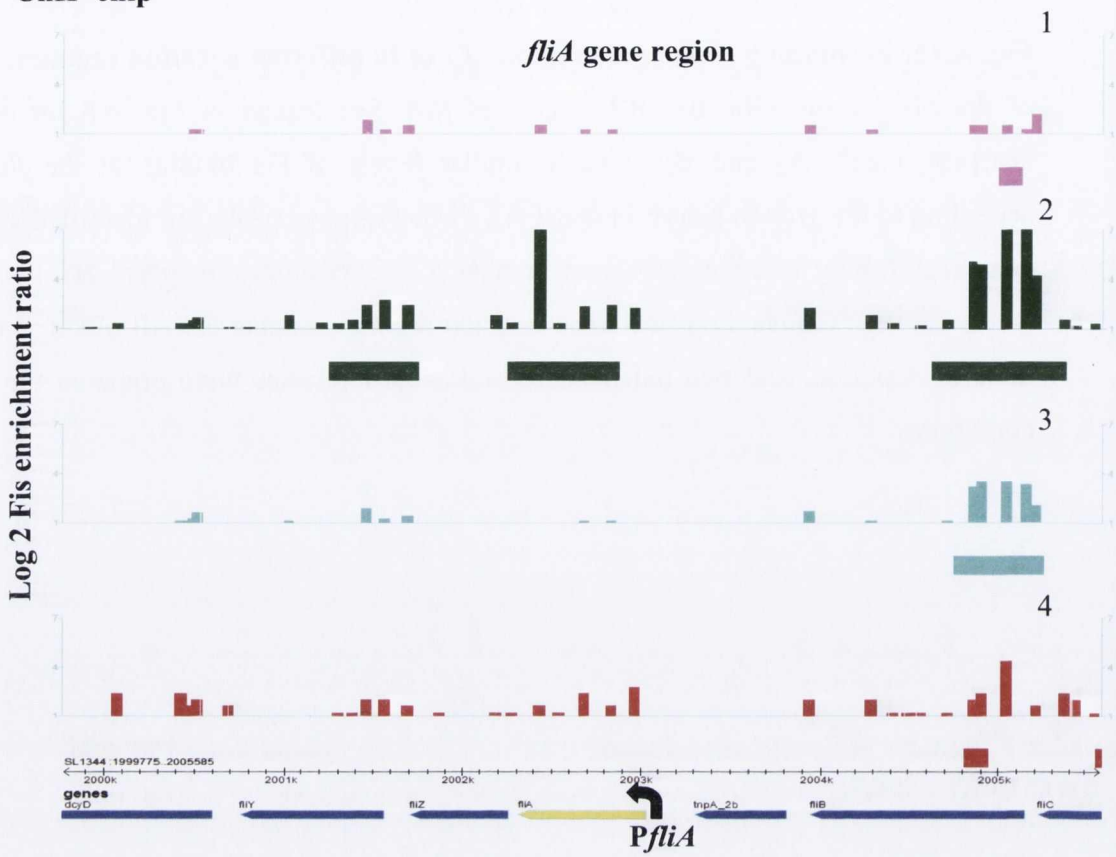


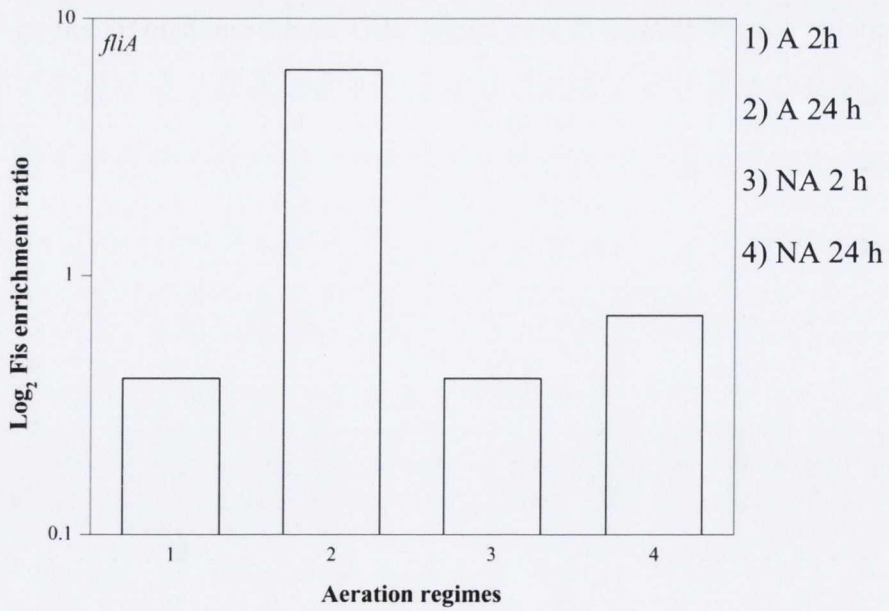
Fig. A.10: Fis binding at the gene region of *fur* in different aeration regimes. Association of the Fis protein with the ORF region of *fur*. See legend in Fig. A.5 for details. Fis enrichment peaks are identified along the *fur* ORF region only in aeration regime number 2.

Fig. A.11: Fis binding at the gene region of *fliA* in different aeration regimes. Association of the Fis protein with the ORF region of *fliA*. See legend in Fig. 6.8 for details. Both analyses, panels A) and B), indicate similar levels of Fis binding at the *fliA* promoter according to the growth phase. In panel A), Fis enrichment peaks are identified along the *fliA* gene region only in aeration regime number 2. Consistently, in panel B), Fis enrichment levels are high (above 1) in the same aeration regimes number 2. All qPCR reactions were done in duplicate, and two independent biological replicates were prepared for each set of conditions.

A) ChIP-chip



B) qPCR



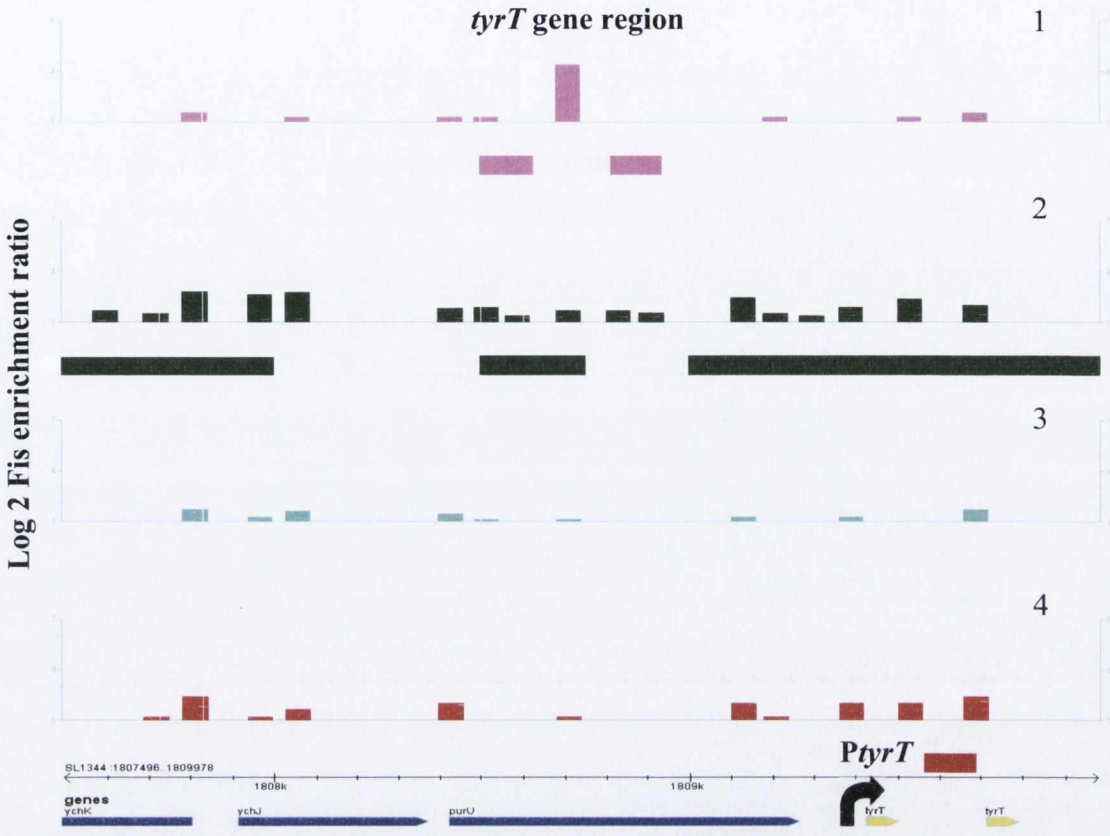
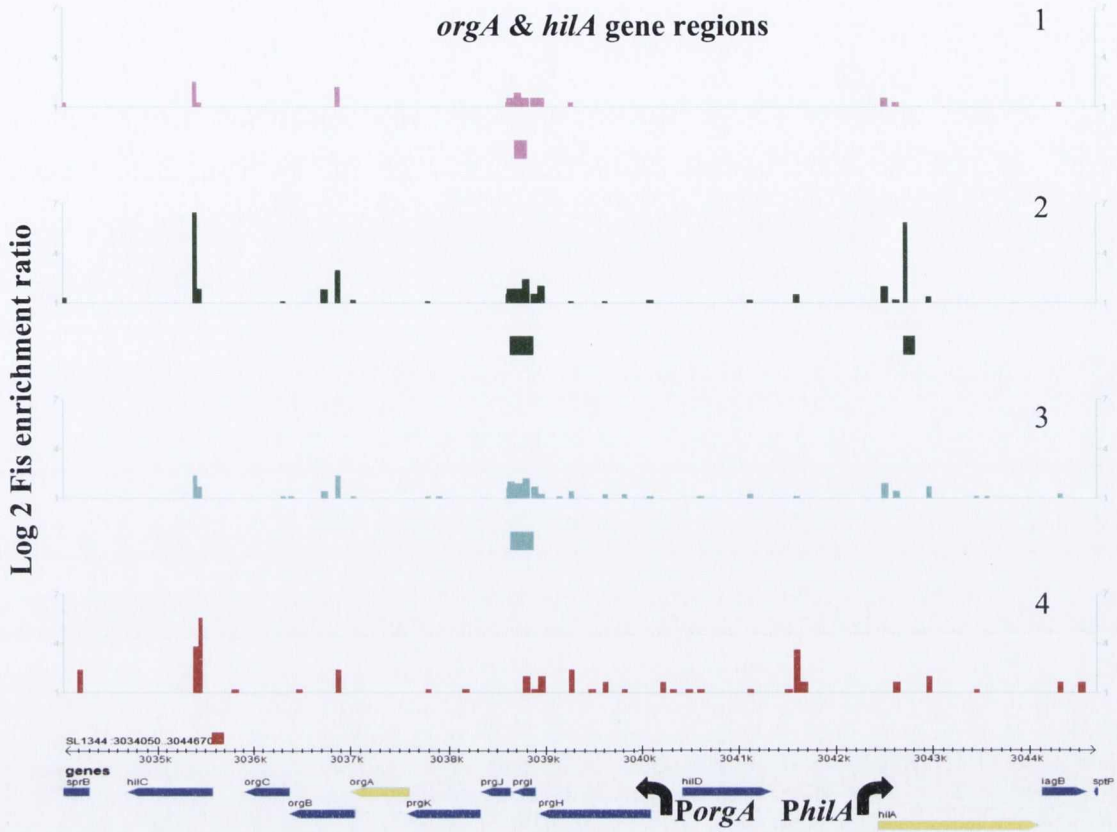


Fig. A.12: Fis binding at the gene region of *tyrT* in different aeration regimes. Association of the Fis protein with the promoter and ORF regions of *tyrT*. See legend in Fig. A.5 for details. Fis enrichment peaks are identified along the promoter and ORF regions of *tyrT* in only aeration regimes 2 and 4.

Fig. A.13 & A.14 A/B: Fis binding at the gene regions of *hilA* and *orgA* in different aeration regimes. Association of the Fis protein with the gene regions of *orgA* and *hilA*. See legend in Fig. 6.8 for details. For the *orgA* gene region (Fig. A.14 A & B), both analyses, panels A) and B), indicate similar levels of Fis binding at the *orgA* gene region in every growth condition, 1-4. In panel A), Fis enrichment peaks are identified along the *orgA* gene region in aeration regimes numbers, 1, 2 and 3, but not in 4. Consistently, in panel B), Fis enrichment levels are high (above 1) for aeration regimes 1, 2 and 3, but not in 4. While for the *hilA* gene region (Fig. A.13 A) Fis binding is revealed in only the growth condition number 2. All qPCR reactions were done in duplicate, and two independent biological replicates were prepared for each set of conditions.

A) ChIP-chip



B) qPCR

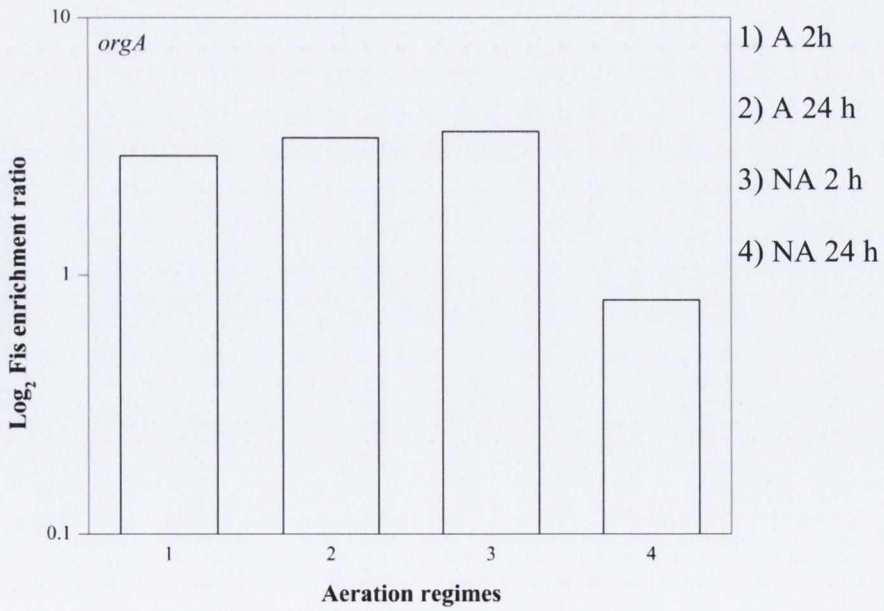
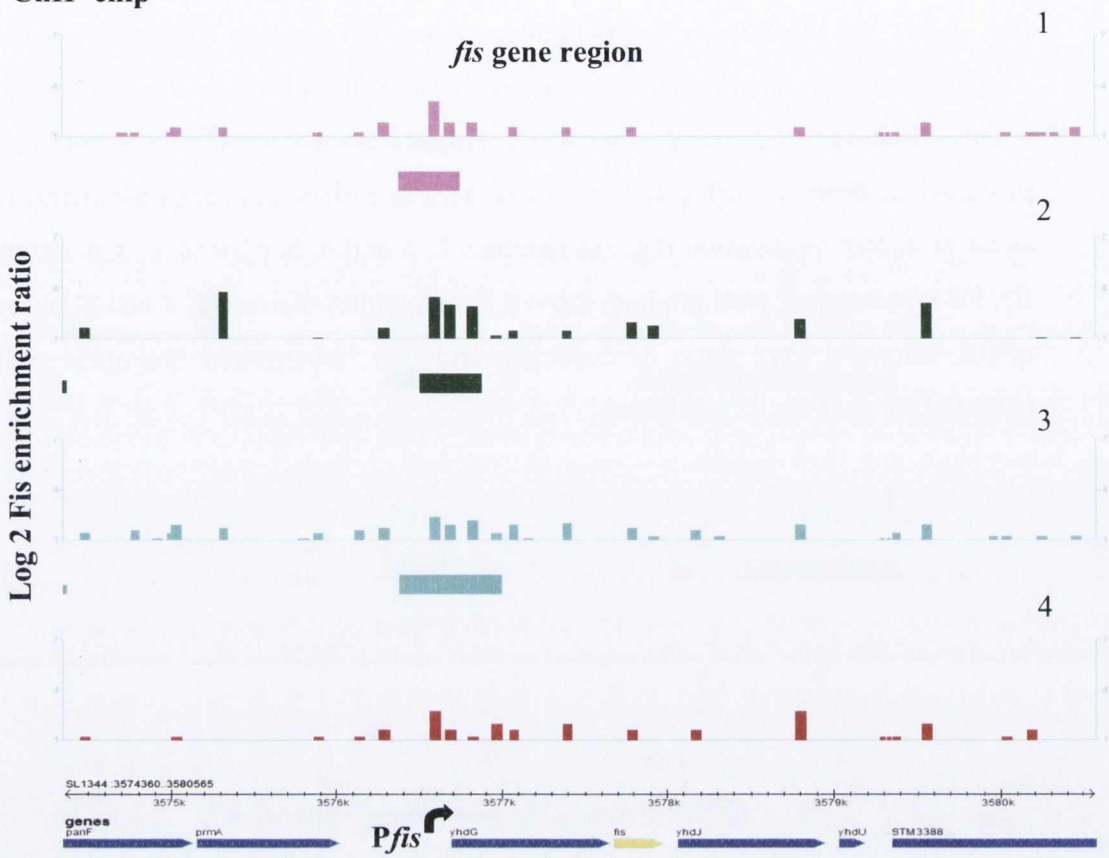


Fig. A.15: Fis binding at the promoter region of *fis* in different aeration regimes. Association of the Fis protein with the promoter region of *fis*. See legend in Fig. 6.8 for details. Both analyses, panels A) and B), indicate similar levels of Fis binding at the *fis* promoter according to the growth phase. In panel A), Fis enrichment peaks are identified along the *fis* promoter in aeration regimes number 1, 2 and 3, but not in 4. Consistently, in panel B), Fis enrichment levels are high for aeration regimes 1, 2 and 3, but not in 4. All qPCR reactions were done in duplicate, and two independent biological replicates were prepared for each set of conditions.

A) ChIP-chip



B) qPCR

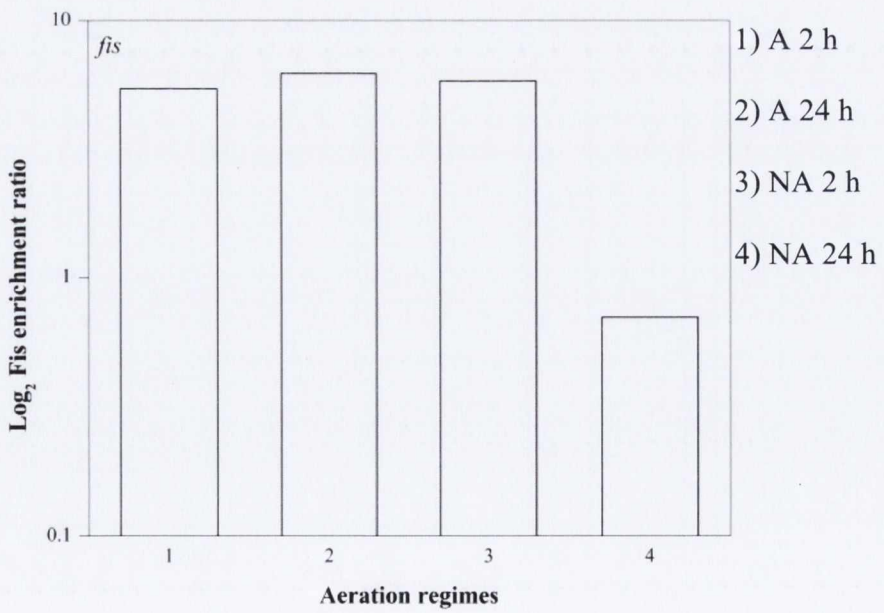
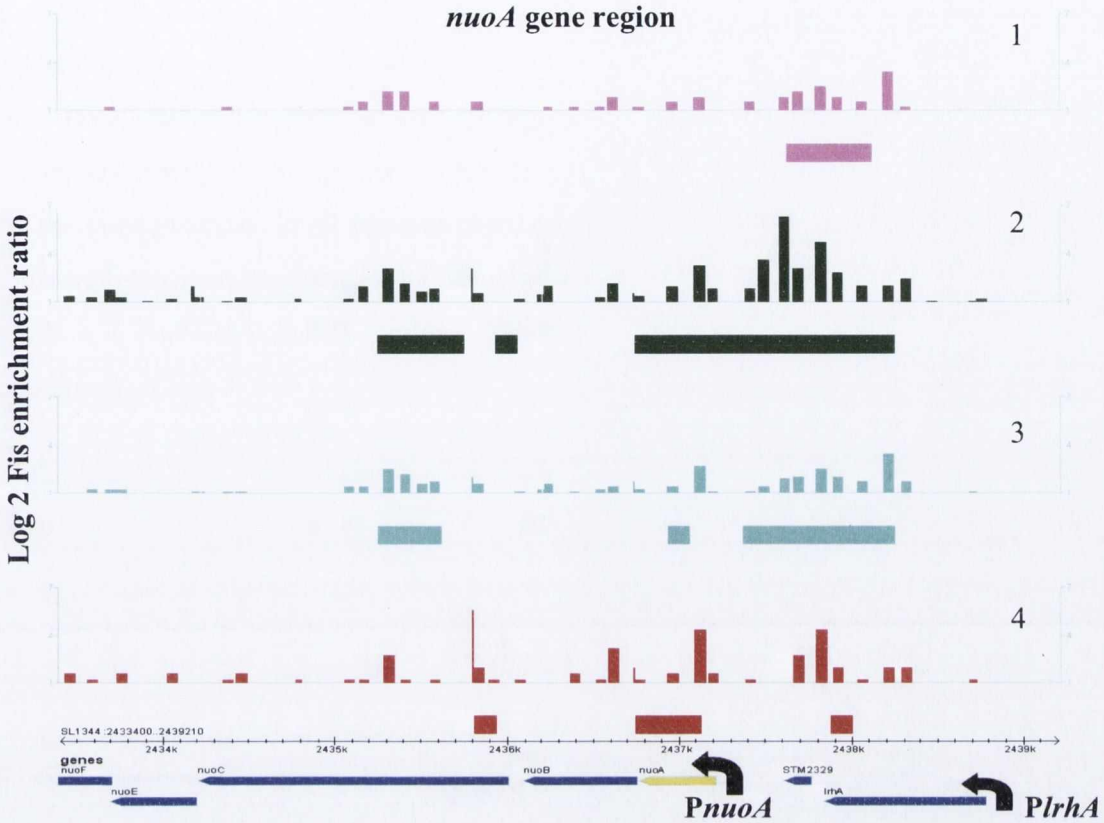
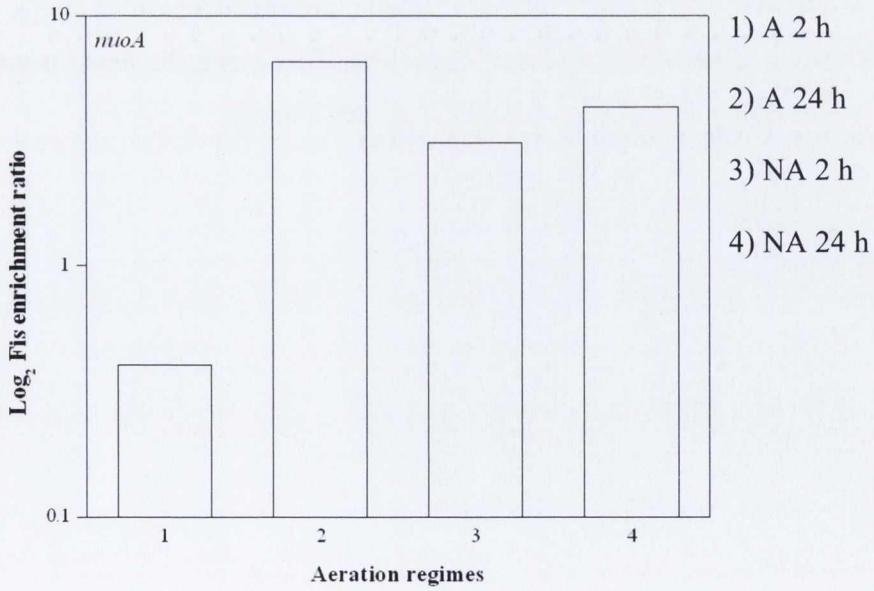


Fig. A.16: Fis binding at the promoter region of *nuoA* in different aeration regimes. Association of the Fis protein with the promoter region of *nuoA*. See legend in Fig. 6.8 for details. Both analyses, panels A) and B), indicate similar levels of Fis binding at the *nuoA* promoter in every growth phase. In panel A), Fis enrichment peaks are identified along the *nuoA* promoter for aeration regimes numbers 2, 3 and 4, but not in 1. Consistently, in panel B), Fis enrichment levels are high (above 1) in aeration regimes 2, 3 and 4, but not in 1. All qPCR reactions were done in duplicate, and two independent biological replicates were prepared for each set of conditions.

A) ChIP-chip



B) qPCR



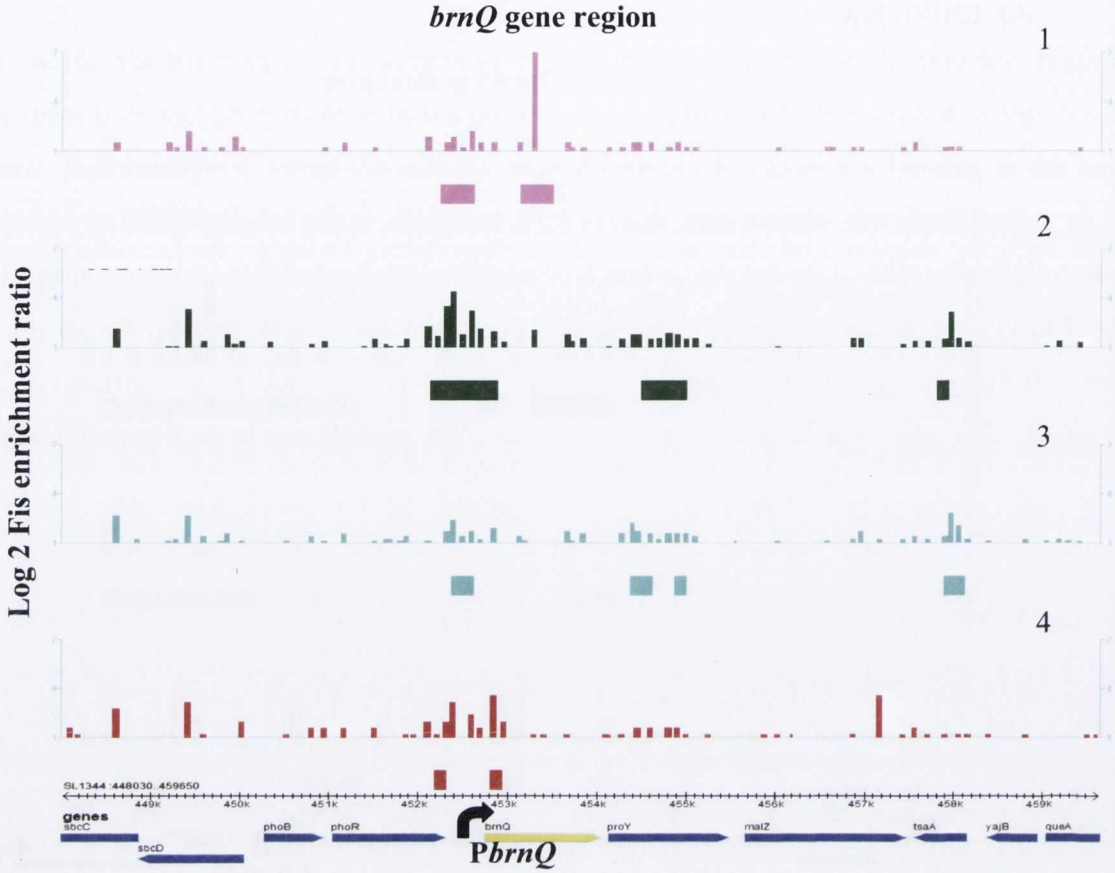
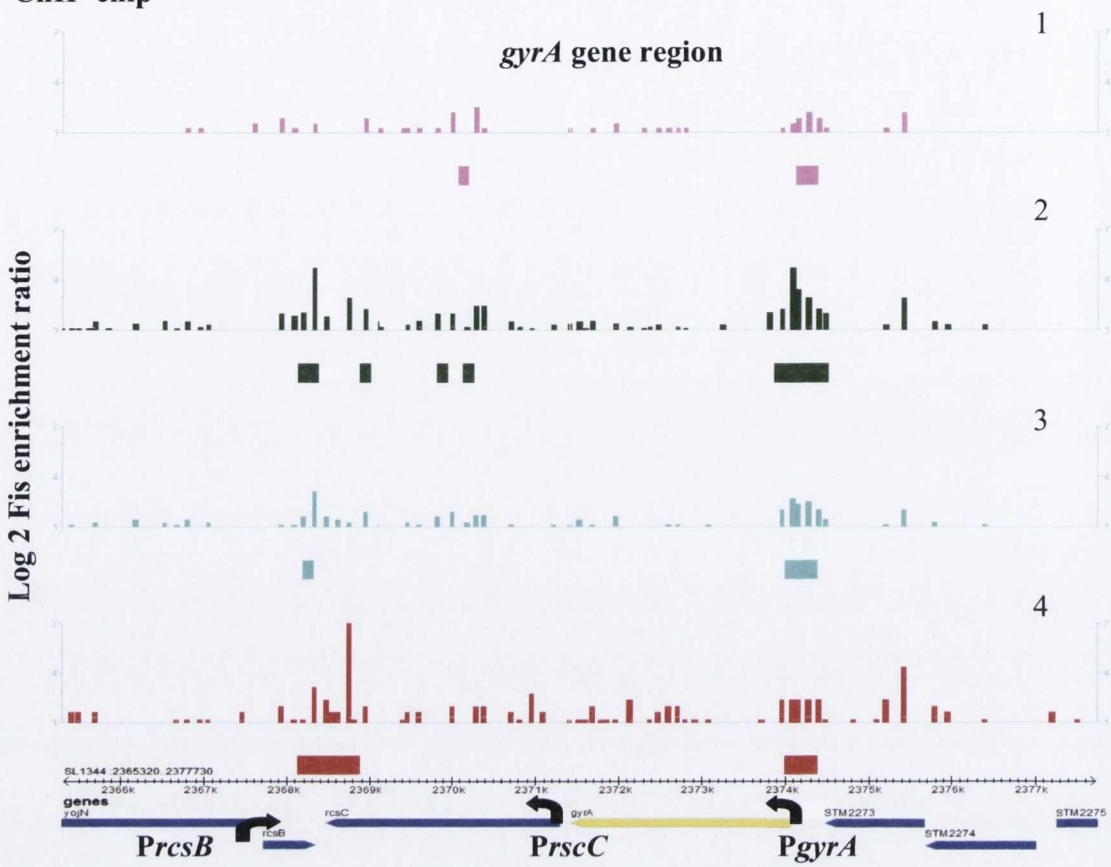


Fig. A.17: Fis binding at the promoter region of *brnQ* in different aeration regimes. Association of the Fis protein with the promoter region of *brnQ*. See legend in Fig. A.5 for details. Fis enrichment peaks are identified along the *brnQ* promoter in all growth aeration regimes, but most remarkably in regimes 2 and 3.

Fig. A.18: Fis binding at the promoter region of *gyrA* in different aeration regimes. Association of the Fis protein with the promoter region of *gyrA*. See legend in Fig. 6.8 for details. Both analyses, panels A) and B), indicate similar levels of Fis binding at the *gyrA* promoter according to the growth phase. In panel A), Fis enrichment peaks are identified along the *gyrA* promoter in all aeration regimes 1-4. Consistently, in panel B), Fis enrichment levels are higher than the threshold (above 1) in all aeration regimes 1-4. All qPCR reactions were done in duplicate, and two independent biological replicates were prepared for each set of conditions.

A) ChIP-chip



B) qPCR

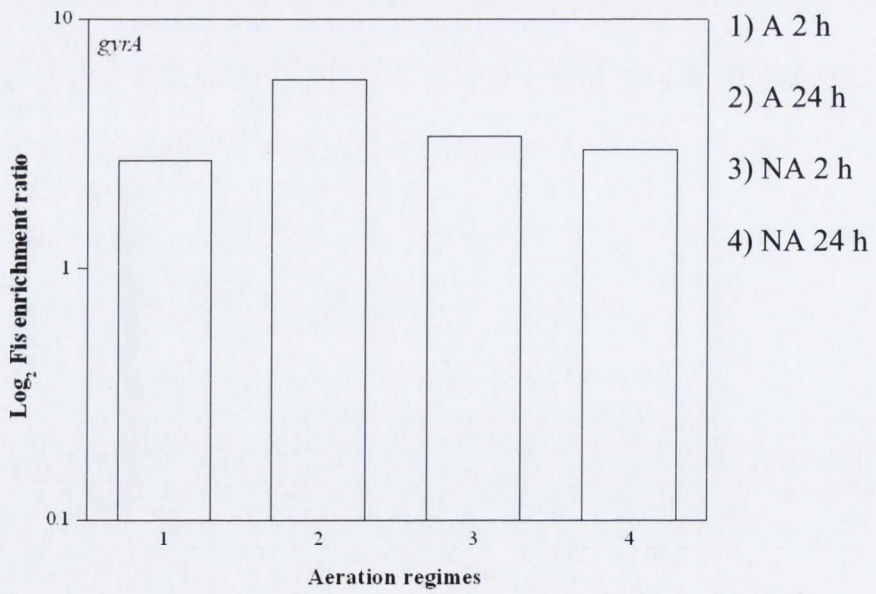
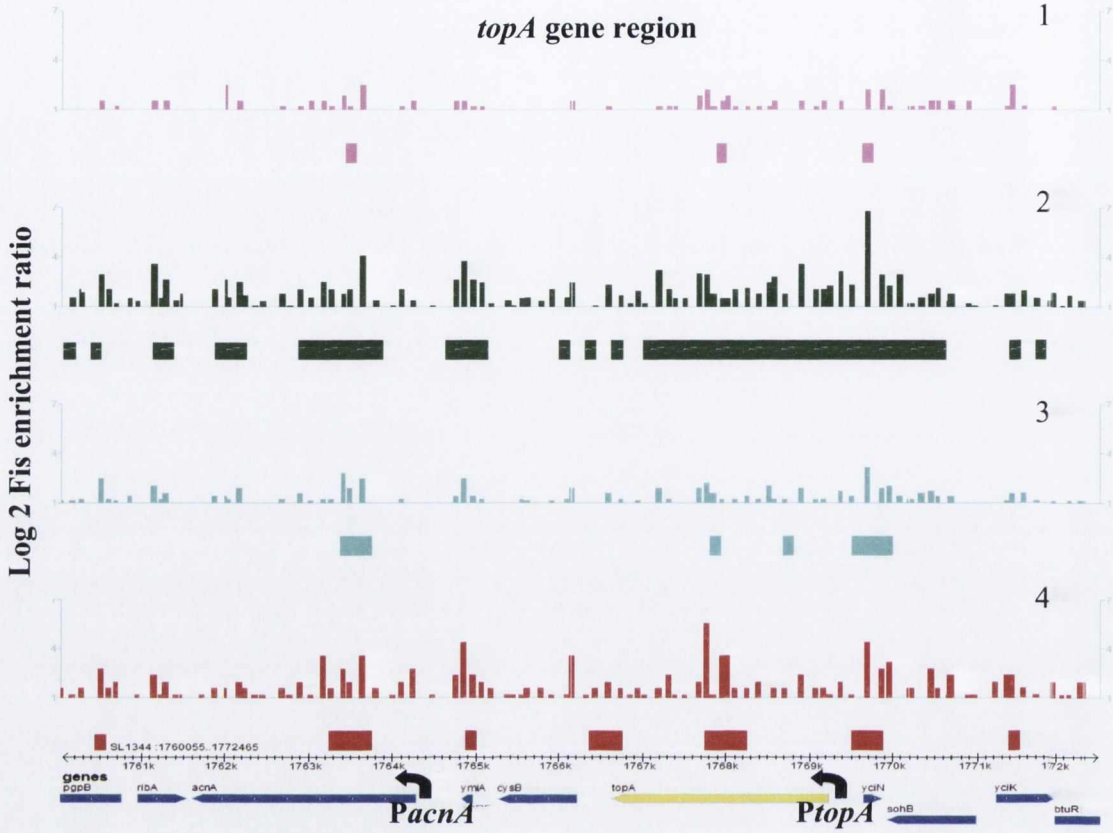


Fig. A.19: Fis binding at the gene region of *topA* in different aeration regimes. Association of the Fis protein with the ORF region of *topA*. See legend in Fig. 6.8 for details. Both analyses, panels A) and B), indicate similar levels of Fis binding at the *topA* ORF region in all four growth conditions. In panel A), Fis enrichment peaks are identified along the *topA* ORF in all aeration regimes 1-4. Consistently, in panel B), Fis enrichment levels are higher than the threshold (above 1) in all aeration regimes 1-4. All qPCR reactions were done in duplicate, and two independent biological replicates were prepared for each set of conditions.

A) ChIP-chip



B) qPCR