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The Control of DNA Gyrase Expression and its Role in Deletion Formation in *Salmonella enterica*

By

Orla Mary Keane

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

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October 2002

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Declarations

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

Massey, R.C., P.B. Rainey, B.J. Sheehan, O.M. Keane, and C.J. Dorman. 1999. Environmentally constrained mutation and adaptive evolution in *Salmonella*. Curr. Biol. **9:**1477-1480.

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For my parents

with thanks

Summary

Salmonella typhimurium strain CJD671 can undergo the deletion of a 5 kb segment of DNA from its large virulence plasmid. This results in a transcriptional fusion between the *rlgA* gene, encoding a putative site-specific resolvase, and the *spvR* virulence gene regulator. The strong activity of the *rlgA* promoter results in the overexpression of a *lacZ* reporter gene in the *spv* locus. Deletions are selected for by lactose and only occur when the bacteria are grown at elevated osmolarity.

This deletion event was found to be independent of the RecA protein demonstrating that it does not require the cellular homologous recombination machinery. The need for elevated osmolarity was shown to be a requirement for enhanced DNA gyrase activity as the DNA gyrase inhibiting antibiotic novobiocin prevented the formation of the deletion in a dose-dependent manner. DNA gyrase is thought to participate indirectly in this deletion event (although a direct role has not been completely ruled out). The levels of the Fis DNA-binding protein were also shown to modulate the frequency at which the deletion could occur. Transposon mutagenesis of the CJD671 genome failed to identify additional factors responsible for deletion formation. However it identified some novel regulators of the *spv* virulence genes.

The *S. typhimurium gyrB* promoter was previously cloned and sequenced. The cloning and sequencing of the *S. typhimurium gyrA* promoter is described in this work. This promoter differs significantly in sequence from the *E. coli gyrA* promoter. The *S. typhimurium* DNA gyrase genes are repressed by the Fis protein and this is thought to be important for the homeostatic regulation of DNA gyrase activity. It may also account for the effect of Fis on deletion formation. The *S.typhimurium gyrA* and *gyrB* genes are also induced upon uptake of the bacteria by macrophages. This is thought to help the bacteria adapt to the intracellular environment. Fis does not appear to play a role in repressing *gyrA* and *gyrB* during growth in macrophage.

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Chapter 1

General Introduction

1.1: Introduction

To survive each stage of life enteric bacteria must adapt to a series of ecological niches. Each new environment encountered by the bacterium can be defined in terms of temperature, nutrient availability, osmotic pressure, oxygen status, and pH. To adapt to these varying conditions the bacterium must firstly analyse the environmental parameters and then transduce this information to the DNA. The cell must then respond in a manner that enhances the fitness of the bacterium within the specific environment. This often requires co-ordinate expression of a large number of genes scattered about the genome.

This adaptation to environmental conditions involves the regulation of gene expression. Often such regulation is carried out by proteins, that usually act by binding the region of DNA close to the gene being regulated, thus either enhancing or repressing transcription of that gene (Perez-Rueda and Collado-Vides, 2000). Many regulators, such as H-NS, Fis, IHF and RpoS control a large number of genes (Vincente *et al.*, 1999). Some regulators, such as IHF and Fis bind a common recognition sequence upstream of the gene to be regulated (Craig and Nash, 1984; Finkel and Johnson, 1992) while others, such as H-NS, bind DNA in a non-sequence specific manner (Schröder and Wagner, 2002). The promoters of many genes, such as *gyrA*, *gyrB*, *fis* and *tyrT* are also sensitive to the negative superhelicity of the DNA (Menzel and Gellert, 1987a; Free and Dorman, 1994; Schneider *et al.*, 2000). Some promoters are repressed by negative supercoiling while others are activated. DNA supercoiling therefore also has the potential to regulate a large number of unlinked genes in the genome (Dorman, 2002). Some genes are also regulated at the post-transcriptional level. This can be done by affecting mRNA stability, translation or post-translational modifications or protein stability (Ishihama, 1997). Altering the expression profile of genes corresponds to non-heritable changes in the cell. Adaptation to environmental conditions has also been shown to occur as a result of stochastic genetic events and these events often improve the fitness of the organism in a given environment. There are many examples of this such as the switching between H1 and H2 flagellar subunits in *Salmonella typhimurium* (van de Putte and Goosen, 1992) and pilin gene variation in *Neisseria gonorrhoeae* (Seifert, 1996) which allow the bacteria avoid the host immune system. Bacteria have also been shown to acquire

resistance to antibiotics to which they are exposed via genetic alterations in their genome or by horizontal transfer of resistance genes located on various types of mobile DNA elements (Normark and Normark, 2002). The mechanisms by which bacteria adapt to conditions and differing environments are many-fold. The versatility of bacteria has evolved as a means to overcome hostile environments, which must be colonised for the successful replication of the bacterium. The diversity and number of mechanisms employed indicate the importance of adaptation to environmental conditions to bacterial populations.

This chapter aims to provide some background to the genetics of virulence in *S. typhimurium*. It also aims to introduce DNA supercoiling and its role in regulating gene expression. It provides a review of the major bacterial nucleoid associated proteins and their role in constraining negative supercoils. Finally it discusses the concept of adaptive mutation, describes some of the systems used to study adaptive mutation and details some of the molecular mechanisms proposed to explain the phenomenon of adaptive mutation.

1.2: *Salmonella typhimurium*

Salmonella enterica serovar Typhimurium (hereafter called *Salmonella typhimurium*) is a Gram negative eubacterium belonging to the enteric group. It is a facultative anaerobe and has a G+C content of 53% (McClelland *et al.*, 2001). It can be distinguished from other enteric bacteria on the basis of its biochemical properties. Unlike *Escherichia coli* or *Shigella*, *S. typhimurium* can utilise citrate as a carbon source, is unable to metabolise lactose, is urease negative and is unable to produce indole from tryptophan (Stanier *et al.*, 1987). *Salmonellae* have been divided into 2 species *Salmonella bongeri* and *Salmonella enterica*. The species *S. bongeri* is supposed to be phylogenetically older than *S. enterica* and only very rarely associated with human disease (Hensel, 2000). *Salmonellae* are significant not only as an ongoing threat to world-wide public health, but also as a model system for the study of fundamental mechanisms of bacterial pathogenesis. The genomes of both *S. typhimurium* LT2 and *Salmonella typhi* CT18 have been sequenced (McClelland *et al.*,

2001; Parkhill *et al.*, 2001) and gene regulation circuits in *Salmonella* have been studied for decades.

1.2.1: *Salmonella* pathogenesis

The principal clinical syndromes associated with *Salmonella* infection are typhoid fever and gastroenteritis. Typhoid fever is a protracted systemic illness that results from infection with the exclusively human pathogens *S. typhi* and *S. paratyphi*. Typhoid fever is still a major health problem in developing countries. According to World Health Organisation estimations, 16 million cases of typhoid fever occur annually world-wide with approximately 600,000 deaths (www.who.int/vaccines/en/typhoid.shtml). Clinical manifestations include fever, abdominal pain and transient diarrhoea or constipation. The pathological hallmark of enteric fever is mononuclear cell infiltration and hypertrophy of the reticuloendothelial system, including the intestinal Peyer's patches, mesenteric lymph nodes, spleen and bone marrow. Without treatment mortality is 10-15% (Ohl and Miller, 2001). In contrast, the many non-typhoidal *Salmonella* strains, such as *S. enteriditis* and *S. typhimurium* infect a wide range of animal hosts, and usually cause self-limiting enteritis in humans. Initial symptoms include nausea and vomiting, which are followed by abdominal pain and diarrhoea. Enteritis induced by non-typhoidal *Salmonella* represents a major economic and welfare problem affecting both man and food-producing animals. The nature and severity of the disease is largely dependent on the *Salmonella* serotype and host species involved (Darwin and Miller, 1999). Very young and immunocompromised individuals are particularly susceptible to severe forms of enteritis and infection may spread to systemic sites with a high associated mortality (Wallis and Galyov, 2000). In healthy adults, enteritis is typically self-limiting and often results in a subclinical carrier status. In certain inbred mouse strains however *S. typhimurium* infection produces an illness resembling enteric fever that serves as an experimental model for systemic *Salmonella* infections (Kingsley and Baeumler, 2000).

Salmonella infections are acquired by the ingestion of contaminated food or water. The bacteria must survive the low pH of the stomach before entering the small

intestine where they adhere to gut epithelial cells. Here they trigger uptake of the bacteria by the host cell by a process known as bacterial mediated endocytosis (Francis *et al.*, 1992). This involves extensive host cell actin rearrangements in the vicinity of the adhering bacterium, which disrupts the normal epithelial brush border. The subsequent formation of membrane ruffles encloses the bacteria in large vesicles. In mice *Salmonella* appear to adhere preferentially to and enter the M cells (Jones *et al.*, 1994), however in bovine ligated ileal loops *Salmonella* invade both M cells and enterocytes (Frost *et al.*, 1997). It has also been reported that *Salmonella* may passively cross the intestinal epithelial barrier following phagocytosis by migrating CD18-positive phagocytes (Vazquez-Torres *et al.*, 1999). Following bacterial internalisation a fraction of the *Salmonella*-containing vesicles transcytose to the basolateral surface and reach the Peyer's patches. Here *Salmonella* encounter submucosal macrophages. *Salmonella* serotypes that cause systemic infection enter macrophages by bacterial mediated endocytosis and are able to survive and grow inside these cells (Alpuche-Aranda *et al.*, 1994). Migration of infected phagocytes to other organs of the reticuloendothelial system allows dissemination of bacteria in the host.

As the pathogen enters and moves through its animal host, it encounters a series of unique environments. Each host environment possesses distinct chemical and physical properties, such as temperature, pH, osmolarity and nutrient availability. In each new environment the pathogen also confronts various components of the host innate immune system. The intestinal epithelium represents an efficient barrier against pathogens. Exclusion of the pathogens is not only a result of the continuous physical barrier formed by the tightly bound epithelial cells. Other associated factors are also involved in the defence process such as the mucus layer, intestinal peristalsis, and an array of innate antibacterial factors such as lactoferrin, lysozyme and cryptins (Sansonetti, 2002). Pathogens must sense these changing surroundings and respond by co-ordinating gene expression to provide an adaptive advantage in each new host environment. A large number of gene products are required for *Salmonella* virulence. Many of these genes are encoded on the "pathogenicity islands" found in *Salmonella* (Hensel, 2000). These pathogenicity islands are segments of DNA which have a different G+C content to the genome as a whole and are not found in closely related non-pathogenic species. Pathogenicity islands are often found integrated at tRNA loci

and are thought to be horizontally acquired (Blanc-Potard *et al.*, 1999). In addition to several small pathogenicity islets, five large pathogenicity islands have been identified in *Salmonella.*

1.2.2: *Salmonella* pathogenicity island 1

Salmonella pathogenicity island-1 (SPI-1) encodes genes necessary for invasion of intestinal epithelial cells and induction of intestinal secretory and inflammatory responses (Watson *et al.*, 1995; Galyov *et al.*, 1997). The SPI-1 chromosomal region encodes for more than 30 proteins, which include regulatory proteins, structural components of a type III secretion system (TTSS) and several secreted proteins and their chaperones (Wallis and Galyov, 2000). Type III secretion systems are specialised virulence devices that have evolved to modify host-cell function through direct translocation of bacterial virulence proteins into the host cell cytoplasm. These translocated bacterial proteins alter such basic host-cell functions as signal transduction, cytoskeletal architecture, membrane trafficking and cytokine gene expression (Hueck, 1998). The delivery of bacterial proteins into the host cell clearly represents a complex task, as translocated proteins must cross the bacterial inner and outer membrane as well as the host cell plasma membrane. The SPI-1 TTSS is composed of a cytoplasmic bulb followed by a disc like structure that spans the inner and outer membrane. A needle like structure extends outside the outer membrane. SipB and SipC then form a pore-like translocator that accounts for the intracellular transfer of other effectors (Sansonetti, 2002). A large number of effector proteins are translocated into the host epithelial cell. The precise function of many of these effectors remains to be elucidated. SopE is known to directly activate *cdc42* and *rac-1*, two members of the Rho family of GTP binding proteins (Hardt *et al.*, 1998). This influences cytoskeletal rearrangements thereby promoting epithelial cell invasion. Another secreted effector protein SptP functionally opposes SopE and inactivates Cdc42 and Rac-1 and stabilises the eukaryotic cell membrane after bacterial endocytosis (Fu and Galan, 1999). SopE and SptP must be therefore temporally regulated. The SipA effector protein is also proposed to play a role in actin rearrangement at the site of bacterial endocytosis (Zhou *et al.*, 1999). SopB is another effector translocated by the SPI-1 TTSS (Galyov *et al.*, 1997). It promotes intestinal

inflammation and fluid secretion by subverting inositol phosphate signalling pathways (Norris *et al.*, 1998). However the precise role of many of the SPI-1 TTSS effectors still remains to be elucidated. A role for the Fis protein in the regulation of SPI-1 has been demonstrated. Fis has been shown to be required for expression of HilA and InvF two regulators of SPI-1. *Salmonella* strains lacking Fis have a 50-fold decreased ability to invade HEp-2 tissue culture cells and are attenuated 100-fold when administered orally to mice (Wilson *et al.*, 2001).

1.2.3: *Salmonella* pathogenicity island 2

Salmonella pathogenicity island-2 (SPI-2) encodes genes required for survival and growth of the bacteria inside macrophages and to cause systemic infection (Ochman *et al.*, 1996; Shea *et al.*, 1996). *Salmonella* strains that are mutant for SPI-2 are attenuated when administered by the oral, intravenous or intraperitoneal routes. *Salmonella* pathogenicity island-2 is approximately 40 kb in size and encodes 42 open reading frames. SPI-2 also encodes a type III secretion system (Shea *et al.*, 1996). The following nomenclature for SPI-2 virulence genes applies, genes encoding components of the type III secretion apparatus were designated *ssa* (secretion system apparatus), genes encoding effectors or their chaperones were designated *sse* (secretion system effector) or *ssc* (secretion system chaperone) respectively and genes encoding regulators of SPI-2 virulence genes were designated *ssr* (secretion system regulator) (Hensel *et al.*, 1997). SPI-2 genes were originally identified as genes that were induced intracellularly (Valdivia and Falkow, 1997). As this TTSS activates within the phagosome and translocates effector proteins into the host cell cytosol, it represents a new paradigm for the function of TTSS. Once again much remains to be elucidated about the function of the effector proteins translocated by the SPI-2 TTSS, however they are proposed to inhibit maturation of *Salmonella*-containing phagosomes to phagolysosomes and to avoid killing of intracellular *Salmonella* by reactive oxygen species (Uchiya *et al.*, 1999; Vasquez-Torres *et al.*, 2000). Recently a role has been demonstrated for Fis in the regulation of the *ssrA* and *ssaK* virulence genes encoded on SPI-2 (Carroll *et al.*, 2002).

1.2.4: Regulation of virulence genes

The PhoP/PhoQ two component signal transduction system controls a large regulon of genes, both positively and negatively, that are required for virulence (Groisman *et al.*, 1989; Miller *et al.*, 1989). PhoQ is an inner membrane sensor protein that senses Mg^{2+}, Ca^{2+} or Mn^{2+}. The PhoP protein is a cytoplasmic DNA binding protein that controls the transcription of many genes. *Salmonella phoP* or *phoQ* mutants are highly attenuated for virulence as they are unable to survive inside macrophages (Fields *et al.*, 1986; Miller *et al.*, 1989) and have increased susceptibility to antimicrobial peptides, bile salts and pH (Fields *et al*, 1989; Miller *et al.*, 1990; Bearson *et al.*, 1998; Van Velkinburgh and Gunn, 1999). The role of PhoP/Q in regulating SPI-2 remains controversial with conflicting reports about whether PhoP/Q is required for activation of the SPI-2 TTSS upon uptake of *Salmonella* by macrophage (Deiwick *et al.*, 1999; Beuzon *et al.*, 2001; Miao *et al.*, 2002). PhoP/Q is required for repression of SPI-1 genes (Pegues *et al.*, 1995). PhoP/Q is also required for activating the *mgtA*, *mgtB* and *mgtC* genes, which function in Mg^{2+} acquisition and are required for growth in low Mg^{2+} concentrations and intramacrophage survival (Blanc-Potard and Groisman, 1997). PhoP/Q also indirectly regulates another two component system *pmrA/pmrB* via the PmrD protein (Kox *et al.*, 2000). This two component system senses extracytoplasmic Fe^{3+} levels (Wösten *et al.*, 2000). Regulation of the SPI-2 genes is also under the control of the SPI-2 encoded two component regulatory system SsrA/SsrB (Shea *et al.*, 1996). The signals to which the SsrB sensor responds have not yet been fully elucidated. The SsrA/SsrB system is itself under the control of the OmpR/EnvZ two component system (Lee *et al.*, 2000). SPI-1 genes are also under the control of a number of regulators namely HilA, InvF, HilC and HilD. HilA directly regulates a number of genes such as *prgH*, a componentof the SPI-1 TTSS. HilA also indirectly regulates a number of genes due to its effect on the expression of the AraC like transcriptional regulator InvF (Lucas and Lee, 2000).

1.2.5: *Salmonella* plasmid virulence genes

Other *Salmonella* genes known to be required for virulence are the salmonella plasmid virulence (*spv*) genes. These genes are encoded on the large virulence plasmid (pSLT)

found in non-typhoid *Salmonella* strains. This plasmid can vary in size from 50-100 kb and is estimated to be present at 2.75 copies per cell (McClelland *et al.*, 2001). Strains of *S. typhimurium* become attenuated once the *spv* genes are deleted and can no longer cause infection (Gulig *et al.*, 1993). The *spv* system consists of a LysR-like positive regulator, SpvR, which activates both its own expression and that of the structural genes (Heinkoff *et al.*, 1988). There are 4 structural genes *spvABCD*. These structural genes form an operon and are all transcribed from the *spvA* promoter although some post-transcriptional modifications do occur (El Gedaily *et al.*, 1997). SpvR has been shown to bind to the *spvA* promoter in a hierarchical fashion at two sites, with binding to a proximal site being dependent on binding to a more distal site (Sheehan and Dorman, 1998). The *spvR* gene is located immediately upstream of *spvABCD*. It is transcribed from its own promoter in the same direction as the structural genes. The precise function of the *spv* structural genes is unknown although *spvB* has recently been identified as a mono-ADP-ribosyl transferase (Otto *et al.*, 2000). SpvB ADP-ribosylates actin and inhibits polymerisation of G actin to F actin thereby destabilising the cytoskeleton of the host cell (Lesnick *et al.*, 2001; Tezcan-Merdol *et al.*, 2001). How SpvB is translocated to the host cell cytosol remains to be determined as subcellular localisation of the proteins found SpvB present in the bacterial cytoplasm (El-Gedaily *et al.*, 1997). The same study found SpvA located in the outer membrane, SpvC located in the cytoplasm and SpvD secreted into the supernatant. *In Vivo* Expression Technology (IVET) has shown the *spv* genes to be induced in mice (Heitoff *et al.*, 1997) and Signature Tagged Mutagenesis (STM) has shown them to be required for virulence (Hensel *et al.*, 1995). The alternative sigma factor RpoS is required for stationary phase activation of the *spv* genes (Kowarz *et al.*, 1994; Chen *et al.*, 1995). The histone-like protein H-NS has been shown to repress *spv* gene expression also in stationary phase (O'Byrne and Dorman, 1994b) as have CRP and LRP (O'Byrne and Dorman, 1994a; Marshall *et al.*, 1999). IHF has been shown to be a positive regulator of the *spv* system (Marshall *et al.*, 1999). A diagram of the factors that positively and negatively regulate *spv* gene expression can be seen in Fig. 1.1. Increasing the level of negative supercoiling has also been reported to decrease transcription of the *spv* structural genes (O'Byrne and Dorman, 1994b). Environmental conditions that affect DNA supercoiling, either positively or negatively may therefore affect transcription of the *spv* genes.

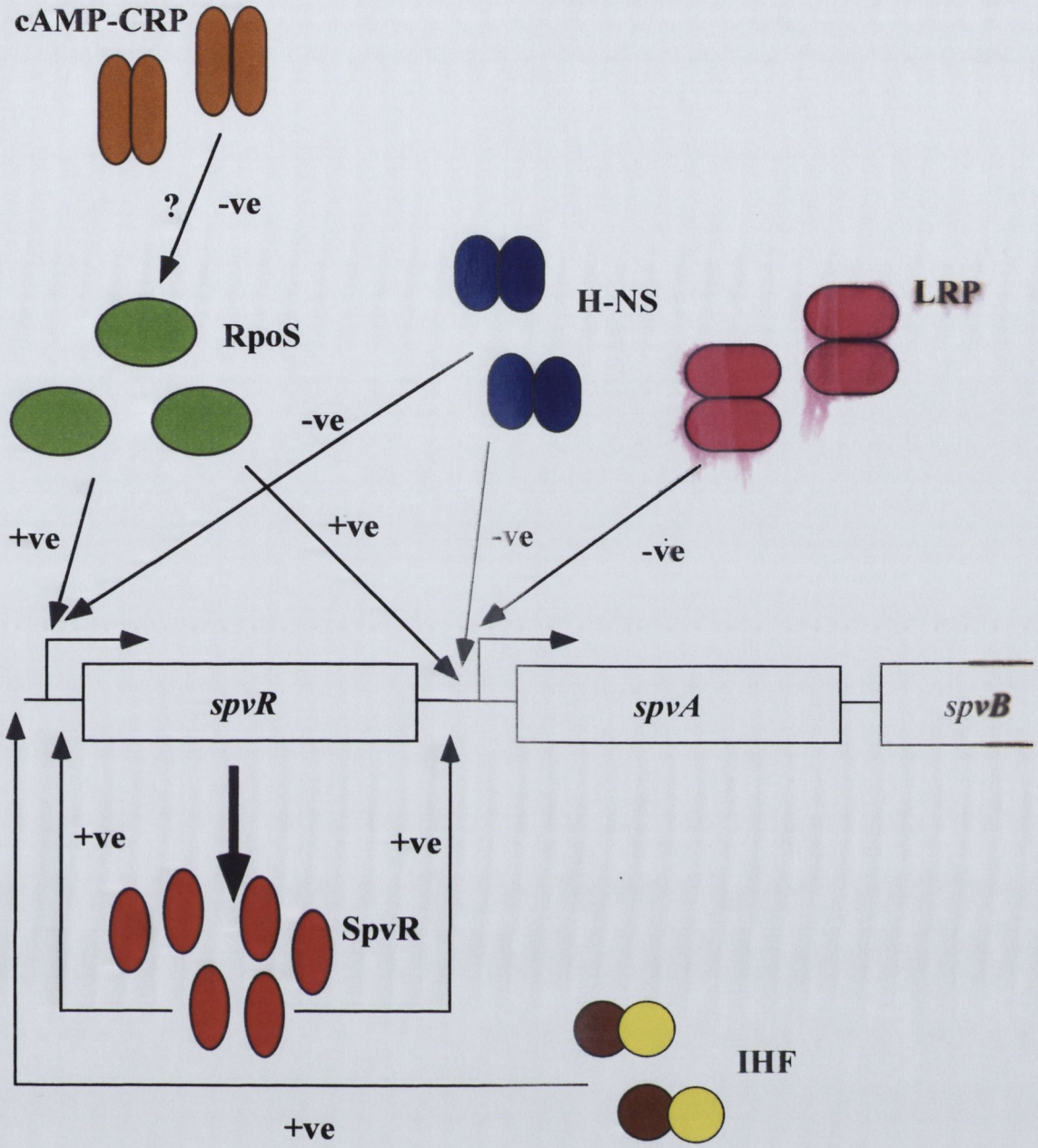


Fig. 1.1. **Factors affecting *spv* gene expression.** The SpvR protein acts as a positive regulator of its own transcription and of *spv* structural gene transcription. RpoS is required for stationary phase activation of the *spv* genes. H-NS acts as a repressor of the *spv* genes. This is independent of its effect on RpoS. IHF is a positive activator of *spvR* transcription and LRP is a repressor of *spvA* transcription. cAMP-CRP has a negative effect on *spv* gene transcription and this is thought to occur via its effect on RpoS.

1.3: DNA supercoiling

The highly compacted DNA in eubacteria is maintained in an underwound or negatively supercoiled state, which imparts torsional stress to the molecule. This stress confers free energy on the DNA, assisting in a number of important cellular functions that require DNA strand separation such as transcription, replication and recombination (Tse-Dinh *et al.*, 1997). The supercoiling of a closed circular DNA molecule can be defined by the following equation:.

$$Lk = Tw+Wr \text{ or } \Delta Lk = \Delta Tw+\Delta Wr$$

Lk is the linking number and refers to the number of times the two strand of closed circular DNA are linked i.e. the number of times one strand of the helix winds completely around the other in a duplex. Twist (Tw) refers to the way in which two individual DNA strands coil around each other and thus describes the number of double-helical turns a molecule contains along the length of the duplex. Writhe (Wr) refers to the turning of the axis of the duplex in space. As linking number is dependent on the length of the DNA molecule it is of limited value when comparing degrees of supercoiling between molecules of different length. For this reason the size-independent parameter of superhelical density (σ) is used. This is defined as:

$$\sigma = \Delta Lk/Lk^{\circ}$$

where Lk° is the linking number of the relaxed molecule (reviewed by Drlica, 1992).

1.3.1: DNA topoisomerases

For most processes that require the information stored in the DNA the two strands of the DNA must separate, either transiently, as in transcription and recombination or permanently, as in DNA replication. DNA topoisomerases solve the topological problems associated with DNA replication, transcription, recombination and chromatin remodelling by introducing temporary breaks in the DNA. These breaks can be single stranded (type I topoisomerases) or double stranded (type II topoisomerases). These enzymes also maintain the cellular homeostatic balance of supercoiling that is needed for appropriate gene expression. Vigorous growth of the bacterial cell only occurs within a $\pm$15% range of supercoiling (Drlica, 1992). This highlights the importance of

maintaining the homeostatic balance in DNA supercoiling. DNA topoisomerases are divided into 2 families. The type I topoisomerases pass one DNA strand through a break in the opposing strand while the type II family introduce a break in both strands of the DNA through which a region of duplex DNA from the same or a different molecule can be passed (Champoux, 2001). The genome of *S. typhimurium* encodes 4 topoisomerases (McClelland *et al.*, 2001), each of which carries out its own specialised function in the cell.

1.3.2: DNA topoisomerase I

DNA topoisomerase I is a type I DNA topoisomerase. This enzyme is a monomeric protein 97 kDa in size, and is encoded by the *topA* gene (Wang, 1971). Cells that are deficient in TopA are viable but grow poorly and often acquire compensatory mutations. These mutations either reduce DNA gyrase activity or overexpress topoisomerase IV by amplification of the *par* locus (DiNardo *et al.*, 1981; Dorman *et al.*, 1989b). The *topA* promoter itself is sensitive to the level of DNA supercoiling in the cell and *topA* is induced when the level of negative supercoiling increases (Tse-Dinh, 1985). This enzyme brings about strand passage that is required for DNA relaxation by cleaving a single strand of DNA and hence changes the linking number of the DNA in steps of 1. The enzyme holds onto both of the DNA ends at the site of cleavage. A tyrosine residue located at position 319 of TopA is required for covalent attachment to the cleaved 5' phosphate of the DNA. The cleaved 3' end of the DNA is proposed to occupy a nucleotide binding site within the enzyme (Lynn and Wang, 1989; Champoux, 2001). The intact strand is then passed through the gap in the cleaved DNA and the cleaved strand is religated (Tse and Wang, 1980; Brown and Cozzarelli, 1981). This reaction occurs without an external energy source such as ATP. DNA topoisomerase I opposes the action of DNA gyrase and prevents negative supercoiling in the cell reaching excessively high levels. This helps maintain the steady state level of negative supercoiling required for the initiation of replication and transcription (Snoep *et al.*, 2002). DNA topoisomerase I also removes positive supercoils generated during transcription (Masse and Drolet, 1999).

1.3.3: DNA gyrase

DNA gyrase is a type II DNA topoisomerase. It is composed of two subunits GyrA and GyrB with molecular weights of 105 kDa and 95 kDa respectively. DNA gyrase has a tetrameric A_2B_2 structure (Klevan and Wang, 1980; Sugino *et al.*, 1980). The genes encoding the 2 subunits, *gyrA* and *gyrB* are unlinked on the genome and must be co-ordinately regulated. Cells deleted for *gyrA* or *gyrB* are not viable. The *gyrA* and *gyrB* promoters are also sensitive to the overall level of DNA supercoiling being induced when the DNA becomes relaxed and repressed by negative supercoiling (Menzel and Gellert, 1983, 1987; Snoep *et al.*, 2002). Recently it has been proposed that axial distortion in the region of the *gyrA* and *gyrB* promoters acts as a sensor of supercoil changes (Unniraman and Nagaraja, 2001). DNA gyrase is the only known enzyme that can introduce negative supercoils into DNA and this is an energy consuming reaction as it requires the hydrolysis of ATP (Gellert *et al.*, 1976a). Negative supercoiling is also required for compaction of the nucleoid (Stuger *et al.*, 2002). DNA gyrase binds duplex DNA and cleaves both strands of the DNA with a four base pair stagger. Cleavage involves covalent attachment of each GyrA subunit to the 5' end of the DNA through a phosphotyrosine bond. This prevents free rotation of the DNA and loss of supercoils. A conformational change pulls the two ends of the cleaved duplex DNA apart to create an opening in the DNA. A second region of duplex DNA, from either the same or a different molecule, is passed through the gap in the DNA, changing the linking number by -2. The DNA is then resealed (Champoux 2001). An N-terminal 59 kDa fragment of *E. coli* DNA gyrase has been crystallised (Morais Cabral *et al.*, 1997). This is the minimal fragment that when complexed with GyrB has DNA cleavage activity (Reece and Maxwell, 1991). The C-terminal fragment is required for the introduction of negative supercoils. A ribbon diagram of this N-terminal fragment can be seen in Fig. 1.2. The GyrB subunit of DNA gyrase is responsible for ATP hydrolysis (Staudenbauer and Orr, 1981).

1.3.4: DNA topoisomerase III

DNA topoisomerase III is a type I DNA topoisomerase, encoded by the *topB* gene (Dean *et al.*, 1983). It is a monomeric protein of 73 kDa and strains carrying

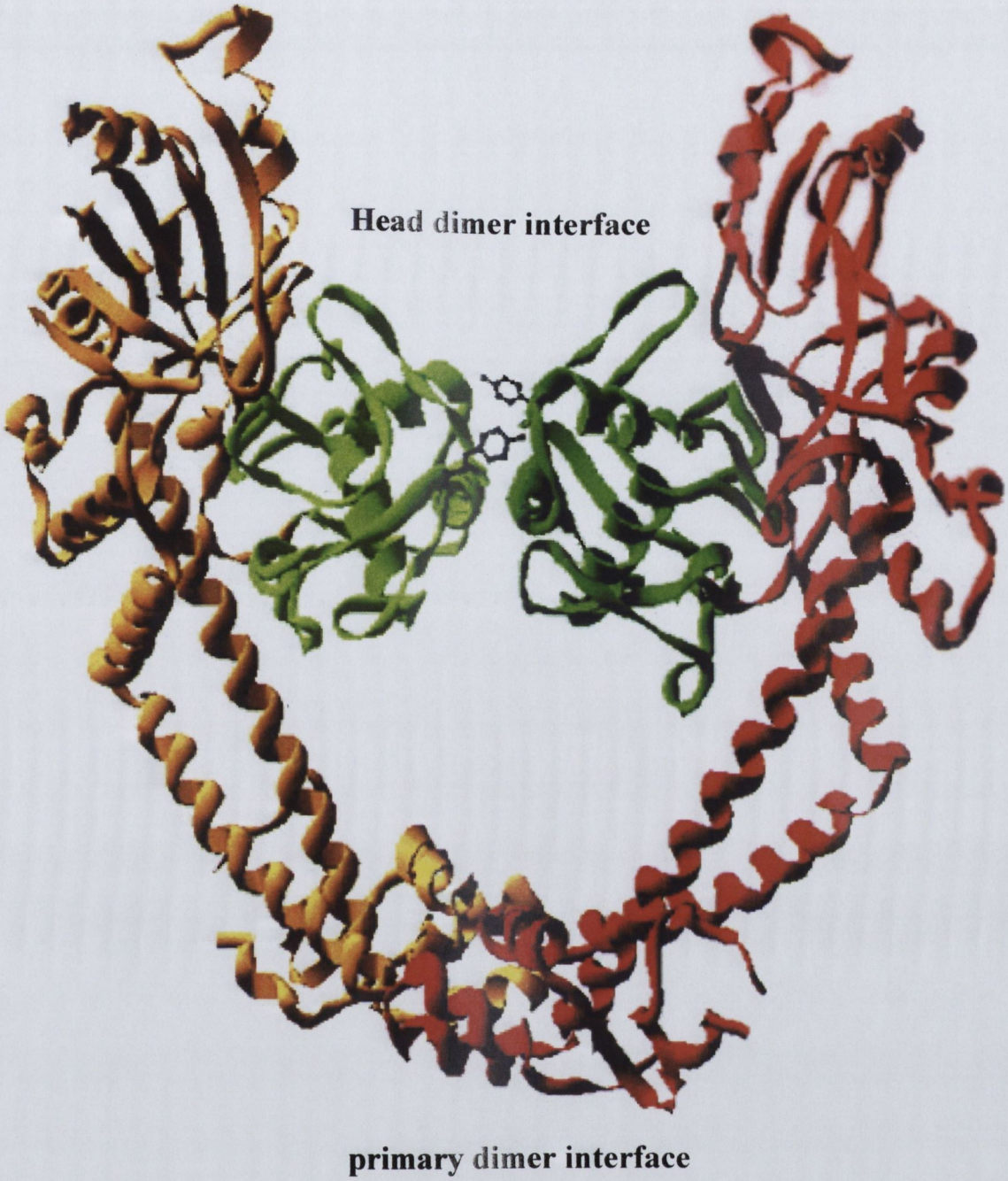


Fig. 1.2. **Ribbon diagram of 59 kDa N-terminal GyrA fragment of *E. coli*.** This is the minimal fragment, which complexed with GyrB will cleave DNA. The helix-turn-helix motifs of the homodimer are coloured light and dark green. These motifs contain the active site tyrosines which are shown in black as a ball and stick model. The remainder of the fragment monomers are shown in red and orange. Figure adapted from Champoux, 2001.

mutations in *topB* are viable with no obvious growth defects (Zhu *et al.*, 2001). This enzyme, like Topo I, works by binding single stranded DNA and cleaving this strand. Another strand of DNA can then be passed through this gap in the DNA altering the linking number of the DNA by one (Champoux, 2002). The crystal structure of *E. coli* topoisomerase III in a non-covalent complex with a single-stranded DNA molecule has been solved (Changela *et al.*, 2001). DNA topoisomerase III appears to function primarily on hypernegatively supercoiled DNA and is much more efficient at decatenation of DNA after replication than Topo I. This decatenation ability requires a 17 amino acid domain present in Topo III but not Topo I (Li *et al.*, 2000). DNA topoisomerase III appears to play a role in removing negative supercoils that are generated behind the replication fork during DNA replication (DiGate and Marians, 1988). It also has the ability to cleave and decatenate RNA molecules although whether this has any physiological relevance remains to be determined (DiGate and Marians, 1992; Wang *et al.*, 1996).

1.3.5: DNA topoisomerase IV

DNA topoisomerase IV is encoded by the *parC* and *parE* genes (Kato *et al.*, 1990) and has a heterotetrameric structure with subunits of 75 kDa and 70 kDa respectively. The ParC and ParE proteins are homologous to GyrA and GyrB respectively. There is 36% identity and 60% similarity between ParC and GyrA, while ParE and GyrB are 42% identical and 62% similar (Huang, 1996). The genes encoding *parC* and *parE* are located close to one another on the *E. coli* and *Salmonella* genomes however they are not adjacent to one another and do not form an operon. Like DNA gyrase, topoisomerase IV is essential for cell survival. The enzyme cleaves both strands of the DNA with a 4 base pair stagger and functions similarly to DNA gyrase, the other type II topoisomerase (Champoux, 2001). Topo IV plays a role in decatenation of sister chromosomes after replication and also in relaxing negative supercoils in the cell. Topo IV, along with Topo I and gyrase maintains steady state supercoiling levels in the bacterial cell (Zechiedrich *et al.*, 2000) and also functions very efficiently at decatenating DNA (Zechiedrich *et al.*, 1997; Deibler *et al.*, 2001). Topoisomerase IV is primarily responsible for unlinking 2 daughter duplexes that occur behind the replication fork as well as the catenates that are generated at the end of replication

(Kato *et al.*, 1990; Peng and Marians, 1993). Topo IV requires ATP hydrolysis for efficient relaxation of supercoils. The properties of the four topoisomerases are summarised in Table 1.1.

1.3.6: DNA supercoiling and transcription

The effect of DNA supercoiling on the activity of some bacterial promoters *in vivo* has been known for many years. Fluctuations in the level of supercoiling, either by topoisomerase inhibition or by genetic means alters the level of expression of many genes, including *topA*, *gyrA*, *gyrB*, *proU*, *tyrT*, rRNA operons and a large number of virulence loci (Oostra *et al.*, 1981; Menzel and Gellert, 1983; Tse-Dinh, 1985; Menzel and Gellert, 1987a; Higgins *et al.*, 1988; Galan and Curtiss III, 1990; Dorman, 1991; Free and Dorman, 1994). Some promoters are activated by increases in negative supercoiling while other promoters are repressed. The molecular mechanism of this regulation has not been fully elucidated in all cases however negative supercoiling can facilitate promoter melting in some cases (Ohlsen and Gralla, 1992). Transcription can also affect supercoiling levels. According to the twin domain model, transcription in eubacteria and other organisms results in a domain of positive supercoils generated ahead of the transcription complex and a domain of negative supercoils generated behind the transcription complex if the transcription complex is not free to rotate (Liu and Wang, 1987). Such a barrier to rotation could be provided by DNA binding proteins (Leng and McMacken, 2002). If not removed these supercoils will impede movement of the transcription complex. DNA topoisomerase I plays a role in removing the negative supercoils generated behind the transcription complex, while DNA gyrase plays a role in relaxing positive supercoils preceding the transcription complex. This twin domain model allows the transient appearance of highly supercoiled domains on the chromosome. This could have important consequences for the regulation of neighbouring genes. A diagram of the twin domain model of Liu and Wang can be seen in Fig. 1.3.

Evidence that locally supercoiled domains can influence transcription of a neighbouring gene initially came from studies carried out with the *leu500* promoter of *S. typhimurium*. This is a mutant promoter of the leucine biosynthetic operon, which

Protein	Gene	Mass (kDa)	Structure	Function
Topoisomerase I	*topA*	97	monomer	Relaxation of negative supercoils
DNA gyrase	*gyrA* *gyrB*	105 95	heterotetramer	Introducion of negative supercoils
Topoisomerase III	*topB*	73	monomer	Decatenation after DNA replication. Decatenation of RNA?
Topoisomerase IV	*parC* *parE*	75 70	heterotetramer	Decatenation of daughter chromosomes

Table 1.1. **Summary of the topoisomerases of *S. typhimurium*.** Their properties and functions are also listed.

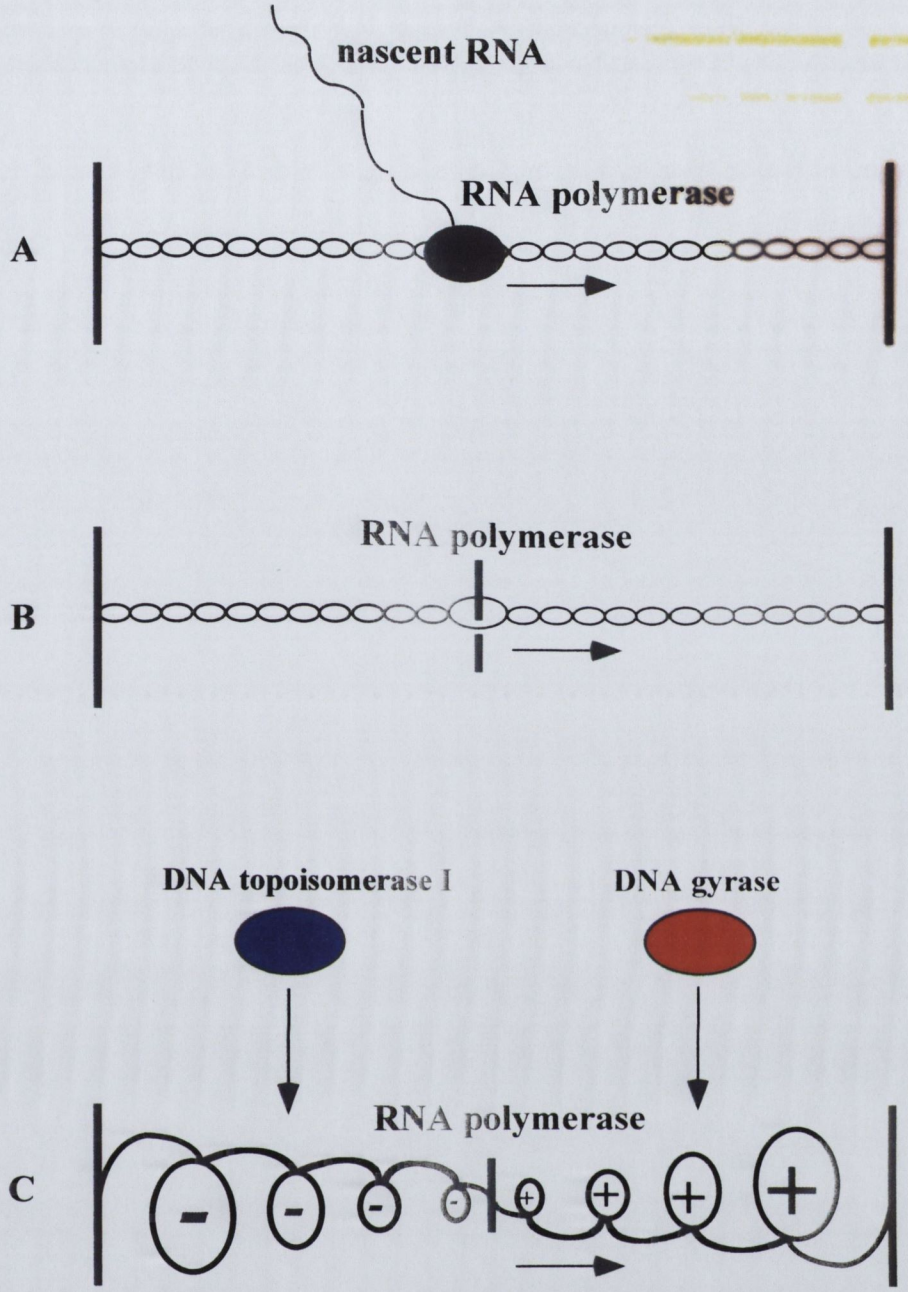


Fig. 1.3. **Liu and Wang model of transcription induced supercoils.** (A) A non-rotatable transcription complex, including RNA polymerase moves in the direction of the arrow along the DNA. The DNA is anchored at both ends (represented by solid bars). (B) This divides the helical DNA into two parts with (C) positive supercoils generated ahead of the RNA polymerase and negative supercoils generated behind it. Figure adapted from Liu and Wang, 1987.

has an A to G mutation in the –10 region of the promoter. This prevents open complex formation at the promoter and gives rise to leucine auxotrophy (Gemmill *et al.*, 1984). In a *topA* mutant leucine prototrophy is restored, as the increased negative supercoiling in this strain provides the additional energy required for promoter melting (Richardson *et al.*, 1988). In a plasmid based system however the *topA* mutation could not act as a suppressor of the *leu500* mutation unless a functional divergent *tetA* gene was located upstream of the promoter. Both transcription and translation of *tetA* were required (Chen *et al.*, 1992). It was proposed that membrane anchorage of the TetA nascent peptide prevents the loss of negative supercoils generated by transcription of *tetA* as it prevents free rotation of the plasmid. These negative supercoils then provide the energy needed to melt the *leu500* promoter. This model was shown to hold true for a chromosomal *leu500* mutation also. Transcription of a divergently transcribed gene, located up to 5 kb upstream, could stimulate *leu500* promoter activity on the chromosome (El Hanafi and Bossi, 2000). The genes encoding many LysR-like transcription factors are arranged such that they are divergently transcribed from other genes (Opel *et al.*, 2001). The *ilvY* transcriptional regulator is divergently transcribed from the *ilvC* structural gene and these genes have been shown to be transcriptionally coupled through DNA supercoiling (Rhee *et al.*, 1999; Opel and Hatfield, 2001). Therefore promoter activity can be modulated by the activity of a neighbouring gene via DNA topology. Another study examined the effect of the DNA gyrase inhibitor novobiocin on gene and protein expression. Microarray data and 2D-PAGE analysis showed that 37% of all *Haemophilus influenzae* genes showed a change in expression in response to novobiocin treatment and are therefore potentially supercoiling sensitive (Gmuender *et al.*, 2001).

The term DNA supercoiling is often used to describe the global properties of the DNA molecule. However as in the case of the *leu500* promoter described above, local distortions of the DNA helix are also an important consideration in the activity of many promoters. DNA topology refers to both the global level of DNA supercoiling and local conformational parameters such as bends and loops. Changes in the local conformation of the DNA can be brought about by domains of positive or negative supercoiling generated by transcription of a neighbouring gene or by the introduction of bend or kinks in the DNA. Bending of the DNA often occurs as a consequence of the binding of proteins to the DNA. This bending usually occurs over a small length

of DNA that includes the protein binding site. Proteins such as IHF can induce bends of up to 180° in the DNA (Rice *et al.*, 1996). Such bending can be important for bringing together distant sites on the DNA and has an important role in the regulation of many genes including the *ilvGMEDA* operon and *pspF* (Dworkin *et al.*, 1998; Engelhorn and Geiselmann, 1998).

1.3.7: Environmental regulation of DNA supercoiling

In vivo studies have demonstrated that several environmental factors influence the level of supercoiling in the bacterial cell. These parameters include nutrient availability, pH, osmolarity, temperature, and anaerobic conditions. It has been shown that DNA becomes more relaxed following nutrient starvation and that supercoiling increases when growth resumes upon nutrient upshift (Balke and Gralla, 1987). A shift from acid to alkaline pH has been shown to result in an increase in negative supercoiling in *S. typhimurium* (Karem and Foster, 1993). Increasing the osmolarity of growth media also results in an increase in bacterial DNA negative superhelicity (Higgins *et al.*, 1988; Hsieh *et al.*, 1991a). DNA also becomes more negatively supercoiled under anaerobic conditions (Yamamoto and Droffner, 1985; Dorman *et al.*, 1988). The effect of temperature on supercoiling seems to be species specific. In *E. coli* and *Yersinia enterocolitica* negative supercoiling increases as temperature increases (Goldstein and Drlica, 1984; Rohde *et al.*, 1994). In *S. typhimurium* and *Bacillus subtilis* negative supercoiling decreases as temperature increases (Dorman *et al.*, 1990; Grau *et al.*, 1994). How exactly environmental modulation of DNA supercoiling is carried out at the molecular level is still unclear. However it appears depend on the cellular [ATP]/[ADP] ratio, as gyrase activity is stimulated by ATP and repressed by ADP and DNA supercoiling is reported to depend on the phosphorylation potential in *E. coli* (van Workum *et al.*, 1996). A number of environmental signals such as transition from aerobic to anaerobic growth or salt shock affect this potential. Moving bacteria from aerobic growth to anaerobic growth results in a transient drop in the [ATP]/[ADP] and a concomitant transient decrease in negative superhelicity while increasing the osmolarity of the bacterial growth medium results in an increase in both [ATP]/[ADP] and negative superhelicity (Hsieh *et al.*, 1991 a,b). The activity of DNA

gyrase *in vitro* also depends strongly on the [ATP]/[ADP] ratio (Westerhoff *et al.*, 1988).

In order to infect a host successfully, bacteria must be able to adapt to changes in the environment that occur during the transition from the free-living to the host-associated state. On entering a host, bacteria may encounter increases in temperature or osmolarity or anaerobic conditions, factors that alter DNA superhelicity. Therefore variations in DNA supercoiling, caused by these environmental parameters may allow the bacterium adapt to the new environment of the host and survive there. Evidence that this is indeed the case comes from a number of studies. *S. typhimurium topA* mutants are known to be less invasive of epithelial cells in tissue culture than wild-type *Salmonella* (Galan and Curtiss III, 1990). Upon uptake of *S. typhimurium* by mouse macrophage-like cells DNA is strongly relaxed and this results in induction of the gyrase genes (Marshall *et al.*, 2000). A number of virulence genes in *Salmonella* and other organisms, such as *E. coli*, *Yersinia*, *Vibrio* and *Bordetella*, have also been shown to be supercoiling sensitive (Dorman *et al.*, 1990; Dorman, 1991; Parsot and Mekalanos, 1992; Graeff-Wohlleben *et al.*, 1995; Beltrametti *et al.*, 1999; Rohde *et al.*, 1999; Daigle *et al.*, 2000; Bang *et al.*, 2002).

1.3.8: Inhibitors of DNA gyrase

As well as a regulator of global gene expression, DNA gyrase is also very important as an antibiotic target. Two types of drugs target DNA gyrase activity. These are coumarins, such as novobiocin and quinolones, such as oxolinic acid. As both types of antibiotics are used in this study to inhibit DNA gyrase activity their mode of action is described. Quinolone antibiotics are broad-spectrum bacteriocidal antibiotics in clinical use, which target type II topoisomerases. They easily penetrate bacterial cells, have good pharmacokinetic properties and show minimum toxicity towards eukaryotic cells (Couturier *et al.*, 1998). Quinolone antibiotics cause cell death by inhibiting DNA synthesis, and at higher concentrations RNA synthesis. They do this by binding to the DNA gyrase (or topoisomerase IV)-DNA complex. They stabilise DNA strand breaks created by gyrase or topoisomerase IV. Ternary complexes of drug, enzyme and DNA then block progression of the replication fork leading to cell death. The

precise molecular nature of the interaction of quinolones with their target enzymes is incompletely understood. However all quinolone resistance mutations map to amino acids that are clustered in α-helices near the active site tyrosine (Hooper, 2001). Novobiocin is a coumarin antibiotic. It is a competitive inhibitor of the gyrase ATPase reaction. Novobiocin binds to the N-terminal part of the GyrB subunit of DNA gyrase thereby inhibiting DNA gyrase (Maxwell, 1993). This prevents the introduction of negative supercoils into the DNA. Due to its mode of action treatment with novobiocin does not have the same pleiotropic effects on the cell as treatment with quinolones.

1.4: The bacterial nucleoid

Much of our knowledge of the structure of the bacterial nucleoid has come from studies on *E. coli* K-12. However the information gleamed from such studies is also relevant to the enteric pathogen *S. typhimurium*. The bacterial nucleoid consists of the chromosome together with mobile genetic elements, bacteriophages and plasmids and the proteins complexed with them. Because of the large size of the chromosome and the small volume of the bacterial cell, the DNA in the nucleoid must be highly organised and compacted. Some of this compaction is achieved by negative supercoiling *in vivo* (Stuger *et al.*, 2002). About half the negative supercoils *in vivo* are available to facilitate processes that require DNA strand separation. The other half are constrained by nucleoid associated proteins and RNA polymerase (Pettijohn and Pfenninger, 1980; Bliska and Cozzarelli, 1987). However it must be noted that the bacterial nucleoid is dynamic and is constantly altering in structure. Nucleoid-associated proteins, such as IHF, Fis and H-NS, modulate the conformation of the genome as a whole (Dame *et al.*, 2000; Holbrook *et al.*, 2001; Schneider *et al.*, 2001). They can also have a more direct effect at specific DNA sequences either by altering the local conformation of the DNA or by binding of the DNA directly (O'Byrne and Dorman, 1994b; Marshall *et al.*, 1999; Schneider *et al.*, 1999, 2000).

1.4.1: The HU protein

HU (heat unstable nucleoid protein) is a small basic heterodimeric protein considered to be a prokaryotic homologue of eukaryotic histones. The 2 subunits of HU, HUα and HUβ, are encoded by the *hupA* and *hupB* genes, which are unlinked on the chromosome. They encode polypeptides of 9.2 kDa and 9.5 kDa respectively. *S. typhimurium* is one of the few species of bacteria that has two distinct though highly homologous subunits that form HU (Claret and Rouvière-Yaniv, 1997). HU is known to restrain negative supercoils *in vivo* and along with Fis is the most abundant nucleoid-associated protein in *E. coli* being present at around 20,000 to 30,000 dimers per genome equivalent in exponentially growing cells. The number of HU dimers falls in stationary phase cells to approximately 7,500 (Talukder *et al.*, 1999). Both subunits are not co-ordinately expressed. Actively dividing cells contain approximately 4 times more HUα than HUβ while in stationary phase the amount of each subunit is approximately equal. Therefore exponentially growing cells contain a mixture of homodimeric α2 and heterodimeric αβ forms while stationary phase cells contain predominantly the αβ form (Claret and Rouvière-Yaniv, 1997). HU is a non-sequence specific DNA binding protein and can bind to single-stranded DNA, double-stranded DNA and RNA with high affinity (Talukder *et al.*, 1999; Talukder and Ishihama, 1999). Upon binding, HU wraps the DNA into nucleosome-like particles. The amino acid sequence of HU only differs by one amino acid for each polypeptide between *E. coli* and *S. typhimurium* highlighting the importance of conserving this protein (Hillyard *et al.*, 1990). Indeed HU-like proteins can be found in a wide range of bacteria. Cells that are deleted for either *hupA* or *hupB* behave like wild-type for all functions tested, due to the ability of the protein to form functional homodimers . The 2 subunits HUα and HUβ share approximately 70% amino acid identity (Rouvière-Yaniv and Kjeldgaard, 1979). However cells that are deleted for both *hupA* and *hupB* have reduced viability and perturbed cell division. These double mutants also have an altered chromosome structure and lose approximately 10% of plasmid supercoiling. HU also plays a role in homologous recombination and *hupA hupB* double mutants are sensitive to UV-light (Hillyard *et al.*, 1990).

1.4.2: The IHF protein

The IHF (Integration Host Factor) protein was first identified by its role in the integration and excision of the phage λ (Nash and Robertson, 1981). IHF is a small heterodimeric protein composed of IHFα and IHFβ subunits of 11.2 kDa and 10.7 kDa respectively. These subunits are encoded by the *ihfA* and *ihfB* genes, which are unlinked on the chromosome. IHF is closely related to HU and shares approximately 30% amino acid identity with HU (Drlica and Rouvière-Yaniv, 1987). IHF is an abundant protein being present at approximately 6,000 dimers per cell in exponential phase. The number of IHF dimers increases to approximately 25,000 dimers per cell during the transition from exponential to stationary phase before dropping again late in stationary phase (Talukder *et al.*, 1999). This indicates a potential role for IHF in the structural and functional conversion of the nucleoid during the phase transition of growth. IHF is a sequence specific DNA binding protein and recognises a 13 bp conserved sequence within a 35 bp binding site. The IHF consensus binding sequence is 5'-WATCAANNNNTTR-3' (where W=A/T, R=purine and N=any base) (Craig and Nash, 1984). On binding DNA, IHF introduces bends in the DNA of up to 180° and this can bring together distant sites on the genome (Rice *et al.*, 1996). IHF is known to play a role in transcription, transposition, site-specific recombination and replication initiation (Chalmers *et al.*, 1998; Dhavan *et al.*, 2002; Nasser *et al.*, 2002).

1.4.3: The Fis protein

Fis (Factor for Inversion Stimulation) protein was first identified as a host factor required for stimulation of the homologous Gin and Hin recombinases of bacteriophage Mu and *Salmonella* respectively (Johnson and Simon, 1985; Kahmann *et al.*, 1985). Fis is a small basic homodimeric DNA binding protein with subunits of 11.2 kDa. The Fis protein sequence is identical in *E. coli* and *S. typhimurium* (Beach and Osuna, 1998). Fis is very abundant in early exponential phase when approximately 30,000 dimers are present per cell however this number decreases rapidly and by stationary phase there are less than 50 dimers per cell (Ball *et al.*, 1992; Talukder *et al.*, 1999). Upon binding DNA Fis can induce bends in the DNA (Perkins-Balding *et al.*, 1997). Fis binds DNA via a helix-turn-helix motif in the C-terminus of

the protein and does so in a sequence specific manner. A highly degenerate binding site has been proposed. The proposed core consensus sequence is 5'-GNNYRNNTNNYRNNC-3' (Y=pyrimidine R=purine) (Hubner and Arber, 1989; Finkel and Johnson, 1992). The crystal structure of the wild-type Fis protein was solved however disorder in the N-terminal 25 amino acids prevented resolution of this domain (Yuan *et al.*, 1991). A K36E Fis mutant protein was also crystallised. This mutant protein was deficient in promoting site-specific recombination catalysed by Hin and had an ordered N-terminal domain (Safo *et al.*, 1997). A ribbon diagram of the K36E Fis homodimer can be seen in Fig. 1.4. The two N-terminal β-sheets are involved in activation of DNA inversion by the Hin and Gin recombinases. The α-helices labelled C and D form the C-terminal helix-turn-helix DNA binding domain (Safo *et al.*, 1997). Fis can bind to and regulate a number of genes *in vivo*. The *fis* gene is organised in an operon with a gene of unknown function and this operon is repressed by Fis itself. It is also activated by IHF. Fis activates transcription of many stable RNA promoters and the *fis* gene is negatively regulated by the stringent response (Ross *et al.*, 1990; Ninnemann *et al.*, 1992; Pratt *et al.*, 1997). Fis also plays a role in chromosome replication as it inhibits initiation of DNA replication at *oriC* (Filutowicz *et al.*, 1992).

As a nucleoid associated protein Fis influences DNA topology. However Fis also plays another role in the homeostatic regulation of DNA supercoiling. Fis binds to and represses transcription at the *gyrA* and *gyrB* promoters of *Escherichia coli* (Schneider *et al.*, 1999). After nutrient upshift the [ATP]/[ADP] ratio in the cell increases leading to increased DNA gyrase activity. Nutrient upshift also induces *fis* expression and repression of *gyrA* and *gyrB* by this protein prevents supercoiling reaching detrimentally high levels. Fis also stabilises a plasmid sub-population of intermediate superhelical density after nutrient upshift by binding to the plasmid (Schneider *et al.*, 1997). This again prevents excessive negative supercoiling after nutrient upshift. Fis may also prevent hypernegative supercoiling of the chromosome after nutrient upshift by a similar mechanism although this has not been directly demonstrated. The *fis* gene itself is also supercoiling sensitive. Expression of *fis* is maximal at high levels of supercoiling. This allows Fis to function in maintaining a homeostatic balance in cellular supercoiling levels (Schneider *et al.*, 2000).

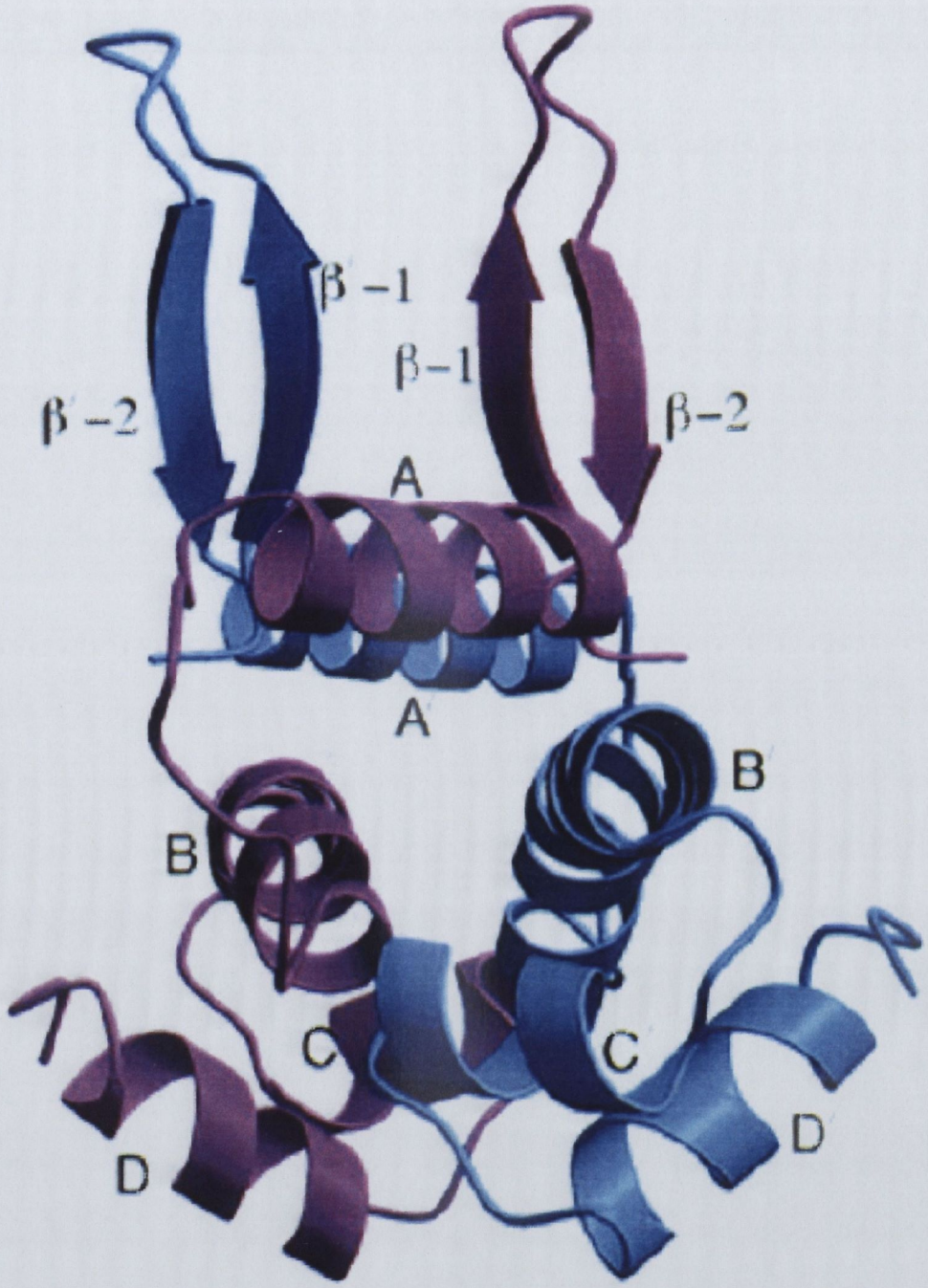


Fig 1.4. **Ribbon diagram of Fis homodimer.** This illustrates the structure of the homodimeric K36E Fis mutant. One monomer is coloured purple and the other blue. α-helices C and D form the C-terminal helix-turn-helix motif involved in DNA binding. The β–sheets marked β1 and β2 are required for the activation of inversion by the Hin and Gin recombinases. Reproduced from Safo *et al.*, 1997.

1.4.4: The H-NS protein

H-NS (Histone-like Nucleoid Structuring protein) is a small heat-stable nucleoid associated protein. The *hns* gene encodes a polypeptide of 15.6 kDa, which can exist as a dimer or a higher order oligomer (Talukder and Ishihama, 1999). Estimations of the number of H-NS molecules in the cell differ significantly with one study measuring approximately 4,000 protein molecules in exponential phase and 20,000 molecules in stationary phase (Spassky *et al.*, 1984). Another study estimated there to be approximately 20,000 molecules per cell in exponential phase and this decreased to 8,000 in stationary phase (Talukder *et al.*, 1999). More recent studies have shown the levels of H-NS to remain constant throughout the growth phase (Deighan, 2001). H-NS binds DNA in a non-sequence specific manner but appears to have a preference for curved DNA (Bracco *et al.*, 1989; Yamada *et al.*, 1990; Zuber *et al.*, 1994). H-NS can also bind RNA albeit with a lower affinity than for DNA (Friedrich *et al.*, 1988). The binding of DNA by H-NS compacts the DNA and constrains negative supercoils as well as regulating the transcription of a large number of genes, usually negatively (Atlung and Ingmer, 1997; Dame *et al.*, 2000). Proteome and transcriptome analysis demonstrated that H-NS plays a major role in bacterial physiology by directly or indirectly controlling the expression of many genes (Hommais *et al.*, 2001). The expression of approximately 5% of the genes in the *E. coli* genome was shown to be altered in a *hns*$^-$ strain. Many of these genes encoded proteins involved in bacterial adaptation to changes in environmental conditions (Hommais *et al.*, 2001; Schröder and Wagner, 2002). The genes regulated by H-NS are scattered around the genome and include the *E. coli* fimbrial genes *pap* and *fim* (Olsen and Klemm, 1994; White-Ziegler *et al.*, 2000), the stationary phase sigma factor RpoS (Barth *et al.*, 1995; Yamashino *et al.*, 1995) and the *Salmonella* plasmid virulence genes (*spv*) (O'Byrne and Dorman, 1994b).

1.4.5: Other nucleoid associated proteins

In addition to the four major nucleoid associated proteins described above, a number of other such proteins exist in the cell. These include Hfq (Host Factor for phage Q_β), LRP (Leucine-responsive Regulatory Protein) and StpA (Supressor of *td*$^-$ phenotype

A). Hfq is a small protein originally identified as a host factor required for phage Q_β replication (Kajitani and Ishihama, 1991). The levels of Hfq are quite high during exponential phase and decrease as the cells reach stationary phase (Talukder *et al.*, 1999). Hfq binds DNA in a non-sequence specific manner (Talukder and Ishihama, 1991) and is essential for *rpoS* mRNA translation (Muffler *et al.*, 1996). LRP functions as a homodimer with subunits of 19 kDa (Cui *et al.*, 1996). The amount of LRP in the cell is higher in exponential phase and decreases as the cells enter stationary phase. LRP binds DNA in a sequence specific manner and is know to regulate the expression of many genes both positively and negatively (Talukder *et al.*, 1999; Talukder and Ishihama 1999). This regulation may be modulated by leucine (Chen *et al.*, 2001). StpA functions as a homodimer with each subunit being 15.3 kDa. StpA can also form heterodimers with the H-NS subunit protein with which StpA has 58% sequence identity (Johansson and Uhlin, 1999; Johansson *et al.*, 2001). The expression of *stpA* is quite high in early exponential phase however it decreases throughout the growth phase (Free and Dorman, 1997). StpA is thought to function primarily as a "molecular backup" for H-NS however its superior RNA binding ability indicates that this protein may carry out some specialised functions also (Deighan *et al.*, 2000). The properties of the major bacterial nucleoid-associated proteins are summarised in Table 1.2.

1.5: Adaptive Mutation

The concept of neo-Darwinian evolution is based on the idea that mutations arise spontaneously, as a result of DNA polymerase replication errors, and the fittest mutants are then selected for by the environment. This proposes that mutations only arise at random and without regard for utility. Therefore the agents of selection play no role in causing mutations, but affect only the relative reproductive success of organisms with different genotypes. The classical experiments of Luria and Delbrück in studying the origin of phage resistant mutants supported this concept (Luria and Delbrück, 1943). They found that the final number of phage resistant mutants after attack by bacteriophage T1 was highly variable from culture to culture, consistent with a rare spontaneous mutation occuring during growth of the cultures prior to selection. If the mutation occurred early in the growth of the culture then a large number of

Protein	Gene	Mass (kDa)	Structure	Preferential Expression
HU	*hupA* *hupB*	9.2 9.5	heterodimer	Exponential and stationary phase
IHF	*ihfA* *ihfB*	11.2 10.7	heterodimer	Entry into stationary phase
Fis	*fis*	11.2	homodimer	Exponential phase
H-NS	*hns*	15.6	Homodimer/ oligomer	Exponential and stationary phase
Hfq	*hfq*	11.2	Monomer/ hexamer	Exponential phase
LRP	*lrp*	19	homodimer	Exponential phase
StpA	*stpA*	15.3	homodimer	Exponential phase

Table 1.2. **Summary of the major nucleoid associated proteins of *S. typhimurium*.**

phage resistant colonies were found. If the mutation occurred later in the growth of the culture then less phage resistant colonies were found. If the mutations had arisen after selection and in response to attack by the bacteriophage then the number of mutants found in each of a large number of independent cultures should form a Poisson distribution. As this was not found they therefore concluded that mutation to phage resistance is the result of rare spontaneous events, occuring during growth of the cultures prior to selection (Luria and Delbrück, 1943). However this experiment did not rule out an adaptive mechanism of mutation because the selective procedure used to determine the number of mutants was lethal for any bacterium that was not already expressing the mutant phenotype. Therefore in order to to study the effect of selection on mutation non-lethal selection must be used. In this case the selection pressure rewards mutants by letting them multiply but allows all the other non-mutant cells to survive so that they can at least have the opportunity to mutate. The controversial topic of selection-induced or adaptive mutation was first introduced by Cairns, Overbaugh and Miller in 1988 (Cairns *et al*, 1988). Cairns *et al.* showed that when *E. coli* was subjected to prolonged, non-lethal selection, advantageous mutations arose in the population while non-advantageous mutations did not. Furthermore, the mutations did not arise in the absence of specific selection for them. This phenomenon has been called directed, adaptive, selection-induced and stationary-phase mutation. Cairns *et al.*, hypothesised that the bacterial cell could affect the sequence of its DNA as a result of an experience or condition it is exposed to and therefore choose which mutations to produce. Stationary phase mutation is thought to be important in a number of processes including the development of antibiotic resistance (Martinez and Baquero, 2000), phase variation in bacterial pathogens (Rosenberg, 2001) and colonisation of new hosts (Giraud *et al.*, 2001). Stationary phase mutations are also studied as a model for mutations that initiate cancer in cells that are not growing actively (Cairns, 1998).

1.5.1: Systems for studying adaptive mutation

The primary model for adaptive mutation, and that studied by Cairns *et al.* is the RecA-dependent reversion of a *lacZ* amber mutation in the *E. coli* strain FC40. FC40 is deleted for the *lac* operon on the chromosome and has a revertible *lacZ* (+1)

frameshift mutation on an F' plasmid. This strain is therefore phenotypically Lac^-. This strain was plated on minimal plates containing lactose and reversion to a Lac^+ phenotype monitored by the ability to grow on this plate. After 2 days growth on the plate a number of Lac^+ colonies had appeared and were attributed to random DNA replication errors that occurred during non-selective growth of the cells prior to plating. This occurred at a rate of 10^{-8} per cell per division. However after another 2 days, more Lac^+ colonies began to appear and continued to do so for 6 days at a constant rate. This is as a result of late genetic events occurring on the plates when the bacteria are in the presence of lactose and under strong selection pressure to become Lac^+. The plated population did not grow during this time and did not accumulate an unselected mutation (valine resistance). Stationary phase reversion to a Lac^+ phenotype also appears to differ mechanistically from growth phase reversion to Lac^+. The late reversion to Lac^+ consists almost entirely of –1 deletions. This is in contrast to Lac^+ reversions that occur during growth, which are heterogeneous (Foster and Trimarchi, 1994). This behaviour suggests that selection directs mutations to growth-promoting sites (Cairns *et al.*, 1988).

Another model system used to study adaptive mutation is the *araB-lacZ* model described by Shapiro (Shapiro, 1984). This system consists of the regulatory region of the arabinose operon and the carboxy-terminal region of the *lacZ* gene separated by a defective Mu bacteriophage. The strain is both Lac^- and Ara^- and is designated $Lac(Ara)^-$. Cells with deletions that fuse *lacZ* in frame with *araB* can utilise lactose if arabinose is present as an inducer. These cells are called $Lac(Ara)^+$. Shapiro found that after plating $Lac(Ara)^-$ cells on lactose-arabinose minimal medium at 32°C, $Lac(Ara)^+$ colonies did not appear until 5-6 days later. If the deletion had occurred during growth of the cells in non-selective conditions prior to plating the colonies should have arisen in 2 days. The frequency of $Lac(Ara)^+$ colony appearance after selection was 3 orders of magnitude greater than the frequency of $Lac(Ara)^+$ colony formation prior to selection. Again in this system the mechanism for the generation of stationary phase $Lac(Ara)^+$ colonies appears to differ from growth phase generation of $Lac(Ara)^+$ (Maenhaut-Michel and Shapiro, 1994; Maenhaut-Michel *et al.*, 1997).

Certain bacteria are classified as late fermenters because they develop the ability to utilise unusual (for them) sugars only after long incubation in the presence of these

sugars. We now know that *E. coli* and other bacteria contain cryptic genes that can be mutationally activated. In some cases more than one mutation is required. The frequency with which such multiple mutants arise exceeds the frequency predicted if the mutations arose independently. Activation of the cryptic evolved β-galactosidase gene *ebgA°* results in hydrolysis of lactose in the absence of *lacZ*. This activation requires two mutations, one in the repressor of *ebgA*, *ebgR*, and one in a particular site in *ebgA* to make the enzyme capable of utilising lactose. Growing cells on lactose minimal plates appears to induce lactose utilisation by EbgA as both mutations occur at a frequency much higher than that predicted for two rare independent events (Hall, 1999). This adaptive mutation event has been shown to be regulated by the PhoP/Q two component regulatory system (Hall, 1998).

1.5.2: Molecular mechanisms of adaptive mutation

Several theories have been suggested as to a possible mechanism for the adaptation of bacteria to non-lethal selection. However as adaptive mutation is a strategy to overcome adversity, not a mechanism it is likely that it proceeds by more than one mechanism (Foster, 1998). Therefore cells may have many diverse ways of achieving this goal. It has been postulated that transcription may be mutagenic. Cells in stationary phase may not be replicating, but genes will still be transcribed. The regions of single-stranded DNA produced by transcription may be more sensitive to DNA damaging agents (Davis, 1989). A number of recent studies have concentrated on elucidating the molecular mechanism of the *lacZ* frameshift mutation in *Escherichia coli* FC40.

A suggestion was put forward by Cairns *et al* in 1988 that there was potentially some sort of reverse flow of information in cells of FC40. This could be that the cell produces a highly variable set of mRNA molecules and then reverse-transcribes the one that makes the best protein (Cairns *et al.*, 1988). This was to account for the apparent direction of the mutation towards specific targets. This theory was quickly discounted (Foster and Cairns, 1992). A number of other theories have since been proposed to explain *lacZ* frame-shifting in FC40 in the presence of lactose. The apparent directionality of the mutation has also been called into question in recent

years. Cells that mutated to give Lac^+ colonies were originally tested for unselected mutations in the chromosomal *rpoB* locus. It was found that mutations did not accumulate at this locus indicating that growth in lactose minimal medium was directing mutations towards the *lac* locus (Foster, 1994). However a subsequent study by the same author found that unselected mutations at another site on the F' plasmid did accumulate. A Tn*10* element, encoded on the F' plasmid was mutated to give tetracycline sensitivity. This sensitivity arose due to a frameshift mutation in the tetracycline resistance gene. The reversion of this element to tetracycline resistance was then measured. It was found that Tet^r mutants do accumulate in Lac^- cells when those cells are under selection to become Lac^+. Therefore the mutation is not directed to the *lac* genes (Foster, 1997). It was also reported that the Lac^- allele needed to be located on the F' episome in order for the mutation to occur. Conjugal functions were also required to be expressed although actual conjugation did not need to take place (Foster and Trimarchi, 1995; Galitski and Roth, 1995; Radicella *et al.*, 1995). It was proposed that nicking of the F' plasmid at the conjugal origin *oriT*, generating single-stranded DNA is the initiating event for adaptive mutation in FC40. Stationary-phase frameshifting of *lacZ* in FC40 has also been shown to require functional proteins of the RecBCD double-strand break repair recombination system. Growth-dependent reversion of the same allele does not (Foster *et al.*, 1996; Harris *et al.*, 1996) thereby showing that they proceed by different mechanisms.

1.5.3: Hypermutation under stress

The possibility that cells generated beneficial mutations by entering a hypermutable state under stress was first proposed by Hall (1990). He proposed that a subset of cells could enter such a state in which they accumulate mutations throughout their genome at a high rate. If a cell produces a useful mutation it could exit the hypermutable state and replicate. Evidence for this model was provided by both Foster and Rosenberg and colleagues (Foster, 1997; Torkelson *et al.*, 1997). Foster showed that unselected Tet^r mutants arose in strain FC40 during selection for Lac^+ adaptive mutations as described above. Rosenberg and colleagues found high frequencies of unselected frameshift reversion, loss of function, and temperature-sensitive mutations at sites on a small multicopy plasmid, on the bacterial chromosome and on the F' plasmid during

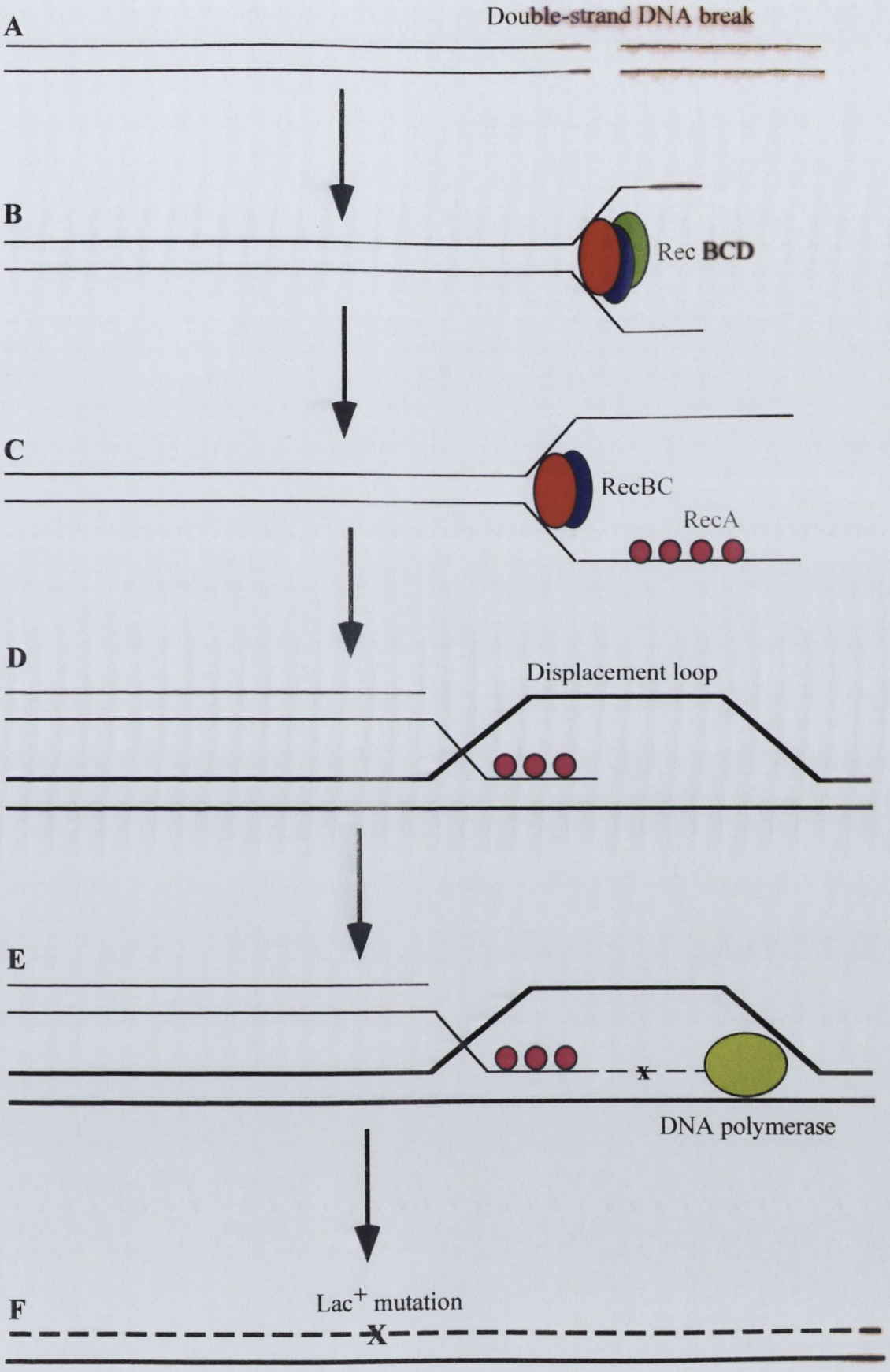
recombination-dependent stationary-phase Lac^+ reversion (Torkelson *et al.*, 1997). They also showed that hypermutation occurred only in a subpopulation of cells by comparing unselected mutations in Lac^+ revertants, Lac^- cells that were exposed to prolonged selection on lactose minimal medium and Lac^- cells that were never exposed to selection. They found that Lac^+ revertants displayed frequencies of unselected mutations 10-100 times greater than either of the two control populations. This indicated that they experienced hypermutation. They also showed that hypermutation in the subpopulation is transient as most Lac^+ revertants did not possess heritable mutator phenotypes.

Rosenberg and colleagues proposed a mechanism for adaptive frameshifting of *lacZ* in FC40 based on recombination (Bull *et al.*, 2000). They proposed that double-stranded breaks occur in the DNA. Such breaks can be generated by nicking of the DNA at *oriT*, disintegration or stalling of a replication fork or some other mechanism. This double-strand end is processed by the RecBCD enzyme, creating single-stranded DNA ends. RecA binds the single-stranded DNA and catalyses the invasion of a homologous duplex producing a displacement loop. The invading strand then serves as a primer and loading site for DNA polymerase III (Liu *et al.*, 1999). Errors produced during DNA synthesis by DNA pol III remain uncorrected due to a transient deficiency in the mismatch repair enzyme MutL (Longerich *et al.*, 1995; Harris *et al.*, 1997). The error then becomes genetically fixed giving a Lac^+ phenotype. A diagram of this mechanism can be seen in Fig. 1.5.

1.5.4: Gene amplification underlies adaptive mutability

Recently Roth and colleagues have proposed another mechanism for reversion of the *lacZ* frameshift mutation in FC40 (Andersson *et al.*, 1998; Hendrickson *et al.*, 2002). They proposed that gene amplification was the mechanism by which the *lac* operon underwent adaptive mutation. This model involved the selected amplification of the mutant *lac* locus, which retained some residual function. The growth of cells with multiple copies of the *lac* region increases the yield of detected mutations by increasing the number of targets. This can occur without any change in the mutation rate per gene copy. The *lacZ* frameshift allele in FC40 produces about 1% of the β-

Fig. 1.5. **Model for recombination-dependent reversion of a *lacZ* frameshift mutation.** (A) A double-stranded break occurs in the DNA. (B) This double-stranded break is processed by the RecBCD enzyme complex. (C) RecA binds the single-stranded DNA subsequently produced and (D) invades homologous DNA. The invading strand serves as a primer for DNA synthesis by DNA polymerase III. (E) This DNA replication may result in errors in DNA synthesis which remain uncorrected due to a temporary deficiency in the cellular mismatch repair function. (F) The error, X, which results in a Lac^+ phenotype becomes genetically fixed in the cell. Newly synthesised DNA is represented by dashed lines. Figure adapted from Bull *et al.* (2000).


A
Double-strand DNA break
B
Rec BCD
C
RecBC
RecA
D
Displacement loop
E
DNA polymerase
F
Lac+ mutation

galactosidase made by the reverted gene. Spontaneous duplication of the mutant *lac* operon increases β-galactosidase activity in proportion to copy number. Cells with a *lac* duplication initiate slow-growing microcolonies in which further amplification is selected. This enhances the probability that some copy of the *lac* operon will acquire a lac^+ reversion event. Selection then favours descendants of this cell that maintain the lac^+ allele and lose the mutant copies by recombination between repeats. They found that use of a *galKT* mutation, which reduces by half the amount of energy that can be derived from lactose, substantially reduced the yield of late mutants (Andersson *et al.*, 1998). Quantitive Southern blotting was also used to show *lac* amplification. Roth and colleagues also proposed that the high frequency of unselected mutations seen in Lac^+ revertants is a result of recombination between repeated sequences. Such segregation releases DNA fragments that are degraded into single-stranded DNA. This induces the SOS response and its error prone polymerase *dinB* (section 1.6). The mismatches produced by DNA polymerase IV exceed the capacity of the mismatch repair system (MMR), and genome-wide general mutation ensues. This mutagenesis continues until cells lose the mutant *lac* copies and hence the source of SOS induction (Hendrickson *et al.*, 2002). This model differs from others in that it suggests that stress has no direct effect on the general mutation rate, reversion does not occur in stationary phase cells and mutation does not reflect error prone recombination. Additional support for this model comes from the fact that late reversion to Lac^+ consists almost entirely of –1 frameshifts. Such –1 deletions are characteristic of slipping of DNA polymerase and implies that DNA synthesis is taking place. The role of RecA in reversion is due to its requirement for formation and segregation of amplifications and for SOS induction. Roth and colleague also proposed that the requirement for the *lacZ* frameshift mutation to be located on the F' plasmid is due to the fact that duplication formation and high level amplification are rare for chromosomal sites (Slechta *et al.*, 2002).

1.5.5: A mechanism for directed evolution

A mechanism by which mutation, and hence evolution could be directed towards specific sites was proposed by Barbara Wright (Wright, 2000). When cells enter stationary phase they undergo major metabolic transitions, requiring the transcription

of many genes (Cashel *et al.*, 1996). There is also a sharp increase in local DNA supercoiling associated with this transcription. This may produce DNA secondary structures that are precursors of mutations. Therefore a gene may be hypermutable because it contains a hot-spot due to a particular DNA sequence. Its hypermutability *per se* is undirected since it is unrelated to the selective conditions and the function of the gene. These random mechanisms resulting in hypermutation are in essence serendipitous relationships. Transcription exposes single-stranded DNA. The unprotected single-strand in the transcription bubble is significantly more vulnerable to mutations than the transcribed strand, which is protected as a DNA-RNA hybrid (Fix and Glickman, 1987). Transcription also drives localised supercoiling (section 1.3.6) this can result in the formation of stem-loop structures at regions of high superhelicity, which could result in localised hypermutation. Mutability of specific sites in the genome can vary by orders of magnitude. This reflects particular characteristics of the DNA sequence associated with hot-spots (Benzer, 1961). Environmental stress targets specific genes for derepression. These genes are transcribed, which exposes the non-transcribed strand and drives local supercoiling. Secondary structures then form in the DNA, which can create unpaired and mispaired bases. This causes hypermutation at vulnerable sites and increases the number of variants. Some of these variants can survive the environmental stress and the environment selects for the fittest variant. This suggests a sensitive directed feedback mechanism initiated by different kinds of stress that can facilitate and accelerate the adaptation of organisms to new environments. Such mutations do not require the RecA protein (Wright, 2000).

Loci that undergo mutations more readily in the adaptive process have been called contingency loci and have been described as hypermutable sequences that allow bacteria "to explore precipitous and unpredictable aspects of their environment, while minimising deleterious effects on fitness" (Moxon and Thaler, 1997). Certain pathogens have subsets of genes that are excessively prone to mutation through slipped-strand mispairings, gene conversions and point mutations. Frameshift mutations appear to be highly relevant to bacterial evolution. Many bacteria regulate the expression of contingency genes used in their battles against host defences by frequent frameshift mutations in simple repeated sequences. Frameshifting is a mechanism of phase variation employed by serogroup B *Neisseria meningitidis* to vary capsular polysaccharide production. This bacterium can generate +1 or –1 frameshifts

in a run of C residues in the coding sequence of the polysialtransferase gene *siaD*. This results in either the production of functional SiaD or a truncated non-functional version. Capsular polysaccharide is only produced by full-length SiaD (Hammerschmidt *et al.*, 1996). *N. gonorrhoeae* also varies expression of the *pilC* gene by a similar mechanism (Jonsson *et al.*, 1991). This mechanism turns genes on and off by switching reading frames and can also be used to activate promoters.

1.6: The SOS response

The SOS system was the first *E. coli* regulatory network to be identified that is induced by DNA damage. This response is due to the induction of *din* (damage inducible) genes (Walker, 1996). The expression of genes in the SOS regulatory network is controlled by a complex circuitry involving the RecA and LexA proteins. In an uninduced cell the LexA protein acts as a repressor of many genes by binding to operator sequences upstream of each gene (Lewis *et al.*, 1994). Regions of single-stranded DNA are the signal that triggers the SOS response. The binding of RecA to single-stranded DNA converts RecA to an activated form RecA*. The interaction of RecA* with LexA results in the proteolytic self-cleavage of LexA. This allows the genes in the SOS regulon to be expressed (Yasuda *et al.*, 1998). Genes controlled by LexA include the *uvr* genes required for excision repair after exposure to UV-light and the *polB* and *dinB* genes that encode DNA polymerase II and DNA polymerase IV respectively (Kenyon and Walker, 1981; Iwasaki *et al.*, 1990; Wagner *et al.*, 1999). DNA polymerase IV is an error prone polymerase responsible for introducing mutations into cells during times of stress (Wagner *et al.*, 1999). When the DNA is repaired and no single-stranded DNA is being released, RecA is no longer activated, LexA is not digested and the SOS genes are repressed in the cell (Walker, 1996). RecA also functions in homologous recombination. The essential steps in homologous recombination are homologous pairing of two DNA chromosomes and exchange of DNA between them. The recombination event begins with a linear duplex DNA generated by double-strand breakage (or conjugation) that is either unwound by a helicase or degraded by a nuclease to produce a single stranded DNA tail. RecA then polymerises onto the ssDNA region to form a helical nucleoprotein filament, which then searches for its homologous duplex DNA target. RecA coated ssDNA searching

for its homologous sequence buried in non-RecA-coated dsDNA initiates the homologous recombination process (Cai *et al.*, 2001).

1.7: Overview

This study examines mechanisms by which bacteria can adapt to new environmental conditions. Firstly it looks at the adaptation of stationary phase *S. typhimurium* strain CJD671 to a lactose-containing environment. This adaptation occurs via a stochastic genetic event in some cells and results in the upregulation of a *lacZ* gene that has been inserted into the *spvB* virulence gene of CJD671. This event is shown to require increased cellular DNA supercoiling, carried out by DNA gyrase and to occur within a restricted range of environmental conditions.

This study also looks at the regulation of the *S. typhimurium* DNA gyrase genes. DNA gyrase is also known to facilitate bacterial adaptation to new environmental conditions by differentially regulating subsets of genes in response to environmental conditions. The *S. typhimurium gyrA* promoter is shown to differ from that of *E. coli* although both retain regulation by Fis. The differences may be important for the adaptation of *Salmonella* to environments where *E. coli* are at a disadvantage such as inside macrophages.

Chapter 2

Materials and Methods

2.1: Chemicals and growth media

2.1.1: Chemicals, reagents and radionucleotides

The supplier for all chemicals and reagents used in this study is indicated in parenthesis after the product. DNA restriction and modifying enzymes were obtained from New England Biolabs or Roche Molecular Biochemicals. Radionucleotides were supplied by Amersham Pharmacia Biotech. Custom automated sequencing was performed by MWG Biotech, Anzingerstr. 7, Ebersberg, D-85560, Germany. In addition, several molecular biology kits were used during this study. The basic principle of each kit is briefly described in the appropriate sections below, without giving an exhaustive protocol.

2.1.2: Growth media

Salmonella and *Escherichia coli* can grow rapidly in minimal media such as M9-broth which contains a carbon source, (generally glucose which also acts as an energy source), and salts which supply nitrogen, phosphorus and other trace metals. From these starting materials the bacteria can make all the amino acids, nucleotide precursors, vitamins and other metabolites required for growth. In contrast, all of these are supplied in a rich broth such as Luria-Bertani (LB) broth.

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

LB broth and LB agar plates:

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

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

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

MacConkey Lactose agar plates:

Red mutants arose on this medium and MacConkey Lactose agar plates were used to indicate the levels of β-galactosidase activity of bacterial colonies. A constituent of MacConkey base is phenol red, a pH indicator which turns red under acidic conditions. β-galactosidase expressing colonies are a red colour due to the production of acidic metabolic products during the fermentation of lactose. In contrast colonies not expressing, or expressing very low levels of β-galactosidase are white or a pale pink colour.

MacConkey lactose agar: 40 g MacConkey agar base (Difco), 10 g lactose (added after autoclaving), 15 g agar.

Lactose salt (LS-lac) broth:

This medium was used to induce the appearance of red mutants in broth culture. Following growth in this medium, the bacteria were plated on MacConkey Lactose agar to differentiate between the wild-type and red mutant colonies.

Lactose salt broth: 10 g tryptone, 5 g yeast extract, 21.1 g NaCl, 10 g lactose.

Lactose (L-lac) broth:

Red mutants of CJD661 but not CJD671 could appear in this medium. Following growth in this medium, the bacteria were plated on MacConkey Lactose agar to differentiate between the wild-type and red mutant colonies.

Lactose broth: 10 g tryptone, 5 g yeast extract, 5 g NaCl, 10 g lactose.

SOC medium:

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

SOC medium: 20 g tryptone, 5 g yeast extract, 0.5 g NaCl. After autoclaving 0.95 g $MgCl_2$, 1.2 g $MgSO_4$ and 1.8 g glucose are added.

Green plates:

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

Green plates: 8 g tryptone, 1 g yeast extract, 5 g NaCl. After autoclaving 21 ml 40% glucose, 25 ml 2.5% Alizarin yellow (freshly prepared) and 3.3 ml of 2% Aniline blue are added.

2.1.3: Antibiotics, X-Gal and IPTG

All stock antibiotic solutions were stored in aliquots at –20°C and those prepared in water were sterilised by filtration through 0.2 μm Acrodisc Filters (PALL). Carbenicillin, kanamycin and streptomycin were prepared as 50 mg/ml stock solutions in ddH_2O and used in media at a concentration of 50 μg/ml. Tetracycline was prepared as a 10 mg/ml stock solution in 100% ethanol and used at a final concentration of 10 μg/ml. Chloramphenicol was prepared as a 25 mg/ml stock solution in 100% ethanol, and used at a final concentration of 25 μg/ml. Novobiocin was prepared at a stock concentration of 50 mg/ml in ddH_20 and used at the concentrations indicated. Oxolinic acid was prepared as a 50 mg/ml stock solution in ddH_2O. NaOH was added until the antibiotic went into solution. Oxolinic acid was used at the final concentrations indicated.

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

IPTG (isopropyl-β-D-thiogalactopyranoside), a gratuitous inducer of the *lac* promoter through inactivation of the LacI repressor, was prepared as a 100 mM stock solution in ddH_2O, filter sterilised, and stored at –20°C. IPTG was used at the final concentration indicated in broth cultures to induce expression of genes under the control of the *lac* promoter.

2.2: Bacterial strains and culture conditions

2.2.1: Bacterial strains

All bacterial strains used in this study were derivatives of *S. enterica* serovar Typhimurium LT2, SL1344 or C5 or derivatives of *E. coli* K-12 and are listed in Table 2.1. Bacterial strains were maintained as permanent stocks in 15% glycerol in LB broth and stored at –70°C.

2.2.2: Bacterial culture conditions

Bacterial cultures were grown aerobically in liquid medium at 37°C except where otherwise stated. Cultures were typically grown by inoculating single colonies into 5 ml of LB broth in a sterile test-tube and incubating at the required temperature overnight. When larger volumes were required cultures were generally grown by inoculating fresh media at a dilution of 1:500 in a suitably sized conical flask.

2.2.3: Assay for red mutants

The ability of a strain to form the 5 kb deletion which leads to the *rlgA-spvR* transcriptional fusion and the concomitant upregulation of *lacZ* transcription with the production of red colonies on MacConkey lactose indicator plates was assayed by growing the strain for 4 days in LS-lac media. At 24 hour intervals 10^{-6} and 10^{-7} dilutions of the culture were plated on MacConkey lactose indicator plates. After

Strain	Relevant Genotype	Reference/Source
Salmonella typhimurium		
C5	Wild-type mouse virulent	G. Dougan
CH23	LT2 *recA1*	C.F. Higgins
CJD574	LT2 *hisD::*MudJ	O'Byrne *et al.*, 1992
CJD661	LT2 *spvR::*MudJ	O'Byrne and Dorman, 1994a
CJD671	LT2 *spvB*::MudJ	O'Byrne and Dorman, 1994a
CJD671*fis*$^-$	CJD671 *fis::cat*	This study
CJD671*ihf*	CJD671 *ihfB::cat*	This study
CJD671*rpoS*$^-$	CJD671 *rpoS::amp*	This study
CJD1081	LT2 *tppB::*MudJ	Gibson *et al.*, 1984
CJD1127	5 kb deletion mutant of CJD671	Massey *et al.*, 1999
JF2690	UK1 *rpoS::amp*	Lee *et al.*, 1995
LB5010	LT2 *galE* restriction$^-$ modification$^+$	This lab
LT2	Wild-type	This lab
RM1	5 kb deletion mutant of CJD661. Arose in L-lac	This study
RM2	5 kb deletion mutant of CJD661. Arose in L-lac	This study
RM3	5 kb deletion mutant of CJD661. Arose in LS-lac	This study
RM4	5 kb deletion mutant of CJD661. Arose in LS-lac	This study
SL1344	Wild-type mouse virulent	Dorman *et al.*, 1989a
SL1344*fis*$^-$	SL1344 *fis::cat*	This study
SL1344*hns*$^-$	SL1344 *hns::kan*	This study
SL1344*ihf*	SL1344 *ihfB::cat*	This study

SL1344*lrp*⁻	SL1344 *lrp::kan*	This study
SL1344*rpoS*⁻	SL1344 *rpoS::kan*	This study
TH2285	LT2 *fis::cat*	K.T. Hughes
Escherichia coli		
BL21(DE3)	F⁻ *dcm ompT hsdS gal*	Studier and Moffatt, 1986
CJD898	GM37 *rssB::amp*	Free, 1995
MC4100	F⁻ λ⁻ *araD139* Δ(*argF-lac*)*U169 rpsL150 relA1 flbB5301 deoC ptsF25 rbsR*	Casabadan, 1976
SM10λpir	*thi thr leu tonA lacY supE recA*::RP4-2Tc::Mu λpir	Simon *et al.*, 1983
TM 2	MC4100p671 *sucA::cat*	This study
TM 6	MC4100p671 *rssB::cat*	This study
TM 20	MC4100p671 *sdhD::cat*	This study
TM 27	MC4100p671 *thiE::cat*	This study
TM 28	MC4100p671 with *cat* inserted upstream of *cspC*	This study
TM 31	MC4100p671 *traV::cat*	This study
TM 42	MC4100p671 *PSLT002::cat*	This study
TM 47	MC4100p671 *PSLT001::cat*	This study
XL1 Blue	*recA*1 *endA*1 *gyrA*96 *thi*-1 *hsdR*17 *supE*44 *relA*1 lac [F'*proAB lacI*^{q}ZΔM15Tn*10*] tet^{R}	Stratagene

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

overnight incubation at 37°C the number of red and white colonies could be counted and the percentage of red mutants calculated.

2.3: Plasmids bacteriophages and oligonucleotides

2.3.1: Plasmids

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

2.3.2: Bacteriophages

The bacteriophages used in this study were bacteriophage P22HT105/1 *int*-201 and P1 *vir*, lysates of which were routinely stored at 4°C in the dark in 5 ml volumes over chloroform.

2.3.3: Oligonucleotides

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

2.4: Genetic Techniques

2.4.1: Transduction with P22-Background

Bacteriophage P22 is a temperate phage that specifically recognises *S. typhimurium* by binding to the O-antigen on its outer membrane. After binding, double stranded linear

Plasmid	Relevant Features	Source
p1-65	7 kb *Eco*RI fragment of *Salmonella gyrA* region in pBR322	This study
p671	pSLT from CJD671, *spvB*::MudJ	O'Byrne and Dorman, 1994a
pACYC177	p15A replicon, Amp^R Kan^R	Chang and Cohen, 1978
pACYC184	p15A replicon, Cm^R Tet^R	Chang and Cohen, 1978
pBR322	pMB1 replicon, Amp^R Tet^R	Bolivar *et al.*, 1977
pBSL181	R6Kγ replicon, Amp^R Cm^R	Alexeyev and Shokolenko, 1995
pDIA17	*lacI* repressor, Cm^R	P. Bertin
pFisC	*fis* + 6 histidine tag cloned downstream of IPTG inducible T7 promoter in pET22b	This study
pGB637	pACYC184 containing *rssB* and *galU*, Tet^R	Barr *et al.*, 1992
pGyrA-lacZ	*gyrA* promoter from SL1344 cloned upstream of promoterless *lacZ* in pQF50	This study
pGyrB-lacZ	*gyrB* promoter from SL1344 cloned upstream of promoterless *lacZ* in pQF50	Marshall *et al.*, 2000
pQF50	pMB1 replicon, *lacZ* promoter trap vector, Amp^R	Farinha and Kropinski, 1990
pUC18	ColE1 replicon, Amp^R	Yanisch-Perron *et al.*, 1985
pUHE25-2	*fis* under the control of P_{tac} Amp^R	G. Muskhelishvili

Table 2.2. **Plasmids used in this study.**

Table 2.3. **Oligonucleotides used in this study.** Restriction endonuclease cleavage sites are underlined.

Primer name	Primer sequence
delMF	5'-CAGACAACCTTTCTCGCCTG-3'
delMR	5'-GAATGTTGAGTTGACCGCAG-3'
PvuF	5'-CATGCGCTATCTCAGCAAG-3'
PvuR	5'-CTGTTCTGAGCAGTGAATG-3'
KpnF	5'-CAGTATGGCAACGCGACC-3'
KpnR	5'-GTACCTGTGGATCTACACC-3'
CatFwd	5'-CACTGGATATACCACCGTTG-3'
CatRev	5'-GCCACTCATCGCAGTACTG-3'
Catseq	5'-ATGGCTTCCATGTCGGCAG-3'
delTMF	5'-GATCGGCATCGCTCAGTCC-3'
delTMR	5'-CTATGAATGTTGAGTTGACC-3'
EcogyrAF	5'-TTGCCACCTGCAATGGCTC-3'
EcogyrAR	5'-TCTCGCAAGGTCGCTCATG-3'
gyrAmidF	5'-GGCAATGACTGGAACAAAGCC
gyrAprobeR	5'-GAACCGAAGTTACCCTGACC-3'
gyrAbam	5'-CGCGGATCCTATGTCGCGCCGGAACAG-3'
gyrAkpn	5'-CGGGGTACCCTAACCGCTATCCCTCTAG-3'
lacZ50	5'-CCCAGTCACGACGTTGTAAAACGACGGCC-3'
Ndelfis	5'-GGAATTCCATATGTTCGAACAACGCGTAAATTC-3'
C-TERrev	5'-CCGCTCGAGGTTCATGCCGTATTTTTTTAATTT-3'
InspvRF	5'-GGCTGGCCGGAACACCTG-3'
InspvRR	5'-CTCCCGTTTCTTGGTCGG-3'
gyrABS	5'-ACAGCGCTTGCGCTAACC-3'
DNase2R	5'-ATACCTCCCGTGTATACCC-3'
gyrBBSF	5'-GGAGCGAAGCTTTGAGCG-3'
gyrBBSR	5'CATCCAGCCCTTTCAGGAC-3'
DNasegyrAF	5'-AGTAACTCGCAGCCAACG-3'
DNaselacZR	5'-GTTTTCCCAGTCACGACG-3'
DNasegyrBPF	5'-TGTGCGCCTTACGTCTG-3'
DNasegyrBPR	5'-GTCGCCGATATACATACC-3'

GGATCC = *Bam*HI, GGTACC = *Kpn*I, CATATG = *Nde*I, CTCGAG = *Xho*I

DNA is injected into the host. The DNA circularises and is replicated first by O replication and then by rolling circle replication which generates long concatemers of double stranded P22 DNA. These concatemers are resolved by cleavage by a phage encoded nuclease, which cuts the DNA at specific sequences, 44 kb apart, called Pac sites. This DNA is packaged into new phage particles, which are released from the host by lysis after 50-100 of these particles have been produced. In this study the P22 derivative used for generalised transduction in *S. typhimurium* was P22 HT105/1 *int*-201. The high transducing frequency of this phage results from its nuclease having a lower specificity for the Pac sequence. This results in a high proportion of the phage heads carrying chromosomal DNA. Approximately 50% of the P22 HT (high transducing) phage heads carry random transducing fragments of host DNA. The *int* mutation prevents the formation of stable lysogens.

2.4.2: Preparation of a P22 lysate

P22 lysates were routinely prepared as follows: the donor strain was grown overnight in 2 ml of LB broth at 37°C, with antibiotic selection as appropriate. This culture was used to inoculate 5 ml of fresh broth at a 1:200 dilution. This culture was incubated at 37°C with shaking until the OD_{600nm} reached approximately 0.15. At this point 10 µl of P22 phage stock (titre of approximately 10^{10} pfu/ml) was added and incubation continued for a further 4 hours. At this point 500 µl of chloroform was added, the culture mixed by vortexing and stored for 1 hour at 4°C. Cellular debris was removed by centrifugation in a bench-top centrifuge at 6,000 r.p.m. for 20 min. The supernatant, containing the lysate was transferred to a clean tube and stored over chloroform.

2.4.3: Transduction with P22

Genetic markers were mobilised using the general transducing properties of P22 lysates as follows: the recipient strain was grown overnight at 37°C in 2 ml of LB broth. 100 µl of the recipient culture was removed to a sterile Eppendorf tube

containing 100 µl of the donor P22 lysate. The mixture was incubated at 30°C without shaking for 30 min. 800 µl of LB broth was then added and the culture was incubated at 37°C with shaking for a further 30 min. The transduced cells were then harvested by centrifugation in a microfuge for 4 min at 14,000 r.p.m. 900 µl of the supernatant was then removed and the pellet resuspended in the remaining broth. Transduced cells were selected for by plating on agar plates with the appropriate antibiotic. The plates were incubated at 37°C overnight. True lysogens were then distinguished from pseudo-lysogens by 3 repeated single colony purifications on Green agar plates. On these plates true lysogens appear light green while pseudo-lysogens appear dark green. At this point transductants were deemed suitable for further experimentation.

2.4.4: Transduction with P1 *vir*-background

Bacteriophage P1 *vir* was used to transduce bacterial DNA from a donor to a recipient *E. coli* strain. For phage P1, between 110 kb and 115 kb of bacterial DNA (~2.3% of the *E. coli* genome) can be encapsidated into phage particles during lytic growth of the phage in the donor cell. Transducing particles represent between 0.3% and 6% of total phage particles in any P1 lysate and comprise less that 5% phage DNA.

2.4.5: Preparation of a P1 *vir* lysate

P1 *vir* lysates were made of the required donor strain as follows: 5 ml of LB broth containing 0.2% glucose and 5 mM $CaCl_2$ was inoculated with 50 µl of an overnight culture of the donor strain. This was incubated at 37°C with aeration for 1 hour. 0.1 ml of a P1 *vir* lysate was then added (titre approximately 5 x 10^8 pfu/ml) and incubation continued for 2-3 hours until the cells lysed. 0.1 ml of chloroform was added and the suspension vortexed. The suspension was then centrifuged at 5,000 r.p.m. in a bench top centrifuge for 10 min to pellet cell debris. The lysate was then transferred to a clean tube where it was stored over chloroform at 4°C.

2.4.6: Transduction with P1 *vir*

P1 *vir* lysates were used to transduce marked alleles from one *E. coli* strain to another. The recipient strain was grown overnight in 5 ml of LB broth. The cells were then centrifuged at 5,000 r.p.m. in a bench top centrifuge for 10 min and resuspended in 2.5 ml 10 mM $MgSO_4$, 5 mM $CaCl_2$. The cells were then incubated at 37°C for 20 min. Recipient cells and donor phage lysate were then added to 5 sterile test-tubes in the following ratios:

Tube No.	Volume recipient cells	Volume P1 lysate
1	100 µl	-
2	100 µl	10 µl
3	100 µl	50 µl
4	100 µl	100 µl
5	-	100 µl

Following mixing the cells were incubated in a static 30°C waterbath for 30 min to allow phage adsorption to cells. 100 µl of 1 M sodium citrate was then added followed by 1 ml of LB broth. The cells were then incubated at 37°C for 1 hour to allow phenotypic expression of the transduced marker. Sodium citrate chelates Ca^{2+} and Mg^{2+} ions thus preventing loss of transductants by further superinfection with viral particles. Cells were then pelleted, resuspended in 100 µl LB broth, spread onto selection plates containing 10 mM sodium citrate and incubated at 37°C overnight. Plates corresponding to tubes 1 and 5 control for recipient cells and phage lysate contamination respectively. Transductants from plates 2-4 inclusive were streaked to purity 3 times on LB plates to obtain phage-free cells.

2.4.7: Transformation of bacterial cells with plasmid DNA using the calcium chloride method

For routine transformation of *E. coli* strains with plasmid DNA or ligation mixes the calcium chloride method was employed. An overnight culture of the strain to be made competent for transformation was used to inoculate 250 ml of LB broth and was grown to an OD_{600nm} of approximately 0.4. The cells were incubated on ice for 30 min and then pelleted by centrifugation at 3,500 r.p.m. for 10 min, and the bacterial pellet resuspended in 100 ml of ice-cold $CaCl_2$ solution (60 mM $CaCl_2$, 15% glycerol, 10 mM PIPES pH 8.0). The cells were spun again for 10 min at 2,500 r.p.m., the supernatant removed and resuspended in 100 ml of ice-cold $CaCl_2$ solution. After incubation on ice for 30 min cells were again harvested as described above, and resuspended in 10 ml ice-cold $CaCl_2$ solution. At this stage cells were distributed into 500 µl aliquots and used directly, or stored at –70°C.

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

2.4.8: Transformation of bacterial cells by electroporation

Typically 250 µl of an overnight culture of the strain to be made electrocompetent for transformation was used to inoculate 250 ml of LB broth and grown to an OD_{600nm} of 0.6. Cells were incubated on ice for 30 min and then pelleted by centrifugation at

5,000 r.p.m. for 10 min and the bacterial pellet resuspended in 100 ml of sterile, ice-cold ddH_2O. The cells were again pelleted by centrifugation at 5,000 r.p.m. for 20 min and resuspended in 100 ml cold ddH_2O. Following another centrifugation step, cells were resuspended in 500 µl of ice-cold 10% (v/v) glycerol. Aliquots (120 µl) were used directly or stored at –70°C.

The DNA to be electroporated (50–200 ng in 5 µl sterile water) was added to a 40 µl aliquot of electrocompetent cells and incubated on ice for 1 min. The mixture was then transferred to a pre-chilled electroporation cuvette (EquiBio, 0.2 cm gap width). The cuvette was placed in the Gene Pulser chamber (Bio-Rad) and an electroshock delivered. To the cuvette 1 ml of pre-warmed SOC broth was added and the contents transferred to a sterile tube and incubated at 37°C with aeration for 1 hour. This incubation allows phenotypic expression of the antibiotic resistance marker. Subsequently 100 µl of the transformation mix was plated onto appropriate selection plates. When the *Salmonella* large virulence plasmid (pSLT) was being electroporated, after 1 hour incubation the electroporation mix was spun at 14,000 r.p.m. for 4 min, 900 µl of supernatant was removed and the pellet resuspended in the remaining medium. This was then plated onto agar plates with appropriate antibiotic selection. Cells to which no DNA had been added were treated in the same way and thus served as a control for contamination. Following overnight incubation at 37°C, transformants were single colony purified.

2.4.9: Analysis of *recA* mutants

The presence of a *recA* mutation was confirmed by a simple test that measured the sensitivity of the mutant to ultraviolet radiation. The RecA protein is induced upon exposure to ultraviolet radiation and cleaves the LexA repressor protein, which allows the expression of a large number of genes known as the SOS regulon. The products of these genes facilitate a cellular response, the SOS response, which helps protect the cell against the effects of ultraviolet radiation and other DNA damaging agents (Walker, 1996). RecA mutants, being unable to induce the SOS response, are more

sensitive to ultraviolet radiation that wild-type strains. This sensitivity was tested qualitatively as follows:

Strains to be tested were streaked in parallel across LB plates. The plates were divided into 4 segments by parallel lines drawn down the plate with a ruler and labelled 1 to 4. The plate was placed upside down on an ultraviolet transilluminator, with a piece of card positioned to protect all but segment 1 from ultraviolet radiation. The transilluminator was then switched on for 30 seconds. The card was then repositioned so that both segments 1 and 2 were exposed, and the plate irradiated for a further 15 seconds. This procedure was repeated until segments 1 to 4 had been irradiated for a total of 60, 30, 15 and 0 seconds respectively. The plates were then incubated at 37°C overnight. Wild-type cells grew in sections of the plate irradiated for 30 seconds or less, although the number of cells in the area irradiated for 30 seconds was severely reduced. In contrast, the *recA* mutant exhibited no growth in any segment irradiated with ultraviolet radiation.

2.5: Assays based on spectrophotometry

2.5.1: Monitoring bacterial growth

The growth of bacterial cultures was monitored by measuring the optical density of the culture at a wavelength of 600nm (OD_{600nm}). For routine measurement of OD_{600nm}, including estimation of cell quantity for β-galactosidase assays (section 2.5.2), 0.2–1 ml of the culture was transferred into a plastic disposable cuvette (Greiner), and brought to a final volume of 1 ml with LB broth. The OD_{600nm} value was measured in a spectrophotometer against a cuvette containing only LB broth as a blank. This value was linear in the range 0.1–0.8 and was multiplied by the dilution factor if necessary.

2.5.2: Assay of β-galactosidase activity

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

The β-galactosidase assay used in this study was a modified, scaled-down version of that described by Miller (1992). The bacterial culture (100 µl) to be assayed was transferred in duplicate into tubes containing 675 µl Z-buffer, 50 µl $CHCl_3$ and 25 µl of 0.1% SDS. Tubes were vortexed briefly to enhance permeabilisation, and then incubated for 10 min at 28°C before addition of 150 µl of ONPG (4 mg/ml in ddH_2O). Incubation was continued until a straw yellow colour was obtained (typically corresponding to an OD_{420nm} reading of 0.1-0.6). The reaction was stopped with the addition of 375 µl 1 M Na_2CO_3, and the tubes were centrifuged at 14,000 r.p.m for 5 min to pellet cellular debris. Of the supernatant 1 ml was transferred into a plastic cuvette and the OD_{420nm} determined. The amount of β-galactosidase activity was expressed in arbitrary Miller units and calculated as follows:

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

t = Reaction time in min (from addition of ONPG to addition of Na_2CO_3)

V = Volume of cells added in ml.

Each assay was performed in duplicate, and the mean values were determined from at least 3 independent experiments. Unless otherwise indicated, standard deviations were less than 8%.

Z buffer (1 litre): 60 mM $Na_2HPO_4.2H_2O$, 40 mM $NaH_2PO_4.2H_2O$, 50 mM β-mercaptoethanol, 10 mM KCl, 1 mM $MgSO_4.7H_2O$

2.5.3: Determination of nucleic acid concentration

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

An OD_{260nm} value of 1 corresponds to -

50 μg/ml of double-stranded DNA

40 μg/ml of single-stranded DNA or RNA.

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

2.5.4: Determination of protein concentration by the Bradford assay

Protein concentration was determined using a Bio-Rad Protein Assay which is based on the method of Bradford (1976), and measures the differential colour change (shift in absorbance from 465 to 595 nm) of Coomassie Brilliant Blue G-250 when protein binding occurs. The concentration of His-tagged purified Fis (section 2.17) was determined by measuring several serial dilutions. The resulting OD_{595nm} measurements were compared to a standard curve determined by measuring several known concentrations of lysozyme.

2.6: Preparation of RNA, plasmid DNA and chromosomal DNA

2.6.1: Small-scale isolation of plasmid DNA

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

2.6.2: Large-scale preparation of high purity plasmid DNA

The QIAGEN midi-plasmid purification kit was used to extract plasmid DNA from 100 ml overnight cultures of *Salmonella* or *E. coli*, according to the guidelines provided. Purification is based on a modified alkaline lysis procedure similar to that described in section 2.6.1, followed by binding of plasmid DNA to a column-based anion-exchange resin under low salt and pH conditions. A medium salt wash removes RNA, proteins, and other impurities, and the plasmid DNA is eluted with a high-salt buffer. The DNA is then precipitated with isopropanol, desalted, and resuspended in 150 µl ddH_2O.

2.6.3: Purification of chromosomal DNA

Purification of chromosomal DNA for Southern blot analysis was performed using the AGTC Bacterial Genomic DNA Purification Kit (Edge Biosystems). A 5 ml sample of an overnight culture was used for DNA extraction according to the guidelines provided. Briefly the procedure involves conversion of bacteria to sphaeroplasts

(spherical cells from which most of the cell wall is removed) by incubation in a Tris-buffered solution containing lysozyme (cleaves peptidoglycan), sucrose (osmotic stress), and EDTA (chelates divalent metal ions, which are necessary cofactors for protease and DNase activity). Efficient lysis is then achieved by heating to 65°C in the presence of SDS (protein denaturant), NaCl (osmotic shock) supplemented with RNase. Latex beads are added that bind and clump denatured proteins and cellular debris. The mixture is centrifuged (debris pellets) and chromosomal DNA is extracted from the supernatant by precipitation with isopropanol. Chromosomal DNA is desalted, dried and uniformly resuspended in a final volume of 100 µl ddH_2O.

2.6.4: Isolation of RNA

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

Total RNA was extracted from 7 ml samples of exponential phase cultures (OD_{600nm} of 0.6) by the hot-phenol method similar to that described previously (Krieg, 1996). Cells were harvested by centrifugation and resuspended in 0.5 ml of boiling lysis buffer (2% SDS, 10 mM EDTA, 50 mM sodium acetate (pH 5.3)). Hot phenol (0.5 ml, pH 5.3) was added, and the samples vortexed vigorously before incubation at 90°C for 5 min. After a 10 min incubation on ice, the aqueous layer was extracted twice with 1:1 phenol:chloroform and once with chloroform, and the RNA precipitated with ethanol at –70°C. Following resuspension, RNA concentration and purity was assessed by A_{260nm} and A_{280nm} measurements as described in section 2.5.3

2.7: Manipulation of DNA *in vitro*

2.7.1: Restriction endonuclease cleavage of DNA

Typically 0.5–2.0 µg of plasmid DNA or purified PCR product were cut with 10 U of restriction enzyme in a 30 µl volume containing the reaction buffer supplied with the enzyme. For double digests involving simultaneous cleavage of DNA by two endonucleases, a suitable buffer was chosen in which both enzymes had >75% activity. Alternatively, double digests were performed sequentially in suitable buffers with ethanol precipitation of the DNA between digestions. Reactions were incubated at 37°C for 1–2 hours unless otherwise recommended. Chromosomal DNA was digested for 6 hours with 30 U of restriction enzyme in a 40 µl volume at 37°C.

2.7.2: Phosphatase treatment of restriction endonuclease-cleaved DNA

Cleavage of DNA by restriction enzymes leaves a 5'-protruding and 3'-recessive or, 5'-blunt end phosphoryl groups on the DNA. These phosphoryl termini are required for formation of phosphodiester bonds between adjacent ends of DNA (inter- or intra-molecular). Consequently, removal of the 5'-phosphoryl group of vector DNA molecules prior to use in cloning reactions prevents self-ligation and decreases the vector background in cloning strategies. Alkaline phosphatase was used to dephosphorylate 5'-phosphoryl groups when a blunt-ended cloning was performed, or if cloning into a single restriction site. Vector DNA was digested with restriction endonucleases for 1–2 hours and then 1 U of calf intestinal alkaline phosphatase (New England BioLabs) and 1 x dephosphorylation buffer (supplied with the enzyme) were added, and incubation at 37°C continued for an additional 60 min.

2.7.3: Purification of linear DNA

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

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

2.7.4: Ligation of DNA molecules

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

2.7.5: Phenol extraction and ethanol precipitation of DNA/RNA

Contaminating proteinaceous debris was removed from DNA/RNA solutions by phenol/chloroform extractions, and then concentrated by ethanol precipitation as detailed in Ausubel *et al.* (1990). For nucleic acid extractions, an equal volume phenol/chloroform (1:1 (v/v) sodium acetate buffered phenol (pH 5.2)/chloroform) was mixed with the DNA or RNA sample (adjusted to a minimum volume of 400 μl if

necessary), vortexed, and centrifuged for 4 min at 14,000 r.p.m. The aqueous (top) layer was carefully removed and an equal volume of chloroform was added to remove traces of phenol. The contents were mixed and then separated by centrifuging at 14,000 r.p.m. for 4 min. The DNA or RNA was concentrated by ethanol precipitation. 0.1 volumes of 3.5 M sodium acetate (pH 5.2) and 2 volumes of 100% ice-cold ethanol were added to the nucleic acid suspension. The contents were mixed, and incubated at –20°C for 15 min, before centrifugation at 14,000 r.p.m. for 10 min. The pellet was washed in 500 µl of 70% ethanol, air-dried and resuspended in a suitable volume of ddH_2O.

2.8: Polymerase Chain Reaction

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

2.8.1: Amplification of DNA

Two different thermostable polymerases were used in this study. *Taq* DNA polymerase (Roche Molecular Biochemicals) is a highly processive 5'–3'-DNA polymerase purified in recombinant form, free of endo- or exo-nucleases. *Taq*

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

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

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

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

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

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

2.9: Southern blotting

The basic principle of Southern blotting (Southern, 1975) involves digestion and separation of DNA by agarose gel electrophoresis. The DNA is then denatured *in situ* and transferred to nylon membrane. The DNA is the fixed to the membrane. Immobilised DNA is hybridised with a Digoxigenin-(DIG) labelled probe that can be detected by a chemiluminescent immunoassay and revealed by autoradiography.

2.9.1: Electrophoresis and transfer to membrane

Chromosomal DNA 1–2 µg was cut overnight with an excess of appropriate restriction endonuclease(s) to ensure complete digestion, and subsequently separated by electrophoresis through a 1% agarose gel overnight. The resolved DNA was then *in situ* depurinated (250 mM HCl, 10 min), denatured (1.5 M NaCl, 0.5 M NaOH, 45 min) and neutralised (1.5 M NaCl, 1.5 M Tris-HCl (pH 7.4), 45 min), prior to overnight capillary transfer to Biodyne B nylon membrane (PALL), according to the method outlined in Sambrook *et al.* (1989). The *in situ* treatment of the DNA allows transfer to proceed more efficiently by cleaving larger fragments, and makes detection more sensitive by denaturing double-stranded DNA. DNA was immobilised on the membrane by UV-crosslinking.

2.9.2: Preparation of a DIG (Digoxigenin) labelled probe

A DIG-labelled probe was synthesised by the polymerase chain reaction (section 2.8.1) using the PCR DIG Labelling Mix (Roche Molecular Biochemicals). The mix contains 2 mM dATP, 2 mM dCTP, 2 mM dGTP, 1.9 mM dTTP and 0.1 mM DIG-11-dUTP. Of this 5 µl was added to a 50 µl volume PCR reaction instead of standard dNTP mix. The PCR products synthesised, incorporate the DIG-labelled nucleotides.

The labelled probes were purified as described in section 2.7.3, and eluted in 100 µl ddH_2O. Typically 2 µl was used per 10 ml hybridisation solution. Hybridisation and detection of probe bound to immobilised targets, was carried out as outlined in section 2.9.3 with the exception that the probe was denatured by heating to 95°C for 10 min then incubated on ice for 10 min prior to use.

2.9.3: Hybridisation and detection of probe bound to immobilised targets

Hybridisation and detection of DIG-labelled DNA probes (used for Southern and Northern blotting) were carried out following the detailed instructions in the DIG System User's Guide for Filter Hybridisation (1995). Briefly, the procedure involves overnight incubation of the probe with the nylon membrane at 68°C (50°C for Northern blotting). Non-specific hybridised probe is removed by a series of stringency washes (decreasing salt concentration, increasing temperature). The membrane is then incubated with an Anti-DIG-alkaline phosphatase-antibody conjugate (Anti-DIG-AP-conjugate, Roche Molecular Biochemicals), which recognises the immobilised DIG moiety that is incorporated into the probe. In the presence of a suitable chemiluminescent substrate (CDP-Star, Roche Molecular Biochemicals), the alkaline-phosphatase-mediated enzymic dephosphorylation reaction results in a luminescent signal that visualises hybridised molecules by autoradiography (section 2.13).

2.10: Northern blotting

The principle of Northern blotting is that a transcript within immobilised RNA can be quantified after hybridisation with a specific probe. The procedure involves (i) resolution of an RNA sample by denaturing gel electrophoresis (section 2.10.1), (ii) transfer of the resolved RNA to nylon membrane (section 2.9.1) and (iii) hybridisation and detection of the RNA with labelled probes (probe synthesis described in section 2.9.2, hybridisation and detection procedures described in section 2.9.3).

2.10.1: Denaturing electrophoresis of RNA

RNA molecules can contain a high degree of secondary structure and are consequently electrophoresed under denaturing conditions. In this study the denaturant used was formaldehyde for agarose gel electrophoresis.

Agarose gels (1%) containing formaldehyde were cast by mixing 1 g agarose, 10 ml 10 x MOPS, 73 ml DEPC-treated ddH_2O in a clean conical flask. The agarose was dissolved, allowed to cool to 60°C, and then 17 ml of 37% formaldehyde was added (gel concentration of formaldehyde is 6.3%). Samples of total RNA (25 μg) were denatured prior to loading in 3 volumes of denaturing solution (50% formamide, 1 x MOPS) at 70°C for 5 min, transferred to ice for 5 min, then loaded with 0.1 volumes of loading buffer. Gels were run in 1 X MOPS buffer at 100 V for 1–3 hours.

10 x MOPS: 41.85 g 4-morpholinepropanesulphonic acid (MOPS), 6.8 g sodium acetate-3 H_2O, 20 ml 0.5 M Na_2EDTA, made to 1 L with DEPC-treated ddH_2O, pH 7.0

10 x TBE: 108 g Tris, 55 g Boric acid, 40 ml 0.5 M Na_2EDTA (pH 8.0) made to 1 L with DEPC-treated ddH_2O

Sample buffer: 50% (v/v) glycerol, 1 mM EDTA pH8.0, 0.25% bromophenol blue

After electrophoresis the RNA is transferred directly to a Biodyne B nylon membrane (PALL) as described in Sambrook *et al.*, (1989), immobilised by UV cross-linking, hybridised to a DIG labelled probe and the specific transcript detected as described in section 2.9.3.

2.11: Colony hybridisation

Colony hybridisation is a method devised by Grunstein and Hogness (1975) by which bacterial colonies can be lysed *in situ* on nylon filters and the released DNA can be attached to the filter and detected by hybridisation of a labelled probe. This procedure was used to detect mutants with deletions of DNA upstream of the *spv* operon and also to detect *gyrA*$^+$ clones. The probes used were amplified as described in section 2.9.2.

The primers used were delMF/delMR to detect mutants with deletions upstream of *spv* and gyrAmidF/gyrAprobeR to detect *gyrA*$^+$ clones.

2.11.1: Transfer of cells to nylon filter

The bacteria to be screened were toothpicked onto LB agar plates with appropriate antibiotic selection such that 100 colonies grew on each plate after overnight incubation at 37°C. The nylon filters were cut to fit inside the agar plate. Using a sterile forceps the filter was laid on the surface of the agar such that contact with each colony was made. The filter was lifted from the surface and placed face-up on a piece of Whatman 3MM paper saturated in 10% SDS. After 5 min the filter was placed on a piece of Whatman 3MM paper saturated in denaturation solution (0.5 N NaOH, 1.5 M NaCl) for 5 min. The filter was then removed to a third piece of Whatman 3 MM paper saturated in neutralisation solution (1.5 M NaCl, 0.5 M Tris-HCl [pH 7.4]) and incubated for 5 min. The filter was then placed on a dry piece of Whatman 3 MM paper and microwaved in an 850 Watt microwave oven for 30 seconds at high power. The filter was vigorously washed in 2 x SSC to remove all cellular debris. Prehybridisation, hybridisation and autoradiography were carried out as described in sections 2.9.3 and 2.13 respectively.

2.12: DNA sequencing

The nucleotide sequences of DNA fragments of interest were determined using the dideoxy chain termination method described by Sanger *et al.* (1977). The chain termination method utilises DNA polymerase to synthesise a complementary copy of a single-stranded DNA template, primed from a specific annealed oligonucleotide. When the polymerase selects a deoxynucleotide analogue, dideoxynucleotide (ddNTP), for incorporation by base-pair matching to the template DNA, chain elongation is terminated. Chain growth depends on the formation of phosphodiester bond between the 3'-OH group at the end of the growing primer and the 5'-phosphate group of the incoming deoxynucleotide (dNTP). ddNTPs are efficiently recognised by DNA polymerase, however lack a 3'-OH, and consequently prevent further chain

elongation. This method of sequencing is designed such that in doing 4 separate reactions, a ddNTP (G, A, C or T) is introduced at every position of the complementary DNA corresponding to the template. Incorporation of [α-^{32}P] dATP or [α-^{35}S] dATP in the reaction mixtures allows newly synthesised DNA to be labelled. Reactions are then electrophoresed side-by-side on a high-resolution denaturing polyacrylamide gel allowing the sequence to be read following autoradiography. Plasmid denaturation, primer annealing, labelling and terminations reactions were carried out using the T7 Sequencing Kit (Pharmacia Biotech) according to the guidelines supplied.

2.12.1: Denaturing polyacrylamide gel electrophoresis

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

2.13: Autoradiography

In this study autoradiography was used to visualise and quantitate on X-ray film; (i) radio-emissions from molecules that incorporated [α-^{35}S]dATP, [α-^{32}P]ATP or [γ-

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

2.14: Primer Extension

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

2.14.1: 5'-end labelling of DNA using γ^{32}P-ATP

Primer lacZ50 was labelled with [γ-^{32}P]dATP using Bacteriophage T4 polynucleotide kinase (T4 PNK; New England BioLabs), which catalyses the transfer and exchange of

a phosphate group from the γ-position of ATP to the 5'-hydroxyl terminus of the primer. To this 20 pmoles of lacZ50 (1 pmol/µl) was mixed with 50 µCi of [γ^{32}P]dATP (10 mCi/ml, 5,000 Ci/mmol), 3 µl 10 x kinase buffer (supplied with enzyme) and 15 U T4 PNK in a 1.5 ml screw-cap Eppendorf tube. The reaction was incubated for 30 min at 37°C. The labelled oligonucleotide was then precipitated with 3 µl sodium acetate (3.5 M pH5.2) and 90 µl 100% ethanol at –20°C for 10 min. Following centrifugation, the pellet was washed with 70% ethanol, dried, and resuspended in 20 µl DEPC-treated ddH_2O (approximately 1 pmol/µl).

2.14.2: Primer-RNA annealing and cDNA synthesis

Total cellular RNA was isolated from mid-exponential phase cultures of SL1344+pQF50 and SL1344+pGyrA-lacZ as described in section 2.6.4. Approximately 50 µg of each RNA sample was mixed with 2 pmol of ^{32}P-labelled oligonucleotide lacZ50 (Table 2.3) and 40 U RNase inhibitor (Gibco-BRL) in a final volume of 50 µl. RNA was denatured by incubating at 90°C for 5 min. The template and primer were annealed by cooling on ice for 15 min. Synthesis of cDNA was performed with the addition of the following to the template-primer reactions; (14 µl of ddH_2O, 1 mM of each dNTP, 10 mM dithiothreitol, 20 µl First Strand Buffer, 200 U of Superscript II RT reverse transcriptase (Gibco BRL)). Reactions were incubated at 42°C for 90 min. RNA was then degraded by the addition of 50 U RNase (DNase free) (Roche Molecular Biochemicals) for 30 min. The cDNA extension products were purified by phenol/chloroform and chloroform extractions and concentrated by ethanol precipitation (section 2.7.5). The DNA pellets were resuspended in 6 µl of ddH_2O and 4 µl of PE loading dye. Extension products were analysed by denaturing PAGE (section 2.12.1) alongside the DNA sequencing reactions.

5 x First Strand Buffer:	250 mM Tris-HCl pH 6.8, 375 mM KCl, 15 mM $MgCl_2$
PE loading dye:	95% (v/v) formamide, 20 mM EDTA, 0.05% (w/v) bromophenol blue, 0.05% (w/v) xylene cyanol FF

2.15: Gel mobility shift analysis of protein:DNA interactions

The association of purified Fis protein with the *gyrA* and *gyrB* promoter regions was analysed by gel mobility shift assay. A 493 bp *gyrA* probe and an 453 bp *gyrB* probe were amplified by PCR with *Pfx* polymerase (Gibco-BRL), using oligos gyrAbam/gyrAkpn and gyrBBSF/gyrBBSR (Table 2.3), and *S. typhimurium* SL1344 chromosomal DNA as template. A 322 bp *gyrA* promoter proximal probe and a 277 bp *gyrA* promoter distal probe were amplified with the oligos gyrABS/gyrkpn and gyrbam/DNase2R respectively and *S. typhimurium* SL1344 chromosomal DNA as template. A 280 bp *spvR* probe was amplified with the oligonucleotides InspvRF/InspvRR (Table 2.3) and purified p671 as template. Amplified probes were purified as detailed in section 2.7.3, and labelled as described in section 2.14.1. Approximately 5 ng of probe was incubated with increasing concentrations of His-tag purified Fis (section 2.17) from 0–100 ng (0–450 nM) for 15 min at room temperature in 20 µl reaction cocktail containing 20 mM Tris-HCl [pH 7.5], 80 mM NaCl, 1 mM EDTA, 100 ng BSA, 25 µg/ml poly-[d(I-C).(dI-dC)], 10% glycerol and 1 mM DTT. Protein-DNA complexes were resolved by electrophoresis through a 7% polyacrylamide gel, for 5 hours at room temperature. The gel was dried and examined by autoradiography.

A second gel mobility shift procedure was also used to examine binding of H-NS to the *gyrA* promoter region. This procedure involves the use of an excess of competitor DNA (Bertin *et al.*, 1999). Plasmid pRR322 was digested into 8 fragments with *Taq*I and *Ssp*I restriction enzymes. One restricted fragment is 217 bp, and contains the sequence of the curved *bla* promoter. This promoter is bound efficiently by H-NS due to the preference of H-NS for curved DNA (Bracco *et al.*, 1989). The *gyrA* promoter region was amplified and purified as a 493 bp PCR product using primer pair gyrkpn/gyrbam (Table 2.3) and as a 322 bp promoter proximal fragment with the oligos gyrABS and gyrkpn (Table 2.3). These probes and digested pBR322 were incubated with 0–2 µM H-NS in the reaction buffer 40 mM HEPES (pH 8.0), 100 mM potassium glutamate, 10 mM magnesium aspartate, 0.022% NP40 and 0.1 mg/ml BSA. The products were electrophoresed through 3% molecular screening agarose (Roche

Molecular Biochemicals) for 5 hours at 100 V. The gel was stained with 1 μg/ml ethidium bromide and viewed under UV illumination.

2.16: Mapping Fis binding sites

Deoxyribonuclease I (DNase I) footprinting was introduced by Galas and Schmitz (1978) to identify the DNA sequences that constitute binding sites for site-specific DNA-binding proteins. The basis of footprinting techniques is that DNA-bound proteins protect the phosphodiester backbone of the DNA from cleavage by DNase I. In a footprinting experiment, mixtures of radiolabelled DNA and different concentrations of the protein are exposed to DNase I. The DNase I reactions are quenched, the DNA is denatured, cleavage products separated by sequencing gel electrophoresis and visualised by autoradiography.

2.16.1: Preparation of probes for DNase I footprinting

A fragment of the *gyrA* promoter region was amplified from pGyrA-lacZ with the primers DNasegyrAF and DNaselacZR (Table 2.3). A fragment of the *gyrB* promoter region was amplified from pGyrB-lacZ with the primers DNasegyrBPF and DNasegyrBPR (Table 2.3). The PCR products were purified as described in section 2.7.3 and end-labelled as described in section 2.14.1. These fragments were then digested with the enzymes *Hind*III (*gyrA* fragment) and *Eco*RI (*gyrB* fragment) for 2 hours in a reaction volume of 60 μl. The probes, labelled at one extremity, were then purified by extraction from a 6% TBE polyacrylamide gel following electrophoresis. The probes were eluted in 3 ml of elution buffer (10 mM Tris-HCl, (pH 8.0), 1 mM EDTA, 300 mM Sodium acetate (pH 5.2), 0.2% SDS) at 37°C overnight. The eluted probes were then extracted once with an equal volume of phenol:chloroform and ethanol precipitated. The probes were then resuspended in 100 μl ddH_2O.

2.16.2: DNase I footprinting

2 µl of labelled probe was used in each footprinting experiment. Protein-DNA complexes were formed in 50 µl of footprinting buffer (20 mM Tris-HCl (pH 7.5), 80 mM NaCl, 1 mM EDTA, 100 µg/ml BSA, 10% glycerol and 1 mM DTT) at 37°C for 30 min. 50 µl of 10 mM $MgCl_2$, $CaCl_2$ was then added and incubation continued for a further 10 min. 2 µl of DNase I (0.01 U Roche Molecular Biochemicals) was then added and digestion allowed to proceed for 1 min before the reaction was stopped by the addition of 90 µl Stop solution (200 mM NaCl, 30 mM EDTA (pH 8.0), 1% SDS, 100 µg/ml yeast tRNA). Samples were then extracted once with an equal volume of phenol:chloroform, ethanol precipitated and resuspended in 6 µl of PE loading dye (section 2.14.2). After denaturation at 95°C for 3 min samples were subjected to electrophoresis on a 7% urea-polyacrylamide gel (section 2.12.1) alongside DNA sequencing reactions.

2.17: Purification of Fis

Plasmid pFisC (Table 2.2) is a pET22b-based plasmid (Novagen) which harbours a C-terminal six histidine fusion to the *fis* gene cloned downstream of the T7 polymerase promoter. Induction of *fis* expression was performed in strain BL21(DE3)+pDIA17. *E. coli* strain BL21(DE3) (Table 2.1) contains the gene coding for T7 RNA polymerase under the control of the *lacUV5*-inducible promoter. This construct was integrated at the λ*att* site using the lambda cloning vector DE3 (Studier and Moffatt, 1986). In addition, strain BL21(DE3) is deficient in the OmpT and Lon proteases. Plasmid pDIA17 (Table 2.2) encodes the LacI repressor that completely silences the *lacUV5* promoter prior to induction, giving tight control over *fis* induction. Upon addition of the gratuitous inducer IPTG, the LacI-mediated repression of the *lacUV5* promoter is effectively removed and T7 RNA polymerase is produced. T7 RNA polymerase then initiates selective transcription from the plasmid-borne T7 promoter sequence upstream of the *fis* gene.

A one litre culture was grown at 30°C to mid-exponential phase in LB broth, and *fis* expression induced by addition of 0.5 mM IPTG for 2 hours. Cells were pelleted by centrifugation, and resuspended in binding buffer (60 mM imidazole, 500 mM NaCl, 20 mM Tris-HCl (pH 7.9), 1 mM PMSF) supplemented with 200 μg/ml lysozyme. The mixture was frozen on dry ice and left at –20°C overnight. After thawing, complete lysis was achieved by sonication. Cell debris was pelleted by centrifugation for 10 min at 14,000 r.p.m., and the soluble extract (15 ml) retained.

Fis purification from the crude extract was performed by metal affinity chromatography using the His-Bind Kit (Novagen). The His-Bind resin within the column contains immobilised Ni^{2+} ions that can retain the His-tag sequence by electrostatic interaction. Imidazole serves as a competitor with histidine (His-tag) for the Ni^{2+} ions and therefore an increasing concentration of imidazole will elute the target protein from the column.
The soluble extract was passed over a pre-equilibrated His-Bind 900 column (Novagen). The column was washed with binding buffer, and then with wash buffer (100 mM imidazole, 0.5 M NaCl, 20 mM Tris-HCl (pH 7.9), 1 mM PMSF) to remove proteins weakly associated with the resin. Fis-H6 was recovered by elution with 500 mM imidazole and was estimated to be <90% pure by SDS-PAGE (section 2.18) followed by Coomassie staining (section 2.18.1). Purified Fis was dialysed into storage buffer (0.5 M NaCl, 20 mM Tris (pH 7.5), 0.1 mM EDTA, 50% Glycerol) and stored in aliquots at –20°C.

2.18: SDS-PAGE

Proteins were separated by discontinuous polyacrylamide gel electrophoresis as described in Sambrook *et al.* (1989). The discontinuous buffer system uses buffers of different pH and composition in the stacking and separating gels. Consequently protein migration through the large pores in the stacking gel (5% acrylamide) is fast and the proteins become concentrated into a narrow band. However, migration through the narrow pores of separating gel (8%-16% acrylamide) is according to size. Both gels were prepared with 0.1% SDS. Since most proteins bind SDS in a constant

weight ratio, this leads to identical charge densities for denatured proteins, and allows proteins to migrate according to size, not charge.

Separating gels (8%, 12% or 16%) were prepared according to Sambrook *et al.* (1989). The separating gel was then overlaid with 200 µl propan-2-ol (excludes oxygen) and allowed to polymerise for 30 min. The 5% stacking gel was made by the mixing of 0.833 ml Protogel (National Diagnostics), 1.25 ml 0.5 M Tris-HCl (pH 6.8), 50 µl 10% SDS and 2.87 ml of ddH_2O. The gel was electrophoresed in 1 x Tris-glycine running buffer (25 mM Tris-HCl, 250 mM glycine (pH 8.3), 0.1% (w/v) SDS). Prior to loading, 15 µl protein samples (section 2.19.1) were denatured at 90°C for 10 min, centrifuged at 14,000 r.p.m. for 1 min. Electrophoresis was performed at 150 V for 60–90 min.

2.18.1: Staining of proteins

Gels were washed in ddH_2O prior to staining with GelCode Blue Stain Reagent (Pierce) for 1 hour according to the guidelines supplied. Gels were then destained in ddH_2O. This stain uses the colloidal properties of Coomassie G-250 for protein binding and staining, but has no affinity for the polymerised gel.

2.19: Western immunoblot analysis

Western immunoblottng is a sensitive technique whereby proteins (antigens) are (i) solubilised with SDS and β-mercaptoethanol (section 2.19.1) and separated by SDS-PAGE (section 2.18), (ii) irreversibly transferred to nitrocellulose membrane (section 2.19.2), (iii) the membrane is incubated with primary antibody, and the antigen-antibody complexes detected with a secondary antibody and revealed by a chemiluminescent assay (section 2.19.3).

2.19.1: Preparation of total cellular extracts

Crude protein extracts for Western immunoblot analysis were prepared as described below. The OD_{600nm} of each culture was measured. A volume of cells, which corresponded to 2 ml of culture per 1 OD_{600nm} unit, was taken for each culture. Bacteria were pelleted and washed in PBS. The pellets were then resuspended in 50 µl PBS and 50 µl 2 x final sample buffer (FSB), and boiled for 10 min. Typically 15 µl of this crude extract was used for immuno-detection of Fis, GyrA or σ^s in Western immunoblot analyses.

2 x FSB: 150 mM Tris-HCl (pH 6.8), 1.2% SDS, 30% glycerol, 15 ml β-mercaptoethanol, made to 100 ml with ddH_2O.

2.19.2: Transfer of proteins to nitrocellulose membrane

Following SDS-PAGE (section 2.18), gels were electroblotted to 0.2 µm PROTAN nitrocellulose membrane (Schleicher and Schuell) using a Mini Trans-blot electrophoretic transfer cell (Bio-Rad) filled with transfer buffer (25 mM Tris, 192 mM glycine, 20% methanol), at 80 V for 2 hours at 4°C. Protein equal loading and consistent transfer to the nitrocellulose membrane was confirmed by staining the membrane with 6 ml Ponceau S solution (2 g Ponceau S (Sigma), 1 ml glacial acetic acid) for 5 min followed by extensive washing with ddH_2O.

2.19.3: Detection of bound antigens

Nitrocellulose membranes were blocked for 1 hour in blocking buffer (5% (w/v) nonfat dry milk, in phosphate-buffered saline (PBS) or 5% milk in Tris-buffered saline (TBS) with 0.1% Tween for detection of GyrA). Anti-Fis (1:1,000), anti-GyrA (1:200) and anti-RpoS (1:3,000), antisera were diluted appropriately in blocking buffer and incubated with the membrane overnight at 4°C. The membrane was washed 3 x 10 min with PBS+1% milk (or TBS+0.1% Tween + 1% milk) and incubated in blocking

buffer containing HRP-linked anti-rabbit IgG (or anti-mouse IgG for detection of GyrA) for 1 hour. The blot was washed as before, and in the presence of a suitable chemiluminescent substrate, the horseradish-peroxidase-mediated enzymic reaction results in a luminescent signal that visualises the antigen-antibody complex, which can by detected by autoradiography (section 2.13). Typical exposures were from 5 min to 1 hour.

2.20: Infection of macrophage cell line

The J772A.1 murine, macrophage-like cell line was obtained from the American Type Culture Collection (ATCC) and was maintained in tissue culture medium (TCM) comprising Dulbecco's Modified Eagles Medium (DMEM) containing 10% heat-inactivated fetal calf serum, 100 U penicillin and 100 μg/ml streptomycin (Gibco). For gene expression assays, cells were seeded 72 hours prior to infection in 12 well plates, at a density of 2.5×10^5 cells per well in TCM. Overnight broth cultures of the bacterial strains for infection were diluted 1:50 into LB broth and grown with shaking at 37°C until the OD_{600nm} reached 1.0. Bacterial cells were harvested and resuspended in antibiotic free TCM. The TCM was removed from the macrophage monolayers and the *S. typhimurium* TCM added to the monolayers at a multiplicity of infection of 100. Infected monolayers were incubated at 37°C and 0.5% CO_2 for 45 min to allow phagocytosis of the *Salmonella* to occur. Infected monolayers were washed twice with TCM and incubated in fresh media containing 100 μg/ml gentamicin to kill any remaining extracellular bacteria. This was time = 0. 30 minutes post-infection the first time point was harvested by washing the monolayers twice with TCM and then lysing them with 0.5% Triton X-100 in PBS. The TCM containing 100 μg/ml gentamicin was removed from the remaining monolayers and replaced with TCM containing gentamicin 20 μg/ml. The monolayers were incubated in this media for the remainder of the experiment. At 1 hour intervals post-infection the monolayers were harvested by washing twice with TCM and then lysing them with 0.5% Triton X-100 in PBS. The numbers of intracellular bacteria was determined by diluting and plating lysates from triplicate wells. The remaining lysates were spun down to remove PBS and were assayed for β-galactosidase activity as described in section 2.20.1

2.20.1: β-galactosidase assays

β-galactosidase activity from intracellular bacteria was assayed using the MUG (4-methylumbelliferyl-β-D-galactopyranoside) assay. Samples were mixed with 0.5 ml ABT (100 mM NaCl, 60 mM K_2HPO_4, 40 mM KH_2PO_4, 0.1% Triton X-100) and incubated at 37°C for 15 min. 50 µl of 4-methylumbelliferyl-β-D-galactopyranoside (MUG) was added and the reaction incubated in the dark at room temperature for 60 min. 0.5 ml of 0.4 M Na_2CO_3 was then added to stop the reaction. Serial dilutions of the samples were then made in ABT + 50% 0.4 M Na_2CO_3 and placed in 96 well plates. The quantity of 4-methylumbelliferone (4-MU) produced was determined by reading luminesence with an excitation wavelength of 365 nm and emission wavelength of 455 nm in an LS 50B luminesence spectrometer (Perkin-Elmer).

2.21: Analysis of DNA topoisomer distributions

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

Plasmid DNA was isolated by Qiagen preparation (section 2.6.2) and quantified by OD_{260nm} reading as described in section 2.5.3. The DNA was then electrophoresed through a 1% agarose gel containing 25 µg/ml chloroquine. At this concentration of chloroquine relaxed DNA takes up the most chloroquine and supertwists into a positively supercoiled molecule which runs faster than a negatively supercoiled

molecule which only takes up enough chloroquine to relax itself. The samples were electrophoresed for 20 hours at 3 V/cm in TBE containing the same concentration of chloroquine as the gel itself. Following electrophoresis the gel was washed in distilled water for 4 hours to remove chloroquine. The gel was then stained with ethidium bromide (5 μg/ml) and the topoisomers visualised by illumination with UV-light.

2.22: Mapping DNA gyrase cleavage sites

Gyrase-chromosome interactions can be examined using oxolinic acid, an inhibitor of the A subunit of gyrase. The drug traps a gyrase-DNA reaction intermediate such that DNA breaks are revealed when drug-gyrase-DNA complexes are treated with a protein denaturant (Gellert *et al.*, 1977; Sugino *et al.*, 1977).

2.22.1: Purification of chromosomal DNA treated with oxolinic acid for mapping DNA gyrase cleavage sites

Salmonella strains were treated with oxolinic acid according to the method of O'Connor and Malamy (1985). Duplicate 20 ml cultures were inoculated with 150 μl of an overnight culture and grown to an OD_{600nm} = 0.8 at 37°C in conical flasks. Oxolinic acid was added to one flask to give a final concentration of 50 μg/ml, while the other was left untreated. Incubation was continued for a further 30 min at 37°C. 10 ml aliquots of the cultures were centrifuged at 5,000 r.p.m. for 10 min and the cells resuspended in 200 μl 50 mM Tris-HCl (pH 8.0), 15% (w/v) sucrose. Lysozyme (50 μl of a 10 mg/ml solution in 0.25 M Tris-HCl (pH 8.0)) was added and the mixture incubated at room temperature for 10 min. 50 μl of 0.25 M EDTA was added and the mixture incubated at room temperature for a further 10 min. 50 μl 10% SDS was added followed by proteinase K (20 μl of a 2 mg/ml solution) and the mixture incubated at 37°C for 3 hours. The lysate was then extracted twice with phenol and phenol/chloroform and then precipitated with 100% ethanol (section 2.7.5). The pellet was resuspended in 200 μl ddH_2O and 2 μl RNase (500 μg/ml; Roche Molecular Biochemicals) was added. The mixture was incubated for 1 hour at 37°C.

2.22.2: Southern blotting of oxolinic acid treated DNA

Chromosomal DNA was digested with appropriate restriction enzymes as described in section 2.7.1. The restriction fragments were separated by agarose gel electrophoresis and Southern blotted as described in section 2.9. The largest hybridising fragment corresponded to the full-length restriction fragment, while shorter fragments corresponded to products cleaved at one end by the restriction endonuclease and by DNA gyrase at the other end. These sites could now be mapped as follows: the largest hybridising fragment is the full-length restriction fragment. Smaller fragments are generated by cleavage of the DNA by DNA gyrase and the length of these fragments is estimated using standard DNA size markers. These fragments retain the sequence to which the probe binds and so must be cleaved by DNA gyrase at the opposite end. The difference (in bp) between the large fragment and the shorter fragment is the distance (in bp) from the end of the DNA molecule not bound by the probe to the gyrase cleavage site.

2.23: Transposon mutagenesis

Transposon mutagenesis was used to identify *trans*-acting factors involved in deletion mutation formation in CJD671. The recipient strain was made electrocompetent as described in section 2.4.8. 1 μg of pBSL181, purified by Qiagen preparation (section 2.6.2), was electroporated into the freshly prepared electrocompetent cells as described in section 2.4.8. After incubation at 37°C for 1 hour to allow phenotypic expression of the antibiotic resistance marker, 9 ml of SOC containing 0.3 mM IPTG was added. This induces expression of the transposase (*tnp*) gene. Transposition was allowed to continue for 4 hours before serial dilution of the recipient were plated on chloramphenicol plates to determine the frequency of transposition. These plates were incubated at 37°C overnight. The remaining recipient culture was stored overnight at 4°C. After overnight incubation the number of cells appearing on the plates were counted and an appropriate dilution determined that gave 100-1,000 colonies per agar plate. Approximately 20,000 colonies containing transposon insertions were then screened from the remaining population by plating on chloramphenicol MacConkey lactose indicator plates and looking for a red phenotype.

Chapter 3

Analysis of factors involved in deletion formation in CJD671

3.1: Introduction

CJD671 (Table 2.1) is a strain of *Salmonella enterica* serovar Typhimurium that contains a MudJ insertion in the *spvB* (*Salmonella* plasmid virulence gene B) virulence gene on the large virulence plasmid. This creates a *spvB-lacZ* transcriptional fusion that can be used to monitor *spv* gene expression. When plated on MacConkey lactose indicator plates CJD671 gives pale pink colonies, indicating low levels of β–galactosidase, and hence *spv*, expression (O'Byrne and Dorman, 1994a). It was noticed however that when colonies of CJD671 matured on such plates, dark red papillae developed. These "red mutants" could be isolated by single colony purification and were shown to breed true (Massey *et al.*, 1999). Such mutants were shown to develop by 3 distinct genetic events:

1. The deletion of 5 kb of DNA between *spvR*, the positive regulator of the *spv* system and an upstream gene *rlgA* (resolvase like gene A). This gene encodes a putative resolvase with 13% sequence identity and a further 20% sequence similarity to the Tn*3* class of site-specific resolvases. Its *in vivo* target sequence remains unidentified however. This deletion event took 2 days to arise and accounted for approximately 45% of all red mutants.
2. Point mutations within MudJ, which leads to increased *lacZ* expression. Such mutations took 3 to 4 days to arise.
3. Large, approximately 17 kb deletions upstream of *spvR*. These deletions also took 2 days to arise however they have not been characterised further.

A detailed analysis of the 5 kb deletion event showed that it removes the *spvR* operator sequence and places *spvR* under the control of the *rlgA* promoter. The deleted region encodes two putative integrase genes and a putative phosphoribulose kinase/uridine kinase. The deletion also removes the 3' end of the *rlgA* gene. This encodes a putative helix-turn-helix motif responsible for autorepression of *rlgA* (Massey *et al.*, 2000). Hence *spvR* is now under the control of a derepressed *rlgA* promoter. This leads to increased levels of SpvR protein, and hence upregulation of the *spv* structural genes (Massey *et al.*, 1999). This deletion event is not site-specific as the deletion end-points of 6 independently arising mutants were sequenced and in no case were they found to coincide (Massey, 1998). As this deletion event is neither site-specific nor involves homologous sequences it is an example of illegitimate recombination. In each case the

deletion mutant has approximately a 70-fold higher level of β–galactosidase expression from *spvB-lacZ* than the wild-type strain. The structure of the *spv* region before and after the deletion event can be seen in Fig. 3.1.

As such a deletion event did not occur in LB media, this led to the identification of moderate osmolarity (0.36 M) and the presence of lactose as essential factors for the deletion event to occur (Massey *et al.*, 1999). Lactose provides a selective advantage to the mutant cells as they now have an increased capacity to scavenge lactose at a time when other nutrients are rare. This deletion event cannot be detected in the presence of any other sugar except lactose (Massey, 1998). This renders the deletion event similar to adaptive mutation, described as those mutations that occur in non-dividing cells during selection and are specific to the selection (Foster, 1998). In the case of CJD671 the cells mutate in order to be able to utilise the lactose present in the media. Non-useful mutations are not detected. This appears to contradict the Darwinian dogma, which states that mutations arise spontaneously without regard for their usefulness and advantageous mutants are selected for. Indeed the deletion mutants were shown to be relatively fitter than the wild-type cells when rare in the population (Massey *et al.*, 1999).

The deletion event was also shown to require a final salt concentration in the range of 0.2 M to 0.6 M NaCl. The highest number of red mutants arose at a salt concentration of approximately 0.36 M NaCl. It was discovered that salt could be substituted by sucrose that exerted a similar osmotic pressure showing that it was osmotic pressure and not salt *per se* that was required for the deletion event. In this case it was shown that the effect of osmotic pressure was not to increase the fitness of the mutants but to allow a genetic constraint impeding formation of the deletion to be overcome (Massey *et al.*, 1999). A broth-based assay was developed to quantify the percentage of red mutants that could arise under different conditions. This assay involves growing the strain to be tested in LB broth supplemented with 1% lactose and 0.275 M NaCl (LS-lac media) for 4 days. Each day an appropriate dilution is plated on MacConkey lactose indicator plates and the percentage of red mutants counted (section 2.2.3).

The aim of this work was to identify factors within the cell, which gave rise to the *rlgA-spvR* fusion in CJD671 upon prolonged incubation in LS-lac media.

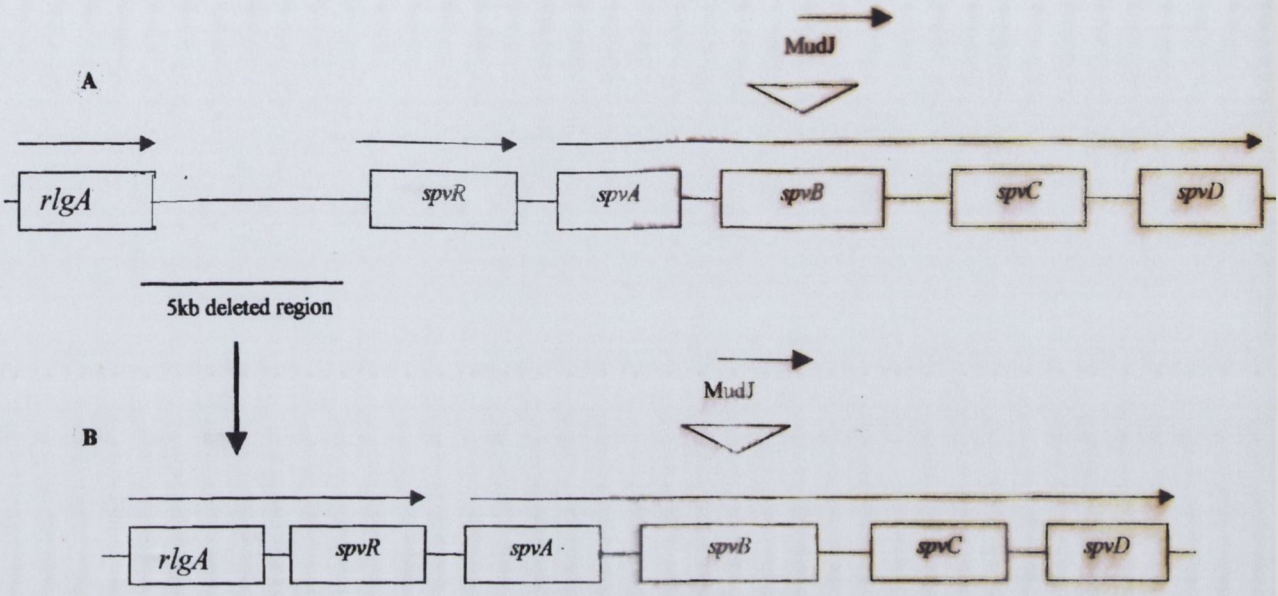


Fig. 3.1. **Structure of the *spv* operon region of CJD671 before (A) and after (B) the deletion event.** MudJ is inserted into the *spvB* structural gene in CJD671. The deletion event removes approximately 5 kb of DNA upstream of *spvR* including the *spvR* operator sequence and the 3' end of *rlgA*, which is responsible for autorepression. This deletion creates a *rlgA-spvR* transcriptional fusion, which in turn leads to upregulated *spv* structural gene expression. (Figure not to scale)

3.2: Results

3.2.1: Examination of the role of RecA in deletion formation

CH23 (Table 2.1) is a *S. typhimurium* LT2 strain with a point mutation in the *recA* gene, which inactivates the RecA protein. This mutation could not be transduced into CJD671 due to the lack of a selectable marker. Therefore the large virulence plasmid of CJD671 was isolated by Qiagen preparation (section 2.6.2) and electroporated into CH23 and selected by growth on kanamycin agar plates. In order to confirm the *recA* mutation, both CH23p671 and wild-type CJD671 were streaked on an agar plate and exposed to ultra-violet light for increasing amounts of time (section 2.4.9). As can be seen in Fig 3.2 the wild-type strain could recover from exposure to UV-light for up to 30 seconds while the *recA* mutant could not, thereby confirming the mutation. Both strains were incubated for 4 days in LS-lac and 10^{-6} and 10^{-7} dilutions plated each day and the percentage of red mutants calculated. For the wild-type strain CJD671 a total of 7,008 cells were counted, 1,083 of them giving red mutants. This gives a percentage of red mutants of 15.5%. For CH23p671 a total of 3,914 cells were counted. It was found that 668 of these cells were red mutants giving a percentage of red mutants of 17.1%. Therefore both strains gave approximately equal proportions of red mutants. A DIG-labelled PCR probe specific for sequences within the deleted region was generated using the primers delMF and delMR (Table 2.3). 100 red mutants from each strain were then picked onto a fresh agar plate and colony blotted using this probe. This distinguishes between deletion mutants, which do not hybridise to the probe on colony blotting as the homologous sequence has been removed by the deletion event and point mutants, which retain this sequence and still hybridise to the probe. Both deletion and point mutants could be found derived from the wild-type and the *recA* mutant demonstrating that RecA protein is not required for the deletion event or point mutations to occur.

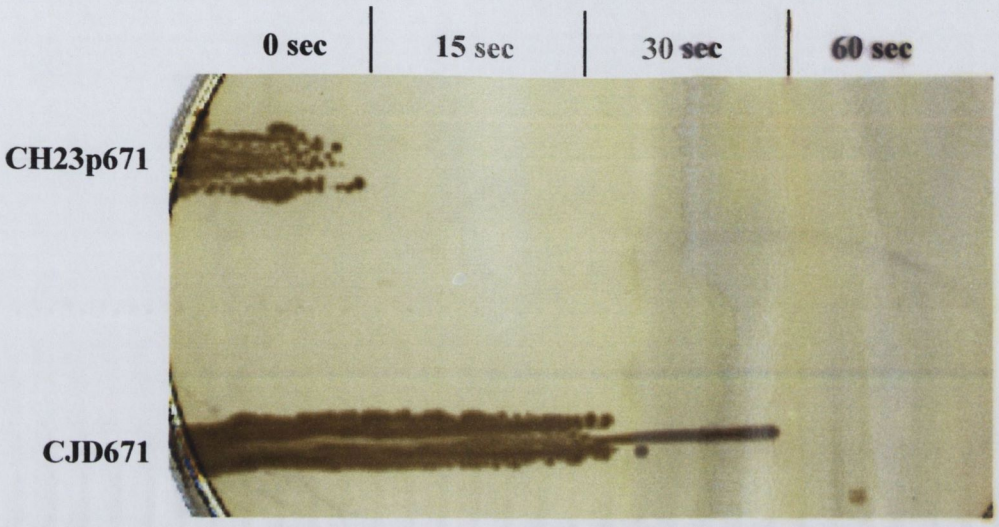


Fig. 3.2. **Confirmation of CH23p671 *recA*⁻ phenotype.** CH23p671 and CJD671 were exposed to UV-light for increasing amounts of time. CJD671 could recover from exposure to UV-light for up to 30 seconds however CH23p671 could not recover from exposure to UV-light for 15 seconds or more thus confirming it is a *recA* mutant.

3.2.2: Effect of global regulators on the deletion event

Some global regulators are known to play a role in the emergence of adaptive mutants, most notably the roles of RpoS and H-NS in the appearance of Lac(Ara)$^+$ strains of *E. coli* MCS2 (Gomez-Gomez *et al.*, 1997). This strain contains a Mu bacteriophage inserted between the *araB* and *lacZ* genes. Mu-mediated DNA rearrangements can result in in-frame fusions of *lacZ* and *araB*. This gives Lac(Ara)$^+$ bacteria capable of growing on lactose in the presence of arabinose as an inducer (Shapiro, 1984). RpoS, the stationary phase sigma factor, is required for the expression of many genes under starvation conditions, including the *spv* genes themselves (Kowarz *et al.*, 1994) and is required for the appearance of Lac(Ara)$^+$ mutants in MCS2. H-NS, a histone–like protein is involved in regulating many promoters, usually negatively, and its effect is generally the opposite of RpoS. The absence of H-NS leads to an increase in the appearance of Lac(Ara)$^+$ fusion clones in MCS2. The strain CJD671*rpoS*$^-$ was constructed by transducing the ampicillin resistance marker from JF2690 (Table 2.1) into CJD671 by P22-mediated transduction. That RpoS was absent from this strain was confirmed by Western immunoblotting with anti-RpoS antiserum and can be seen in Fig. 3.3 (A). CJD671 and CJD671*rpoS*$^-$ were then incubated for 3 days in LS-lac and each day appropriate dilutions were plated and the percentage of red mutants calculated. For the wild-type strain between 9% and 28% of the cells had formed red mutants by day 2. For CJD671*rpoS*$^-$ between 5% and 21% of the cells had formed red mutants by day 2. It therefore appears that RpoS is not required for the deletion event to occur. CJD671*ihf* (Table 2.1) was also constructed and the role of IHF in deletion formation examined. A lesion in the *ihfB* gene was found to have no effect on the formation of red mutants as for CJD671*ihf* between 10% and 24% of the cells formed red mutants compared to between 9% and 28% for CJD671.

3.2.3: Examination of the role of Fis in deletion formation

The role of the Fis protein in deletion formation in CJD671 was also examined, as it is known that Fis is required in certain cases for illegitimate recombination (Shanado *et al.*, 1997). Strain CJD671*fis*$^-$ was constructed by transducing the chloramphenicol marker from TH2285 (Table 2.1) into CJD671 by P22-mediated transduction. The

mutant status was confirmed by Western immunoblotting using early exponential phase cells (OD_{600nm} = 0.2) and anti-Fis antiserum and can be seen in Fig 3.3 (B). The *fis* mutation was also complemented by transforming CJD671*fis*⁻ with the plasmid pUHE25-2 (Table 2.2). This plasmid contains the *fis* gene under the control of an IPTG inducible promoter. The next step was to determine what concentration of IPTG should be used to induce Fis from pUHE25-2. CJD671*fis*⁻+pUHE25-2 was grown up with IPTG concentrations of 0 mM, 0.1 mM, 0.5 mM and 1 mM. CJD671 and CJD671*fis*⁻ were also grown. Growth was monitored by measuring the OD_{600nm}. A graph of OD_{600nm} against time can be seen in Fig. 3.4 (A). All the complemented strains appear to grow equally well and as well as the wild-type strain. There does not appear to be any toxicity associated with either overexpressing Fis or the inclusion of IPTG in the media. The *fis* mutant grows a little slower than wild-type in exponential phase but reaches a similar density to the other strains in stationary phase. At appropriate intervals during growth, early exponential phase (OD_{600nm} = 0.15), late exponential phase (OD_{600nm} = 0.8), early stationary phase (OD_{600nm} = 1.4) and late stationary phase (OD_{600nm} = 2.5) samples were taken and total protein extracted. These samples were then used in a Western blotting experiment to quantify the amount of Fis protein present in each sample. This Western blot can be seen in Fig. 3.4 (B). As reported previously (Ball *et al.*, 1992) the amount of Fis protein decreased throughout the growth phase for the wild-type strain CJD671 (lanes 1, 7, 13 and 19). No Fis was present at any stage for CJD671*fis*⁻ (lanes 2, 8, 14 and 20). Fis protein levels increased throughout the growth phase for the complemented mutant however. This occurred irrespective of IPTG concentration. Indeed similar amounts of Fis were present in the uninduced sample and the maximally induced sample showing that the promoter is very leaky and *lacI* should be supplied *in trans* in order to repress *fis*. It was therefore not deemed necessary to add IPTG to the media in order to complement the *fis* lesion. CJD671, CJD671*fis*⁻ and the complemented mutant were then incubated for 4 days in LS-lac media and appropriate dilutions plated daily in order to calculate the percentage of red mutants. The absence of Fis resulted in no change in the percentage of red mutants detected on day 2, however as can be seen in Fig. 3.5 the absence of Fis resulted in a decrease in the percentage of red mutants on day 3. Therefore Fis may affect the appearance of point mutants that upregulate *lacZ* activity. Alternatively the absence of Fis could affect the fitness or persistance of the red mutants over time resulting in their elimination from the media. Overexpressing Fis from pUHE25-2

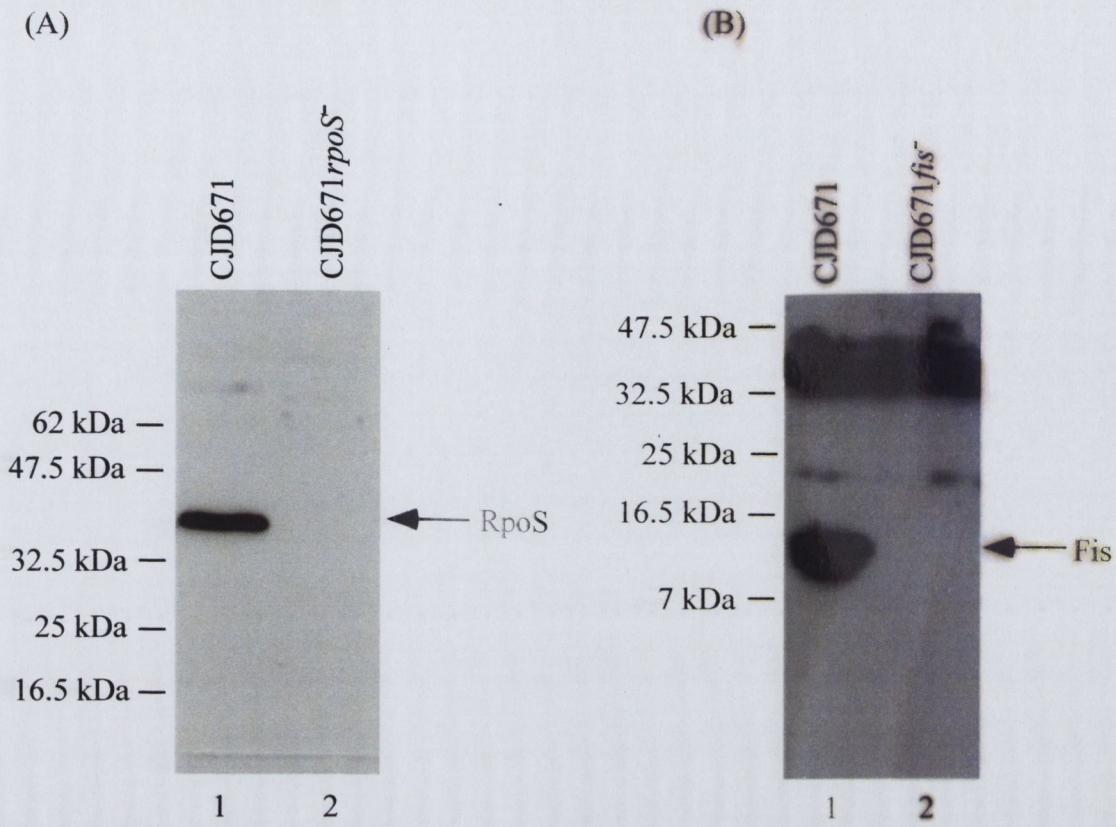
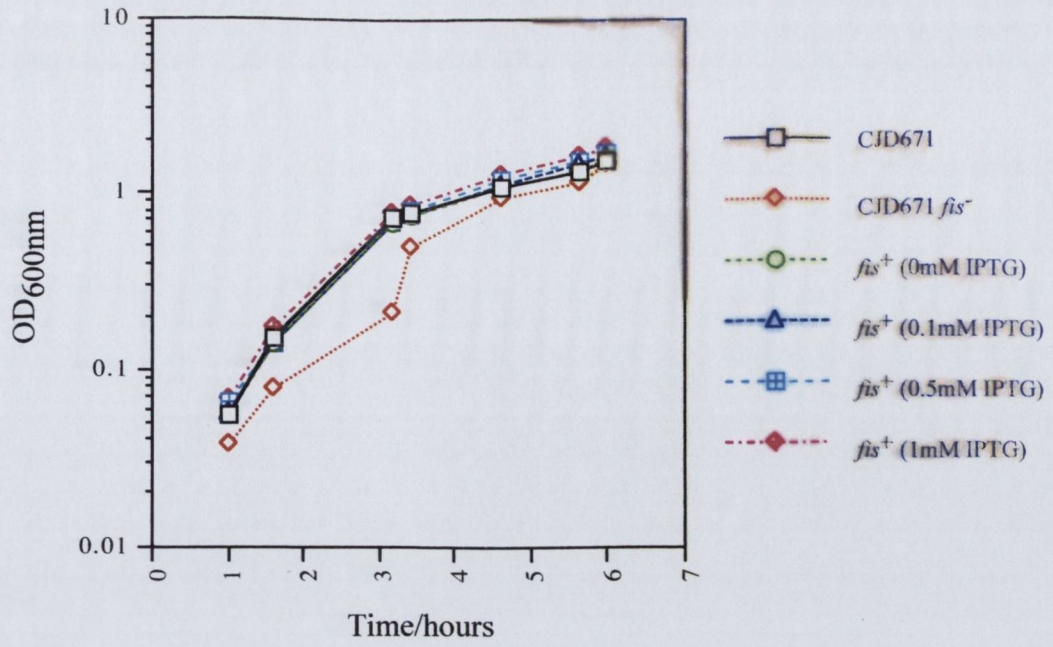


Fig. 3.3. **Confirmation of CJD671*rpoS*⁻ and CJD671*fis*⁻ mutant phenotypes.**

(A) Western immunoblot of total protein extracts from CJD671 and CJD671*rpoS*⁻ probed with anti-RpoS antiserum. In lane 1 wild-type CJD671 shows the appearance of an RpoS specific band. In lane 2 CJD671*rpoS*⁻ shows no RpoS specific band thereby confirming the mutation.

(B) Western immunoblot of total protein extracts from CJD671 and CJD671*fis*⁻ probed with anti-Fis antiserum. In lane 1 wild-type CJD671 shows a Fis specific band which is absent from CJD671*fis*⁻ (lane2) thereby confirming the mutation.

(A)



(B)

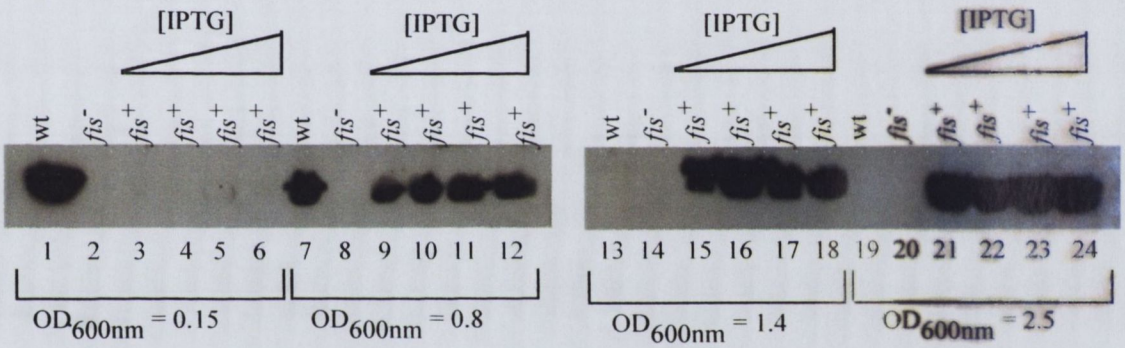


Fig. 3.4. (A) **Effect of *fis* mutation and overexpressing Fis on growth rate of CJD671.** Growth curve of CJD671, CJD671*fis*$^-$ and the complemented mutant (*fis*$^+$) induced with increasing concentrations of IPTG (0 mM, 0.1 mM, 0.5 mM and 1 mM). All strains grow equally well and overexpressing Fis does not appear to be toxic to the cells.

(B) **Expression of Fis from pUHE25-2.** Western immunoblot of CJD671 (wt), CJD671*fis*$^-$ (*fis*$^-$) and the complemented mutant (*fis*$^+$) induced with increasing concentrations of IPTG. Fis expression is as expected in the wild-type strain however the complemented mutant is very leaky and Fis is produced at all stages of the growth phase to equal levels regardless of IPTG concentration.

resulted in a large increase in the amount of red mutants appearing. In this case Fis continues to be produced into stationary phase, as it is no longer under the control of its own growth phase regulated promoter.

3.2.4: Effect of DNA gyrase on the deletion event

It had previously been demonstrated that moderate osmolarity was required in order for the deletion event to occur (Massey, 1998). Changes in osmolarity of the growth media lead to changes in the activity of DNA gyrase and hence to changes in the overall cellular DNA superhelicity (McClellan *et al.*, 1990). As the osmolarity of the media increases so too does the cellular negative superhelicity. Therefore it was hypothesised that DNA gyrase may play a role in bringing about the deletion event. In order to test this hypothesis the DNA gyrase inhibiting antibiotic novobiocin was used at sub-lethal concentrations. Concentrations of novobiocin that result in a decrease in negative supercoiling but still allow the bacteria grow had been previously determined (Free, 1995). Novobiocin affects DNA gyrase activity by inhibiting ATP binding and hydrolysis by the B subunit. It does not have the pleiotropic effects associated with antibiotics, which affect the A subunit of DNA gyrase and lead to gaps in the DNA (section 1.3.8). Using novobiocin in the media negates the increase in DNA supercoiling caused by growth in higher osmolarity media. CJD671 was incubated under a variety of conditions:

1. L-lac (LB broth supplemented with 1% lactose).
2. LS-lac.
3. LS-lac supplemented with novobiocin 50 μg/ml.
4. LS-lac supplemented with novobiocin 75 μg/ml.
5. LS-lac supplemented with novobiocin 100 μg/ml.

Each day for 4 days appropriate dilutions were plated on MacConkey lactose indicator plates and the percentage of red mutants calculated. The results can be seen in Fig. 3.6. As expected red mutants did not arise in L-lac media, as the osmolarity of this media is too low. 23.5% of the cells gave red mutants in LS-lac media. For LS-lac media containing novobiocin the percentage of red mutants decreased in a dose-dependent manner with increasing novobiocin concentration. Therefore as the overall

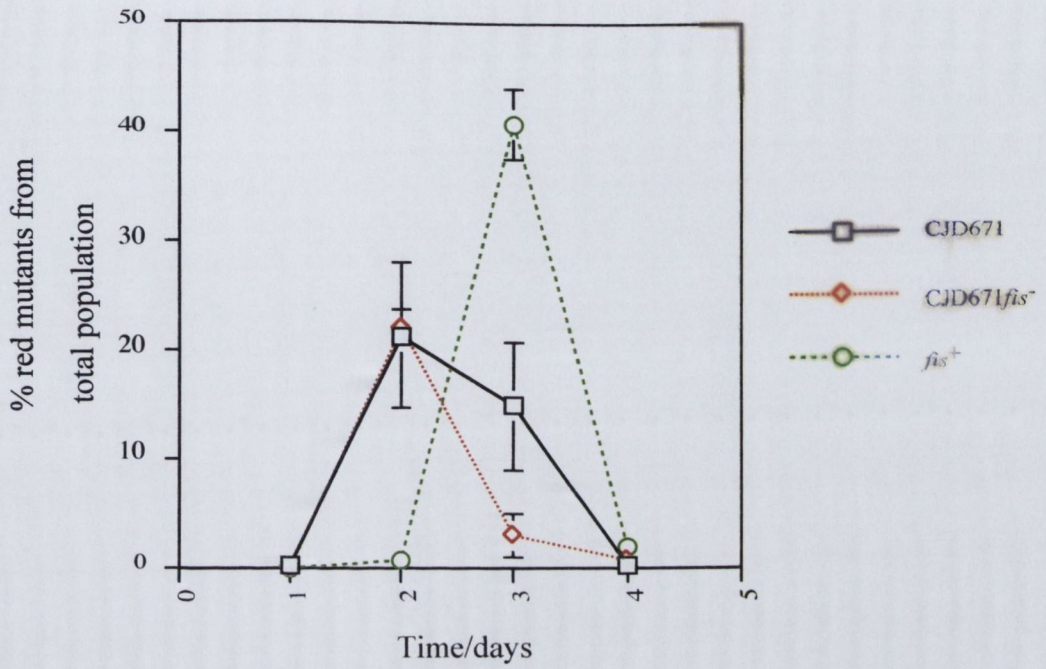


Fig. 3.5. **Effect of Fis on deletion formation.** Percentage of total population that can form red mutants over time for CJD671, CJD671*fis*$^-$ and CJD671*fis*$^-$ complemented with pUHE25-2 (represented by *fis*$^+$).

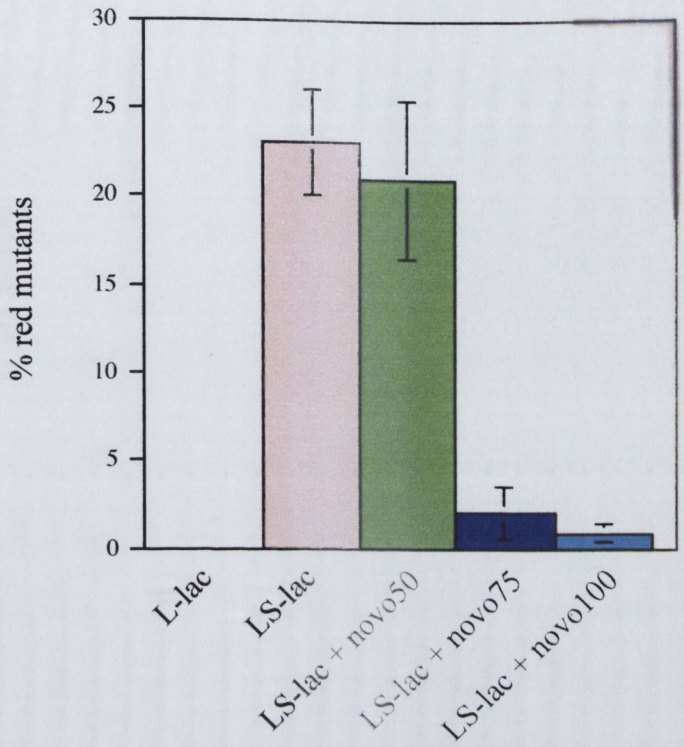


Fig. 3.6. **Effect of salt and novobiocin on deletion formation.** Percentage of red mutants that appear in different media. Salt is required in order for any red mutants to appear. This effect is counteracted by the DNA gyrase inhibiting antibiotic novobiocin (novo) in a dose-dependent manner.

negative superhelicity of the DNA decreases so does the number of red mutants proving that the effect of increased osmolarity in bringing about the deletion event is to stimulate DNA gyrase activity and hence the overall negative superhelicity of the DNA.

3.2.5: Visualisation of the effect of DNA supercoiling

In order to demonstrate the effect of both increased osmolarity and novobiocin on the overall superhelicity of the DNA the reporter plasmid pACYC184 (Table 2.2) was utilised. CJD671 was transformed with this plasmid. The strain was then grown in

1. LB broth.
2. LB broth supplemented with 0.275 M NaCl.
3. LB broth supplemented with 0.275 M NaCl and 100 μg/ml novobiocin.

Lactose was not added to the media as it was presumed not to be contributing to the osmotic pressure required for the deletion event to occur. Evidence for this came from the fact that the deletion could not be detected in LB broth supplemented with 0.275 M NaCl and a range of other sugars which exert a similar osmotic pressure to lactose (Massey, 1998). The reporter plasmid was isolated by Qiagen preparation, quantified by OD_{260nm} reading and equal amounts electrophoresed through an agarose-chloroquine gel. The results are shown in Fig. 3.7. Lane 1 shows plasmid isolated from CJD671 grown in LB media. Plasmid isolated after growth under these conditions shows a broad range of topoisomer distribution. Lane 2 shows plasmid isolated from CJD671 grown in LB broth supplemented with 0.275 M NaCl. Plasmid isolated after growth under these conditions shows an increase in the amount of negatively supercoiled plasmid. Lane 3 shows plasmid isolated after growth in LB broth supplemented with 0.275 M NaCl and 100 μg/ml novobiocin. The inclusion of novobiocin in the media negates the effect of the NaCl and again a broad range of plasmid topoisomers is once more visible.

Negative supercoiling overcomes a genetic constraint for the formation of red mutants. The requirement for increased DNA gyrase activity could be direct or indirect. DNA gyrase introduces negative supercoils into the DNA by introducing a double stranded break in the DNA, DNA is then passaged through this break and the gap resealed.

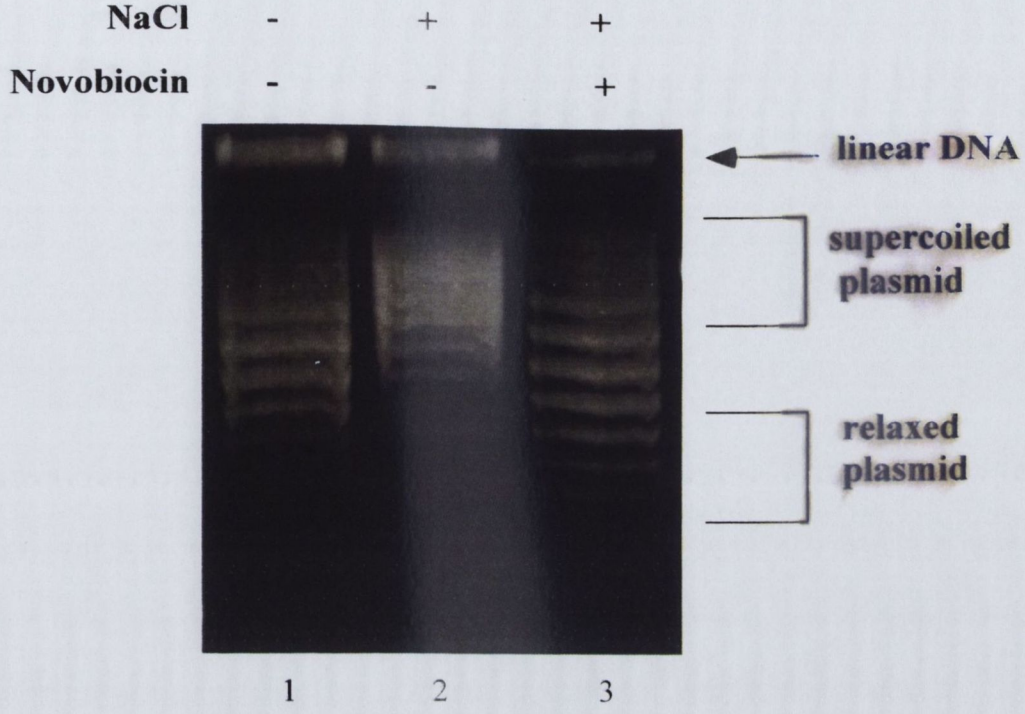


Fig. 3.7. **Effect of salt and novobiocin on topoisomer distribution.** Topoisomer distribution of pACYC184 isolated from CJD671 grown in LB broth (lane 1), LS- broth (lane 2) and LS-broth supplemented with 100 μg/ml novobiocin (lane 3). The addition of NaCl to the media results in an increase in negatively supercoiled plasmid while the addition of novobiocin counteracts this and more relaxed forms of the plasmid reappear.

DNA gyrase has previously been shown to be directly involved in illegitimate recombination, which is RecA independent (Miura-Masuda and Ikeda, 1990). It is proposed to act in illegitimate recombination as follows: 2 gyrase tetramers introduce 2 double stranded breaks in the DNA at the points of recombination. Each protomer binds covalently to the 5' termini of the DNA. If both gyrase tetramers can transiently associate to form an octamer then AB subunit exchange could occur between the 2 tetramers resulting in strand exchange. This would lead to the ligation of 2 new strands and hence illegitimate recombination (Ikeda *et al.*, 1982). Illegitimate recombination carried out by DNA gyrase also does not require sequence homology between the strands being exchanged (Naito *et al.*, 1984). This model was supported by the finding that treatment with oxolinic acid, which would increase the time the DNA and gyrase subunits were in contact, increased the frequency of illegitimate recombination in a cell free system, while treatment with coumermycin did not although it too would affect DNA supercoiling. If DNA gyrase tetramers introduced double stranded breaks at the 3' end of *rlgA* and at the *spvR* operator sequence and these 2 tetramers came together, strand exchange could occur that would ligate the 3' end of *rlgA* to the 5' end of *spvR*. The intervening sequence would be lost. This is represented schematically in Fig. 3.8 (A). Unfortunately the effect of oxolinic acid on the deletion event could not be tested. This is due to the pleiotropic effects of oxolinic acid treatment, which not only inhibits supercoiling but also DNA-dependent processes such as replication and transcription due to the double-stranded breaks in the DNA that it induces.

Alternatively DNA gyrase could be indirectly necessary for the deletion event. Increasing the negative superhelicity of the DNA could allow 2 DNA sequences that are distant on the linear sequence, such as *rlgA* and *spvR*, come into contact with each other. This may then allow another protein, as yet unidentified, act on the sequences to splice them together. A diagrammatic representation of this hypothesis can be seen in Fig 3.8 (B).

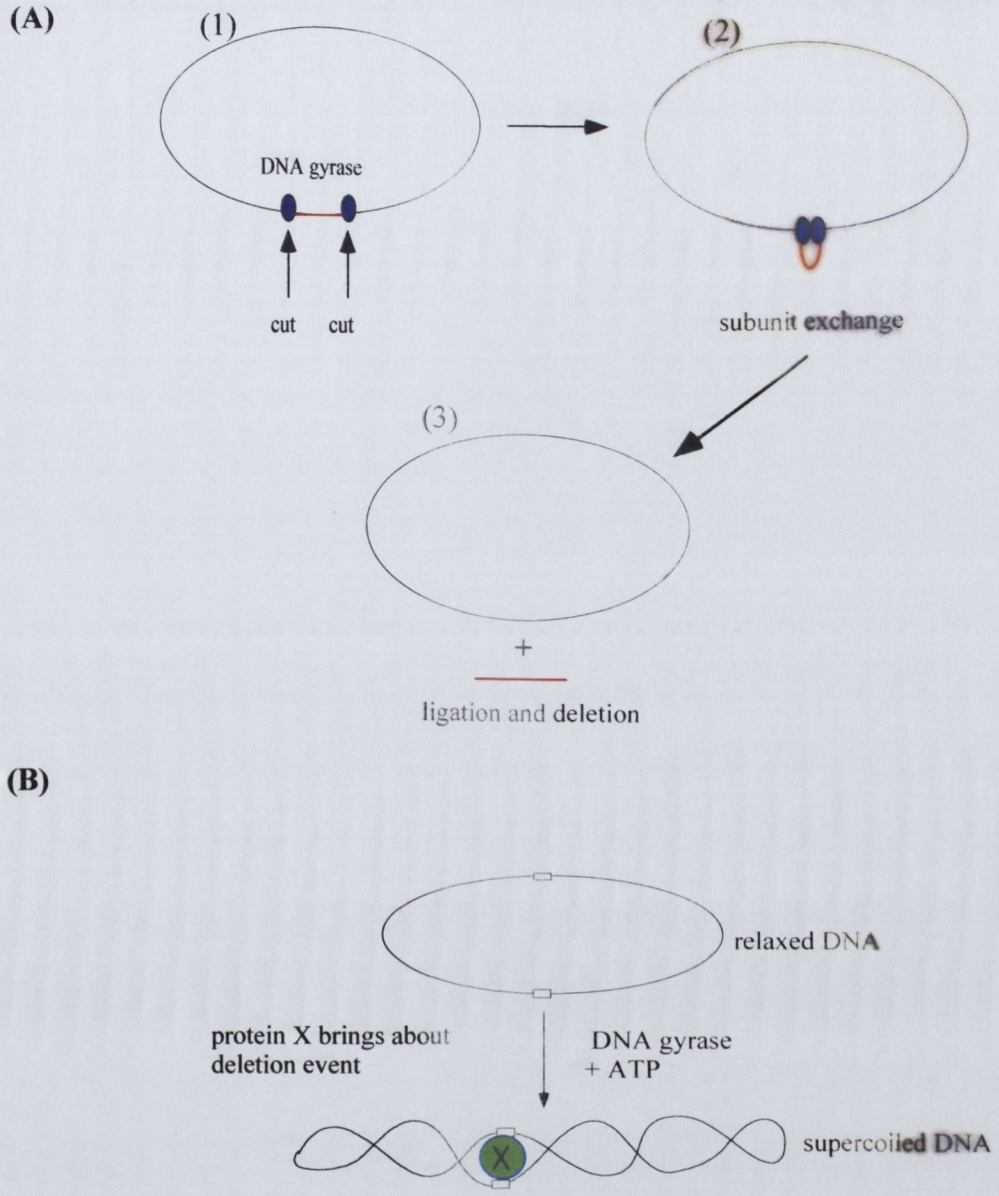


Fig. 3.8. (A) **Schematic illustration of how gyrase could bring about the deletion event directly**. Gyrase binds to the deletion end-points and introduces double stranded breaks at the deletion end-points (1). The 2 gyrase tetramers then associate and subunit exchange occurs (2). The deletion end-points are ligated together with the deletion of the intervening DNA (3).

(B) **Schematic illustration of how gyrase could bring about the deletion event indirectly.** Negative supercoiling brings together the deletion end-points which allows another protein, (X) cut and religate the DNA.

3.2.6: Mapping gyrase cleavage sites

In order to distinguish whether DNA gyrase participates directly or indirectly in the deletion reaction high frequency gyrase cleavage sites were mapped around the *spvR* deletion end point using the method of O'Connor and Malamy (1985). This method involves treating the DNA with oxolinic acid, a DNA gyrase inhibitor. This antibiotic works by allowing gyrase to introduce double stranded breaks into the DNA but it prevents religation of the DNA. This gyrase cleaved DNA is then extracted from the cells and treated with a protein denaturant to remove the bound gyrase. It is then digested with a restriction enzyme, electrophoresed through an agarose gel, transferred to a nylon membrane and probed using a probe specific to one end of the restriction fragment of interest (section 2.22). The probe hybridises to a number of fragments. The largest most intense band corresponds to the full-length restriction fragment. However the probe also hybridises to a number of shorter fragments that are generated by restriction endonuclease digestion at one end and by gyrase cleavage of the DNA at the other end. The position of the gyrase cleavage sites can then be mapped (section 2.22). The limitation of this method is that it only detects high frequency gyrase cleavage sites. Gyrase cleavage sites around the *spvR* deletion end point were mapped as can be seen in Fig. 3.9. Total chromosomal DNA was extracted from CJD671 grown in LB broth, CJD671 grown in LB broth with 50 μg/ml oxolinic acid, CJD671 grown in LB broth with 0.275 M NaCl and CJD671 grown in LB broth with 0.275 M NaCl and oxolinic acid 50 μg/ml. This DNA was quantified by OD_{260nm} readings and digested with either *Kpn*I (Fig. 3.9 (A) Lanes 1-4) or *Pvu*II and *Stu*I (Fig 3.9 (A) Lanes 5-8). The digestion products were electrophoresed through a 1% agarose gel, denatured and transferred to a nylon membrane. This membrane was then divided and each half probed with a probe corresponding to the 3' end of the *spv* containing restriction fragment. The probe hybridised to the *Kpn*I restriction fragment was generated with the oligos KpnF and KpnR (Table 2.3) and the probe hybridised to the *Pvu*II-*Stu*I fragment was generated with the oligos PvuF and PvuR (Table 2.3). The resulting Southern blot can be seen in Fig. 3.9 (A). One major hybridising fragment can be seen which corresponds to the full-length restriction fragment. This fragment is 9,028 bp in length for the *Kpn*I fragment and 3,610 bp in length for the *Pvu*II-*Stu*I fragment. A number of smaller weakly hybridising fragments can also be seen in the oxolinic acid treated lanes. These correspond to fragments generated by restriction

endonuclease digestion at the 3' end and gyrase cleavage at the 5' end. The positions of the high frequency gyrase cleavage sites were then mapped. The map for the *Pvu*II-*Stu*I fragment can be seen in Fig. 3.9 (B). Stars indicate the gyrase cleavage sites and the deletion end-point is indicated by an arrow. As can be seen they do not appear to coincide. A similar result was obtained for the *Kpn*I restriction fragment.

3.2.7: Analysis of the ability of other strains to form red mutants

When grown in LS-lac media CJD671 is under a strong selective pressure to adapt to this new lactose-containing environment in a way that allows it to readily metabolise lactose. We wished to determine if the ability to adapt to such a new lactose-containing environment was unique to CJD671. To this end 3 other strains were examined. The first was CJD574, which contains a *hisD*::MudJ fusion on the chromosome (Table 2.1). This gene encodes a component of the histidine biosynthetic pathway. The second was CJD661, which contains a *spvR*::MudJ fusion on the large virulence plasmid (Table 2.1) and lastly CH1081, which contains a *tppB*::MudJ fusion on the chromosome (Table 2.1). This gene encodes a tripeptide permease outer membrane porin, responsible for the transport of di and tri-peptides into the cell. These strains were chosen as all gave pale pink colonies on MacConkey lactose indicator plates. The strains were incubated for 4 days in LB broth, L-lac and LS-lac media and red mutants assayed for in the usual manner. As can be seen in Table 3.1 neither CJD574 nor CH1081 gave rise to red mutants under any of the conditions tested. CJD661 did give rise to red mutants in both L-lac media and LS-lac media. Therefore it appears that in this strain the genetic constraint that impedes deletion formation in CJD671 is absent. Two red mutants of CJD661 that arose in L-Lac (RM1 and RM2) and 2 red mutants that arose in LS-lac (RM3 and RM4) were single colony purified and retained for further analysis. Large virulence plasmid was prepared from these 4 red mutants as well as CJD671, CJD661 and CJD1127, a characterised 5 kb deletion mutant. This DNA was then digested with *Hpa*I and the resulting fragments electrophoresed through an agarose gel. The digestion pattern obtained is diagnostic for intact or rearranged large virulence plasmids. Cells that have undergone the 5 kb deletion event lose a band of approximately 7 kb and show the appearance of another band of 1.5-2 kb (Massey, 1998). As can be seen in Fig. 3.10 all 4 RM strains (Lanes

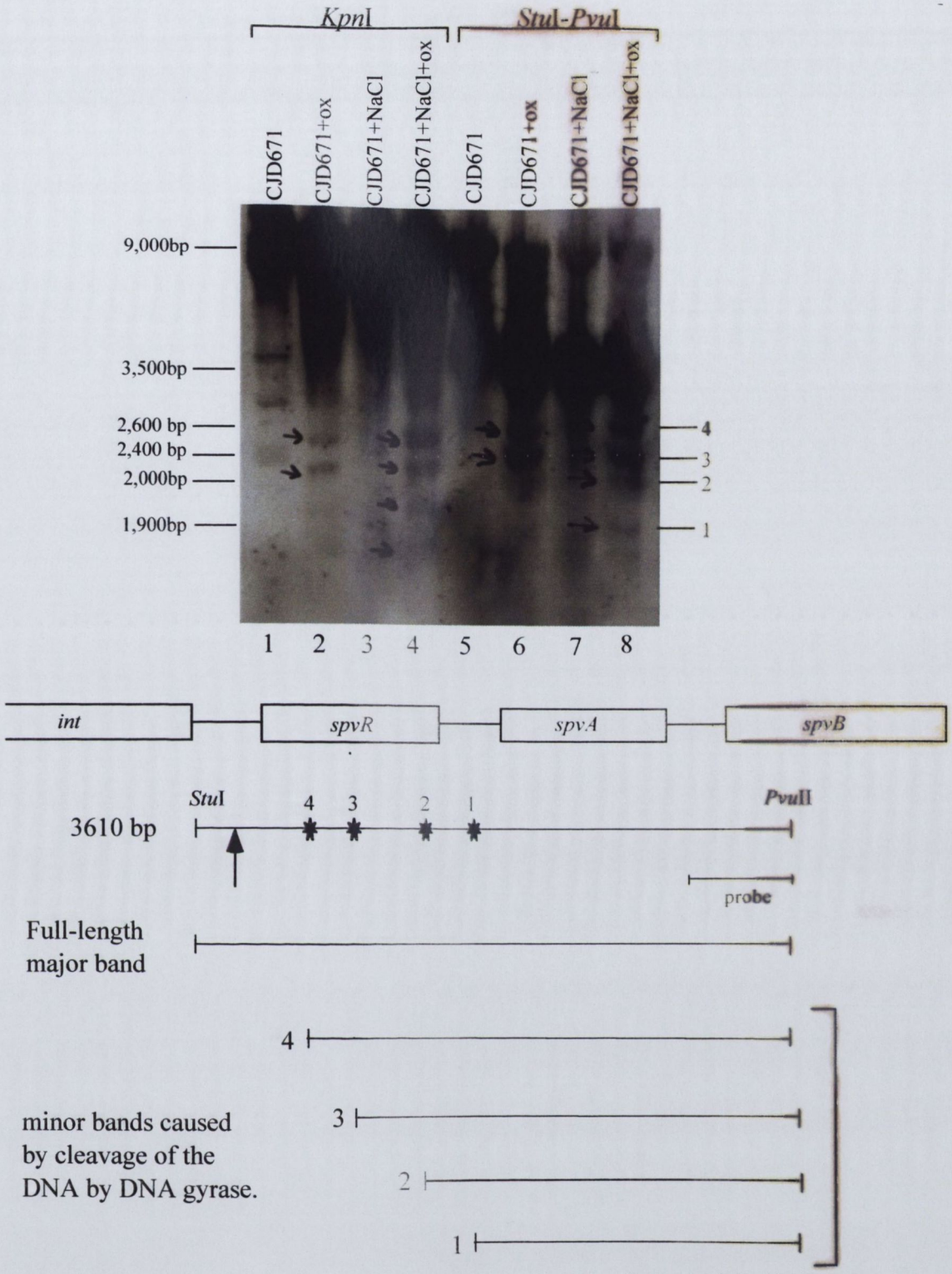


Fig. 3.9. **Mapping of DNA gyrase cleavage sites in the vicinity of the *spvR* deletion end-point.** (A) Southern blot to map high frequency gyrase cleavage sites in the vicinity of the *spvR* deletion end-point by the method of O'Connor and Malamy (1985). (B) Map of where the sites 1-4 locate to. The deletion end-point is indicated by an arrow and the high-frequency gyrase cleavage sites by stars. As can be seen none of the gyrase cleavage sites colocate with the deletion end-point.

	CJD671	CJD574	CJD661	CH1081
L broth	0%	0%	0%	0%
L-lac	0%	0%	26%	0%
LS-lac	21%	0%	18%	0%

Table 3.1. **Percentage of red mutants that arose in CJD671, CJD574, CJD661 and CH1081**. The bacteria were incubated in the media indicated.

5-8) appear to be 5 kb deletion mutants as shown by the disappearance of the 7 kb band and the appearance of a new band of approximately 1.9 kb. This is similar to the pattern seen for CJD1127 (Lane 2) a known deletion mutant. Both CJD671 (Lane 1) and CJD661 (Lane 3) show the larger 7 kb band. This ~7 kb band is not identical in CJD671 and CJD661 due to the different positions of the MudJ insertions in both strains. The approximately 1.9 kb band that appears in each of the red mutants is of slightly different sizes once again showing that the deletion event is not site-specific. The appearance of red mutants in CJD661 could also be inhibited by the DNA gyrase inhibiting antibiotic novobiocin showing that the mechanism of deletion is similar in both strains and involves DNA gyrase.

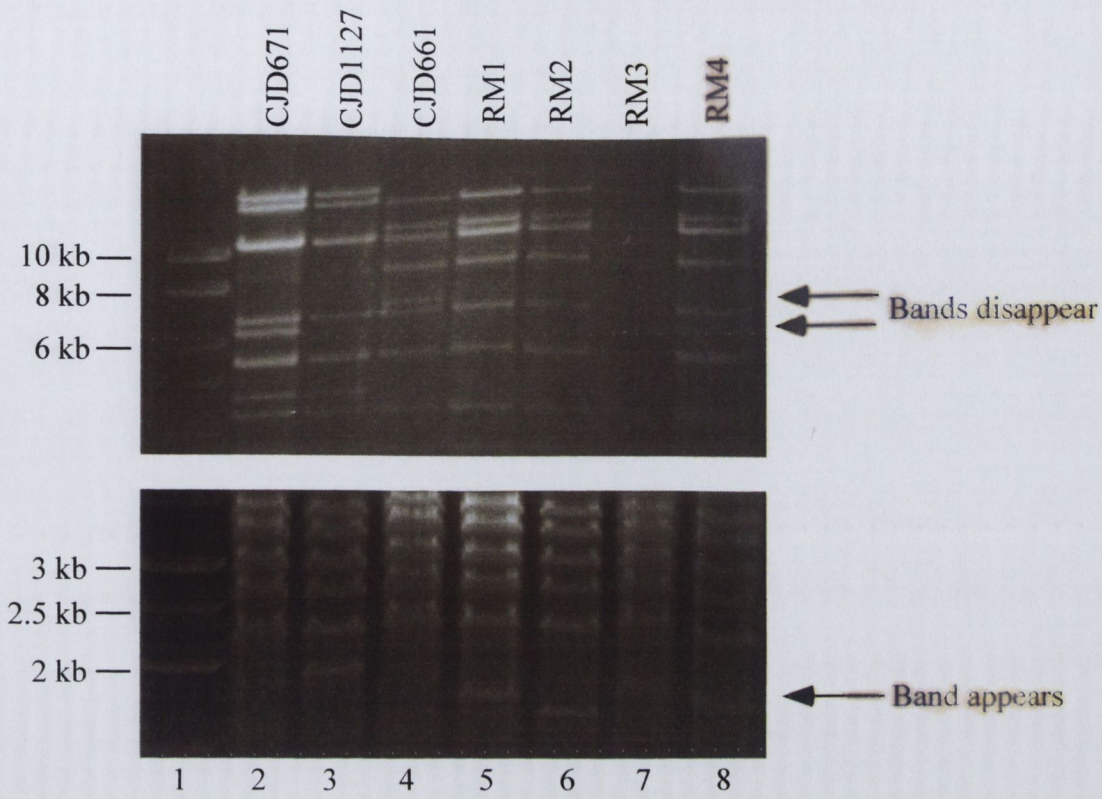


Fig. 3.10. **Analysis of CJD661 red mutants.** *Hpa*I digest of plasmid DNA from CJD671, CJD1127, CJD661 and four red mutants isolated after growth of CJD661 in L-Lac (RM1 and RM2), and LS-lac (RM3 and RM4). All four red mutants appear to have undergone the 5 kb deletion event as shown by the loss of a 7 kb band and the appearance of a 2 kb band, similar to CJD1127. The 2 kb band that appears is of different sizes in each of the 4 red mutants strains indicating that once again the deletion event is not site-specific.

3.3: Discussion

It had previously been shown that *S. typhimurium* strain CJD671 could delete 5 kb of DNA between *rlgA* and *spvR* on the large virulence plasmid upon prolonged incubation in LS-lac media. This deletion event allows the cells to upregulate *lacZ* expression and hence utilise the lactose in the media. This gives the mutant cells a selective advantage over the wild-type cells. This deletion event is reminiscent of adaptive mutation as the cells are grown under non-lethal selection and mutate to adapt to this new selection. Non useful mutations do not appear to accumulate. Other examples of adaptive mutations can be found in the literature (Shapiro, 1984; Cairns *et al.*, 1988; Hall, 1998). Such mutation events are interesting as they appear to contradict the neo-Darwinian theory of evolution, which states that mutations occur randomly by DNA replication errors and that beneficial mutations are selected for. In this case mutations occur in non-dividing cells and the mutation appears to be directed towards a specific locus. However Lamarckian explanations for adaptive evolution have been excluded (Mittler and Lenski, 1990; Maenhaut-Michel *et al.*, 1994; Sniegowski, 1995). There is now some evidence that a small population of bacteria can enter a hypermutable state in order to generate beneficial mutations (Torkelson *et al.*, 1997). In this specific case the environment activates the "deletion machine" which creates the *rlgA-spvR* fusion. Therefore the virulence plasmid has evolved a capacity to exploit this machine. Whether this is serendipity or programmed, we do not know.

In this case it was shown that the deletion event does not require RecA the major protein involved in homologous recombination. It is therefore presumed not to involve the cellular homologous recombination machinery. Neither does it require the product of the *rpoS* gene. This protein has previously been shown to regulate the appearance of Lac(Ara)$^+$ adaptive mutants in *E. coli*. The levels of the Fis protein do however appear to influence the frequency of the deletion event. This was a little surprising as this adaptive mutation event occurs late in stationary phase when Fis protein is presumed not to be present (Ball *et al.*, 1992). Some reports suggest however that molecules of Fis remain in the cell for up to two days after nutrient upshift, although not enough protein remains to be detectable by Western immunoblotting (Keener and Nomura, 1996). The absence of Fis results in a decrease in the percentage of red

mutants detected on day 3 but not day 2. Therefore Fis may be affecting the appearance of other classes of red mutants besides the 5 kb deletion mutants. Alternatively Fis may affect the ability of the mutants to persist in LS-lac. The overexpression of Fis however results in a large increase in the number of red mutants that appear. The fact that overexpressing Fis has a greater effect is probably not surprising given that in this case Fis continues to be expressed throughout the growth phase and into stationary phase. Therefore expression of Fis in the complemented mutant is temporally different to that of the wild type. This is therefore not a straightforward complementation.

The absolute requirement for salt in order for the deletion event to occur in CJD671 was shown to actually be a requirement for increased DNA gyrase activity. This was shown by the fact that novobiocin (a DNA gyrase inhibitor) could negate the effect of increased osmolarity thereby showing that salt was exerting its effect through DNA gyrase. DNA gyrase could be directly or indirectly involved in bringing about the deletion event. The evidence appears to point to an indirect role for DNA gyrase. High frequency DNA gyrase cleavage sites in the vicinity of the *spvR* deletion end-point were mapped and they did not appear to co-locate with the deletion end-point. However this does not definitively exclude the possibility that a low frequency cleavage site exists here. Increasing salt concentration results in increased gyrase activity (McClellan *et al.*, 1990). If gyrase was acting directly then one would assume that there would be no upper osmolarity limit at which this deletion event can occur. The fact that there is an upper limit to the osmolarity at which this deletion event can occur also appears to favour the indirect model. If gyrase was acting indirectly then one would assume that there would be an optimal level of negative supercoiling at which both *rlgA* and *spvR* are in proximity and the deletion event can occur. As one moves further away from this optimal level in either direction one would expect to see a decrease in the frequency of red mutants appearing. This is what is observed in the laboratory giving further credence to the indirect model.

In *E. coli* it is known that the Fis protein is a repressor of the *gyrA* and *gyrB* genes (Schneider *et al.*, 1999). Therefore Fis could be exerting its effect on the deletion event via an effect on DNA gyrase expression. As Fis levels change this alters DNA gyrase activity and hence the deletion frequency. Indeed Fis has been shown to have

an effect on *gyrA* and *gyrB* levels in *E. coli* even in overnight cultures when very small quantities of Fis protein are present (Schneider *et al.*, 1999). In CJD671*fis*⁻ DNA gyrase activity could be increased beyond the optimal level for the deletion event to occur. This would once again support the idea that DNA gyrase participates indirectly in the deletion event. For the complemented mutant negative supercoiling is possibly at an optimal level for the deletion event to occur. This model however assumes that Fis is a regulator of the *Salmonella gyrA* and *gyrB* genes, a fact that will be demonstrated in Chapter 6. Alternatively Fis could exert its influence on the deletion event by binding in the deletion region and constraining negative supercoils or by the formation of a nucleoprotein complex, analogous to that in the *Salmonella* flagellar phase variation system, that is required for deletion formation.

Other *S. typhimurium* strains that contained *lacZ* fusions to weakly expressed genes were also examined for their ability to adapt to a lactose containing environment during starvation. Neither of two strains that contained chromosomal MudJ insertions could do so however CJD661 which contained a *spvR*::MudJ fusion could adapt. The molecular rearrangement that brought about this adaptation was shown to be identical to that of CJD671 i.e. the deletion of 5 kb of DNA between *rlgA* and *spvR* and the creation of a derepressed *rlgA-spvR* transcriptional fusion. However in CJD661 the need for increased osmolarity is abrogated as the deletion can occur in L-lac media. This would once again appear to favour the model that gyrase is acting indirectly. In CJD671 and CJD661 the deletion end-points are similar, therefore if DNA gyrase was acting directly one would presume that increased DNA gyrase activity would be required in order to cleave the DNA in both cases. In CJD661 the 2 deletion end points are potentially capable of coming into contact without increased supercoiling thereby allowing the unidentified protein, protein X, bring about the deletion event. The 2 sequences could be in contact in CJD661 due to the MudJ insertion immediately downstream of the 3' deletion end-point. This would alter the DNA structure in this region and also possibly alter binding by DNA bending proteins thereby potentially allowing *rlgA* and *spvR* come in contact with one another. The presence of novobiocin in the growth media reduces the frequency at which red mutants appear in CJD661. Novobiocin treatment will relax the DNA and this may effect the ability to *rlgA* and *spvR* to contact each other in order to form the deletion.

Chapter 4

Identification of *trans*-acting factors involved in deletion mutant formation

4.1: Introduction

Salmonella typhimurium strain CJD671 (Table 2.1) can undergo the deletion of 5 kb of DNA between the *rlgA* and *spvR* genes when grown for 2 days in the presence of salt and lactose. Its ability to undergo this deletion event is dependent on DNA gyrase but DNA gyrase does not appear to bring about this deletion event directly. In order to identify any *trans*-acting factors that may be involved in the deletion event it was decided to carry out transposon mutagenesis of the CJD671 genome and also to generate an overexpression library of the CJD671 genome. Transposon mutagenesis which inactivated an essential component of the deletion machinery would result in a strain unable to form deletions. Conversely mutagenesis of a repressor of the deletion machinery would form a strain that instantly gave deletion mutants. Overexpression of a positive regulator of the deletion system could also result in a strain that formed red mutants instantly and overexpression of a repressor of the system could result in a strain with an inability to form deletion mutants.

In order to carry out the transposon mutagenesis the vector pBSL181 (Table 2.2) was utilised (Alexeyev and Shokolenko, 1995). This plasmid has a number of salient features and a map of the plasmid can be seen in Fig. 4.1. The plasmid contains a *tnp* gene encoding a Tn*10* transposase with *ats1* and *ats2* mutations. These mutations drastically reduce the "hot-spot" specificity of the wild-type Tn*10* transposase. This Tn*10* transposase is under the control of the P_{tac} promoter and so is IPTG inducible. The plasmid also contains the *lacI*^{q} gene. This encodes the LacI repressor, which represses the P_{tac} promoter in the absence of an inducer such as IPTG. The plasmid contains a β–lactamase gene that encodes resistance to the antibiotic ampicillin. It also contains a 300 bp *mob* or mobility region from the RP4 plasmid, which allows conjugative transfer of the vector in a wide range of Gram negative bacteria. The plasmid replicates from the γ–*ori* of the R6K plasmid. Replication of this plasmid is restricted to those *E. coli* strains that can provide *in trans* the π protein encoded by R6K. This determines the suicidal properties of pBSL181. Finally the plasmid carries a *cml* gene which encodes resistance to the antibiotic chloramphenicol. This *cml* gene is flanked by IS*10* inverted repeats which are the substrate for the Tn*10* transposase. The plasmid also contains a multiple cloning site upstream of the *cml* gene (Alexeyev and Shokolenko, 1995). Another important feature of this plasmid is the presence of a

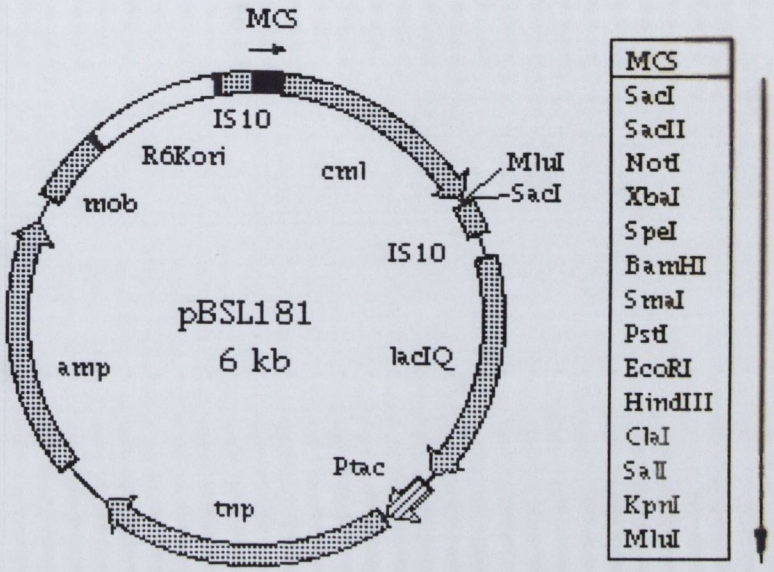


Fig. 4.1. **Map of pBSL181.** The chloramphenicol resistance cassette (*cml*) is flanked by 2 IS*10* sequences. The transposase gene *tnp* is under the control of the P_{tac} promoter. This promoter is repressed in the absence of IPTG by LacI. The plasmid encodes ampicillin resistance and replicates from the γR6K origin of replication. This plasmid map is taken from the catalog of the cloning vector collection 2000.

strong promoter pOUT, in the IS*10* sequence reading outwards from the *cml* gene that can be used to transcribe downstream genes (Simons *et al.*, 1983). This plasmid therefore can be used as a mutagenesis tool and an overexpression tool simultaneously as the insertion of the *cml* cassette into a gene will result in the disruption of the gene. Insertion of the *cml* cassette into an intergenic region downstream of a transcription terminator will also result in the transcription and overexpression of downstream genes. Transposon insertions occur when IPTG is added to strains containing pBSL181 but lacking the π protein. This results in derepression of the *tnp* gene encoding the Tn*10* transposase. This transposase then acts on the IS*10* inverted repeats and transposes the *cml* cassette randomly around the genome. As the plasmid cannot replicate in the absence of π pBSL181 is lost and the *cml* cassette is stably inherited (Alexeyev and Shokolenko, 1995).

Once transposon mutagenesis is carried out in CJD671 then interesting mutants have to be screened for in order to be characterised further. This presents a problem, as deletion formation in CJD671 is a population phenomenon and not a single cell phenomenon. In a population of CJD671 grown for 3 days with salt and lactose approximately 20% to 30% of the population will form deletion mutants. The other 70% to 80% do not as deletion mutants only have a fitness advantage when rare in the population (Massey *et al.*, 1999). Therefore the fact that a cell with a transposon insertion does not form a deletion mutant does not mean that it is incapable of doing so. In order to test if it is incapable of forming deletion mutants the cell would need to be grown in LS-lac broth for 2-3 days and the resulting population tested for the presence of red mutants. There are approximately 4,000 genes in the *E. coli* and *Salmonella* genomes. In order to be statistically confident of inactivating each gene at least once the genome would need to be covered at least 5 fold. Therefore when carrying out transposon mutagenesis in *Salmonella* or *E. coli* 20,000 to 30,000 colonies would need to be tested in order to be confident of having complete genome coverage. To grow 20,000 to 30,000 cultures for 3 days and test each population for an ability to form red mutants is not trivial. Therefore it was decided to screen for mutants that went red at an accelerated rate as these could easily be visualised on MacConkey lactose indicator plates.

CJD671 contains a MudJ insertion in the *spvB* gene generating a *spvB-lacZ* transcriptional fusion. Therefore screening for factors that result in strains that form red colonies instantly may also pick up genes involved in *spv* gene regulation. For example the inactivation of *hns* by transposon mutagenesis will result in red colonies not because of a role for H-NS in the deletion event but rather because H-NS is a negative regulator of *spv* gene expression (O'Byrne and Dorman, 1994b). Therefore any mutant that is red due to upregulated *spv* gene expression will need to be distinguished from mutations that are affecting deletion mutation formation.

The aim of this work was to identify any *trans*-acting factors that are involved in deletion mutation formation in CJD671.

4.2: Results

4.2.1: Transposon mutagenesis of CJD671

In order to harvest large quantities of plasmid pBSL181 this plasmid was transformed into the *E. coli* strain SM10λpir (Table 2.1). This strain is lysogenised with phage λ and expresses the π protein required for replication initiation at the γ-*ori* of pBSL181 (Simon *et al.*, 1983). SM10λpir+pBSL181 was grown overnight and pBSL181 isolated by Qiagen preparation (section 2.6.2). Approximately 60 μg of purified plasmid was obtained. 1 μg of this plasmid was then electroporated into CJD671 and transposition induced by the addition of 0.3 mM IPTG (section 2.23). Transposon mutants were then selected by growth on chloramphenicol agar plates. The yield of transposon mutants by this method was however very low and did not result in complete genome coverage. One potential problem is that the pBSL181 plasmid, isolated from *E. coli* is recognised as foreign DNA and is restricted by *Salmonella* restriction endonucleases on entry into CJD671. The reciprocal restriction barrier between *E. coli* K-12 and *S. typhimurium* LT2 is known to reduce electrotransformation efficiency approximately 100-fold (Binotto *et al.*, 1991). Normally this problem is overcome by passaging the plasmid through a *Salmonella* restriction$^-$ modification$^+$ strain such as LB5010 (Table 2.1). However in this case this was not possible as pBSL181 would not be capable of replicating in such a host due to the absence of the π protein. Therefore in order to overcome the restriction barrier the large virulence plasmid of CJD671 (p671) was moved into the *E. coli* strain MC4100 (Table 2.1) by electroporation giving the strain MC4100p671. MC4100 was the strain of choice as it is *lac*$^-$. This ensures that all the β-galactosidase activity measured in MC4100p671 must be coming from the *spvB-lacZ* fusion. The next step was to ensure that this strain could form red mutants similar to CJD671. MC4100p671 and CJD671 were grown for 3 days in LS-lac and each day appropriate dilutions were plated on MacConkey lactose indicator plates and the percentage of red mutants that appeared was calculated. MC4100p671 was found to give red mutants at a frequency similar to wild-type CJD671 showing that the factors involved in deletion mutation are either common to *E. coli* and *Salmonella* or are encoded on the large virulence plasmid itself.

1 μg of pBSL181 DNA was then electroporated into MC4100p671, transposition was induced by the addition of 0.3 mM IPTG and transposon mutants were selected by growth on chloramphenicol agar plates. Approximately 20,000 transposon mutants of MC4100p671 were screened for their ability to form red deletion mutants at an accelerated rate. This is a transposon insertion approximately every 230 bp or a 5-fold coverage of the entire *E. coli* genome (~4,600,000 bp in *E. coli* genome/20,000 mutants screened = 1 insertion every 230 bp; ~4,000 genes and 20,000 screened = 5-fold coverage of the genome). From this screen 49 colonies (hereafter named TM 1-49) that were red on MacConkey lactose indicator plates were single colony purified and retained for further analysis. These 49 strains were streaked on ampicillin agar plates to test for ampicillin sensitivity. All were found to be ampicillin sensitive. This ensures that all the mutants arose from a true transposition event and not from integration of the entire pBSL181 plasmid into the genome of MC4100p671 (Alexeyev and Shokolenko, 1995).

4.2.2: Marker rescue of transposon mutants

In order to confirm that the transposon insertion was responsible for the red phenotype in TM 1-49 a marker rescue experiment was carried out. Bacteriophage P1 *vir* transducing lysates were made of each of the transposon mutants TM 1-49. These lysates were then used to transduce MC4100p671 to chloramphenicol resistance. The recipient strain was then plated out on MacConkey lactose indicator plates in order to check that the red phenotype co-transduced with chloramphenicol resistance. In each case it was found that the red phenotype was indeed a result of the transposon insertion as the red phenotype cotransduced with the *cml* cassette with 100% frequency. However for three of the transposon mutants P1 *vir* transducing lysates could not be made. These mutants were TM 1, TM 29, and TM 49. The reason transducing lysates could not be made from these three strains is unknown. For a number of the other mutants only a small number of transductant colonies were obtained however in each of these cases the red phenotype co-transduced with the *cml* cassette.

4.2.3: Southern blotting of transposon mutants

There was a possibility that in some cases the transposon had inserted into the same site in the genome twice therefore giving rise to 2 transposon mutants with insertions in the same gene. In order to eliminate such overlaps and simplify identification of the mutants it was decided to carry out a number of Southern blots to determine where on the genome the chloramphenicol resistance cassette had inserted. Strains with insertions in the same site in the genome could then be eliminated. Total genomic DNA was isolated from all 49 transposon mutants. This genomic DNA was then digested singly with both *Bam*HI and *Pst*I. Both these enzymes cut once within the multiple cloning site at the 5' end of the *cml* cassette of pBSL181. This will generate a restriction fragment containing the *cml* cassette along with downstream DNA until the next restriction cut site. The restriction fragments were electrophoresed through 1% agarose gels. After transfer to nylon membranes the restriction fragments were probed for the *cml* cassette using a DIG-labelled probe generated from the oligonucleotides catFwd and catRev (Table 2.3). The resulting Southern blots can be seen in Figs. 4.2 and 4.3 for *Bam*HI and *Pst*I respectively. The size of each hybridising fragment was estimated and is tabulated in Table 4.1. From these results mutants that gave hybridising bands of similar size and therefore were potentially identical to each other were grouped together and are shown in Table 4.2. Genomic DNA from TM 1-49 was then digested with *Eco*RI. This enzyme also cuts in the MCS of pBSL181. These digestion products were then electrophoresed through 1% agarose gels and Southern blotted using the *cml* probe as before. Mutants that were potential duplicates of each other were loaded beside one another. This was done in order to overcome the difficulty of estimating if fragments are of equal size when they are loaded on different gels. The result of this Southern blot can be seen in Fig. 4.4. A number of the potential duplicates gave similarly sized *Eco*RI restriction fragments thereby providing further evidence that they contained transposon insertions in the same site in the genome. These were TM 1, TM 8, TM 15, TM 18, TM 21 and TM23; TM 3, TM 7 and TM 33; TM 4 and TM 22; TM 5, TM 12, TM 13, TM 16, TM 17, TM 25, TM 39 and TM 49; TM 9, TM 14, TM 34, TM 43 and TM 48; TM 10, TM 30 and TM 40; and TM 44 and TM 46. Digestion with *Eco*RI also showed that TM 19 and TM 24 were not identical to TM 5, TM 32 and TM 47 were not identical to TM 10 and TM 2 and TM 37 were not identical to each other as predicted previously.

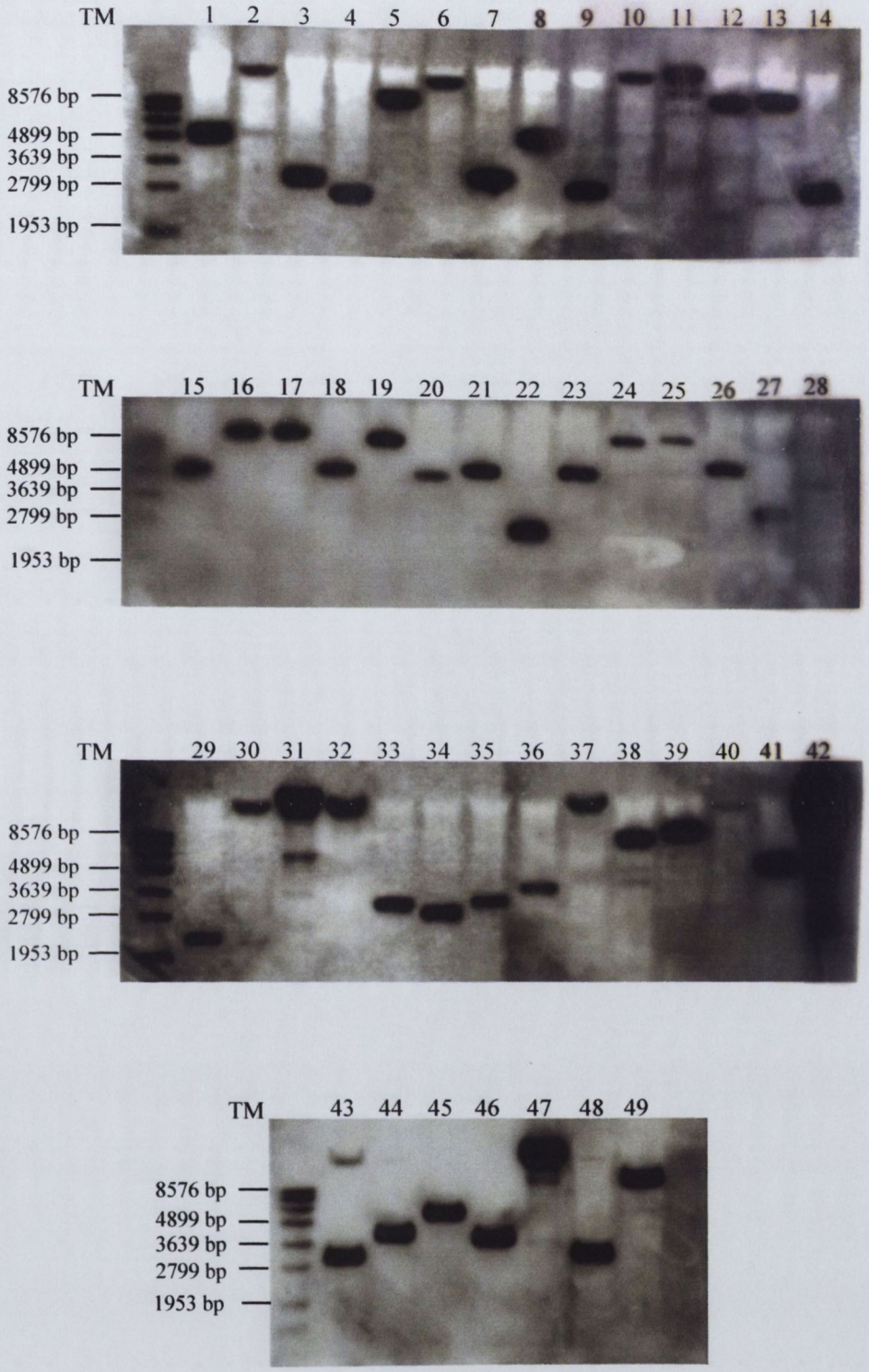


Fig. 4.2. **Analysis of transposon mutants by *Bam*HI Southern blot.** Southern blot of genomic DNA from TM 1-49 restricted with *Bam*HI and probed with a DIG-labelled *cml* probe.

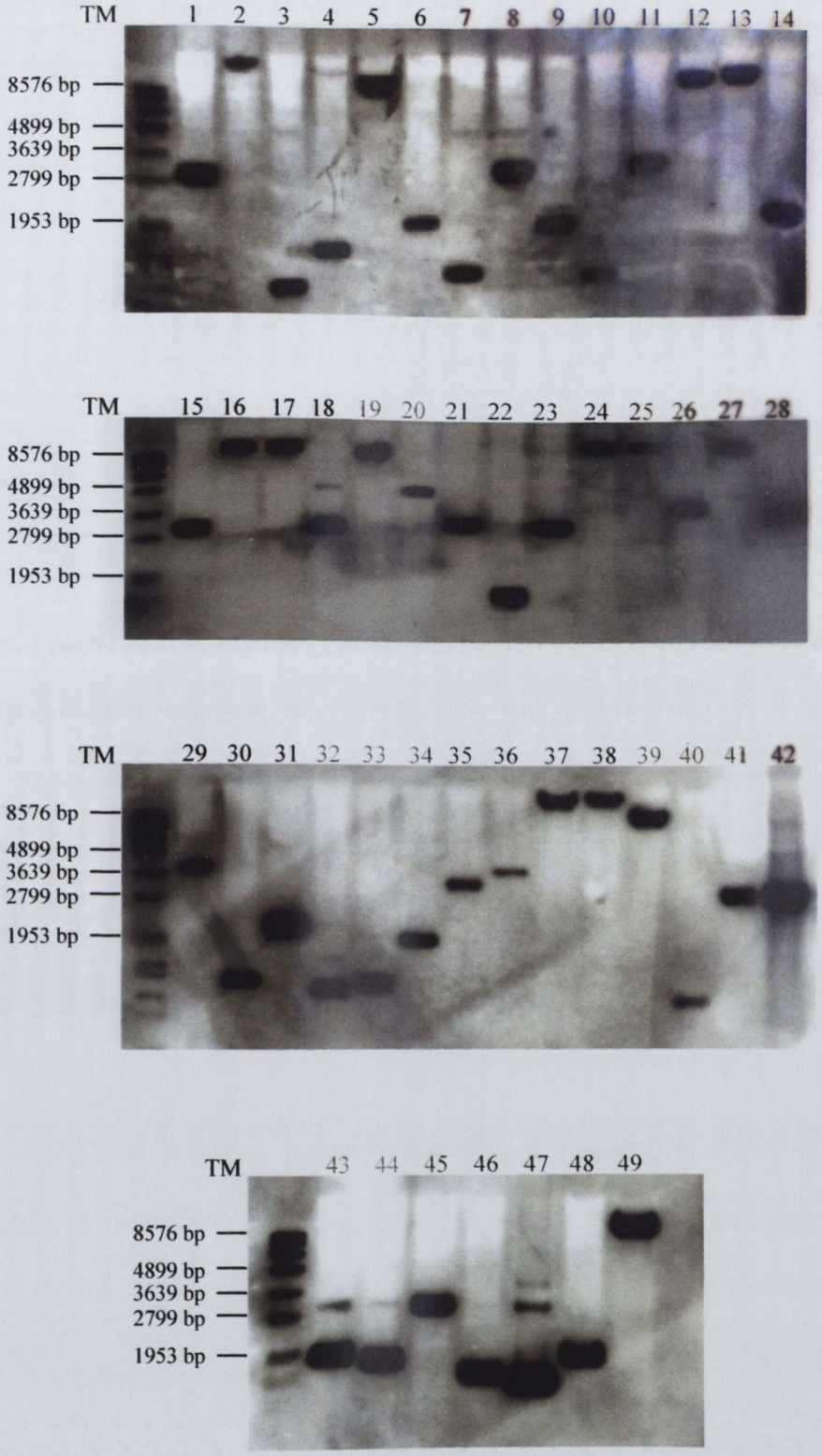


Fig. 4.3. **Analysis of transposon mutants by *Pst*I Southern** blot. **Southern blot of** genomic DNA from TM 1-49 restricted with *Pst*I and probed with **a DIG-labelled** *cml* probe.

Transposon mutant	*cml* containing *Bam*HI restriction fragment length/bp	*cml*-containing *Pst*I restriction fragment length/bp	β-galactosidase activity/ Miller units
TM 1	5500	3000	1662
TM 2	>>9000	>>9000	80
TM 3	3200	1000	1842
TM 4	2700	1500	1008
TM 5	>9000	>9000	1010
TM 6	>9000	2100	91
TM 7	3200	1250	1666
TM 8	5500	3200	1609
TM 9	3000	2100	366
TM 10	>9000	1250	74
TM 11	>9000	3200	843
TM 12	>9000	>9000	1242
TM 13	>9000	>9000	2849
TM 14	2800	2300	447
TM 15	5000	3200	1371
TM 16	>9000	>9000	1368
TM 17	>9000	>9000	1424
TM 18	5000	3200	1456
TM 19	>9000	>9000	307
TM 20	4800	5000	154
TM 21	5000	3200	1730
TM 22	2500	1500	920
TM 23	5000	3200	1575
TM 24	>9000	>9000	173
TM 25	>9000	>9000	1404
TM 26	5500	4300	505
TM 27	3200	>9000	40
TM 28	3800	4000	359
TM 29	2350	4200	245

TM 30	>9000	1500	72
TM 31	>9000	2300	93
TM 32	>9000	1200	241
TM 33	3400	1200	1545
TM 34	3200	2100	388
TM 35	3600	3600	168
TM 36	4000	4000	111
TM 37	>>9000	>>9000	71
TM 38	>9000	>>9000	37
TM 39	>9000	>9000	1199
TM 40	>9000	1500	64
TM 41	7200	3200	1010
TM 42	>9000	3200	319
TM 43	3000	2000	510
TM 44	4500	1800	1432
TM 45	6000	3200	665
TM 46	4500	1600	1288
TM 47	>9000	1500	573
TM 48	3000	2000	468
TM 49	>9000	>9000	1139

Table 4.1. ***Bam*HI and *Pst*I *cml* restriction fragment lengths for TM 1-49 and β–galactosidase activity of TM 1-49.** Column 2 and column 3 list the *Bam*HI and *Pst*I restriction fragment length for the *cml*-containing fragment from TM 1-49. Column 4 lists the β-galactosidase activity in Miller units for TM 1-49.

Transposon mutants that are potentially identical.
TM 1, TM 8, TM 15, TM 18, TM 21, TM 23.
TM 2, TM 37.
TM 3, TM 7, TM 33.
TM 4, TM 22.
TM 5, TM 12, TM 13, TM 16, TM 17, TM 19, TM 24, TM 25, TM 39 TM 49.
TM 6, TM 31.
TM 9, TM 14, TM 34, TM 43, TM 48.
TM 10, TM 30, TM 32, TM 40, TM 47.
TM 11, TM 42.
TM 20.
TM 26.
TM 27.
TM 28, TM 36.
TM 29.
TM 35.
TM 38.
TN 41.
TM 44, TM 46.
TM 45.

Table 4.2. **Potentially identical transposon mutants.** Transposon mutants that give *cml* restriction fragments of similar length when cut with *Bam*HI and *Pst*I are grouped together in rows as these potentially contain transposon insertions in the same site of the genome.

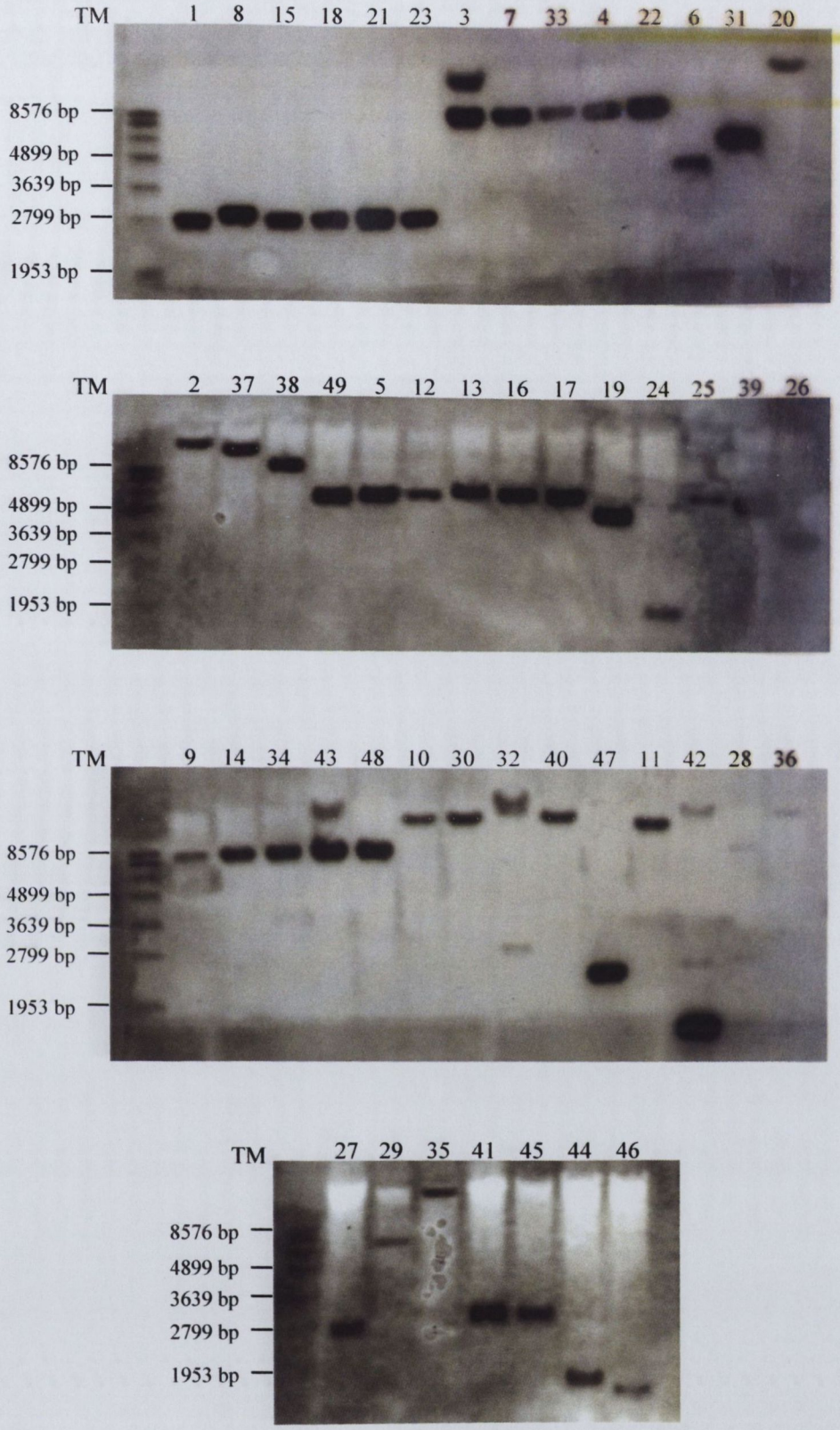


Fig. 4.4. **Analysis of transposon mutants by *Eco*RI Southern blot.** Southern blot of genomic DNA from TM 1-49 restricted with *Eco*RI and probed with a DIG-labelled *cml* probe.

4.2.4: β-galactosidase activity of transposon mutants

It was assumed that 2 mutants with transposon insertions at the same site in the genome would give similar levels of β-galactosidase expression from the *spvB-lacZ* fusion. Therefore stationary phase β-galactosidase assays were carried out on TM 1-49 in order to identify mutants with similar β-galactosidase activity and hence aid in the elimination of identical mutants. Duplicate cultures of each of the transposon mutants were grown overnight in LB broth and each culture was assayed twice for β-galactosidase activity (section 2.5.2). The mean of the 4 values are listed in Table 4.1 for each mutant. Again a number of the transposon mutants had similar levels of β-galactosidase activity and these results correlated well with the Southern blot data. These β-galactosidase data were combined with the Southern blot data and a number of transposon mutants with insertions in the same gene were identified. Mutants were assumed to have transposon insertions in the same gene if they contained similarly sized *cml* hybridising fragments on all 3 Southern blots and had similar β-galactosidase levels. The only anomaly was TM 13. This mutant was predicted to be identical to TM 5 from the Southern blot data however it has a much higher level of β-galactosidase expression. It was therefore decided to sequence this mutant in addition to TM 5. Table 4.3 lists all of the transposon mutants and whether they contain insertions at unique sites in the genome or not. From this table it is clear that 21 transposon mutants can be eliminated from the screen, as they appear to have been isolated more than once. The mutants eliminated were TM 1, TM 15, TM 18, TM 21 and TM 23 as these were all identical to TM 8 (TM 8 was chosen to be sequenced in this case as no P1 *vir* transducing lysate could be made of TM 1 for marker rescue). TM 7 and TM 33 were also eliminated, as they were identical to TM 3. TM 12, TM 16, TM 17, TM 25, TM 39 and TM 49 were excluded from the screen as they contain insertions in the same gene as TM 5. TM 14, TM 34, TM 43 and TM 48 were eliminated, as they are identical to TM 9. TM 30 and TM 40 which are duplicates of TM 10 and TM 46 which is the same as TM 44 were also excluded. TM 38 was also eliminated from the screen as this mutant grew very poorly and died when stored on agar plates at 4°C. TM 4 and TM 22 appear to have transposon insertions in identical genes however it was decided to sequence both of these mutants in order to validate the method for excluding mutants from sequencing.

List of transposon mutants and any duplicates.
TM 1, TM 8, TM 15, TM18, TM 21, TM 23.
TM 2
TM 3, TM 7, TM33.
TM 4, TM22.
TM 5, TM 12, TM 16, TM 17, TM 25, TM 39, TM 49.
TM 6.
TM 9, TM 14, TM 34, TM 43, TM 48.
TM 10, TM 30, TM 40.
TM 11.
TM 13.
TM 19.
TM 20.
TM 24.
TM 26.
TM 27.
TM 28.
TM 29.
TM 31.
TM 32.
TM 35.
TM 36.
TM 37.
TM 38.
TM 41.
TM 42.
TM 44, TM 46.
TM 45.
TM 47.

Table 4.3. **Transposon mutants and their duplicates.** Transposon mutants that are assumed to be identical are listed in the same row. This eliminated 21 transposon mutants from the screen.

4.2.5: Determining if the transposon mutants are deletion mutants

In order to distinguish between transposon mutants that were red on MacConkey lactose indicator plates due to an accelerated deletion event and those that were red due to an effect on *spv* gene regulation PCR analysis of the deletion mutants was carried out. The primers delTMF and delTMR (Table 2.3) amplify 343 bp of sequence upstream of *spvR*. In deletion mutants this sequence is absent and therefore no product is visible after agarose gel electrophoresis. In non-deletion mutants this sequence remains intact, can be amplified and is visible after agarose gel electrophoresis. PCR analysis of all 49 transposon mutants showed amplification of a band of approximately 350 bp (Fig. 4.5). This showed that none of the mutants is the result of accelerated deletion mutation formation. It was therefore presumed that all the transposon insertions were in genes that affect the *spv* system either directly or indirectly.

4.2.6: Cloning and sequencing of transposon mutants

From the Southern blots shown in Figs. 4.2, 4.3 and 4.4 a suitable restriction enzyme was chosen for the cloning of the *cml* cassette and its surrounding DNA from the genome of each of the transposon mutants. This clone was then sequenced and the gene into which the *cml* cassette inserted identified. A suitable enzyme was one, which on Southern blotting gave a hybridising band of between 1.5 kb and 7 kb when probed with the *cml* probe. As the chloramphenicol cassette itself is approximately 1 kb then a minimum of an additional 500 bp of DNA was desired in order to identify the gene into which the transposon had inserted. Fragments larger than 7 kb are generally difficult to clone due to the large size of the recombinant plasmid. The enzyme chosen to clone the chloramphenicol cassette from each transposon mutant is listed in Table 4.4. For TM 2 and TM 37 neither *Eco*RI nor *Bam*HI nor *Pst*I were suitable as all gave hybridising fragments >9 kb on Southern blotting. Genomic DNA from each transposon mutant to be sequenced was digested to completion with the desired enzyme. The enzyme was then removed using the Promega PCR clean up kit (section 2.7.3). Total genomic DNA was then ligated into similarly cut pUC18 or pACYC177 (Table 2.2). The cloning was done in both high and low copy number vectors as it was feared that some target genes when cloned in a high copy number

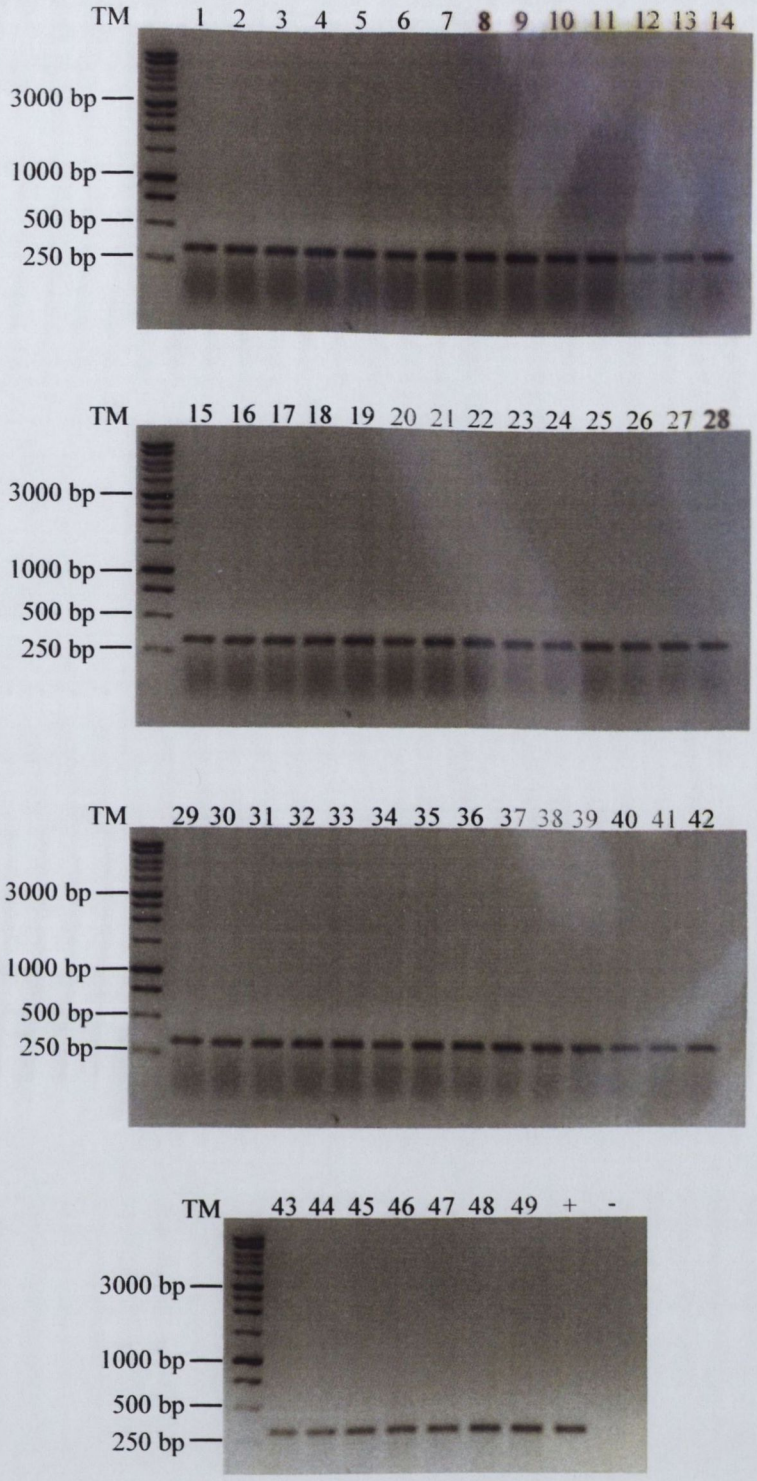


Fig. 4.5. **Detection of *rlgA-spvR* intergenic region by PCR.** Amplification was with the primers delTMF and delTMR, which amplify 343 bp upstream of ***spvR***. All of the transposon mutants show amplification of this band meaning that none of them is a deletion mutant. The positive control was MC4100p671 and the negative control was CJD1127, a known deletion mutant.

Transposon Mutant	Enzyme used to clone
TM 2	All > 9000 bp
TM 3	*Bam*HI
TM 4	*Pst*I
TM 5	*Eco*RI
TM 6	*Pst*I
TM 8	*Pst*I
TM 9	*Pst*I
TM 10	*Pst*I
TM 11	*Pst*I
TM 13	*Eco*RI
TM 19	*Eco*RI
TM 20	*Pst*I
TM 24	*Eco*RI
TM 26	*Pst*I
TM 27	*Bam*HI
TM 28	*Pst*I
TM 29	*Pst*I
TM 31	*Pst*I
TM 32	*Pst*I
TM 35	*Pst*I
TM 36	*Pst*I
TM 37	All > 9000 bp
TM 41	*Pst*I
TM 42	*Pst*I
TM 44	*Pst*I
TM 45	*Pst*I
TM 47	*Pst*I

Table 4.4. **Restriction enzymes used to clone the transposon mutants.**

vector such as pUC18 may be toxic due to overexpression of the gene product. As pACYC177 does not contain an *Eco*RI site any genome cut with this enzyme was only ligated into pUC18. The ligation mix was transformed into XL-1 Blue (Table 2.1) and plated on chloramphenicol LB plates in order to select for those plasmids into which the *cml* cassette and its surrounding DNA had been cloned. After overnight incubation at 37°C chloramphenicol resistant colonies were single colony purified. Colony PCR, using the primers catFwd and catRev (Table 2.3) was carried out to ensure the presence of the *cml* cassette. Plasmid DNA was then isolated by minipreps and digested with the restriction enzyme used for cloning to ensure the presence of an insert. These plasmids were then sent to MWG-Biotech, Germany for sequencing. The primer used for sequencing was Catseq (Table 2.3) which base pairs with the 3' end of the *cml* gene and reads into the downstream gene. This allows identification of the site of insertion of the transposon. By this method all the transposon mutants were cloned with the exception of TM 2, TM 28 and TM 37. TM 2 and TM 37 could not be cloned by this method as *Bam*HI, *Pst*I or *Eco*RI did not give suitably sized bands for cloning. The reason no clone was obtained for TM 28 was not apparent. Alternative enzymes were now needed to clone the remaining 3 mutants. Genomic DNA from these 3 transposon mutants was digested with *Cla*I, *Kpn*I, *Sal*I and *Sma*I. These restriction fragments were then Southern blotted using the *cml* probe in order to identify restriction enzymes suitable for cloning the *cml* cassette from these 3 strains. This Southern blot can be seen in Fig. 4.6. From this Southern blot it was decided to use *Cla*I for cloning the *cml* cassette from TM 2, TM 28 and TM 37 in pACYC177 and *Sal*I for cloning *cml* into pUC18. Genomic DNA from TM 2, TM 28 and TM 37 was digested singly with *Cla*I and *Sal*I, cleaned and ligated into similarly cut pACYC177 and pUC18 respectively. The ligation mix was transformed into XL-1 Blue and plated on chloramphenicol LB plates. Resistant clones were checked once again for the *cml* gene by PCR and plasmid inserts by plasmid isolation and digestion. These plasmids were sent to MWG-Biotech for sequencing.

4.2.7: Identification of transposon mutants

The sequence returned from MWG was used in a BLAST search (http://www.ncbi.nlm.nih.gov/BLAST) to identify where in the genome of

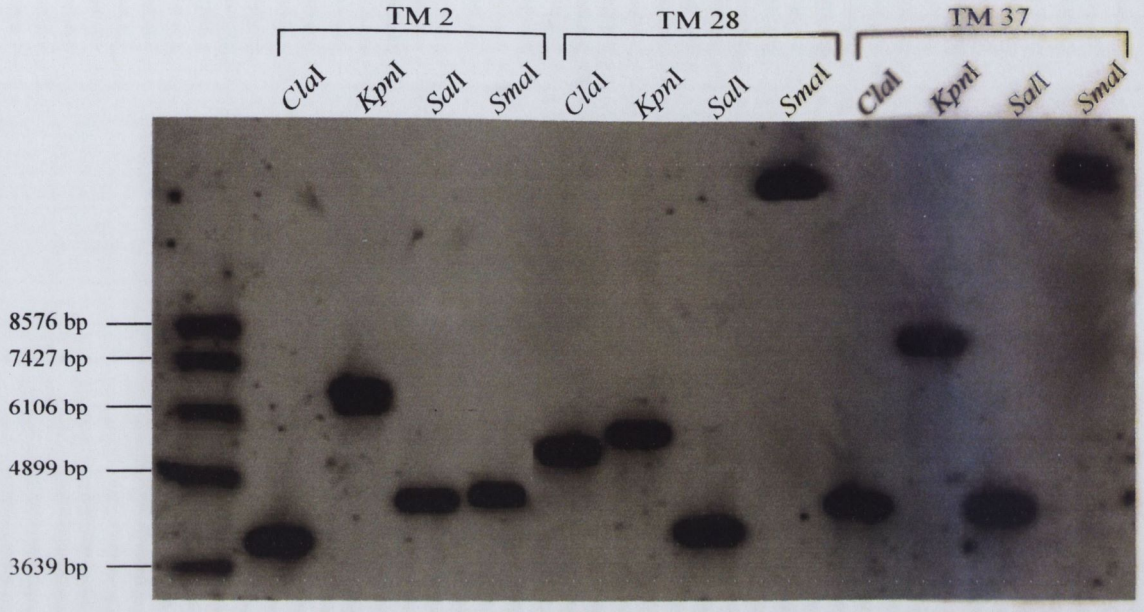


Fig. 4.6. **Southern blot analysis of transposon mutants TM 2, TM 28 and TM 32.** Genomic DNA was digested singly with *Cla*I, *Kpn*I, *Sal*I or *Sma*I as indicated. These fragments were electrophoresed through a 1% agarose gel and transferred to a nylon membrane, which was probed with a DIG-labelled *cml* probe. Hybridising bands between 1.5 kb and 7 kb were deemed suitable for cloning.

MC4100p671 the transposon had inserted. The results obtained are tabulated in Table 4.5 along with the function of the gene into which the transposon inserted. TM 4 and TM 22 both contain transposon insertions in the same gene thereby validating the method used to eliminate mutants from the screen. Many of the transposon insertions are in the large virulence plasmid particularly in the *spv* region. Presumably all these mutants are affecting *spvB-lacZ* expression due to increased *spv* transcription initiated from pOUT, the outward reading promoter located in the *cml* cassette. A number of interesting mutants were isolated. TM 20, TM 35 and TM 36 inactivated either the *sdhA* or *sdhD* genes encoding succinate dehydrogenase. TM 2 inactivated *sucA* encoding 2-oxoglutarate dehydrogenase, another enzyme of the Krebs cycle. Two of the transposon insertions isolated were in genes with biosynthetic functions namely TM 27 which inactivated *thiE* involved in thiamin biosynthesis and TM 29 which inactivated *pheA*, involved in phenylalanine biosynthesis. Another 2 mutants TM 24 and TM 37 contained insertions in the genes, *yneB* and *ybfP* respectively. These genes are of unknown function. Two of the transposon insertions were located immediately beside one another in *PSLT001* and *PSLT002* on the large virulence plasmid approximately 30 kb from the *spv* region. The genes inactivated encode a putative phospholipase D (*PSLT002*) in the case of TM 42 and a putative cytoplasmic protein (*PSLT001*) in the case of TM 47. Another transposon insertion TM 31 mapped to the *traV* gene on pSLT, which is involved in conjugative transfer. The screen also isolated a mutation in *rssB* (also known as *sprE* or *hnrG*) which inactivates a response regulator (TM 6). Finally TM 28 contained a transposon insertion upstream of the cold-shock protein C gene *cspC* and hence overexpresses this gene due to transcription from pOUT. As TM 35 and TM 36 inactivated the genes coding for succinate dehydrogenase, the same enzyme that was affected in TM 20, only TM 20 was characterised further. Also, as TM 24 and TM 37 contained transposon insertions in genes of unknown function they were also not characterised further. TM 29 was not characterised further as the marker rescue experiment could not be carried out for this strain. This left 8 strains to be characterised TM 2 (*sucA*$^-$), TM 6 (*rssB*$^-$), TM 20 (*sdhD*$^-$), TM 27 (*thiE*$^-$), TM 28 (*cspC* overexpressing), TM 31 (*traV*$^-$), TM 42 (*pSLT002*$^-$) and TM 47 (*pSLT001*$^-$). β-galactosidase assays were carried out on stationary phase cultures of the wild-type MC4100p671, TM 2, TM 6, TM 20, TM 27, TM 28, TM 31, TM 42 and TM 47 in order to determine the fold increase in β-galactosidase activity caused by the transposon insertion in each case. Fig. 4.7 shows

Transposon mutant	Transposon insertion site	Gene function
TM 2	*sucA*	2-oxoglutarate dehydrogenase
TM 3	Upstream of *spvA*	*Salmonella* virulence
TM 4	*spvA*	*Salmonella* virulence
TM 5	*trpB* (MudJ)	Tryptophan biosynthesis
TM 6	*rssB*	Response regulator
TM 8	Upstream of *spvR*	*Salmonella* virulence
TM 9	*spvA*	*Salmonella* virulence
TM 10	*lacZ* (MudJ)	Lactose metabolism
TM 11	Upstream of *spvR*	*Salmonella* virulence
TM 13	*spvB*	*Salmonella* virulence
TM 19	*lacZ* (MudJ)	Lactose metabolism
TM 20	*sdhD*	Succinate dehydrogenase
TM 22	*spvA*	*Salmonella* virulence
TM 24	*yneB*	Function unknown
TM 26	*PSLT042*	Putative integrase
TM 27	*thiE*	Thiamine biosynthesis
TM 28	Upstream of *cspC*	Cold shock protein C
TM 29	*pheA*	Phenylalanine biosynthesis
TM 31	*traV*	Conjugative transfer
TM 32	*lacZ* (MudJ)	Lactose metabolism
TM 35	*sdhA*	Succinate dehydrogenase
TM 36	*sdhA*	Succinate dehydrogenase
TM 37	*ybfP*	Function unknown
TM 41	Upstream of *spvR*	*Salmonella* virulence
TM 42	*PSLT002*	Putative phospholipase D
TM 44	*spvR*	Salmonella virulence
TM 45	Upstream of *spvR*	*Salmonella* virulence
TM 47	*PSLT001*	Putative cytoplasmic protein

Table 4.5. **Identification of genes into which the transposon inserted for the different transposon mutants.**

the results. TM 2 shows a 2-fold increase in β-galactosidase expression from *spvB-lacZ* relative to MC4100p671. TM 6 shows a 3.5-fold increase in β-galactosidase levels while TM 20 shows a 5-fold increase. TM 27 expresses only 1.2-fold more β-galactosidase than MC4100p671 and while this is enough to ensure the strain is red on MacConkey lactose indicator plates it is not a significant increase. TM 28 and TM 31 express 13.5-fold and 3-fold more *lacZ* than MC4100p671 respectively. TM 42 and TM 47, which contain transposon insertions in neighbouring genes on pSLT express 12-fold and 17-fold more β-galactosidase than MC4100p671 respectively.

4.2.8: Analysis of growth rate of transposon mutants

As a number of the transposon insertions mapped to genes involved in metabolism or biosynthesis it was decided to check the growth rate of the mutants. Inactivation of genes involved in metabolism or biosynthesis may result in a growth defect in the strain. This would lead to stationary-phase like conditions in the cell. As the *spv* system is stationary phase induced (Fang *et al.*, 1991; Coynault *et al.*, 1992) this could lead to the upregulation of the *spv* genes giving a red phenotype on MacConkey lactose indicator plates. Overnight cultures of wild-type MC4100p671 and each of the 8 transposon mutants to be characterised were diluted 1:1000 in LB broth and their growth monitored by measuring cell density at OD_{600nm} (section 2.5.1). This experiment was carried out in triplicate and in each case a similar result was obtained. A growth curve for each strain is shown in Fig. 4.8. All the mutants appear to grow similarly to the wild-type with the exception of TM 2. This mutant grows as well as the wild-type in early to mid exponential phase however it cannot grow to high cell density in stationary phase. Prolonged stationary phase like conditions in this strain may be leading to the upregulation of the *spv* genes in this strain.

4.2.9: Comparison of RpoS levels in the mutant strains

CspC is a known regulator of RpoS. Overexpressing CspC results in a four-fold increase in the *rpoS* mRNA levels in *E. coli* and a subsequent increase in RpoS protein

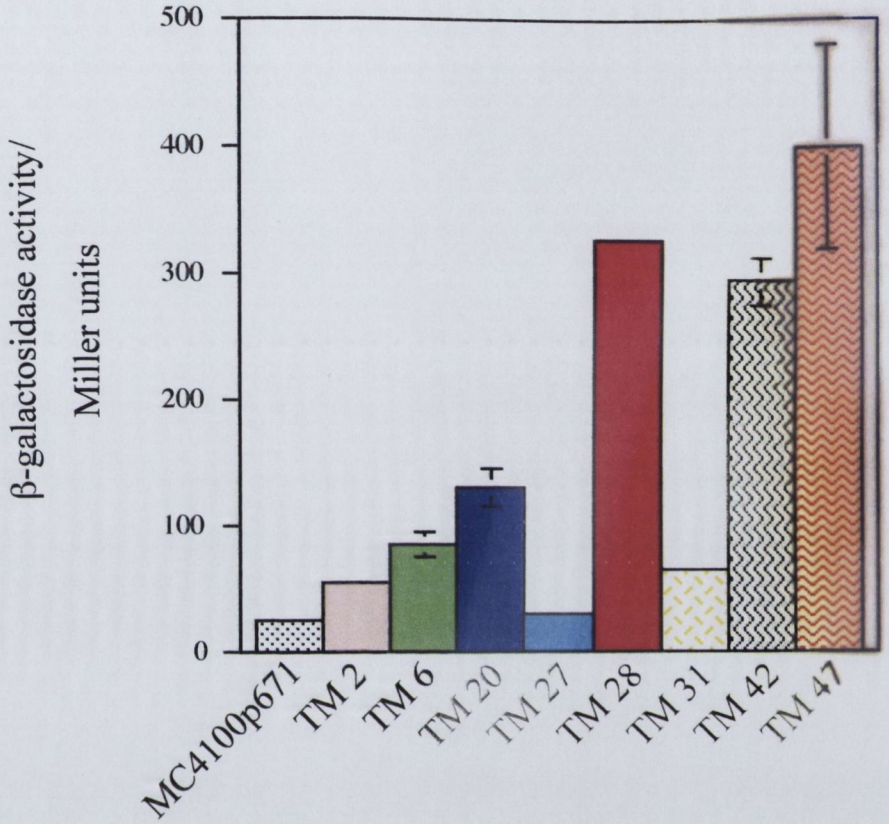


Fig. 4.7. **Stationary phase *lacZ* expression in MC4100p671, TM 2, 6, 20, 27, 28, 31, 42 and 47.** The data, expressed in Miller units, represent the mean of 3 independent experiments. Error bars, which extend to 1 standard deviation from the mean are shown when greater than 8%.

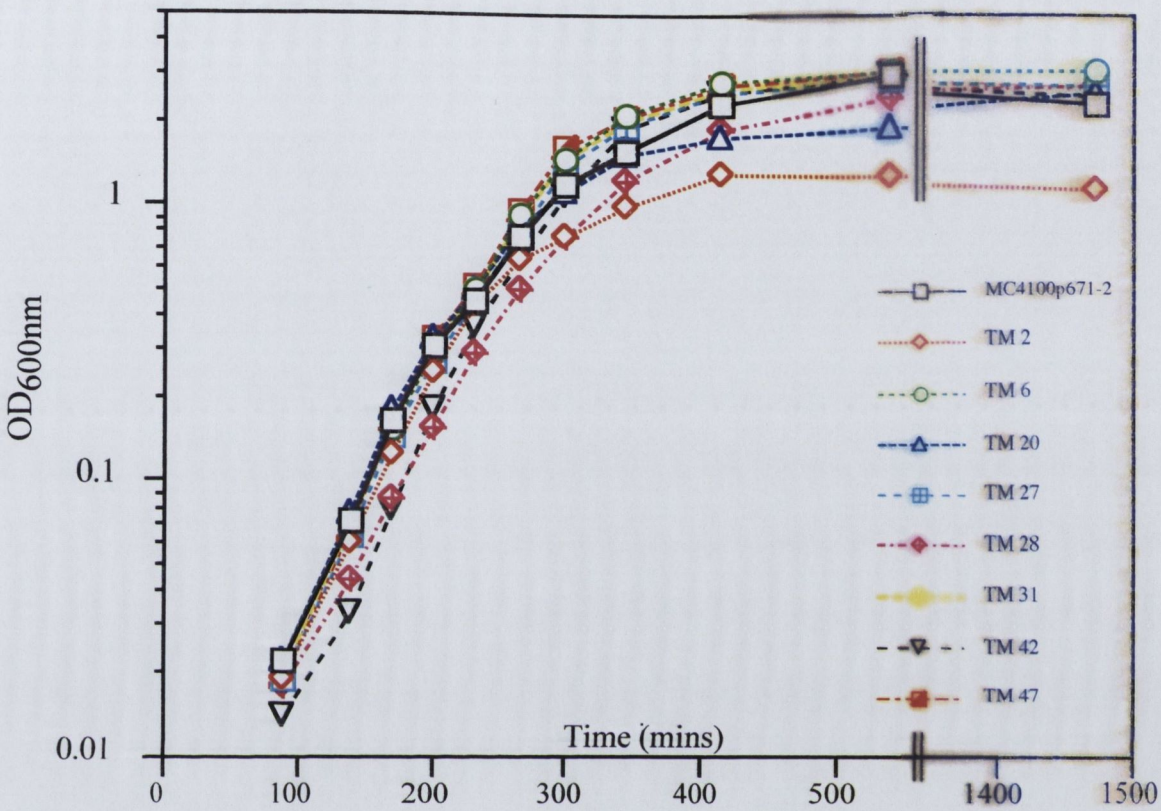


Fig. 4.8. **Growth curve of MC4100p671 and mutant derivatives.** Overnight cultures were diluted 1/1000 in LB broth, grown at 37^0C and bacterial growth monitored by measuring OD_{600nm} at the indicated times.

levels. Overexpressing CspC is also know to increase the half-life of the *rpoS* mRNA (Phadtare and Inouye, 2001). RssB is another known regulator of RpoS. RssB targets RpoS for proteolytic degradation by the ClpXP protease. RssB is responsible for delivery of RpoS to ClpX the regulatory element of ClpXP that is believed to unfold RpoS and eventually feed it to ClpP the proteolytic component (Zhou *et al.*, 2001). It was likely that overexpressing CspC (TM 28) and inactivating *rssB* (TM 6) was affecting *spv* gene expression indirectly via RpoS. Transcription of *spvR* is RpoS dependent (Kowarz *et al.*, 1994) therefore any increase in RpoS levels would lead to an increase in SpvR levels and hence an increase in *spv* structural gene expression. As mentioned previously mutations in genes involved in metabolism or biosynthesis could lead to a "stationary-phase like" state in the cell, which could be reflected by increased RpoS levels. For this reason the amount of RpoS protein in each of the transposon mutants was measured by Western immunoblotting (section 2.19) and compared to wild-type. As can be seen in Fig. 4.9 TM 6 and TM 28 have elevated RpoS levels relative to MC4100p671. None of the other transposon mutants appears to have elevated RpoS levels in fact some appears to have decreased levels of RpoS compared to MC4100p671.

4.2.10: Complementing TM 6

In order to confirm that the transposon insertion in *rssB* was leading to increased RpoS levels plasmid pGB637 (Table 2.2) was transformed into TM 6. This plasmid was also transformed into CJD898 (Table 2.1) a known *rssB* mutant. This plasmid provides RssB *in trans* and so should reduce RpoS levels once more. MC4100p671, TM 6, CJD898, TM 6+pGB637 and CJD898+pGB637 were then subjected to Western blot analysis to determine RpoS levels. The results obtained are shown in Fig. 4.10 (A). Both CJD898 and TM 6 have increased RpoS levels compared to wild-type due to inefficient protease degradation of RpoS by ClpXP. Providing RssB *in trans* from a multi-copy plasmid reduces RpoS levels in both TM 6+pGB637 and CJD898+pGB637. It reduces RpoS levels below that of MC4100p671 to a levels that is barely detectable by Western immunoblotting. This is presumably because increased RssB levels ensure more efficient degradation of RpoS by ClpXP. MC4100p671, TM 6 and TM 6+pGB637 were also assayed for β-galactosidase

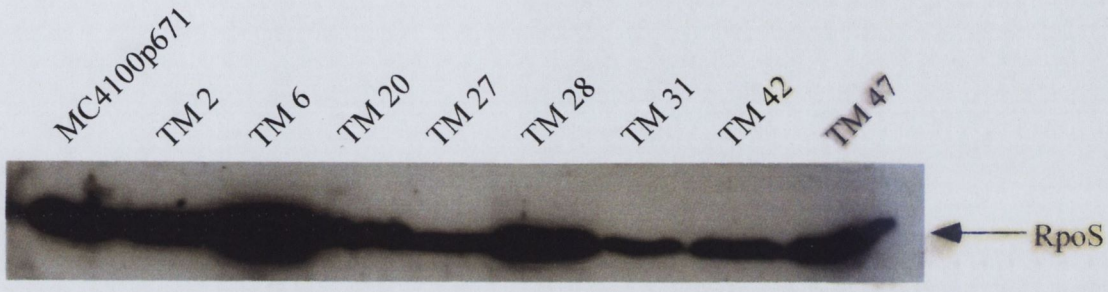


Fig. 4.9. **Western blot of RpoS levels in transposon mutants.** Samples are of stationary phase MC4100p671, TM 2, TM 6, TM 20, TM 27, TM 28, TM 31, TM 42 and TM 47. Cells were grown overnight in LB broth at 37°C. Loadings were normalised according to cell density.

expression from *spvB-lacZ*. As can be seen in Fig. 4.10 (B) the transposon insertion in *rssB* results in elevated *lacZ* expression presumably due to increased *spvR* transcription from its RpoS dependent promoter. Providing RssB *in trans* reduces *lacZ* expression to a level slightly below that of wild-type MC4100p671. This is consistent with TM 6+pGB637 having lower RpoS levels than MC4100p671 and hence lower SpvR levels.

4.2.11: Comparison of pSLT copy number between MC4100p671, TM 31, TM 42 and TM 47

As the transposon insertions in TM 31, TM 42 and TM 47 mapped to the large virulence plasmid it was decided to compare plasmid copy number in these strains relative to MC4100p671. An increase in plasmid copy number in these strains could be responsible for the increased expression of *lacZ* from *spvB-lacZ*. Large virulence plasmid was extracted from an equal number of cells of MC4100p671, TM 31, TM 42 and TM 47. This plasmid was then digested singly with either *Xho*I or *Hpa*I and electrophoresed through a 1% agarose gel. This gel was stained with ethidium bromide, viewed under UV illumination and can be seen in Fig. 4.11 (A). Lanes 1-5 show 2 µl of *Xho*I digested pSLT isolated from MC4100p671, MC4100p671, TM 31, TM 42 and TM 47. The wild-type strain was loaded twice in each case to ensure no loading error. Lanes 6-10 show 6 µl of *Xho*I digested pSLT from MC4100p671, MC4100p671, TM 31, TM 42 and TM 47. Lanes 11-15 show 15 µl of *Hpa*I digested pSLT from MC4100p671, MC4100p671, TM 31, TM 42, and TM 47. This gel was then transferred to a nylon membrane, blotted and hybridised to a DIG-labelled probe generated from the primers InspvRF and InspvRR (Table 2.3). The resulting Southern blot can be seen in Fig. 4.11 (B). All 3 transposon mutants appear to have an increased copy number of pSLT. Densitometric analysis indicated that TM 31 and TM 47 had a 2-fold increase in pSLT levels relative to MC4100p671 while TM 42 contained 4-fold more plasmid than MC4100p671.

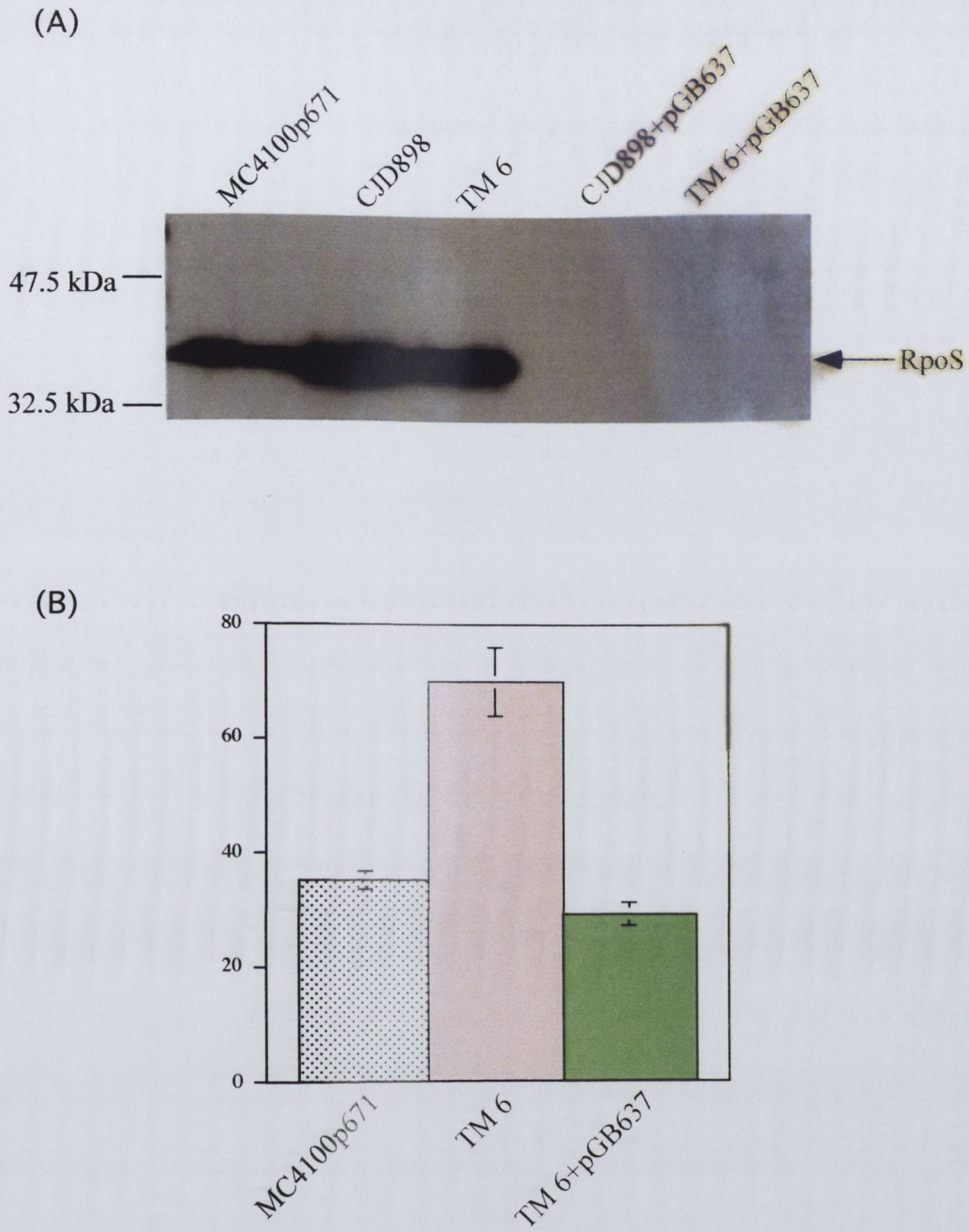


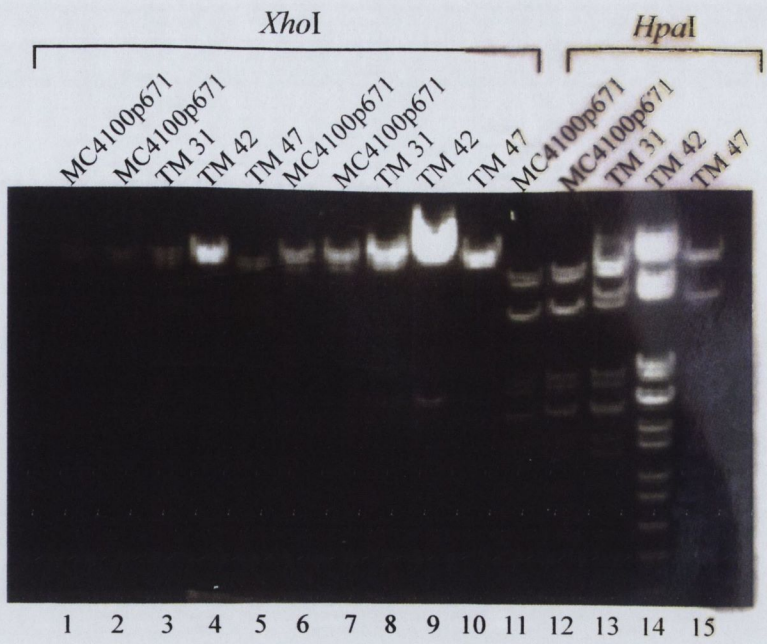
Fig. 4.10 **Complementing the *rssB* lesion in TM 6.**

(A). Western immunoblot analysis of RpoS levels from stationary phase MC4100p671, CJD891 (*rssB*$^-$), TM 6, CJD898+pGB637 and TM 6+pGB637.

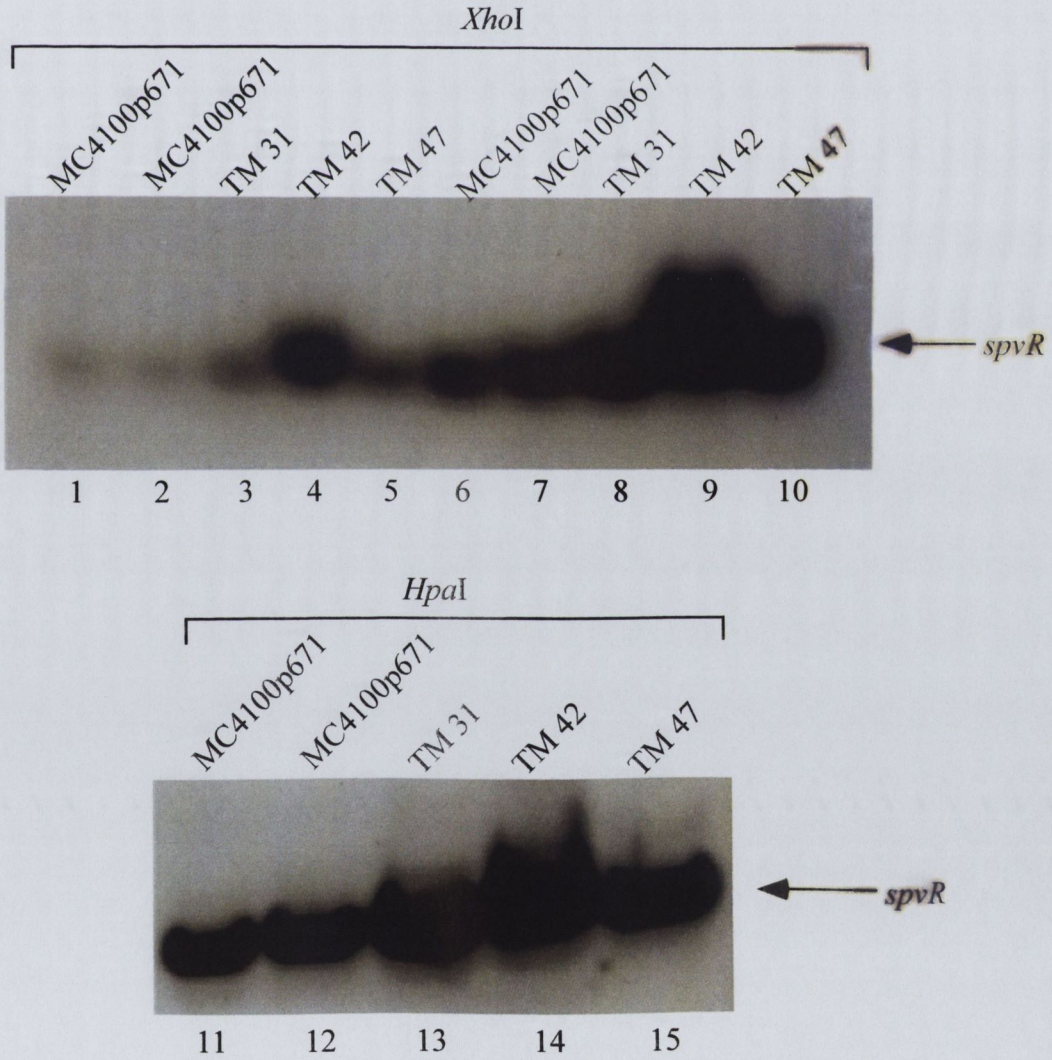
(B) Histogram showing stationary phase β-galactosidase activity for MC4100p671, TM 6 and TM 6+pGB637. The data, expressed in Miller units, represent the mean of 3 independent experiments. Error bars extend to 1 standard deviation from the mean.

Fig. 4.11. (A). **Plasmid pSLT copy number comparison in MC4100p671, TM 31, TM 42 and TM 47.** Large virulence plasmid DNA was extracted by Qiagen preparation from each of the strains mentioned above. Cell numbers were normalised according to cell density. 2 μl (lanes 1-5) or 6 μl (lanes 6-10) of the plasmid was then digested with *Xho*I and 15 μl was digested with *Hpa*I. These restriction fragments were electrophoresed in order to compare band intensity.
(B). The gel shown in (A) was Southern blotted and hybridised to a DIG-labelled *spvR* probe. Band intensity was compared by densitometry in order to determine the relative levels of pSLT.

(A)


XhoI
HpaI
MC4100p671
MC4100p671
TM 31
TM 42
TM 47
MC4100p671
MC4100p671
TM 31
TM 42
TM 47
MC4100p671
MC4100p671
TM 31
TM 42
TM 47
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

(B)


XhoI
MC4100p671
MC4100p671
TM 31
TM 42
TM 47
MC4100p671
MC4100p671
TM 31
TM 42
TM 47
spvR
1 2 3 4 5 6 7 8 9 10
HpaI
MC4100p671
MC4100p671
TM 31
TM 42
TM 47
spvR
11 12 13 14 15

4.3: Discussion

As the combined transposon mutagenesis and overexpression experiment could not be carried out in CJD671 due to the restriction barrier between *E. coli* and *Salmonella* the large virulence plasmid of CJD671 was moved into an *E. coli* MC4100 background forming the strain MC4100p671. This strain gave red deletion mutants at the same frequency as CJD671 showing that the "deletion machinery" is either common to *E. coli* and *Salmonella* or is encoded on pSLT and can be expressed in *E. coli*. Transposon mutants that had lost the ability to form red mutants were not easy to screen for as this would involve screening populations and not single cells. Therefore mutants that went red at an accelarated rate on MacConkey lactose indicator plates were screened for. In total 49 red colonies out of approximately 20,000 were found. However none of these mutants affected deletion formation. There are a number of possible reasons for this. The "deletion machinery" may be an essential component of the cell and inactivation of it may result in cell death. Alternatively it may not be possible to accelerate deletion formation and isolate a strain that forms red mutants overnight. Another possible explanation is simply that due to the preference for transposition into the *spv* region of pSLT not enough colonies were screened. The fact that transposon insertions in known repressors of the *spv* system such as H-NS and Lrp (O'Byrne and Dorman, 1994b; Marshall *et al.*, 1999) were not isolated would support this idea. Any further attempt to knock out the deletion machinery by transposition insertion would need to take into account this bias towards transposition into the *spv* region and adjust the numbers of colonies screened accordingly.

The 49 transposon mutants isolated all gave a red phenotype on MacConkey lactose indicator plates due to an effect on the *spv* locus. 21 of these 49 mutants could be eliminated from the screen as they had been isolated more than once. This was shown by similar sized *cml* hybridising fragments on Southern blots and similar β-galactosidase activity. One mutant was not viable on sub-culturing. The remaining 27 mutants were cloned and sequenced. Fourteen mapped to the *spv* region of pSLT. These transposons inserted in many sites in the *spv* region ranging from upstream of *spvR* to within the MudJ element itself. Presumably these mutants all affect *spvB-lacZ* transcription by increasing transcription of *spvR*, the *spv* structural genes or *lacZ* itself from the pOUT promoter located within the transposon. Evidence for this comes from

the fact that in each case the pOUT promoter was reading into the *spv* genes. It is also worth noting that many of these insertions were isolated more than once. In fact any mutant isolated more than once mapped to the *spv* region with the exception of those mutations which mapped to the genes encoding succinate dehydrogenase. The reason for so many insertions in the large virulence plasmid is unclear. It may be due to the fact that the large virulence plasmid is for some reason more readily available for transposition into. Alternatively more than a single copy of the large virulence plasmid may have been present in the cell when the chloramphenicol cassette was being transposed thereby increasing the probability of transposition into pSLT. The copy number of pSLT in LT2 has been estimated at 2.75 copies per cell (McClelland *et al.*, 2001). This may account for the frequency with which transposon insertions in *spv* were isolated. This preference for transposition into the *spv* region biased the transposon mutagenesis study as complete genome coverage was probably not achieved.

Of the 13 non-*spv* transposon mutants, 8 were characterised further. These were TM 2 (*sucA*$^-$), TM 6 (*rssB*$^-$), TM 20 (*sdhD*$^-$), TM 27 (*thiE*$^-$), TM 28 (*cspC* overexpressing), TM 31 (*traV*$^-$), TM 42 (*PSLT002*$^-$) and TM 47 (*PSLT001*$^-$). TM 27 had only an increase in β-galactosidase expression of 1.2-fold relative to MC4100p671. All the other mutants isolated had increases in β-galactosidase expression between 2-fold and 17-fold. Three of these mutants inactivate genes with known metabolic or biosynthetic functions, namely TM 2, TM 20 and TM 27. Inactivation of these genes could potentially affect growth of the cells. Therefore the mutants were analysed for growth rate as impaired growth of the cells may result in stationary phase conditions in the cell and hence induce expression of *spv*. All the mutants grew at the same rate as wild-type MC4100p671 in exponential phase. In stationary phase one of the mutants TM 2 had impaired growth as it could not grow to the same cell density after overnight incubation at 37°C as all the other strains. The slow growth of TM 2 may cause increased *spv* expression. This is not likely however as TM 20 also contains a transposon insertion inactivating one of the genes of the Krebs cycle and this strain while also having elevated *spv* gene expression does not suffer from impaired growth. Therefore it is unlikely to be the growth defect alone of TM 2 that is leading to increased *spv* expression. The level of RpoS in all the transposon mutants was also measured and compared to wild-type. If these cells were in stationary phase-like

conditions then RpoS levels would be elevated. The RpoS levels of TM 2, TM 20 and TM 27 were not found to be elevated with respect to wild-type. It is therefore unlikely that these cells are experiencing a harsher stationary phase than MC4100p671. The reason that inactivation of the succinate dehydrogenase enzyme increases *spv* gene expression is not clear. It is known that growth of *Salmonella* or *E. coli* in succinate or formate with their subsequent metabolism protects the cells from killing by antimicrobial peptides such as BPI (bactericidal/permeability increasing protein). The precise mechanism of protection remains unclear as yet however it is hypothesised that these antimicrobial proteins affect the respiratory chain at the inner membrane and halt oxygen consumption (Barker *et al.*, 2000). This leads to an imbalance between endogenous catabolism and macromolecular synthesis much like entry to stationary phase (Nyström, 1998; Dukan and Nyström, 1999). Growing the cells in formate or succinate bypasses this inhibition and respiration continues. Succinic acid has also been reported to inhibit the bactericidal activity of neutrophils (Abdul-Majid *et al.*, 1997). One of the genes encoding succinate dehydrogenase *sdhA,* which encodes the flavoprotein subunit is known to be repressed by both Fis and RpoS (Xu and Johnson, 1995). Therefore it is clear that there exists a link between succinate metabolism, stationary phase and virulence however the precise nature of the link remains to be elucidated. From the results presented here, this link may involve *spv* gene expression. The inactivation of succinate dehydrogenase in TM 2 should make this strain more sensitive to killing by antimicrobial peptides such as BPI in the absence of succinate. This may result in the induction of the *spv* locus to compensate for this. Indeed *S. typhimurium* was shown not to be as sensitive to killing by BPI as *E. coli* (Barker *et al.*, 2000) and this may be mediated through the *spv* system. However further work is required in order to elucidate the connection between succinate metabolism, stationary phase and *spv* gene expression.

Three of the mutations mapped to the large virulence plasmid but not the *spv* region. These were TM 31, which mapped to *traV* a gene involved in conjugative transfer of pSLT, TM 42 which mapped to *PSLT002* a putative phospholipase D and TM 47 which mapped to *PSLT001* a putative cytoplasmic protein. Both *PSLT001* and *PSLT002* are located upstream of DNA replication genes involved in replication of pSLT. Therefore pSLT plasmid copy number in these 3 strains was compared to wild-type. It was found that all 3 mutant strains had increased plasmid copy number over

the wild-type. The plasmid copy number of TM 31 was double that of MC4100p671. The largest increase in plasmid copy number was for TM 42. This strain had a 4-fold increase in plasmid copy number compared to MC4100p671. TM 47 had a 2-fold increase in plasmid copy number compared to MC4100p671. In both TM 42 and TM 47 pOUT reads into the replication proteins, which may account for the increase in plasmid copy number. For TM 42 the increase in plasmid copy number is greater than TM 47 as the transposon insertion in TM 42 is downstream of that in TM 47. This means that there are less transcription terminators between the promoter and the *rep* genes and that the RNA polymerase is less likely to dissociate from the DNA. This increase in plasmid copy number will lead to an increase in *spvB-lacZ* transcription simply by virtue of the fact that there are more copies of *spvB-lacZ* in the cell. However TM 47 has slightly higher β-galactosidase levels than TM 42 despite having less copies of pSLT. The quadrupling and doubling of pSLT levels alone does not explain the 12-fold and 17-fold increase in β-galactosidase expression from *spvB-lacZ* in TM 42 and TM 47 respectively. A number of other methods are available for determining plasmid copy number. Reporter genes, under the control of their native promoters, can be cloned onto the plasmid and transcription from these reporter genes used to compare plasmid copy number (Klotsky and Schwartz, 1987). Alternatively cells can be grown in the presence of radiolabelled thyamine. This is then incorporated into the plasmid and genomic DNA. The negatively supercoiled plasmid DNA can then be separated from the chromosomal DNA on a CsCl gradient. After collection of the radiolabelled DNA on Whatman paper scintillation counting is used to calculate the ratio of plasmid DNA to genome equivalent (Womble *et al.*, 1977). However as an increase in plasmid copy number did not fully account for the increase in *lacZ* transcription in TM 42 and TM 47 alternative methods to calculate the plasmid copy number were not employed.

TM 6 contains a transposon insertion in the *rssB* gene. This gene encodes an orphan response regulator as no sensor kinase is encoded alongside it. The environmental signal to which RssB responds, or the sensor kinase that transduces this signal to RssB have not been identified. The gene encoding RssB is located immediately downstream of the *rssA* gene, which encodes a serine esterase/protease. *rssA* and *rssB* form an operon and are cotranscribed although *rssB* can be transcribed from its own weaker promoter (Ruiz *et al.*, 2001). RssB plays a role in bringing RpoS to the ClpXP

protease and hence aids in the degradation of RpoS. This catalytic role is enhanced when RssB is phosphorylated however the sensor kinase that phosphorylates RssB remains unknown (Zhou *et al.*, 2001). In TM 6 RpoS levels are increased as shown by Western blotting. This is due to inefficient degradation of RpoS by ClpXP. As RpoS is a positive regulator of *spvR* (Kowarz *et al.*, 1994) this leads to increased SpvR levels and hence increased transcription from the *spvB-lacZ* fusion. When a multicopy plasmid providing *rssB in trans* was provided this decreased RpoS levels down below that of the wild-type. This strain has lower than wild-type levels of RpoS as the protein is now more efficiently targeted to the ClpXP protease and hence more easily degraded. Plasmid encoded RssB could also reduce β-galactosidase levels in TM 6 back to wild-type levels showing that TM 6 is increasing *spvB-lacZ* expression via an increase in RpoS levels.

TM 28 contains a transposon insertion upstream of the *cspC* gene encoding cold shock protein C. This results in the overexpression of CspC from the pOUT promoter. CspC is constitutively expressed at 37°C throughout the growth phase. This protein was originally identified as a multi-copy supressor of a temperature sensitive chromosome partitioning mutant (Yamanaka *et al.*, 1994). CspC has been shown to selectively bind RNA and ssDNA (Phadtare and Inouye, 1999). The *in vivo* significance of this is not yet known. It has also been shown that the overexpression of CspC results in an increase in RpoS protein levels as well as an increase in OsmY, Dps and UspA (Phadtare and Inouye, 2001). All these proteins are involved in stress responses in *E. coli*. RpoS is the stationary phase sigma factor, UspA is universal stress protein A, Dps is the DNA protection during starvation protein and OsmY is osmotically inducible protein Y. In TM 28 overexpressing CspC results in increased levels of RpoS as shown by Western immunoblotting. This in turn leads to increased levels of SpvR and hence increased transcription of the *spv* structural genes.

Chapter 5

Molecular analysis of the *Salmonella typhimurium gyrA* promoter

5.1: Introduction

DNA gyrase is the bacterial enzyme that introduces negative supercoils into DNA. It is composed of 2 subunit proteins GyrA and GyrB and has a tetrameric A_2B_2 structure. This enzyme is necessary for cell survival and *gyrA* or *gyrB* null mutations are lethal. Gyrase introduces negative supercoils into the DNA by initially binding DNA. The subsequent binding of ATP drives double stranded cleavage of the DNA with an AB dimer bound to the 5' terminus of each cleaved strand to prevent free rotation and loss of supercoils. Double-stranded DNA then passes through the newly created gap and the gap is resealed with the introduction of 2 negative supercoils (Mizuuchi *et al.*, 1980). The A subunit of DNA gyrase is involved in breaking and resealing of the DNA while the B subunit hydrolyses ATP. Negative supercoiling imparts free energy to the DNA and this drives structural transitions in the DNA helix including open complex formation at some promoters. The degree of DNA supercoiling influences DNA replication, recombination and transcription (Dorman, 2002).

DNA gyrase is also very important as an antibiotic target. Two types of drugs target DNA gyrase. Quinolones such as nalidixic acid and oxolinic acid inhibit the A subunit of DNA gyrase. They are proposed to act by binding the exposed single-stranded regions of DNA produced by cleavage of the DNA by gyrase. They then prevent resealing of the DNA after strand passage. This could lead to cell death by inhibition of negative supercoiling or by forming a barrier on the DNA, which would prevent the passage of such molecules as RNA and DNA polymerase (Gellert *et al.*, 1977). Novobiocin and coumarmycin inhibit the B subunit of DNA gyrase. They have no effect on the A subunit. They prevent ATP hydrolysis by the B subunit by blocking ATP binding. Novobiocin treatment therefore specifically inhibits negative supercoiling and does not have the same pleiotropic effects on the cell as treatment with quinolones has (Gellert *et al.*, 1976b; Gilbert and Maxwell, 1994).

The correct regulation of DNA gyrase is of paramount importance to the cell. The genes *gyrA* and *gyrB* are located at 48 and 82 minutes respectively on the *E. coli* genetic map. This means that both genes must be co-ordinately regulated. Together with Topoisomerase I (which relaxes negatively supercoiled DNA) DNA gyrase helps maintain a homeostatic supercoiling balance in the cell which ensures that supercoiling

does not reach detrimentally high or low levels (Menzel and Gellert, 1983). DNA gyrase is growth phase regulated and expression increases during exponential phase, peaking at late exponential phase and then decreases again in stationary phase (Balke and Gralla, 1987; Gomez-Gomez *et al.*, 1996). This is presumably influenced by the increase in the intracellular ATP pool, which occurs after nutrient upshift. ATP serves as a cofactor for DNA gyrase. The rate of synthesis of both the GyrA and GyrB proteins increases 10-20 fold in response to the DNA relaxation that occurs following the addition of the gyrase inhibitor novobiocin (Menzel and Gellert, 1983). Other factors that are known to affect negative supercoiling include temperature change, salt shock and anaerobic shock (Drlica, 1992). Coupled to the fact that supercoiling can drive open complex formation at some promoters this means that many promoters located in different parts of the genome can respond simultaneously to an alteration in the external environment. DNA gyrase in *E. coli* is negatively regulated by Fis (Schneider *et al.*, 1999), positively regulated by the cyclic AMP receptor protein CRP (Gomez-Gomez *et al.*, 1996) and positively regulated by IHF (Gellert *et al.*, 1983) all at the level of transcription. The *topA* gene encoding topoisomerase I has been reported to be negatively regulated by IHF, also at the level of transcription and to have 4 different promoters (Tse-Dinh and Beran, 1988). Transcription from one of these promoters in stationary phase requires the RpoS alternative σ factor (Qi *et al.*, 1997). DNA gyrase has also been reported to play a role in mediating illegitimate recombination in the formation of λ*bio* transducing phage (Shimizu *et al.*, 1997; Ashizawa *et al.*, 1999).

Previous work in this laboratory carried out by Dr. David Marshall had involved the cloning and sequencing of the *S. typhimurium* SL1344 *gyrB* and *topA* promoter regions. These promoters were cloned by polymerase chain reaction using oligonucleotides complementary to the *E. coli gyrB* and *topA* promoters (Marshall *et al.*, 2000). Sufficient homology existed between the *S. typhimurium* and *E. coli gyrB* and *topA* promoters to allow their amplification by this method. All attempts at amplifying the *S. typhimurium gyrA* promoter sequence by this method failed. The *S. typhimurium gyrB* and *topA* promoters were then placed upstream of the promoterless *lacZ* gene in the plasmid pQF50 (Table 2.2). When strains transformed with these plasmids were used to infect the mouse macrophage-like cell line J774A.1 it was found that *gyrB* transcription was induced 5-fold upon uptake of the bacteria by macrophage.

No induction of the *S. typhimurium topA* gene was seen in bacteria that were inside macrophage. Reporter plasmid isolated from macrophage internalised *Salmonella* showed a distribution that became progressively more relaxed with time and this is most likely leading to the induction of DNA gyrase in the internalised *Salmonella* (Marshall *et al.*, 2000). Induction of *gyrA in vivo* could not be confirmed due to the fact that this promoter could not be cloned by the method mentioned above.

The aim of this work was to clone and sequence the *gyrA* promoter from *S. typhimurium* and to compare this promoter to its *E. coli* counterpart. At the time that this work was carried out the genome sequence of *S. typhimurium* or *S. typhi* had not been completed.

5.2: Results

5.2.1: Localisation of the *gyrA* open reading frame

PCR primers EcogyrAF and EcogyrAR (Table 2.3) were designed to amplify the *E. coli* K-12 *gyrA* promoter sequence. These were then used in PCR reactions to try and amplify the *gyrA* promoter sequence from *S. typhimurium* SL1344 (Table 2.1). No amplification product could be detected when SL1344 chromosomal DNA was used as a template in the PCR reaction. A product was clearly visible when *E. coli* K-12 DNA was used as a template showing that the reaction was working (data not shown). This PCR reaction was also attempted with 2 other strains of *S. typhimurium*, LT2 and C5 (Table 2.1) to ensure this was not a phenomenon specific to the strain SL1344. These results indicated that the *S. typhimurium gyrA* promoter region differed in sequence to that of *E. coli* K-12.

In order to localise the *Salmonella gyrA* open reading frame Southern blotting was carried out (section 2.9). Chromosomal DNA from *S. typhimurium* strains LT2, SL1344 and C5 along with *E. coli* K-12 was digested singly with each of the restriction enzymes *Bam*HI, *Eco*RI, *Eco*RV and *Hind*III. The restriction fragments were separated on a 1% agarose gel, transferred to a nylon membrane and Southern blotted. The probe used was gyrAORF, which is a DIG labelled probe specific to the *gyrA* coding sequence. As this probe recognises the 5' end of the *gyrA* coding sequence it was hoped that any large restriction fragment to which it hybridised would also contain the *gyrA* promoter. This probe was generated using the primers gyrAmidF and gyrAprobeR (Table 2.3). The resulting Southern blot can be seen in Fig. 5.1. Lanes 1 and 16 show DIG labelled size markers, lanes 2-5 show a *gyrA* hybridising *Bam*HI restriction fragment from LT2, SL1344, C5 and K-12 respectively. Lanes 6-9 show a *gyrA* hybridising *Eco*RI restriction fragment from LT2, SL1344, C5 and K-12 respectively. Lanes 10-12 show a *gyrA* hybridising *Eco*RV restriction fragment from LT2, SL1344 and C5 and lanes 13-15 show a *gyrA* hybridising *Hind*III restriction fragment from LT2, SL1344 and C5. This Southern blot shows similar sized hybridising bands for all the 3 *S. typhimurium* strains confirming again that there are no major strain specific differences in the *gyrA* region. It also shows that the most

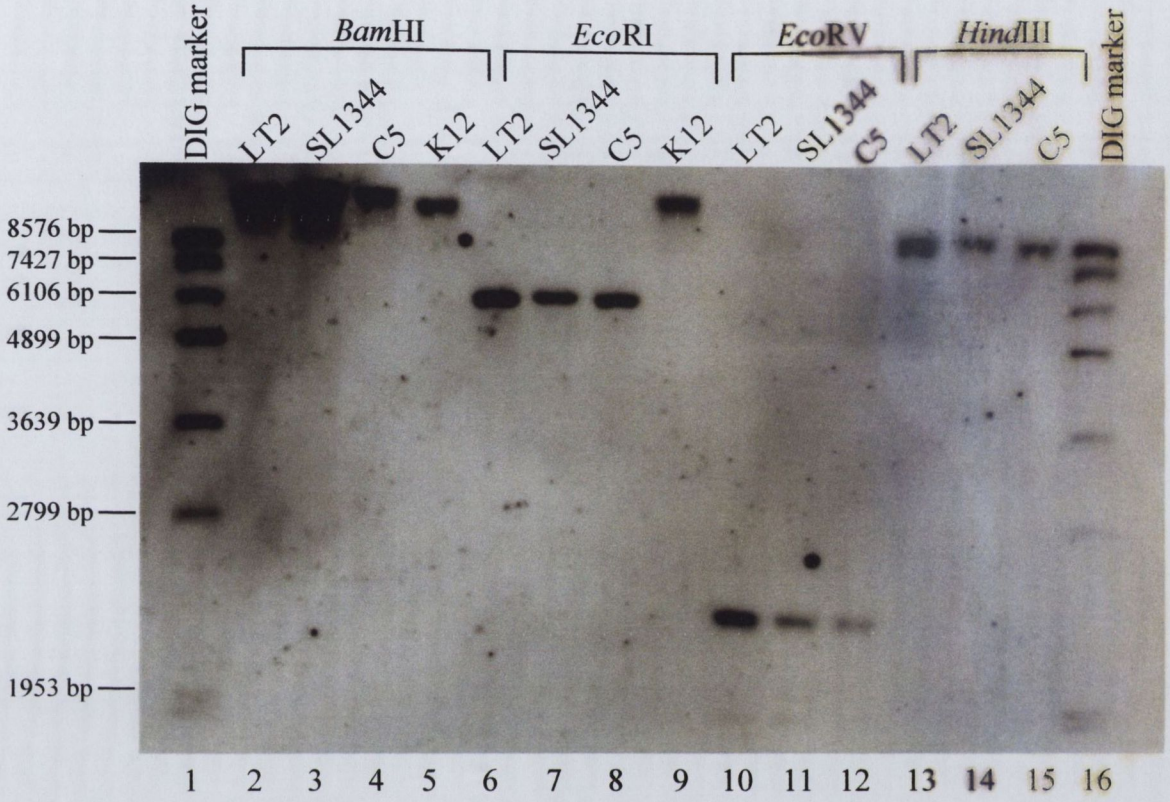


Fig. 5.1. **Localisation of *Salmonella gyrA* ORF by Southern blot.** Southern blot of genomic DNA from *Salmonella* strains LT2, SL1344 and C5 and *E.coli* K12. The DNA was digested with the enzymes indicated and probed with the DIG-labelled **gyrA**ORF probe in order to locate the *Salmonella gyrA* promoter for cloning.

suitable enzymes for cloning the entire *gyrA* open reading frame and promoter are *Eco*RI and *Hind*III which give *gyrA* containing restriction fragments of approximately 6,500 bp and 8,500 bp respectively. The *Bam*HI restriction fragment is a little too large to clone efficiently as it is greater than 10,000 bp in length. The *Eco*RV restriction fragment is approximately 2,500 bp in length and therefore is unlikely to contain the entire *gyrA* coding sequence and upstream regulatory regions.

5.2.2: Cloning of the *Salmonella typhimurium gyrA* gene

Chromosomal DNA from *S. typhimurium* SL1344 was digested singly with the restriction enzymes *Eco*RI and *Hind*III. These restriction fragments were then separated on a 1% agarose gel. A gel slice containing *Eco*RI digested DNA in the size range 6,000 bp to 8,000 bp was then excised from the gel. A gel slice containing *Hind*III digested DNA in the range 8,000 bp to 9,000 bp was also excised from the gel. The DNA was eluted from the gel slice and precipitated. A Southern blot was performed on the eluted DNA using the probe gyrAORF, which confirmed the presence of the *gyr*A gene in the eluate (data not shown). The *Eco*RI or *Hind*III digested DNA was then ligated into similarly cut pBR322 (which had been treated with alkaline phosphatase), transformed into XL-1 Blue and plated on LB ampicillin plates. The vector pBR322 was chosen as it has approximately 25 copies per cell. This aids in identification of *gyrA*-containing plasmids by colony blot due to increased signal intensity over the single chromosomal copy. It also avoids the potentially toxic effect of high copy number plasmids such as pUC18 that oxerexpress proteins to a very high level. After overnight incubation at 37°C colonies were screened for *gyrA*-containing recombinant plasmids.

5.2.3: Selection of *gyrA*$^+$ clones

The selection for *gyrA* containing plasmids was two-fold. First 600 colonies from the *Eco*RI ligation and 200 colonies of the *Hind*III ligation were picked onto fresh agar plates. After overnight incubation these colonies were subjected to colony blotting (section 2.11) with the gyrAORF probe. All colonies contained at least one copy of

the *gyrA* gene, the chromosomal copy and hence the probe hybridised to all colonies. However any colony containing a *gyrA* encoding recombinant plasmids should have multiple copies of the *gyrA* gene and therefore should hybridise much more strongly to the probe. An example of the colony blots obtained can be seen in Fig. 5.2. Of the 100 colonies blotted onto this membrane 2 appear to hybridise strongly to gyrAORF. These colonies are indicated by arrows.

The second selection for *gyrA* containing plasmids involved streaking each recombinant onto agar plates containing 20 μg/ml nalidixic acid and looking for nalidixic acid sensitivity. Nalidixic acid is a DNA gyrase inhibitor which inhibits the A subunit of DNA gyrase. XL-1 Blue is naturally nalidixic acid resistant due to a point mutation in its *gyrA* gene. However drug sensitivity is dominant over drug resistance therefore overexpressing a *gyrA* sensitive allele will restore a nalidixic acid sensitive phenotype (Swanberg and Wang, 1987). In total 8 colonies were picked that were nalidixic acid sensitive and hybridised strongly to gyrAORF. All of these clones contained an *Eco*RI restriction fragment cloned in pBR322. Plasmid DNA was extracted from these 8 colonies and the purified plasmid Southern blotted using the gyrAORF probe. The resulting blot can be seen in Fig. 5.3. Four of the plasmids contained *gyrA* hybridising sequences while the other 4 did not. One of these plasmids, p1-65, was chosen for all further work.

5.2.4: Transcription of *gyrA* from plasmid p1-65

It was suspected that the *Eco*RI restriction fragment encoded the full-length *gyrA* gene complete with regulatory sequences as transforming XL-1 Blue with p1-65 rendered the strain nalidixic acid sensitive. However in order to confirm p1-65 encoded full-length *gyrA* and its promoter Northern blotting was carried out (section 2.10). Total RNA was extracted from SL1344 and SL1344+p1-65. The amount of RNA was quantified at $OD_{260nm.}$ 25 μg of RNA from each strain was then loaded on a formamide denaturing gel and electrophoresed through the gel. After transfer to a nylon membrane the RNA was probed with the DIG labelled DNA probe gyrAORF. The resulting Northern blot can be seen in Fig. 5.4. Lane 1 shows DIG labelled DNA size markers. Lane 3 shows the *gyrA* transcript from wild-type SL1344. This lane shows a

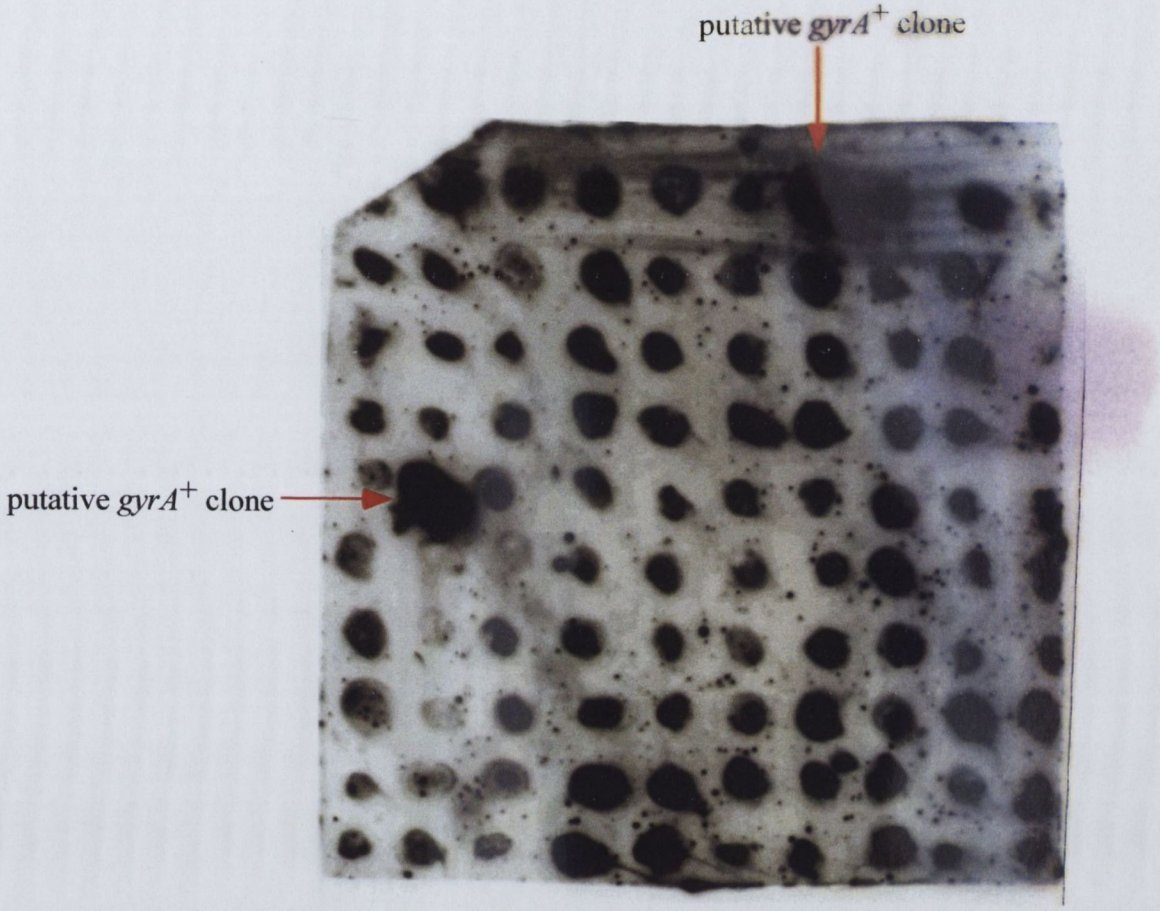


Fig. 5.2. **Colony blot to identify *gyrA*$^+$ clones.** All colonies gave a hybridisation signal due to the chromosomal copy of *gyrA*. Colonies that gave the strongest hybridising signals (arrowed) were selected as these were thought to contain *gyrA*$^+$ plasmids.

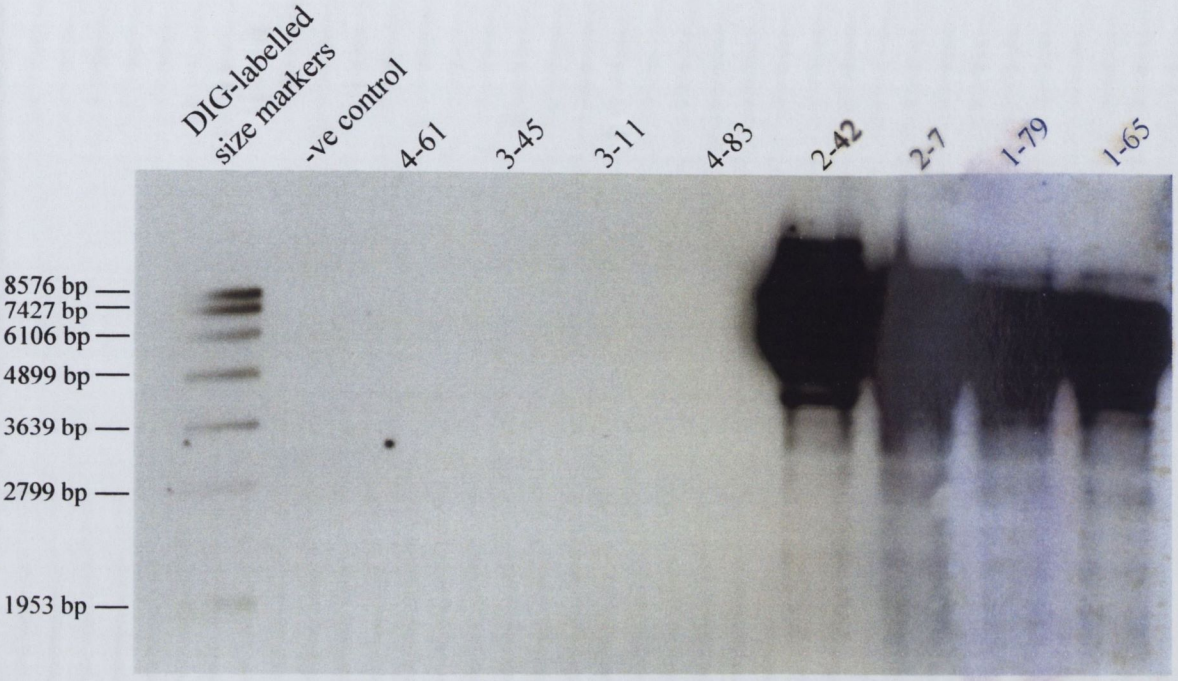


Fig. 5.3. **Analysis of putative *gyrA* clones.** Southern blot of purified plasmid DNA from 8 putative *gyrA*$^+$ plasmids. The DNA was probed with the DIG-labelled gyrAORF probe. 4 out of the 8 plasmids harbour *gyrA* from SL1344. The negative control used was pBR322 DNA.

full-length transcript of approximately 2,700 bp. It also shows a number of degradation products. Lane 4 shows the *gyrA* transcript from SL1344+p1-65. This strain contains much more *gyrA* mRNA then SL1344 consistent with the strain containing a multi-copy plasmid expressing *gyrA*. The p1-65 plasmid encodes full-length *gyrA* as the *gyrA* mRNA extracted from SL1344+p1-65 is the same length as that extracted from SL1344. It also follows the same degradation pattern. This confirms that p1-65 encodes the full *gyrA* gene and all the necessary sequences for its transcription. This promoter region could now be sequenced.

5.2.5: DNA sequence of the *S. typhimurium gyrA* promoter region

Plasmid p1-65 was sent to MWG Biotech for sequencing in order to determine the *S. typhimurium gyrA* promoter sequence. In *E. coli* the *ubiG* gene is encoded approximately 150 bp upstream of *gyrA* and is divergently transcribed. In *S. typhimurium* there appears to be an additional 4 kb of DNA inserted between *ubiG* and *gyrA*. This 4 kb sequence has a G+C content of approximately 42% compared to 53% for the *S. typhimurium* genome as a whole. This indicates that this sequence may have been horizontally acquired. This DNA sequence did not display significant homology to any sequence in the database at the time. It was predicted that this additional *Salmonella* specific sequence would encode one open reading frame. This open reading frame is transcribed in the same direction as *gyrA*. It had no homology to any sequence in the database at the nucleotide level however a TBLASTX (http://www.ncbi.nlm.nih.gov/BLAST) search indicated that the putative protein has significant homology to a number of inner membrane transport proteins most noticeably the TtuB tartrate transporter of *Agrobacterium vitis* (34.4% identity, 51.4% similarity). Bioinformatical analysis indicated that this protein has 12 transmembrane helices and that both the N and C-termini are cytoplasmic. The publication of the complete *S. typhimurium* genome sequence predicted 3 open reading frames in this region. An annotated diagram of the genes encoded in the *S. typhimurium gyrA* region is shown in Fig. 5.5.

As predicted from the PCR data the *Salmonella gyrA* promoter sequence differs from that of *E. coli* although the protein coding sequence remains largely unchanged. An

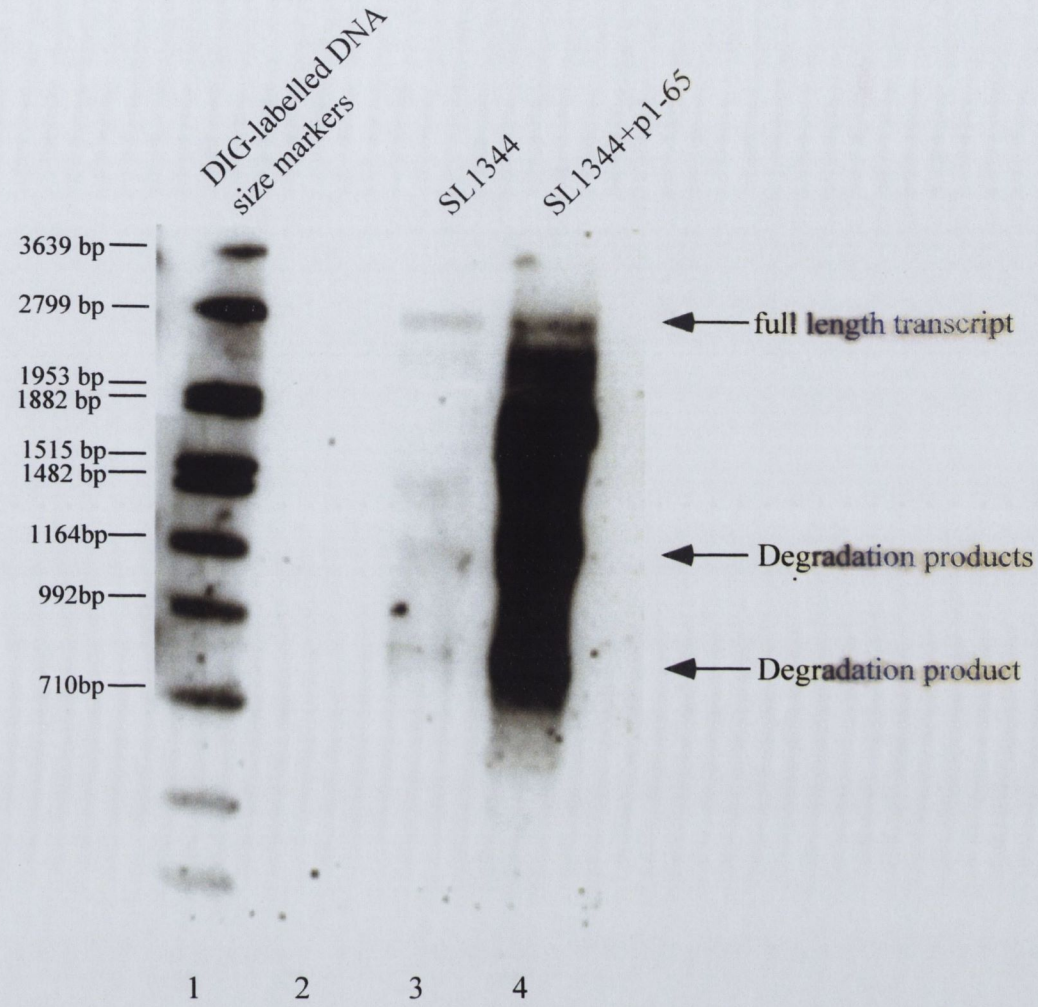


Fig. 5.4. **Transcriptional analysis of *gyrA* expression from plasmid p1-65.** Northern blot of SL1344 and SL1344 transformed with plasmid p1-65. The blot was probed with the DIG-labelled gyrAORF probe. Plasmid p1-65 appears to encode full-length *gyrA* and the sequences required for its transcription.

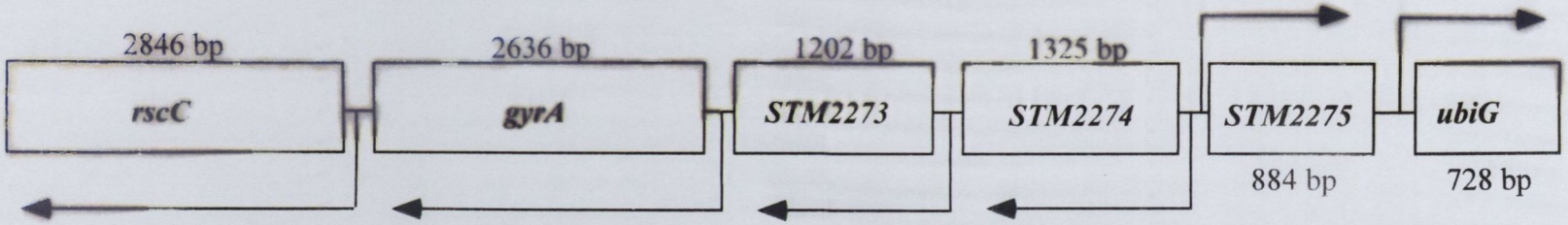


Fig 5.5. **Orientation of the genes in the *gyrA* region of *S. typhimurium*.** The length of each of the genes, in bp, is indicated above or below the gene. The direction of transcription in indicated by an arrow. *rscC* encodes a sensory histidine kinase, *STM2273* encodes a putative dehydratase protein, *STM2274* encodes a putative permease protein with amino acid sequence homology to the TtuB transporter of *A. vitis*, *STM2275* encodes a putative regulator protein of the *gntR* family, and *ubiG* encodes 3-dimethyl ubiquinone-9,3 methyltransferase.

alignment of the 2 promoter sequences can be seen in Fig. 5.6. The sequences differ just upstream of the –10 Pribnow box. Both the *E. coli* and *Salmonella gyrA* promoter sequences contain putative –10 boxes (TATAAT) that perfectly match the consensus for a σ^{70} dependent promoter. The -35 sequence of the *E. coli gyrA* promoter has not been previously positively identified however a sequence that matches the -35 consensus at 4 out of 6 positions can be found 19 bp upstream of the -10 box. Both promoters also contain the pentamer TGTGT immediately upstream of the –10 box. They therefore belong to the class of "extended –10" promoters. At these promoters the first TG, immediately upstream of the –10 hexamer, allows promoter activity in the absence of a –35 region, thus representing, in conjunction with the –10 hexamer the minimal sequence that can function as a promoter (Bown *et al.*, 1997). The second TG has previously been reported to play a role in increasing promoter strength (Burr *et al.*, 2000). The *E. coli* and *Salmonella gyrA* promoters diverge immediately upstream of the TGTGT motif.

5.2.6: Construction of a *gyrA* transcriptional fusion

In order to study the regulation of the *Salmonella gyrA* gene it was decided to clone its promoter into pQF50 upstream of the promoterless *lacZ* gene. The *gyrA* promoter was amplified with the high fidelity enzyme Platinum *pfx* polymerase (section 2.8). The primers gyrAbam and gyrAkpn (Table 2.3) were used for the amplification. These primers contain 5' *Bam*HI and *Kpn*I extensions on the forward and reverse primers respectively. The PCR product was then restricted with *Kpn*I and *Bam*HI and cleaned using the Promega PCR clean up kit (section 2.7.3). This product was then ligated into *Kpn*I and *Bam*HI digested pQF50. The ligation mix was transformed into XL-1 Blue and plated on ampicillin LB plates containing X-gal. This allows the selection of promoter containing recombinant plasmids as these colonies will be blue on LB agar containing X-gal. After overnight incubation at 37°C a number of blue colonies were visible. 12 of these colonies were screened and all were found to contain plasmids with inserts of the correct size. One of these plasmids was sent to MWG-Biotech for sequencing. This confirmed the presence of an intact *gyrA* promoter sequence upstream of *lacZ*. This plasmid was named pGyrA-lacZ (Table 2.2). A map of plasmid pGyrA-lacZ can be seen in Fig. 5.7.

Fig 5.6. **Alignment of *E. coli* and *S. typhimurium gyrA* promoter regions.**

The ATG translation start codon is in red, the -10 hexamer in orange and the TGTG promoter strengthening sequence in blue. The GTTTACC motif, conserved between *gyrA* and *gyrB* and containing the putative -35 sequence is in green. There is little homology between the 2 sequences upstream of the TGTG motif.

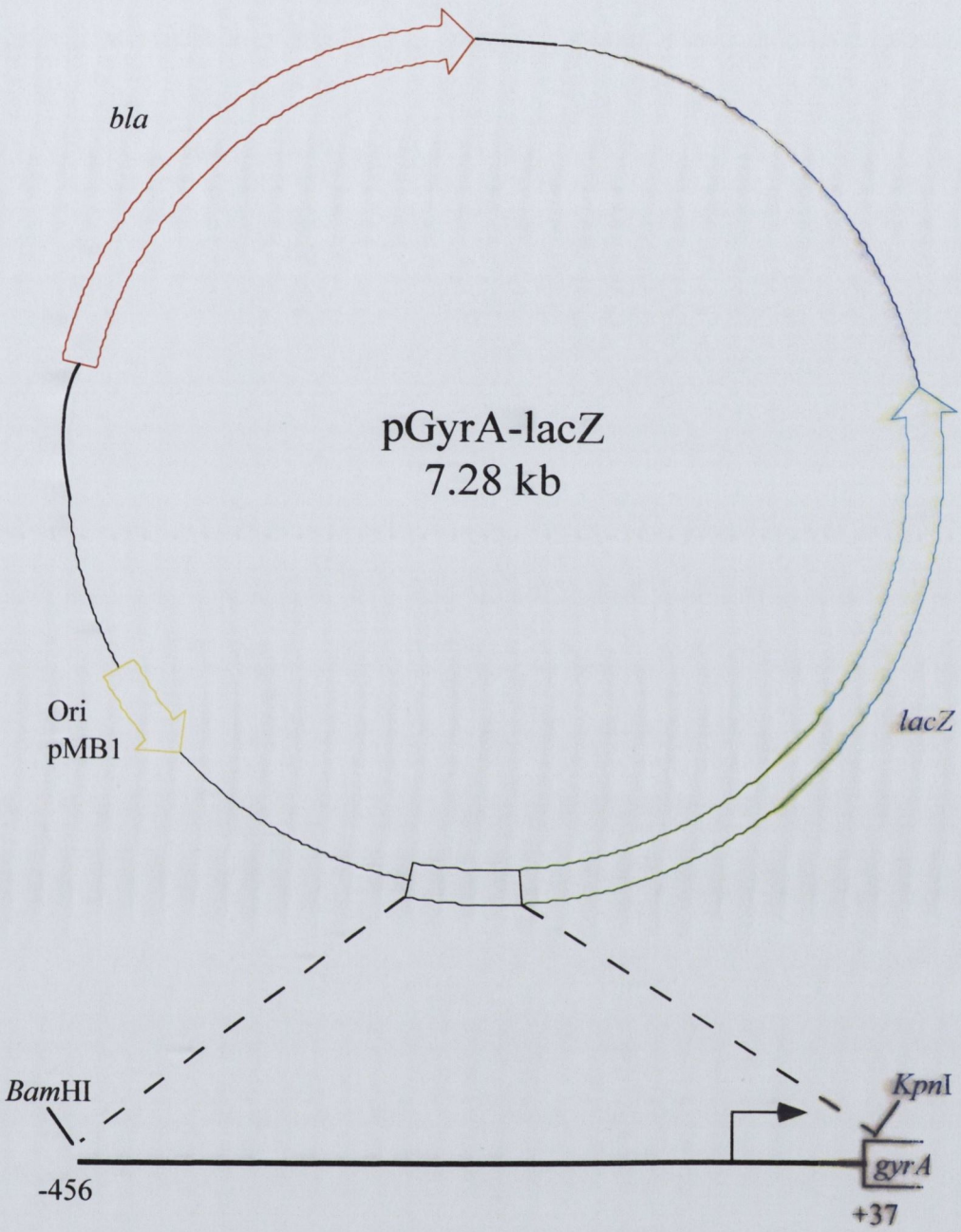


Fig. 5.7. **Map of pGyrA-lacZ plasmid.** See section 5.2.6 for details on pGyrA-lacZ construction. The bases are numbered relative to the transcription start site which is +1.

5.2.7: Determination of the *gyrA* transcription start site

It was decided to map the *S. typhimurium gyrA* transcription start site by primer extension (section 2.14) and determine if it matched that of *E. coli*. Total RNA was extracted from SL1344+pGyrA-lacZ and SL1344+pQF50 (this was used as a negative control). The amount of RNA was quantified by OD_{260nm} readings (section 2.5.3). Equal amounts of RNA was then subjected to primer extension analysis using the primer lacZ50, which bound within *lacZ*. This prevented the primer binding to chromosomal *gyrA* mRNA. The primer extension products were run on a 7% polyacrylamide gel alongside DNA sequencing reactions. The sequencing reactions used the same primer and pGyrA-lacZ as template DNA (section 2.12). The transcription start site was mapped to a C residue 37 bp upstream of the ATG translation start codon (Fig. 5.8). A TATAAT motif, which matches the consensus –10 sequence for a σ^{70}-dependent promoter lies 10 bases upstream. This transcription start site corresponds to that of *E. coli gyrA* (Menzel and Gellert, 1987a).

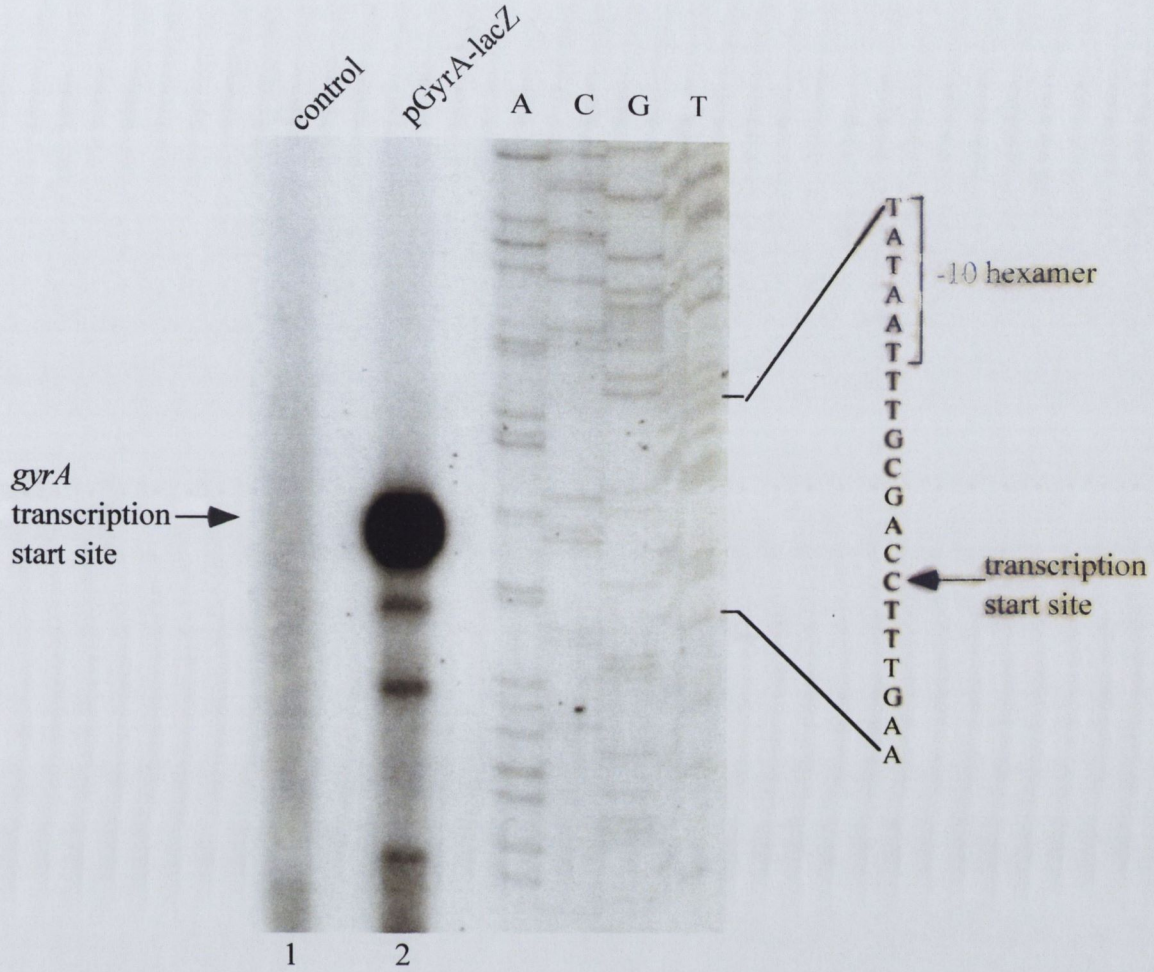


Fig. 5.8. **Determination of *S. typhimurium gyrA* transcription start site by primer extension.** Primer extension was performed using the oligonucleotide lacZ50. Lane 1 SL1344+pQF50. Lane 2 SL1344+pGyrA-lacZ. DNA sequencing reaction using the same primer and pGyrA-lacZ as template were electrophoresed in parallel in order to determine the transcriptional start site and are labelled ACGT.

5.3: Discussion

The *gyrB* and *topA* promoters from *S. typhimurium* SL1344 and *E. coli* K-12 shared sufficient homology to allow the amplification of the *Salmonella* promoters with primers designed from the *E. coli* sequence (Marshall *et al.*, 2000). Attempts at amplifying the *Salmonella gyrA* promoter by this method failed indicating that this promoter differed significantly from *gyrA* of *E. coli*. This was shown to be true for 3 strains of *S. typhimurium* tested, LT2, SL1344 and C5 and so was not strain specific. This may have implications for the regulation of DNA gyrase. The genes for DNA gyrase *gyrA* and *gyrB* are located quite a distance from each other on the chromosome and so are not transcribed as an operon therefore they must be co-ordinately regulated in both *E. coli* and *Salmonella*. The *gyrA* promoters from *E. coli* and *Salmonella* must respond appropriately to the same environmental signals despite their sequence differences. It was therefore decided to clone and sequence the *gyrA* promoter from *S. typhimurium* SL1344 and compare this promoter to its *E. coli* counterpart.

The sequence of *S. typhimurium gyrA* was found to differ significantly from that of *E. coli* although the coding sequence remained largely unchanged. The promoter sequences diverged 5 bases upstream of the –10 hexamer. There appears to be an additional 4 kb of DNA inserted between the *ubiG* and *gyrA* genes in *Salmonella* compared to *E. coli*. This is true for both *S. typhimurium* and *S. typhi* (McClelland *et al.*, 2001, Parkhill *et al.*, 2001). We predicted this DNA to contain a single open reading frame, which encoded an inner membrane transport protein with significant homology to the tartrate transporter TtuB of *Agrobacterium vitis*. Subsequent to this work the *Salmonella* genome sequencing project was completed and predicts 3 open reading frames in this 4 kb of DNA (McClelland *et al.*, 2001, Parkhill *et al.*, 2001).

Some similarities do exist between the *Salmonella* and *E. coli gyrA* promoter regions. Between the –10 hexamer and the ATG translation start codon both sequences are identical. This is significant for a number of reasons. The region between the –10 hexamer and the translation start site has been demonstrated to be responsible for the relaxation stimulated transcription (RST) response (Menzel and Gellert, 1987b; Straney *et al.*, 1994). This results in the induction of DNA gyrase when the DNA becomes more relaxed and the repression of gyrase when the DNA becomes more

negatively supercoiled. This is an important feature in the homeostatic regulation of DNA gyrase activity. It is important that the *Salmonella gyrA* promoter retains the sequence required for this RST response and would be under selective pressure to do so. Both the *Salmonella gyrA* promoter and the *E. coli gyrA* promoter contain a TGTGT motif immediately upstream of the –10 hexamer. A TGTG motif 1 base upstream of a –10 hexamer is known to allow promoter activity in the absence of a good –35 region and to increase promoter strength (Bown *et al.*, 1997; Burr *et al.*, 2000). This ensures that the *gyrA* promoters of *E. coli* and *Salmonella* can be strongly expressed. Such a TGTG motif is not found upstream of the –10 sequence in the *Salmonella* or *E. coli gyrB* promoter however. Both the *Salmonella* and *E. coli gyrA* and *gyrB* promoters contain the sequence GTTTACC 19 bases upstream of the –10 hexamer. This sequence contains the putative -35 sequence of *gyrA* but is found only 11 bp upstream of the -10 box in *gyrB*. The precise function of this sequence is unknown however as this motif is found in both the *gyrA* and *gyrB* promoter regions in *E. coli* and *Salmonella* it is therefore thought to be significant. This sequence is known not to be required for relaxation stimulated transcription (Menzel and Gellert, 1987b). A closely homologous motif (GTTTATC) is also found upstream of *topA*, the gene encoding the major DNA relaxing activity in *E. coli* (Tse-Dinh and Wang, 1986). This motif differs by just 1 base from that found upstream of *gyrA* and *gyrB* and contains a pyrimidine for a pyrimidine substitution in the 6th position. This sequence is not completely conserved upstream of the *topA* gene of *S. typhimurium*, where the sequence (GTGAATC) differs by 3 bases from that found upstream of *gyrA* and *gyrB* and differs by 2 bases from that found upstream of *E. coli topA*. The fact that these 7 bases are conserved between *gyrA* of *Salmonella* and *E. coli* indicates their potential importance, especially as they are situated in a region of the *gyrA* promoter with little overall sequence homology. This would indicate that both *E. coli* and *Salmonella* are under selective pressure to retain this sequence and that it serves an important function.

The *Salmonella gyrA* promoter sequence was cloned upstream of the promoterless *lacZ* gene in the plasmid pQF50 giving plasmid pGyrA-lacZ. This will allow the regulation of *gyrA* to be studied. The transcription start site of the *S. typhimurium gyrA* gene on pGyrA-lacZ was also mapped by primer extension. The start site was mapped to a C residue 37 bases upstream of the ATG translation start codon. This transcription start site corresponds to that of *E. coli gyrA*. This is not surprising given

that the transcription start site is located in the region of homology between the 2 sequences. Both promoters share the same –10 sequence and a TGTG promoter strengthening motif immediately upstream of it. The –10 sequence found in both *E. coli* and *Salmonella* matches the consensus sequence for a σ^{70} dependent promoter perfectly. The –35 sequence does not perfectly match the σ^{70} consensus sequence however.

Chapter 6

Regulation of DNA gyrase expression in *Salmonella typhimurium*

6.1: Introduction

DNA gyrase is an essential enzyme in bacterial cells and null mutations in *gyrA* or *gyrB* are lethal. The genes encoding DNA gyrase are induced in response to relaxed DNA and repressed in the presence of negatively supercoiled DNA (Menzel and Gellert, 1983). This homeostatic regulation of DNA gyrase is of utmost importance to the cell, as the changes in the level of negative supercoiling allows different subsets of genes to be expressed (Dorman, 2002). The topology of the DNA is one of the general factors co-ordinating gene activity with environmental changes (McClellan *et al.*, 1990; Friedman *et al.*, 1995). This allows genes located in various positions around the genome to be co-ordinately regulated in response to a single environmental stimulus. Both *gyrA* and *gyrB* themselves must also be co-ordinately regulated.

A number of studies have been carried out examining the regulation of DNA gyrase in *E. coli* (Gellert *et al.*, 1983; Balke and Gralla, 1987; Menzel and Gellert, 1987a; Gomez-Gomez *et al.*, 1996). One study has shown that the Fis protein negatively regulates *gyrA* and *gyrB* (Schneider *et al.*, 1999). Fis has been shown to bind the *gyrA* and *gyrB* promoter regions and inhibit transcription of these genes. Fis blocks transcription of *gyrA* by binding immediately upstream of the *gyrA* extended –10 hexamer and so prevents RNA polymerase binding. In the case of *gyrB* Fis binds the region –55 to –122, relative to the transcription start site of *gyrB*. This does not occlude the RNA polymerase binding site but prevents the isomerisation of the closed to an open complex and promoter clearance. Inactivation of *fis* results in an increase in *gyrA* and *gyrB* mRNA in *E. coli* of between 1.5 and 2-fold (Schneider *et al.*, 1999). The *fis* gene itself also responds strongly to changes in the negative superhelicity of the DNA. Expression of *fis* is maximal at slightly higher than total physiological supercoiling levels. This is fully consistent with the role of Fis in homeostatic regulation of gyrase, as once supercoiling reaches too high a level, *fis* is expressed which represses *gyrA* and *gyrB*. The GC rich discriminator sequence located between the –10 hexamer of *fis* and the ATG translation start codon, is necessary for stringent control of *fis* and is the primary structural element responsible for the supercoiling sensitivity of the *fis* promoter (Schneider *et al.*, 2000). This GC rich discriminator is also found in the *E. coli tyrT* and *Salmonella hisR* promoter regions. Both of these promoters are also stringently controlled and stimulated by negative supercoiling

(Lamond, 1985; Free and Dorman, 1994; Figueroa-Bossi *et al.*, 1998). Fis has also been shown to delay the increase in the overall level of negative supercoiling of a reporter plasmid after nutrient upshift by binding directly to the plasmid and stabilising the plasmid population of intermediate superhelical density (Schneider *et al.*, 1997). This is therefore independent of the Fis effect on *gyrA* and *gyrB* transcription. By maintaining the topological diversity of DNA in exponentially growing cells Fis increases the structural information required for the fine-tuning of cellular processes involving DNA transactions. The *fis* promoter is stringently regulated and is therefore sensitive to the nutritional condition of the cell (Ninnemann *et al.*, 1992). In this way Fis can adapt global chromosome dynamics to changing growth environments by co-ordinating the expression of other nucleoid-associated proteins such as HU and H-NS (Claret and Rouvière-Yaniv, 1996; Falconi *et al.*, 1996), by regulating DNA gyrase and by direct effects on the topology of bacterial DNA. Fis appears to be involved in fine-tuning the homeostatic control of DNA gyrase, which in turn results in tighter regulation of gyrase (Schneider *et al.*, 1997; 1999; 2000). After nutrient upshift ATP becomes available as a cofactor for DNA gyrase. This results in an increase in DNA gyrase activity and a subsequent increase in the negative superhelicity of the DNA (Balke and Gralla, 1987). Fis is also induced which represses the gyrase genes so negative supercoiling does not reach detrimentally high levels (Schneider *et al.*, 1999). The levels of Fis are then depleted as the cells grow through the growth phase and into stationary phase (Ball *et al.*, 1992).

Fis has also been reported to activate *topA* expression under conditions of oxidative stress (Weinstein-Fischer *et al.*, 2000). This also fits in with the role of Fis in the homeostatic regulation of supercoiling as DNA gyrase and topoisomerase I have opposing functions and when one is repressed the other is induced. Fis is also known to regulate the genes for HU and H-NS (Claret and Rouvière-Yaniv, 1996; Falconi *et al.*, 1996). These histone-like proteins also contribute to the overall level of DNA supercoiling in the cell. HU has been shown to compact the DNA into nucleosome-like structures (Rouvière-Yaniv *et al.*, 1979). H-NS binds preferentially to curved DNA (Bracco *et al.*, 1989; Yamada *et al.*, 1990; Zuber *et al.*, 1994) and when overproduced results in the condensation of the bacterial chromosome (Spurio *et al.*, 1992). Fis and RpoS also comodulate growth-phase dependent gene expression in *E. coli* (Xu and Johnson, 1995).

A number of other factors are known to regulate DNA gyrase expression in *E. coli*. The cyclic AMP receptor protein CRP has been shown to positively control *gyrA* transcription and alter DNA topology after nutrient upshift (Gomez-Gomez *et al.*, 1996). The activity of a *gyrA-lacZ* fusion was monitored in isogenic wild-type and *crp*⁻ *E. coli* backgrounds. An increase in *gyrA* expression during exponential phase was seen in wild-type *E. coli* but not in the *crp*⁻ strain. Glucose was also shown to inhibit *gyrA* expression in exponential phase suggesting that *gyrA* is subject to catabolite control. CRP also appears to play a role in modulating DNA topology in the transition from the lag period to exponential growth (Gomez-Gomez *et al.*, 1996). Whether CRP directly or indirectly regulates *gyrA* remains to be determined. CRP may regulate *gyrA* indirectly as CRP is required to maintain the growth phase pattern of *fis* expression and Fis and CRP synergistically repress *fis* gene expression (Nasser *et al.*, 2001). Integration Host Factor (IHF) has also been reported to play a role in regulating DNA topology in *E. coli*. Along with other nucleoid-associated proteins, IHF can bind DNA and constrain supercoils locally. IHF has also been reported to positively regulate *gyrA* expression, as a Δ*ihfA82* mutation decreased *gyrA* expression about 4-fold in *E. coli* (Gellert *et al.*, 1983). A *ihfA* mutation was also shown to increase *topA* expression showing that IHF can negatively regulate *topA* (Tse-Dinh and Beran, 1988).

The gene encoding *Salmonella* GyrA differs in some of its regulatory region from that encoding GyrA of *E. coli*. Both genes must be able to respond to changes in environmental conditions such as osmolarity, heat shock and anaerobiosis. Both genes must also be homeostatically regulated and induced by relaxed DNA and repressed by negatively supercoiled DNA. Both genes must also co-ordinate their expression with that of *gyrB* the other subunit of DNA gyrase. The promoter sequences of *gyrB* from *Salmonella* and *E. coli* do not differ significantly. Why therefore does the *gyrA* promoter sequence differ between *E. coli* and *Salmonella* and is this difference significant? It has been proposed previously that *gyrA* is more sensitive to alterations in DNA supercoiling than *gyrB* (Neumann and Quinones, 1997). Therefore *gyrA* may require additional regulatory signals to *gyrB*. Also it has been previously noted that *gyrB-lacZ* transcriptional fusions are more strongly expressed than *gyrA-lacZ* transcriptional fusions (Schneider *et al.*, 1999). If there is more GyrB in the cell than GyrA, then GyrA is the limiting factor for generating the DNA gyrase enzyme and

regulation of this component of DNA gyrase is critical. Although DNA gyrase from *E. coli* and *Salmonella* carry out similar functions there are a number of reasons why the regulation of this enzyme may differ between the 2 species. In response to heat shock negative supercoiling increases in *E. coli* (Goldstein and Drlica, 1984). In *S. typhimurium* however negative supercoiling decreases in response to heat shock (Dorman *et al.*, 1990). Therefore the genes for DNA gyrase from *E. coli* and *Salmonella* need to respond differently to heat shock. The gene encoding the B subunit of DNA gyrase *gyrB* is induced when *Salmonella* are internalised by the mouse macrophage-like cell line J774A.1. DNA gyrase is thought to help the *Salmonella* cells adapt to this new intracellular environment by inducing a specific subset of genes required for survival inside macrophage (Marshall *et al.*, 2000). *E. coli* K-12 is not capable of surviving inside macrophages. Therefore the *Salmonella* gyrase genes may require unique regulatory sequences to allow induction of DNA gyrase when the bacteria are internalised by macrophages.

The aim of this work was to examine the regulation of the *S. typhimurium gyrA* and *gyrB* genes and determine if this regulation differed from the regulation of DNA gyrase in *E. coli*.

6.2: Results

6.2.1: Effect of Fis on *gyrA* and *gyrB* transcription

Strain SL1344*fis*⁻ (Table 2.1) was constructed by moving the chloramphenicol resistance marker from strain TH2285 (Table 2.1) into SL1344 by P22-mediated transduction. The *fis* lesion was confirmed by Western immunoblotting with anti-Fis antiserum (data not shown). SL1344 and SL1344*fis*⁻ were then transformed with the plasmids pGyrA-lacZ and pGyrB-lacZ (Table 2.2). The 4 resulting strains were grown overnight and the bacteria assayed for β-galactosidase activity. The results can be seen in Fig. 6.1. The absence of Fis from the cell resulted in an approximately 2-fold increase in the level of *lacZ* expression from pGyrA-lacZ and pGyrB-lacZ. This was a little surprising as the amount of Fis in overnight cultures is known to be very low (Ball *et al.*, 1992). The expression of *lacZ* from pGyrA-lacZ and pGyrB-lacZ was then examined as a function of time after nutrient upshift in wild-type and *fis*⁻ backgrounds. The growth of the bacterial cells was monitored by measuring OD_{600nm}. At various times throughout the growth phase samples were taken and their β-galactodsidase activity measured. A graph of β-galactosidase activity against time can be seen in Fig. 6.2. Throughout the growth phase the level of *lacZ* expression from pGyrA-lacZ was higher in SL1344*fis*⁻ than in SL1344. The level of *lacZ* expression from pGyrB-lacZ was also higher in SL1344*fis*⁻ than SL1344 for all time-points measured except one. The difference between *gyrB* expression in SL1344 and SL1344*fis*⁻ is not as great as the difference in *gyrA* expression in the same backgrounds. This experiment was repeated 3 times and each time similar results were obtained. These results are similar to the results obtained by Schneider *et al.* (1999). The difference in *gyrA* and *gyrB* expression in wild-type and SL1344*fis*⁻ backgrounds is not very large and this is consistent with a role for Fis in the homeostatic regulation of supercoiling and the repression of *fis* itself by negative supercoiling. There also appears to be a larger difference between *gyr* gene expression in wild-type and *fis*⁻ backgrounds when the cells are grown in 5 ml cultures in test-tubes rather than in 50 ml cultures in 250 ml conical flasks (compare Fig 6.1 and 6.2). This may be due to the difference in oxygen levels between the 2 growth conditions as oxygen levels are known to affect DNA supercoiling (Dorman *et al.*, 1988). Anaerobic conditions increase negative

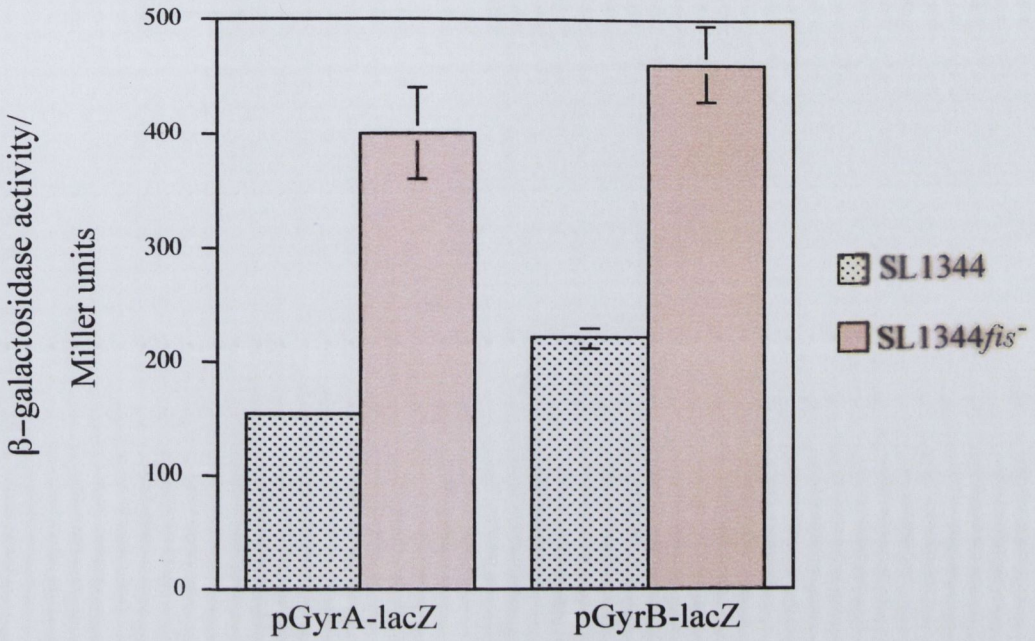


Fig. 6.1. **Stationary phase expression of *gyrA* and *gyrB* in SL1344 and SL1344*fis*$^-$.** The absence of Fis from the cell results in an approximately 2-fold increase in ***gyrA*** and *gyrB* transcription. The *fis* lesion was shown to have no effect on plasmid copy number (data not shown).

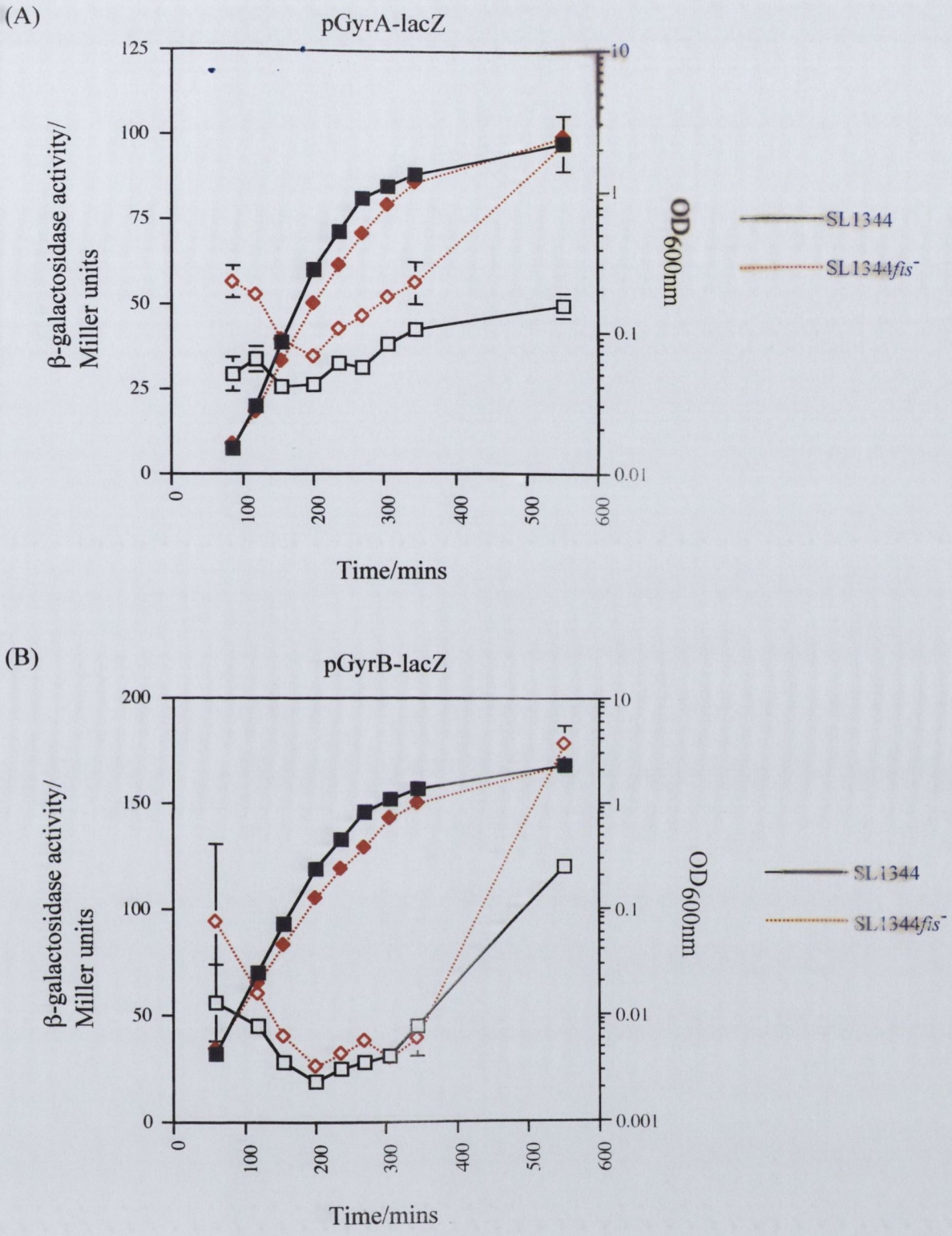


Fig 6.2. **Growth phase expression of *gyrA* and *gyrB* in SL1344 and SL1344*fis*⁻.** The absence of Fis from the cell results in an increase in *gyrA* and *gyrB* transcription throughout the growth curve. Open symbols represent β-galactosidase activity and closed symbols represent OD_{600nm}.

supercoiling. 5 ml cultures in test-tubes are less well aerated and hence do not reach the same optical density as 50 ml cultures grown in 250 ml conical flasks. Therefore it appears that Fis regulates *gyrA* and *gyrB* expression in *Salmonella* as well as *E. coli*. This is very interesting given the fact that the sequence to which Fis binds upstream of the *E. coli gyrA* promoter is not present upstream of *gyrA* from *Salmonella*.

6.2.2: Measurement of Fis throughout growth

As mentioned previously the absence of Fis from the cell results in an increase in *lacZ* expression from pGyrA-lacZ and pGyrB-lacZ. This remains true throughout the growth phase. It was therefore decided to measure the amount of Fis protein in the cell at various points along the growth curve. *Salmonella typhimurium* strains LT2 and SL1344 and *E. coli* K-12 strain MC4100 (Table 2.1) were grown and samples taken at various points along the growth curve. These samples were then subjected to Western immunoblotting using anti-Fis antiserum. The resulting Western blot can be seen in Fig. 6.3. The levels of Fis are highest soon after nutrient upshift and remain quite high for approximately 180 min after nutrient upshift. The levels decrease as the cells grow and Fis is not detectable in the last sample, which was taken 400 min after nutrient upshift and corresponds to an OD_{600nm} of 2.13. The expression pattern of Fis is similar in both *E. coli* and *Salmonella* although Fis levels appear to be depleted slightly quicker in *E. coli*. These results are similar to previously published results on the growth phase dependent expression of Fis (Ball *et al.*, 1992).

6.2.3: Purification of Fis protein

In order to determine if Fis bound directly to the promoter regions of *gyrA* and *gyrB* from *Salmonella* gel mobility shift assays were carried out using total protein extracts from SL1344 and SL1344*fis*$^-$. A radiolabelled fragment of the *gyrA* promoter shifted with both extracts (data not shown). This was probably due to binding of the promoter fragment by proteins such as RNA polymerase and non-specific DNA binding proteins such as H-NS, which are present in both extracts. No Fis-specific shift could therefore

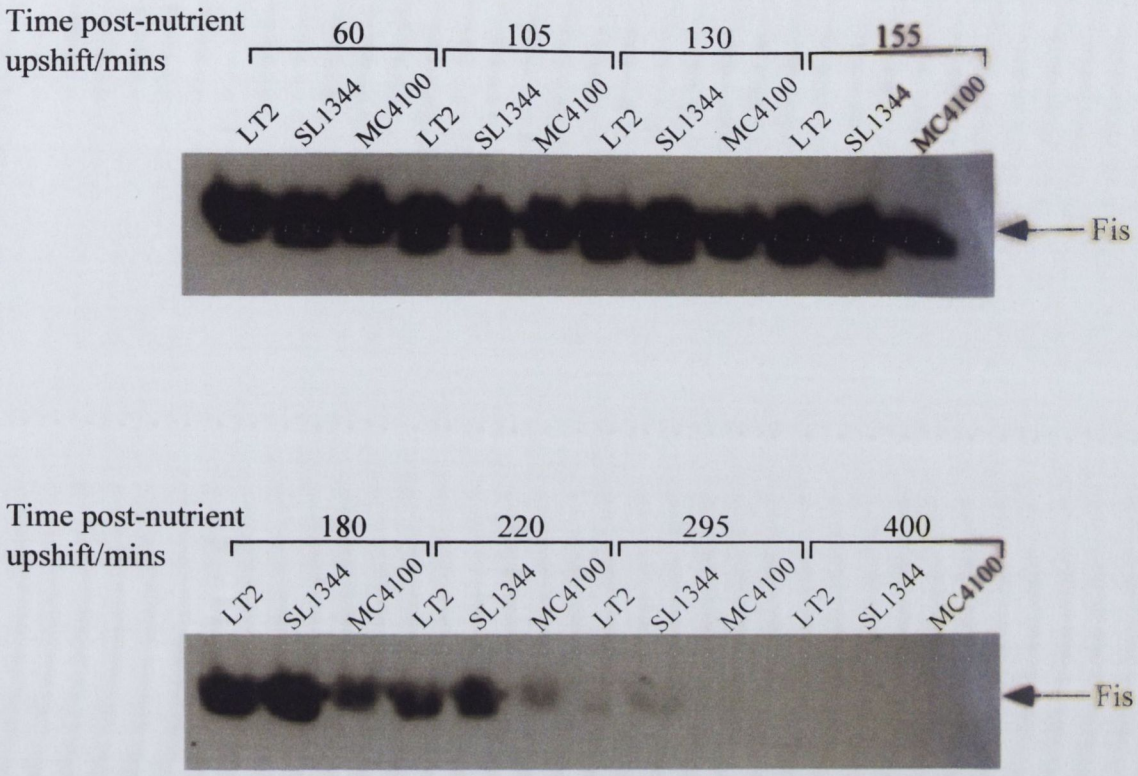


Fig. 6.3. **Measurement of Fis throughout growth.** Western immunoblot of **Fis** levels over time after nutrient upshift. Fis levels decrease over time and Fis is not detectable 400 minutes after nutrient upshift. Fis also appears to be depleted slightly quicker in *E.coli* MC4100 compared to *S. typhimurium* LT2 or SL1344.

be distinguished. It was then decided to purify recombinant His-tagged Fis protein and examine the ability of the purified protein to bind the *gyrA* and *gyrB* promoter regions.

The pET system (Novagen) was used to purify recombinant Fis protein. This system is based on the T7 RNA polymerase expression system (Studier and Moffatt, 1986). In the pET system the gene for the protein to be purified is inserted in frame into a pET vector either upstream or downstream of a number of histidine codons. This results in the expression of either a C-terminal or N-terminal His-tagged protein respectively. The His-tagged gene is also under the control of the inducible T7 promoter and is therefore not expressed in the absence of T7 RNA polymerase. This vector is then transformed into the strain BL21(DE3) (Table 2.1). This strain is deficient in both the Lon and OmpT proteases. BL21(DE3) is also a λDE3 lysogen and encodes T7 RNA polymerase under the control of the *lacUV5* promoter. Upon the addition of IPTG to the growth media the *lacUV5* promoter is derepressed. This results in the production of T7 RNA polymerase and the subsequent production of the His-tagged protein. T7 RNA polymerase is so selective and active that almost all the cell resources are converted to target gene expression (Studier and Moffatt, 1986). The His-tagged protein can comprise over 50% of the total cell protein after a few hours induction. If the protein is soluble it can then be purified by nickel affinity chromatography.

The *fis* coding sequence was amplified using the high fidelity thermostable polymerase Platinum *pfx* polymerase (section 2.8). The primers used were Ndelfis and C-TERrev (Table 2.3), which contain 5' *Nde*I and *Xho*I extensions respectively. The PCR product was then cleaned up using the Promega PCR clean up kit (section 2.7.3) and digested with *Nde*I and *Xho*I in order to generate overhangs. The PCR product was then ligated into *Nde*I-*Xho*I digested pET22b. This generated the plasmid pFisC (Table 2.2), which encoded Fis with 6 C-terminal histidines. The integrity of the *fis* insert was confirmed by sequencing to confirm it was in frame and that it did not contain any PCR errors.

The vector pFisC was then transformed into BL21(DE3)+pDIA17 (Table 2.1). The plasmid pDIA17 carries a copy of the *lacI* repressor and ensures that no T7 RNA polymerase is produced in the absence of inducer. Upon addition of 0.5 mM IPTG for 2 hours clear induction of His-tagged Fis could be seen by SDS-PAGE analysis of total protein extracts and approximately 70% of induced Fis was also found in the soluble

supernatant fraction (data not shown). Fis was purified from this fraction by nickel affinity chromatography, and was retained in the column, as little Fis was evident in the flow-through fraction. Bound Fis was eluted with 500 mM imidazole, which out-competes the C-terminal histidines for the Ni^{2+}(Fig. 6.4 A). In this way full-length Fis protein was purified and judged to be greater than 90% pure by Coomassie staining. The Fis protein was dialysed into a suitable storage buffer and its integrity, purity and concentration confirmed by SDS-PAGE analysis (Fig. 6.4 B).

6.2.4: Fis binds the *Salmonella gyrA* and *gyrB* promoter regions

To determine the ability of Fis to bind to the *Salmonella gyrA* and *gyrB* promoter regions, an electrophoretic mobility shift assay was used. A 493 bp *gyrA* promoter fragment was amplified using the primers gyrAkpn and gyrAbam (Table 2.3). The restriction enzymes were then removed and the PCR fragment end-labelled using T4 polynucleotide kinase and $\gamma\,^{32}$P dATP (section 2.14.1). Increasing amounts of purified Fis protein were incubated with the labelled promoter fragment. Bovine serum albumin and poly-[d(I-C).(dI-dC)] were used as competitors. The complexes were then resolved on a 7% acrylamide gel. As can be seen in Fig. 6.5 (A) Fis clearly binds to the region upstream of the *Salmonella gyrA* gene. There appears to be up to 6 discrete Fis binding sites in the *gyrA* upstream region. The ability of Fis to bind to a region of *spvR* coding sequence generated with the primers InspvRF and InspvRR (Table 2.3), was also examined as a negative control. Fis does not appear to bind to this fragment of DNA showing that non-specific binding of Fis to DNA has been successfully competed out (Fig. 6.5 (B)). These *gyrA* binding sites were then resolved into promoter distal and promoter proximal sites. A 322 bp promoter proximal fragment was amplified with the primers gyrABS and gyrAkpn (Table2.3). A 277 bp promoter distal fragment was amplified with the primers gyrAbam and DNase2R (Table 2.3). These fragments, which overlap by 106 bp, were end-labelled with T4 polynucleotide kinase and $\gamma\,^{32}$P dATP (section 2.14.1). The fragments were then used in a gel-mobility shift assay with the purified recombinant Fis. As can be seen in Fig. 6.6 (A) there are two Fis binding sites in the *gyrA* promoter proximal region. These sites are occupied when the concentration of Fis is approximately 45 nM for the first site and 225 nM for the second site. There also appears to be four Fis binding sites in

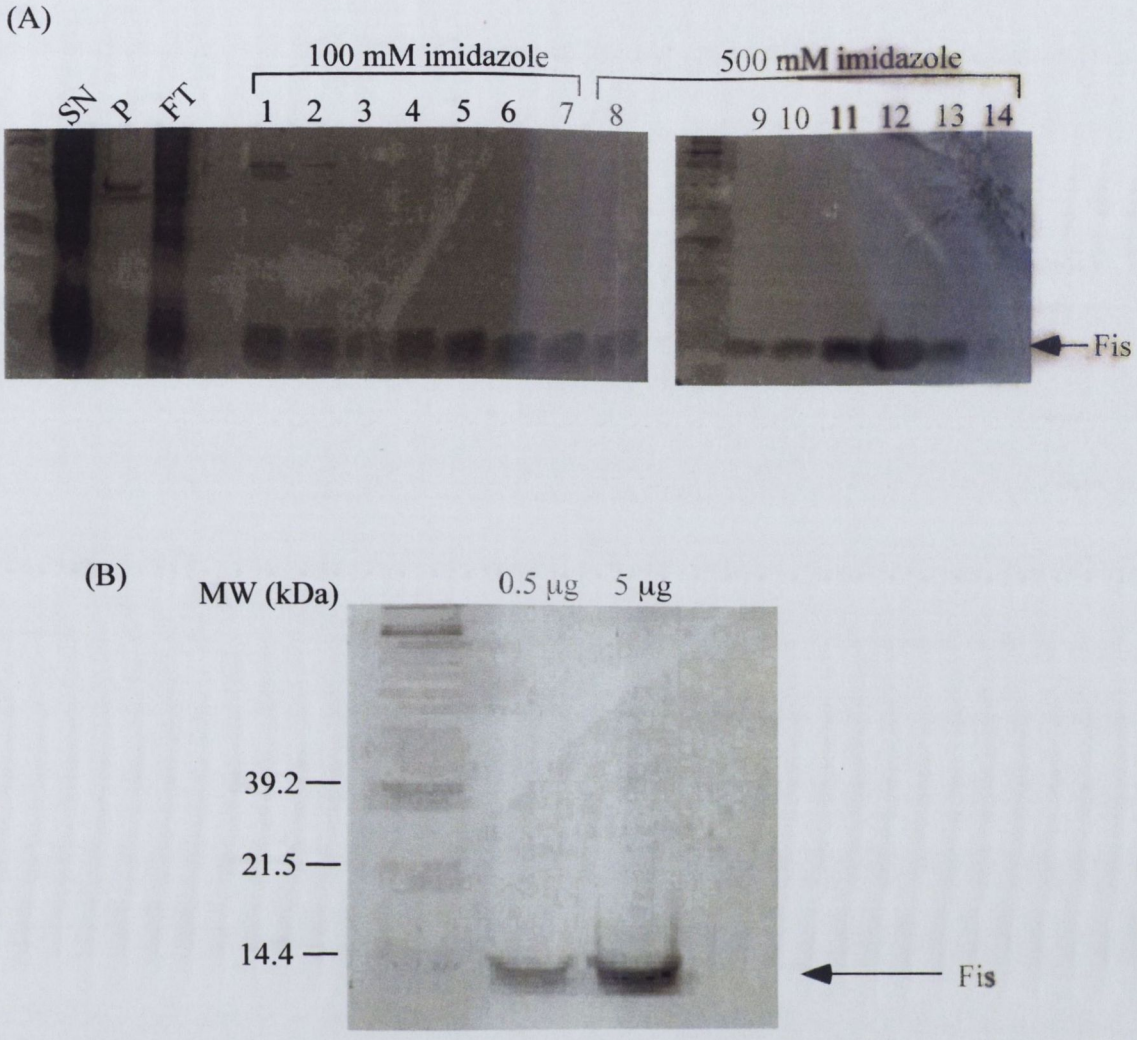


Fig. 6.4. **SDS-PAGE analysis of purified Fis.** (A) SDS-PAGE analysis of steps in the purification of Fis. SN = supernatent fraction, P = pellet fraction, FT = flow through from His bind column, 1-14 are 1 ml fractions of eluate, eluted with 100 mM imidazole for 1-7 and 500 mM imidazole for 8-14. Fractions between 11 and 13 were pooled and purified Fis dialysed into storage buffer.

(B) 0.5 µg and 5 µg samples of purified His-tagged Fis was analysed for protein concentration, purity and integrity. The concentration of the pure protein was calculated to be 2 mg/ml.

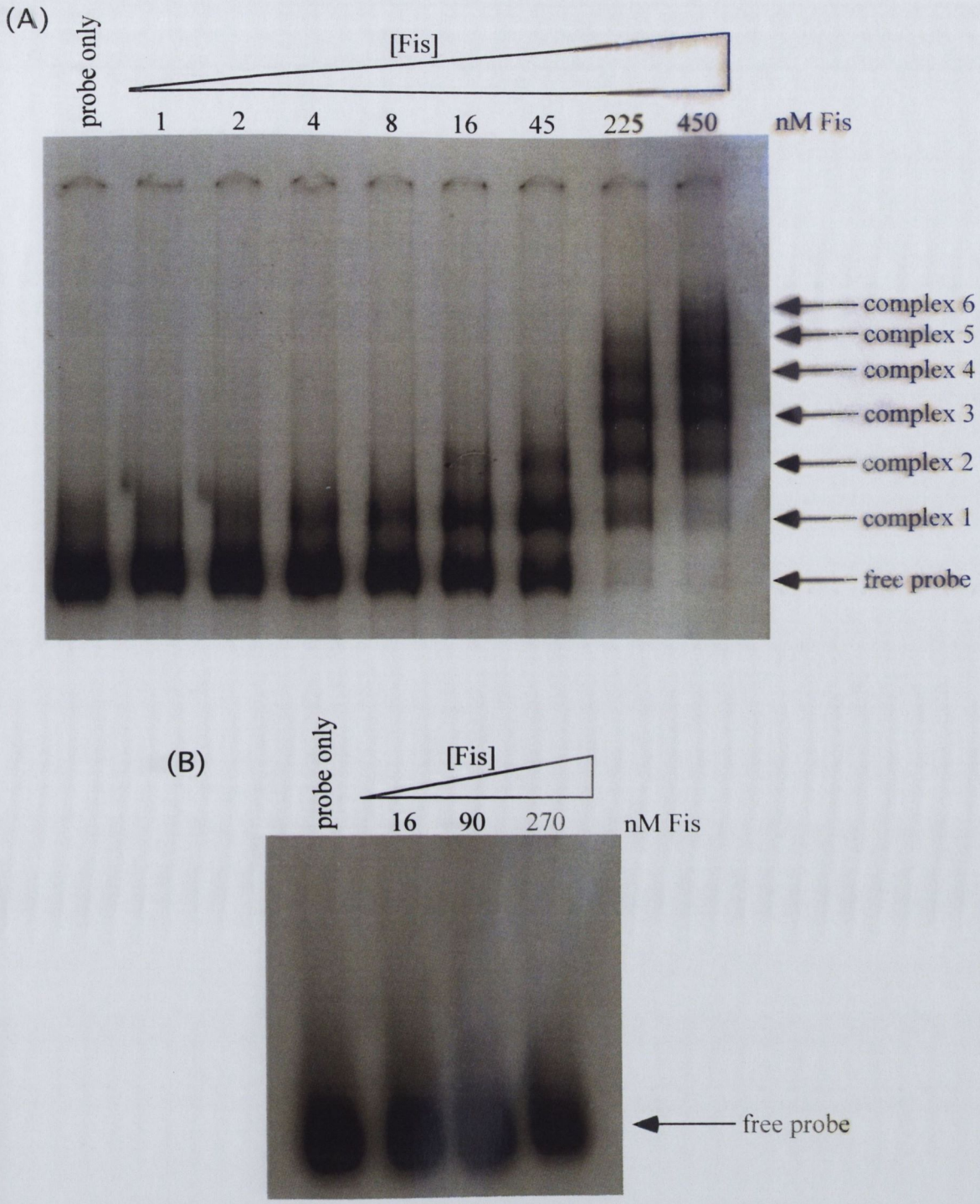
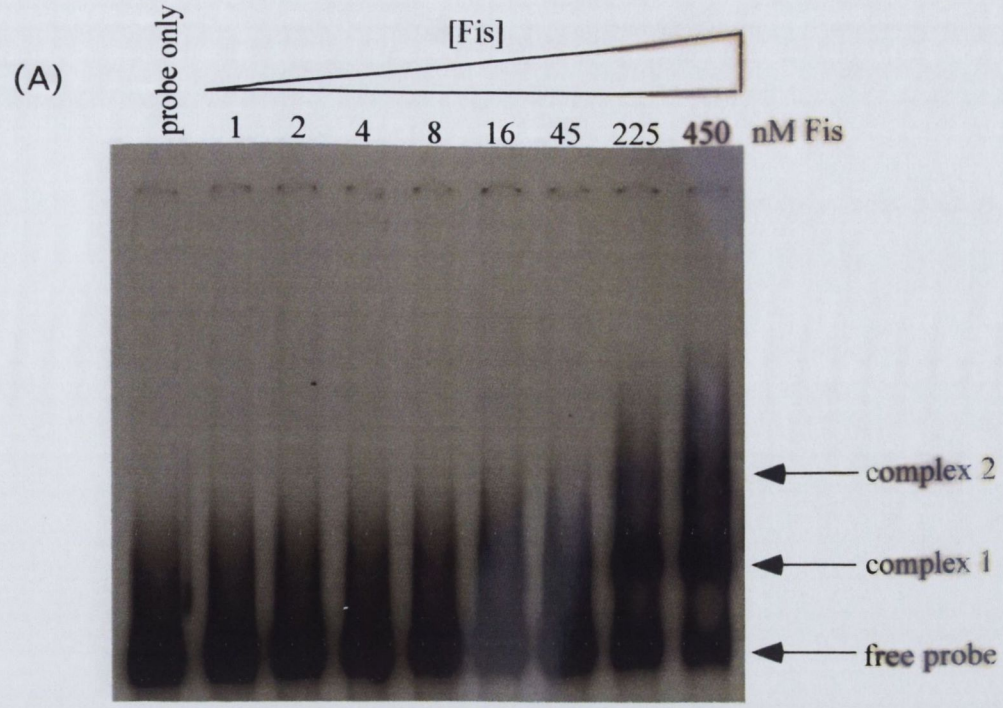


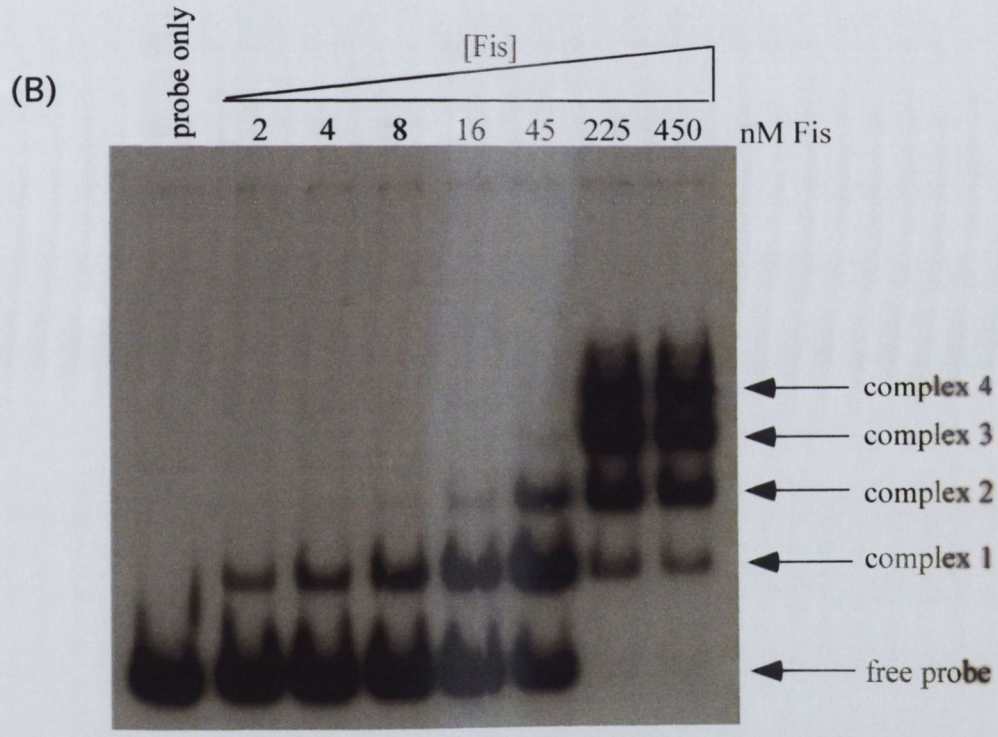
Fig 6.5. **Fis binds the *Salmonella gyrA* promoter.**

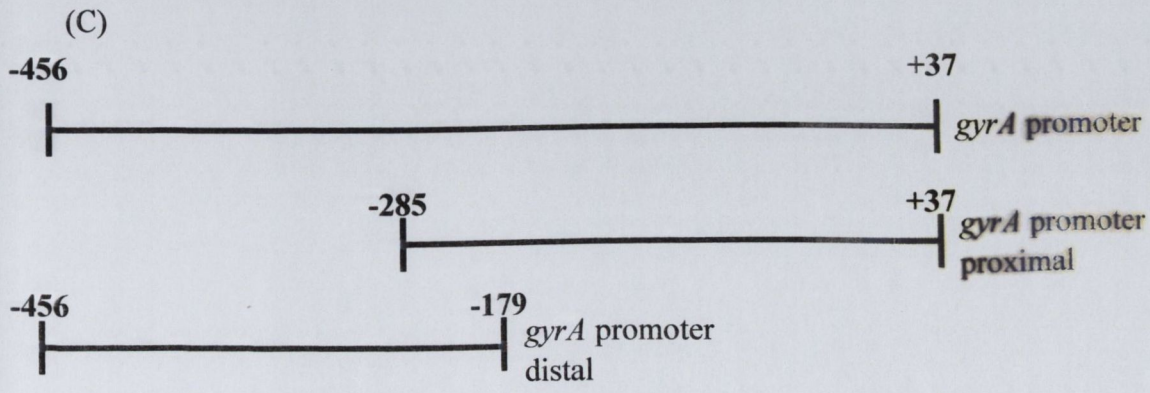
(A) Electrophoretic mobility shift assays were performed with a 493 bp *gyrA* promoter fragment and recombinant Fis used at the concentrations shown. Fis appears to bind up to 6 distinct sites upstream of *gyrA*.

(B) Fis does not bind an internal fragment of *spvR* showing that non-specific DNA binding by Fis has been out-competed.

Fig. 6.6. **Fis binds both the *gyrA* promoter proximal and distal regions.**
(A) Fis binding to the 322 bp *gyrA* promoter proximal fragment *in vitro*. Electrophoretic mobility shift assays was performed with a radiolabelled 322 bp *gyrA* promoter proximal fragment and pure Fis, used at the concentrations shown. Fis appears to have two binding sites in the promoter proximal region.
(B) Fis binding to the 277 bp *gyrA* promoter distal fragment *in vitro*. Fis appears to have 4 binding sites in this region.
(C) Schematic map of the full-length *gyrA* promoter fragment and the *gyrA* promoter proximal and distal fragments. Bases are numbered relative to the transcription start site which is +1.


(A)
probe only
[Fis]
1 2 4 8 16 45 225 450 nM Fis
complex 2
complex 1
free probe


(B)
probe only
[Fis]
2 4 8 16 45 225 450 nM Fis
complex 4
complex 3
complex 2
complex 1
free probe


(C)
-456
+37
gyrA promoter
-285
+37
gyrA promoter proximal
-456
-179
gyrA promoter distal

the promoter distal region (Fig. 6.6 (B)). These sites are occupied at Fis concentrations of approximately 2 nM for the first site, between 16 nM and 45 nM for the second site and 225 nM for the third and fourth sites. Therefore Fis occupies more binding sites in the promoter distal region than the promoter proximal region and it also appears to bind these sites more efficiently. However Fis may bind the entire *gyrA* promoter region co-operatively. In this case separating the promoter proximal site from the promoter distal sites may have led to less efficient binding than *in vivo* binding. The *gyrA* promoter sequence from *Salmonella* retains the ability to be bound by Fis even though its sequence differs from the *E. coli gyrA* Fis binding region.

The ability of Fis to bind the *Salmonella gyrB* promoter region was also examined by gel mobility shift assay. The *Salmonella gyrB* promoter region was amplified with the primers gyrBBSF and gyrBBSR (Table 2.3). These amplify 453 bp of the *Salmonella gyrB* promoter region. This PCR product was then end-labelled with T4 polynucleotide kinase and γ^{32}P dATP (section 2.14.1). This promoter fragment was then used in a gel mobility shift assay with purified Fis. As can be seen in Fig. 6.7 the *Salmonella gyrB* promoter region can also be bound by Fis protein. Fis appears to bind 4 discrete sites in the *S. typhimurium gyrB* promoter region. The first site is occupied at a Fis concentration of 2 nM, the next site is occupied at a concentration of Fis between 16 nM and 45 nM while the third and fourth sites are occupied at concentrations of 225 nM. In this case the promoter region has homology to the *E. coli gyrB* promoter region, which is known to be bound by Fis.

6.2.5: Mapping Fis binding sites upstream of *gyrA* and *gyrB*

Schneider *et al.* (1999) identified a Fis binding site upstream of the transcription start sites of *gyrA* and *gyrB* in *E. coli*. From our bandshift data there appears to be more than one such site in *Salmonella*. We decided to map the Fis binding site closest to the transcription start site of *gyrA* and *gyrB* in *S. typhimurium* SL1344 in order to determine if it matched that of *E. coli*. We wished to determine if Fis was repressing transcription of *gyrA* and *gyrB* in *S. typhimurium* by a similar mechanism to that in *E. coli*. The binding of Fis to the *gyrA* and *gyrB* promoter regions was analysed by DNase I footprinting. The basis of footprinting techniques is that DNA-bound proteins

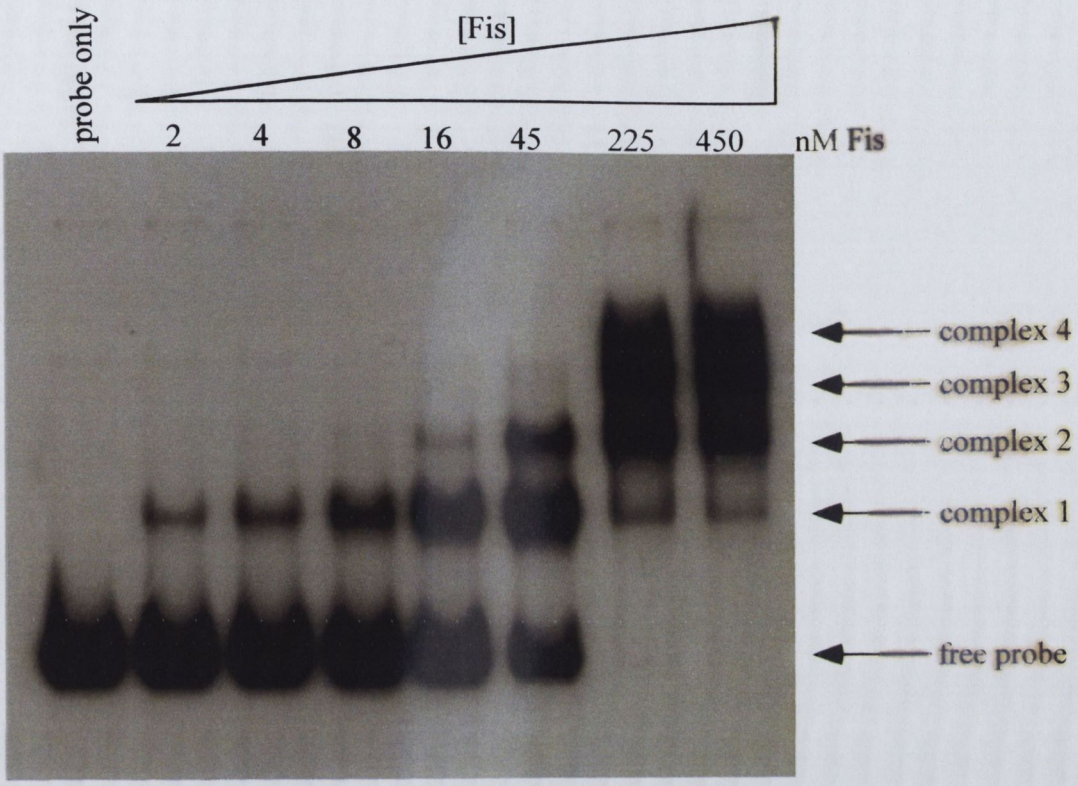


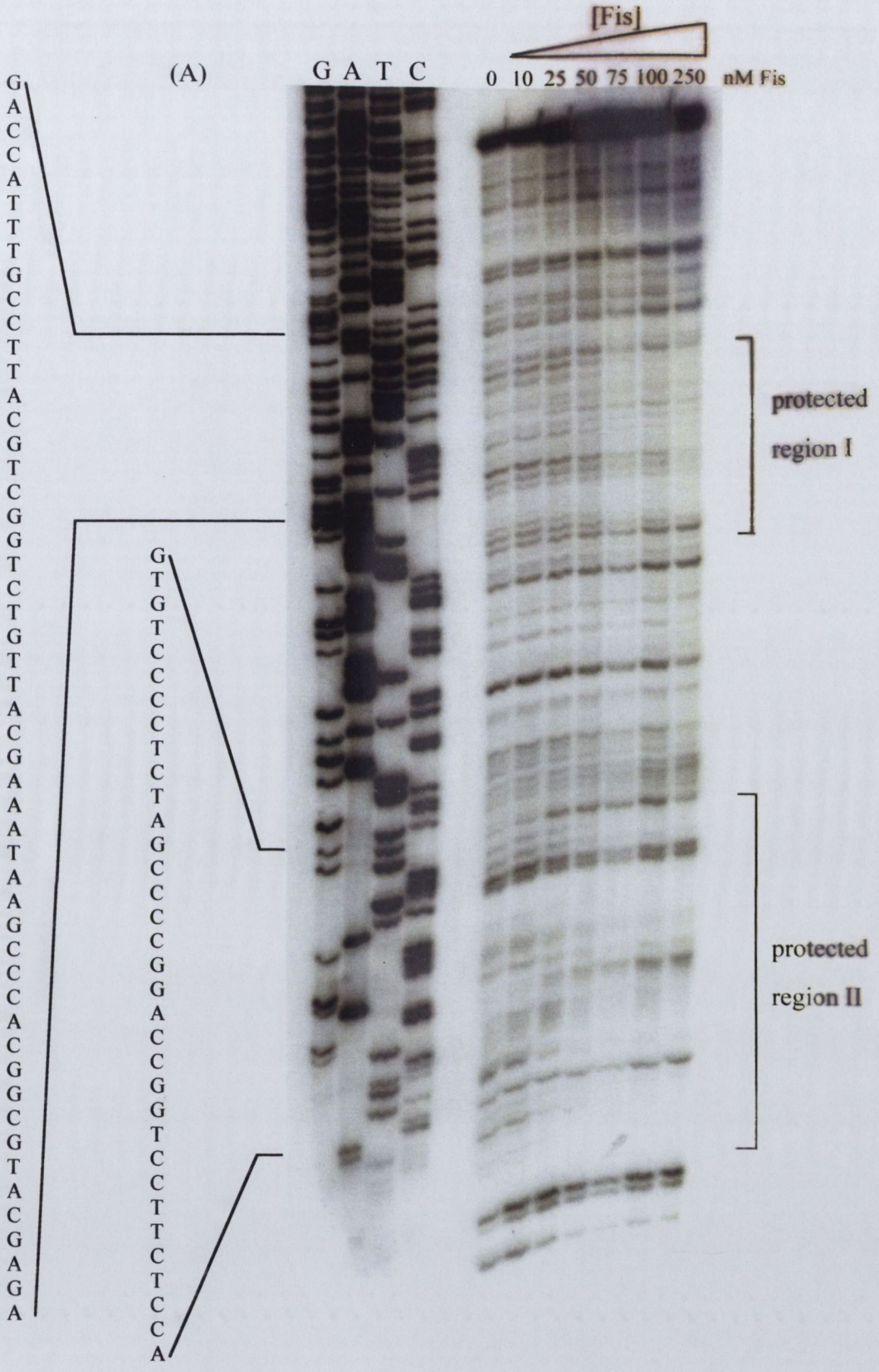
Fig. 6.7. **Fis binds the *Salmonella gyrB* promoter.** Electrophoretic mobility shift assay was carried out with a 453 bp *gyrB* promoter fragment and recombinant Fis used at the concentrations shown. Fis appears to bind 4 sites upstream of the *gyrB* promoter.

protect the phosphodiester backbone of the DNA from cleavage by DNase I. Thus, in a titration experiment in which the protein concentration is systematically varied, the relative amount of cleavage of a particular phosphodiester bond is a measure of the fraction of DNA molecules that are not bound at that location (Galas and Schmitz, 1978). DNase I mapping can therefore be used to locate protein binding sites on a particular DNA fragment.

The *gyrA* promoter proximal fragment from *Salmonella typhimurium* SL1344 was amplified with the primers DNasegyrAF and DNaselacZR (Table 2.3), using pGyrA-lacZ as template DNA. The *gyrB* promoter proximal fragment from *Salmonella typhimurium* SL1344 was amplified using the primers DNasegyrBPF and DNasegyrBPR (Table 2.3) and pGyrB-lacZ as template DNA. The PCR products were gel-purified and end-labelled using T4 polynucleotide kinase and $\gamma\,^{32}$P dATP (section 2.14.1). This labels both 5' ends of the PCR product. In order to have only one strand of the PCR product labelled, the PCR products were digested with the restriction enzymes *Hind*III for the *gyrA* promoter fragment and *Eco*RI for the *gyrB* promoter fragment. Cutting the *gyrA* PCR product with *Hind*III generates a 290 bp *gyrA* promoter fragment and a 75 bp *lacZ* fragment. Cutting the *gyrB* PCR product with *Eco*RI generates a 275 bp *gyrB* promoter fragment and an 81 bp *gyrB* coding sequence fragment. The desired DNA fragment was then purified after separation on a 7% acrylamide gel. This fragment was then incubated with increasing amounts of Fis protein for 20 min at room temperature in order to allow Fis bind the DNA. DNase I was then added for 1 min and the reaction stopped (section 2.16). The resulting fragments were then separated on a 7% acrylamide gel. The Fis binding sites on the *gyrA* promoter proximal fragment can be seen in Fig. 6.8 (A). Fis protects 2 regions of the *Salmonella gyrA* promoter that extend from –31 to –82 and –136 to –169 relative to the transcription start site. These sequences are underlined in Fig. 6.8 (B). A sequence that has good homology with the Fis consensus sequence (5'-GNNYRNNTNNYRNNC-3', Finkel and Johnson, 1992) has also been identified within the protected regions and is indicated in bold type. This is different to the situation in *E. coli* where Fis binds the sequence –16 to –68 immediately upstream of the *gyrA* extended –10 hexamer and blocks RNA polymerase binding. The Fis binding sites in the *Salmonella gyrB* promoter proximal sequence can be seen in Fig. 6.9 (A). Fis appears to have 2 binding sites in this region also. Both sequences are underlined

Fig. 6.8. **Mapping the proximal Fis binding sites upstream of *gyrA* by DNaseI footprinting.** (A) DNase I footprint of Fis binding the *gyrA* promoter proximal region. Radiolabelled *gyrA* promoter was incubated with increasing concentrations of purified Fis before digestion with DNase I. The regions protected by Fis from digestion are bracketed. A DNA sequencing ladder was generated with the primer DNasegyrAF.

(B) Sequence of the *gyrA* promoter proximal region. The transcription start site is indicated in blue and the extended –10 hexamer in red. The regions protected by Fis are underlined. Potential Fis binding sites with good homology to the Fis consensus binding site are indicated in bold type.



(B)

-206 AGTATGACGG GTATACACGG GAGGTATTGA TTTTCTAACC TCTTCCTGGC CAGGCCCCGA

-146 TCTCCCCTGT GTCGCCTCTG AAGCCGATCG CCAATAAACG CCGAGACCAC GCTTATAAAT

-86 GAAGAGAGCA TGCGGCACCC GAATAAAGCA TTGTCTGGCT GCATTCCGTT TACCAGTACG

-26 TGGGCGAATG TGTTATAATT TGCGACCTTT GAATCCGGGA TACAGTAGAG GGATAGCGGT

in Fig. 6.9 (B). The first binding site protects positions –53 to –119 relative to the transcription start site. This corresponds almost exactly with the Fis binding site upstream of the *gyrB* promoter in *E. coli,* which was mapped by Schneider *et al.* (1999) to positions –55 to –122. Schneider *et al.* (1999) also identified 2 sequences closely matching the Fis binding site consensus sequence in this region. These sites were centered at –62 and –109 and are indicated in bold-type in Fig 6.9 (B). The second Fis binding site protects positions –148 to –178 from cleavage by DNase I. This site was not found by Schneider *et al* (1999) however they did not extend their search this far upstream of the transcription start site. Binding of Fis to the *gyrB* promoter region also induces hypersensitivity to DNase I cleavage at position –145.

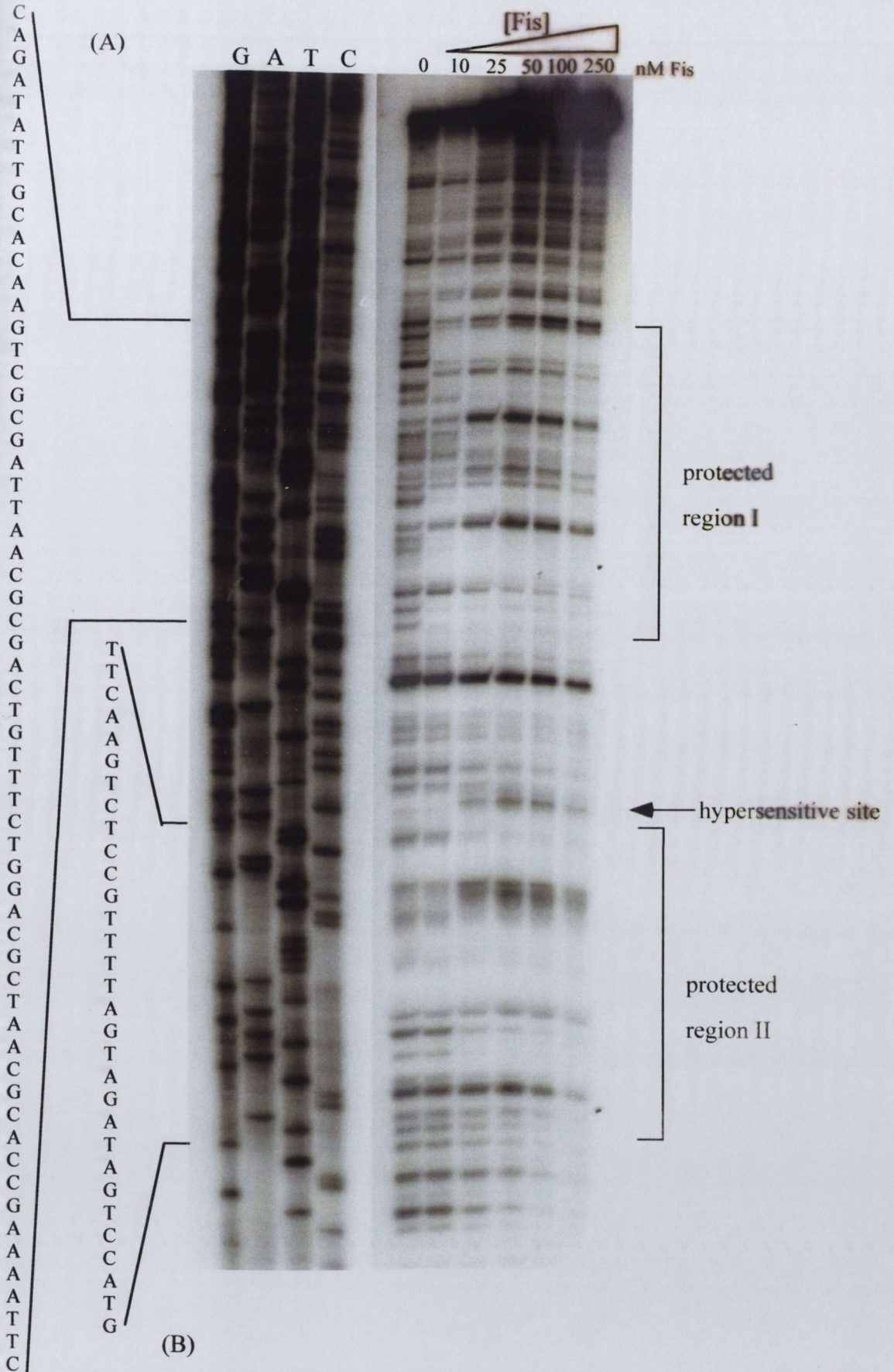
6.2.6: Western immunoblot of GyrA levels

In order to determine the effect of Fis on DNA gyrase protein levels quantitative Western immunoblotting was carried out (section 2.19). This was carried out on SL1344 and SL1344*fis*⁻ and GyrA levels measured. Western blotting was carried out on cells harvested 1 hour after nutrient upshift in order to ensure a high level of Fis in the wild-type SL1344. The results can be seen in Fig. 6.10. One hour after nutrient upshift there is more GyrA in SL1344*fis*⁻ than SL1344 (panel A). This is in agreement with the results of Schneider *et al.* (1999). The amount of Fis present in the cells is also shown in Fig. 6.10 (panel B).

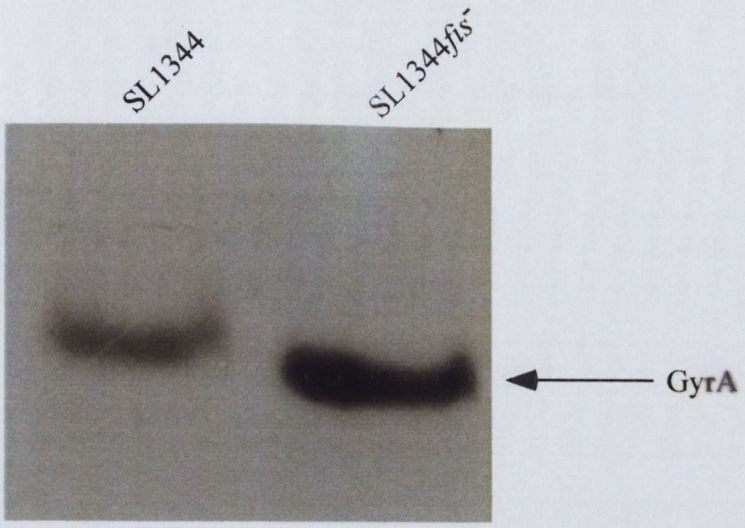
6.2.7: Effect of other global regulators on *gyrA* transcription

As mentioned previously a number of other global regulators such as IHF and CRP are reported to modulate DNA gyrase expression in *E. coli* (Gellert *et al.*, 1983; Gomez-Gomez *et al.*, 1996). It was therefore decided to look at the role of a number of such proteins in regulating the *gyrA* and *gyrB* genes of *S. typhimurium*. Isogenic SL1344 strains were constructed that were each deficient in one of the following proteins, LRP, IHF, H-NS and RpoS (Table 2.1). Each strain was then transformed with the plasmid pGyrA-lacZ and β-galactosidase levels measured and compared to wild-type. A graph of the results can be seen in Fig. 6.11. The presence of a lesion in the *rpoS* or *himD*

Fig. 6.9. **Mapping the proximal Fis binding site upstream of *gyrB* by DNase I footprinting.** (A) DNase I footprint of Fis binding the *gyrB* promoter fragment. Radiolabelled *gyrB* promoter was incubated with increasing concentrations of purified Fis before digestion with DNase I. The regions protected by Fis from digestion are bracketed. A DNA sequencing ladder was generated with the primer DNasegyrBPF. (B) Sequence of the *gyrB* promoter region. The transcription start site is indicated in blue, the –10 hexamer in red and the ATG translation start codon in green. The regions protected by Fis from digestion are underlined. Potential Fis binding sites with good homology to the Fis consensus binding site are indicated in bold type.


(A)
G A T C
[Fis]
0 10 25 50 100 250 nM Fis
protected region I
hypersensitive site
protected region II
(B)
-189 CGGCGCTGCC TGTACCTGAT AGATGATTTT GCCTCTGAAC TTGACGATGC GCGTCGCGGA
-129 CTGCTGGCCA GCCGCTTAAA AGCCACGCAA TCGCAGGTCT TTGTCAGCGC AATTAGCGCT
-69 GAACACGTTA TAGACATGTC GGATGAAAAT TCGAAGATGT TTACCGTGGA AAAGGGTAAA
-9 ATAACGGATT AACCCAAGAT TAAATGAGCG AGAAACGTTG ATGTCGAATT CTTATGACTC

(A)



(B)

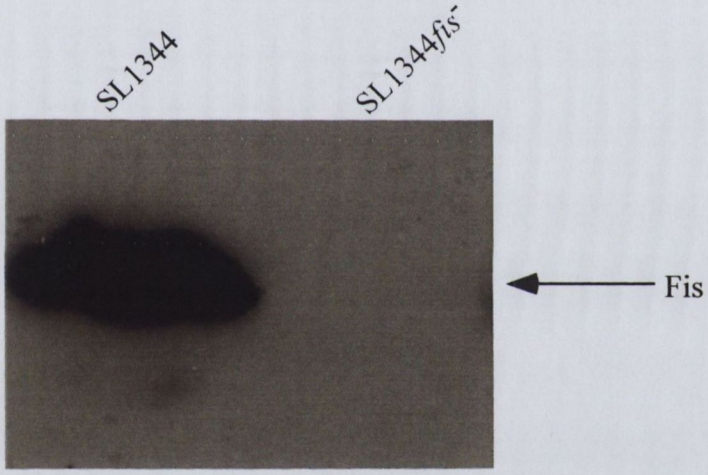


Fig. 6.10. **Expression of GyrA in early exponential phase in SL1344 and SL1344*fis*⁻.** The antibody used was an anti-GyrA monoclonal antibody obtained from John Innes Enterprises Ltd. The absence of Fis from the cell results in an increase in the amount of GyrA protein in the cell.

(B) Western immunoblot showing the amount of Fis present in the cell 1 hour after nutrient upshift.

genes encoding the stationary phase σ factor and IHF respectively appears to have no effect on *gyrA* transcription. Interestingly the presence of a lesion in *lrp* results in a 2-fold increase in *gyrA* expression in this plasmid reporter system. The presence of a lesion in *hns* results in a drastic decrease in the amount of *lacZ* expressed from pGyrA-lacZ. At first glance this would appear to show that H-NS is required for expression of *gyrA*. This would be unusual as H-NS usually functions to repress genes (Ueguchi and Mizuno, 1993). However when the pGyrA-lacZ plasmid copy number in SL1344 and SL1344*hns*⁻ was compared it was found that the *hns* lesion resulted in a large decrease in the plasmid copy number (data not shown), which results in a subsequent decrease in the β-galactosidase activity. Another approach is needed in order to determine if H-NS is involved in the regulation of DNA gyrase in *S. typhimurium*.

6.2.8: Binding of LRP and H-NS to the *gyrA* promoter

In order to determine if LRP directly or indirectly regulates *gyrA* and to determine if H-NS binds to and could regulate *gyrA*, gel mobility shift assays were carried out. A 493 bp *gyrA* promoter fragment from *S. typhimurium* was amplified with the oligos gyrAbam and gyrAkpn (Table 2.3) and radiolabelled as described previously. This was then mixed with purified LRP protein and electrophoresed through a 7% acrylamide gel. No shift of the probe was found (data not shown). This indicates that any effect of LRP on *gyrA* expression is either indirect or that binding of LRP to the *gyrA* promoter is modulated by leucine.

In order to determine if H-NS can bind the *gyrA* promoter region a competitive gel shift assay was used (section 2.15). The 493 bp *gyrA* promoter fragment and the 322 bp *gyrA* promoter proximal fragment were mixed in equimolar amounts with *Taq*I-*Ssp*I digested pBR322. The mixture, containing an excess of competitor DNA, was then incubated with increasing concentrations of purified H-NS. Free DNA and DNA protein complexes were then resolved by electrophoresis through a 3% agarose gel. The resulting bandshift can be seen in Fig. 6.12. H-NS binds to the *bla* promoter and shifts this curved piece of DNA. H-NS also appears to shift both the 493 bp *gyrA* promoter fragment and the 322 bp promoter distal fragment although it appears to bind the 493 bp promoter fragment with higher affinity. It does not shift any of the other 7

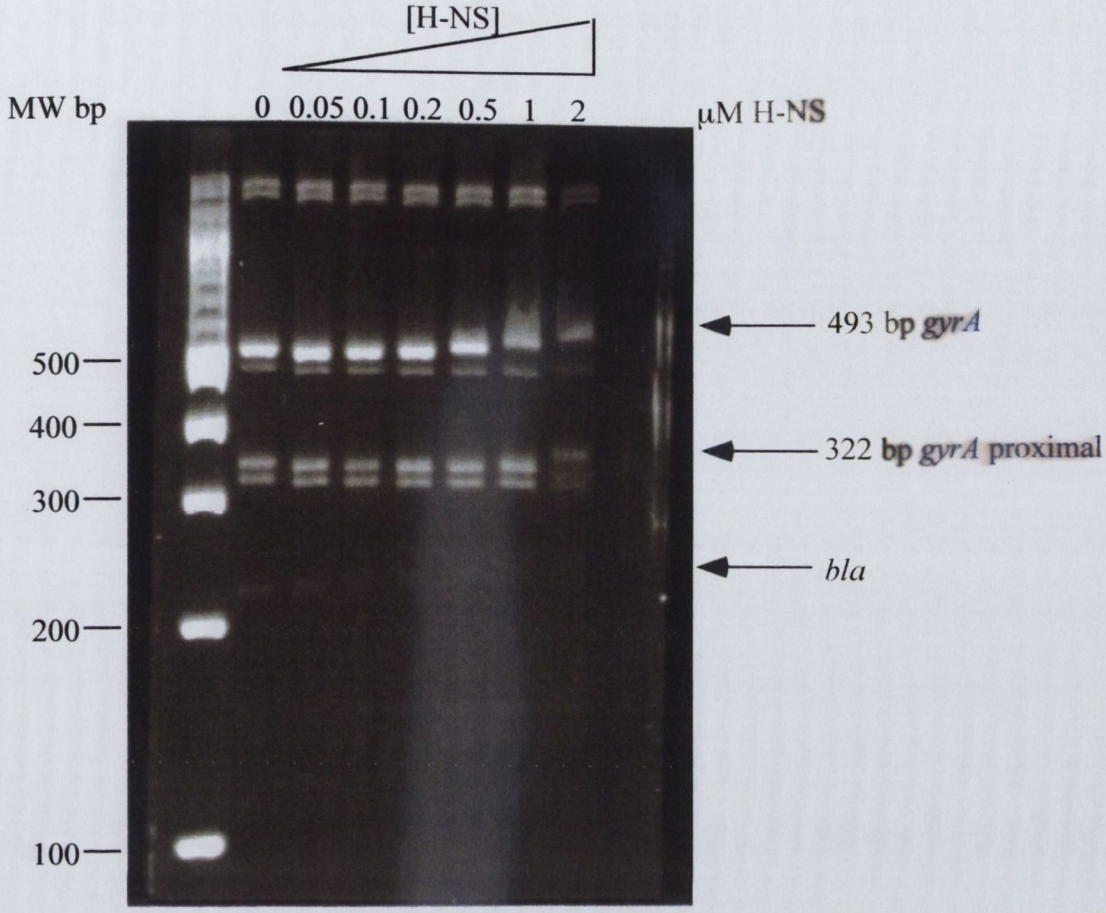


Fig. 6.12. **H-NS binds the *gyrA* promoter region.** Competitive gel mobility shift assay showing the specific binding of H-NS to the *gyrA* promoter region. The DNA fragments from the *Taq*I-*Ssp*I digested pBR322 were mixed with a 322 bp *gyrA* promoter proximal fragment and a 493 bp full promoter fragment. The mixtures were preincubated with purified H-NS at the indicated concentrations. The free DNA and DNA-protein complexes were separated by migration through a 3% molecular screening agarose gel. The migration of ***bla*** and the 2 *gyrA* promoter fragments are indicated.

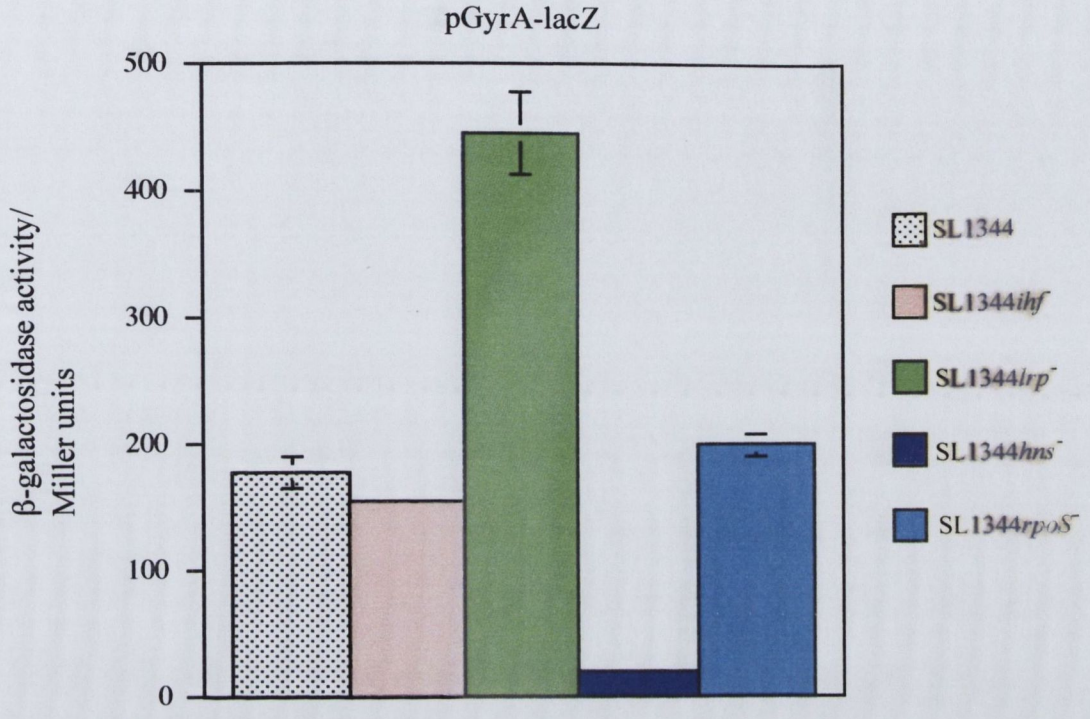


Fig. 6.11. **Expression of *gyrA* in SL1344 and isogenic *ihf*, *lrp*, *hns* and *rpoS* mutants.** The absence of LRP from the cell results in a 2-fold increase in *gyrA* transcription. The absence of H-NS results in a decrease in *gyrA* transcription however this was due to a decrease in plasmid copy number in SL1344*hns*⁻. The absence of IHF or RpoS from the cell appears to have no effect on *gyrA* transcription.

pBR322 fragments showing that the binding and shifting of the *gyrA* promoter by H-NS is specific.

6.2.9: Regulation of DNA gyrase *in vivo*

DNA gyrase is thought to play a role in the adaptation of *S. typhimurium* to life inside human cells such as epithelial cells and macrophages (Galan and Curtiss III, 1990; Marshall *et al.*, 2000). Upon uptake of *Salmonella* by macrophages the bacterial DNA becomes very relaxed, this results in the induction of *gyrB* (Marshall *et al.*, 2000). We wanted to confirm that *gyrA* is also induced when *Salmonella* are engulfed by macrophages and to examine if Fis plays a role in regulating DNA gyrase gene expression *in vivo*. The murine macrophage-like cell line J774A.1 was infected with SL1344+pGyrA-lacZ (section 2.20). Following infection gentamicin was added to the tissue culture media to kill any extracellular bacteria that had not been phagocytosed. At various times post-uptake of the bacteria, the macrophages were lysed and intracellular bacteria recovered. These bacteria were then assayed for *lacZ* expression from pGyrA-lacZ. The level of *lacZ* expression from intracellular bacteria was compared to that from bacteria grown in tissue culture media alone. The level of *gyrA* expression does indeed increase upon uptake of the bacteria by macrophages (Fig. 6.13 (A)). This confirms the result found previously for *gyrB*. SL1344 and SL1344*fis*$^-$ both containing pGyrA-lacZ were also used to infect the cell line J774A.1. β-galactosidase assays carried out on bacteria recovered from the intracellular environment showed that Fis does not appear to repress *gyrA in vivo*. In fact *gyrA* expression was marginally lower in SL1344*fis*$^-$ than SL1344 when inside macrophages (Fig. 6.13 (A)). Similar results are also seen with pGyrB-lacZ (Fig. 6.13 (B)). This would indicate that Fis does not play a role in repressing *gyr* gene expression *in vivo*.

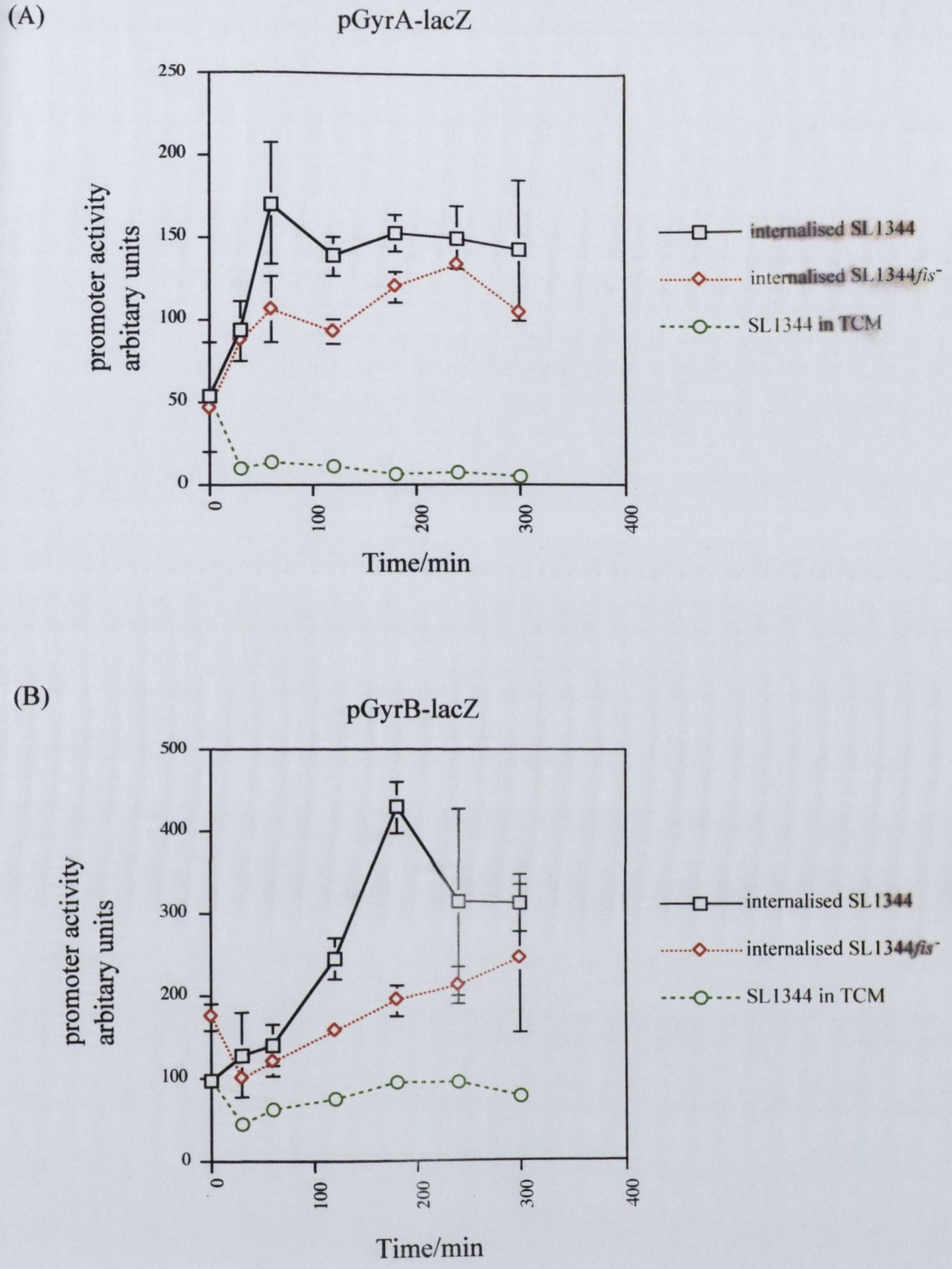


Fig. 6.13. **Transcription of *gyrA* and *gyrB in vivo*.** Promoter activity of pGyrA-lacZ (panel A) and pGyrB-lacZ (panel B) in macrophage internalised SL1344 and SL1344*fis*⁻ and SL1344 grown in tissue culture media alone. Both *gyrA* and *gyrB* are induced upon uptake of SL1344 by macrophage cells. Fis does not appear to play a role in repressing DNA gyrase *in vivo*.

6.3: Discussion

The *Salmonella gyrA* promoter sequence differs from that of *Escherichia coli* K-12. This could allow both genes respond differently to certain environmental signals such as heat shock or uptake by mammalian cells. A number of studies have examined the regulation of the genes for DNA gyrase in *E. coli* by Fis, IHF and CRP (Gellert *et al.*, 1983; Gomez-Gomez *et al.*, 1996; Schneider *et al.*, 1999), however similar studies have not been carried out in *Salmonella typhimurium*. We therefore examined the regulation of *gyrA* and *gyrB* in *Salmonella typhimurium* SL1344. Fis is reported to play an important role in the homeostatic regulation of DNA gyrase in *E. coli* (Schneider *et al.*, 1997; 1999; 2000) however the sequence to which Fis binds upstream of *gyrA* in *E. coli* is not present upstream of *gyrA* in *Salmonella*. The expression of *gyrA* and *gyrB* was however found to be increased approximately 1.5-fold in SL1344*fis*⁻ compared to SL1344. This is similar to what is seen in *E. coli*. Recombinant His-tagged Fis was purified and shown to bind the *Salmonella gyrA* and *gyrB* promoters at a number of sites. The binding site closest to the transcription start site was then mapped for both *gyrA* and *gyrB* by DNase I footprinting. For the *gyrA* promoter sequence the closest Fis binding site was mapped to an extended region from –31 to –82 relative to the *gyrA* transcription start site. A good Fis binding consensus sequence could also be found in this region. This is different to what is seen in *E. coli*. In *E. coli* the Fis binding site maps to the region –16 to –68 relative to the transcription start site closely approaching the –10 hexamer. It represses *gyrA* transcription by occluding the promoter region (Schneider *et al.*, 1999). The *S. typhimurium gyrA* promoter sequence retains the ability to bind Fis and this binding results in repression of *gyrA* transcription however the mechanism of repression has not been fully elucidated. As Fis occludes the *gyrA* –35 sequence it could interfere with RNA polymerase binding, alternatively repression of *gyrA* by Fis in *Salmonella* may be similar to the mechanism of repression of *gyrB* in *E. coli* where Fis blocks open complex isomerisation and promoter clearance. The retention of Fis repression of DNA gyrase in *Salmonella* indicates its evolutionary importance. Although the *gyrA* promoter sequence differs between the 2 species both have retained the ability to bind Fis and be repressed by it. The *Salmonella gyrB* promoter sequence does not differ significantly from that of *E. coli*. Both these sequences are also repressed by Fis. Fis binds the *Salmonella gyrB* promoter from –53 to –119. Two sequences that match the

Fis binding consensus sequence can be found in this region. This corresponds almost exactly to the Fis binding site in the *E. coli gyrB* promoter region. This is not surprising given the similarity between the 2 sequences. Therefore despite the difference between the regulatory region of *gyrA* in *Salmonella* and *E. coli* both can be repressed by Fis.

There also exists the potential for DNA gyrase of *S. typhimurium* to be regulated by LRP and H-NS. A *lrp* lesion resulted in a 2-fold increase in the level of *lacZ* expression from pGyrA-lacZ. No direct binding of LRP to the *gyrA* promoter region was detected however any binding might be modulated by leucine. Alternatively LRP may not regulate *gyrA* directly. The H-NS protein also binds specifically to the *gyrA* promoter and may be involved in regulating DNA gyrase. H-NS probably plays an indirect role in regulating DNA gyrase due to its effect on *fis* expression (Falconi *et al.*, 1996). However it may also play a role in directly regulating DNA gyrase. The plasmid reporter system used to quantify the effect of other proteins on *gyrA* expression could not be used in this case as a lesion in the *hns* gene was shown to result in a drastic decrease in plasmid copy number. In order to determine the effect of H-NS on *gyrA* expression, if any, an alternative method such as Northern or Western blotting will need to be applied.

To survive inside macrophages bacteria must overcome many environmental assaults, including oxidative stress, acid stress and cationic peptides. A previous report showed that infection of a macrophage cell line with *Salmonella* caused the bacterial DNA to become relaxed (Marshall *et al.*, 2000). This is similar to what is seen when bacterial cells are subjected to prolonged starvation (Balke and Gralla, 1987). The effect of this DNA relaxation is a burst in DNA gyrase gene transcription. The changing levels of DNA supercoiling following phagocytosis of the bacteria by macrophages would allow the transcription profile of the cell to be changed as the bacteria adapt to intracellular life. Other reports have shown that vigorous protein synthesis occurs for approximately 2 hours post-infection (Abshire and Neidhardt, 1993). Interestingly DNA topology has also been shown to be important for invasion of epithelial cells by *Salmonella*. In this case however increased negative supercoiling is an essential pre-requisite for expression of invasion function (Galan and Curtiss III, 1990). We have shown that transcription of *gyrA* is induced when *Salmonella* are phagocytosed by

macrophages and confirmed an earlier report that *gyrB* gene transcription is induced intracellularly (Marshall *et al.*, 2000). Induction of *gyrA* and *gyrB* is also seen in a *fis*$^-$ background. Fis is induced when bacteria are phagocytosed by macrophages and continues to be expressed at least 5 hours post-infection (Carroll *et al.*, 2002). The absence of Fis from the bacterial cell does not result in an increase in *gyr* gene transcription relative to the wild-type in intracellular bacteria. Therefore Fis does not appear to repress *gyrA* and *gyrB* in an intracellular environment.

Chapter 7

General Discussion

The ability to adapt to new environments is of utmost importance to the bacterial cell if it is to survive. Whether free-living or associated with a host, bacteria encounter many different environments with variations in temperature, nutrient availability, oxygen availability, pH and osmotic pressure (Colwell, 2000). When associated with a host they also have to deal with host defences such as antimicrobial peptides and host phagocytic cells (Sansonetti, 2002). The environment in which a bacterium finds itself may alter rapidly and the bacterium will be required to adapt accordingly. Bacteria encode many genes that allow such adaptation (Dougan *et al.*, 2001) and have developed many strategies for adapting to adverse conditions. These strategies range from regulation of gene expression to stochastic genetic changes.

There are many documented cases of stochastic genetic changes in bacterial cells that modulate gene expression. One of the best studied examples is the flagellar phase variation system of *Salmonella typhimurium*. *Salmonella* can express either one of two antigenically distinct flagellar subunits, H1 or H2. Switching between H1 and H2 occurs via a site-specific recombination reaction. Reversible inversion of a 993 bp segment of DNA, which contains the promoter, determines whether H1 or H2 is produced and this inversion is carried out by the Hin recombinase (Johnson and Simon, 1985). Recombination requires the binding of Hin dimers to *hixL* and *hixR* the core recombination sites, the binding of Fis to the recombination enhancer sequences and bending of the DNA by HU (Johnson *et al.*, 1986). A synaptic complex called the invertasome is formed. Hin is then able to cleave the middle of the *hix* sites to which it is bound. Exchange of the DNA strands and religation leads to inversion of the DNA. This is mechanistically similar to V(D)J recombination used to generate antibody diversity (Nanassey and Hughes, 1998). Site specific recombination catalysed by Hin also requires DNA supercoiling. Hin can bind to the *hix* sites and protein-protein pairing of Hin bound to these sites can be accomplished at low superhelical density (σ = -0.02) however invertasome formation and DNA inversion can only be accomplished at higher superhelical densities (σ = -0.06) (Lim *et al.*, 1997).

A heritable genetic change that allows strain CJD671 to adapt to a lactose-containing environment has been described in this work. This event is represented schematically in Fig. 3.1. This deletion event is reminiscent of adaptive or stationary-phase mutation (described in section 1.5) as the cells that mutate are presumed to have ceased dividing

and the cells are under selective pressure to mutate. Although this deletion event results in the upregulation of the *spv* genes it is unlikely to play a role in the virulence of the organism as the deletion can only be detected in the presence of lactose as this gives the mutants a selective advantage. The deletion is therefore unlikely to occur in a wild-type strain lacking the *spvB::lacZ* fusion. This deletion event was found to be independent of the bacterial RecA protein, showing that the host homologous recombination functions are not required. The bacterial SOS response is also dispensable for the deletion event to occur as inactivation of RecA results in a stable LexA repressor and the SOS regulon is not stimulated (Walker, 1996). Therefore a specific mechanism appears to exist in the cell that can bring about the deletion. Whether this mechanism is programmed or serendipitous we do not know.

This deletion occurs by illegitimate recombination as it is neither site-specific nor between sequences with any homology. Illegitimate recombination is a major cause of chromosomal aberrations both in prokaryotes and eukaryotes and can lead to duplication, deletion, insertion, or translocation of DNA (Onda *et al.*, 2001). It usually occurs at low frequency but is greatly enhanced by treatment with DNA damaging agents (Ikeda *et al.*, 1995). A system frequently used for studying illegitimate recombination is the formation of λ*bio* transducing phage. Normally λ is precisely excised at the *attR* and *attL* sites however occasionally recombination occurs between substantially non-homologous DNA sequences. This results in the production of specialised transducing phages containing the *E. coli bio* or *gal* genes and lacking the phage *red* and *gam* genes (*bio* and *gal* are adjacent to the *att* site on the *E. coli* chromosome). The proportion of phages that contain such sequences is a measure of the frequency at which illegitimate recombination is taking place (Ikeda *et al.*, 1995). Illegitimate recombination in this system was shown to occur by two mechanisms. The first is short homology independent illegitimate recombination (SHIIR). This type of illegitimate recombination requires no homology between the sequences being recombined and is mediated by DNA gyrase (Ikeda *et al.*, 1982). The mechanism is thought to be similar to that illustrated in Fig. 3.8 (A). The dependence of this mechanism on DNA gyrase could be shown by the fact that treatment with oxolinic acid, which increased the amount of time the gyrase and DNA were in contact, increased the frequency of illegitimate recombination (Shimizu *et al.*, 1995). Mutations in *gyrA* could also be found that conferred a hyper-recombination

phenotype (Ashizawa *et al.*, 1999). The second mechanism by which λ*bio* transducing phage is formed is short homology dependent illegitimate recombination (SHDIR). This mechanism requires short stretches of homology between the sequences being recombined, usually 4-10 bp of homology although the homology may be as short as 1-2 bp (Shimizu *et al.*, 1997). The precise molecular mechanism of SHDIR has not been elucidated but two models currently exist. The first model is a slipped mispairing model where a replication fork slips down a region of the DNA strand during DNA replication resulting in a deletion or other mutation. The second model is a breaking and rejoining model. This model involves a double stranded break being introduced in the DNA, the ends of the DNA being processed and then religated. This model has been supported by the finding that inactivating DNA ligase reduces the frequency of illegitimate recombination while overexpressing ligase increases the frequency of illegitimate recombination and decreases the homology required between sequences for SHDIR (Onda *et al.*, 2001). The formation of λ*bio* transducing phage by DNA gyrase-mediated illegitimate recombination is suppressed by the HU protein (Shanado, *et al.*, 1998). The formation of λ*bio* transducing phage by short homology dependent illegitimate recombination is suppressed by the H-NS protein (Shanado, *et al.*, 2001), by the RecQ helicase (Hanada *et al.*, 1997) and by the UvrAB system of nucleotide repair (Hanada *et al.*, 2000). The RecJ exonuclease promotes illegitimate recombination in this system (Ukita and Ikeda, 1996). The Fis protein is also known to be required for short homology dependent illegitimate recombination (Shanado *et al.*, 1997) as is DNA replication (Hanada *et al.*, 2001).

The deletion event in CJD671 does not require any homology between the *rlgA* and *spvR* deletion end points. This is therefore reminiscent of SHIIR. However in this case DNA gyrase does not appear to be bringing about the deletion event directly. Evidence for this view is obtained from three sources. Firstly high frequency gyrase cleavage sites could not be mapped to the *spvR* deletion end-point even in the presence of 0.36 M NaCl, although it must be noted that this does not exclude the possibility of DNA gyrase cleaving this sites with low frequency. Secondly in strain CJD671 the deletion cannot occur in LB broth without salt and lactose. Lactose provides a selective advantage to the mutant cells. Salt is required to overcome a genetic constraint to the formation of deletions. The ability to form red mutants is dependent on the osmolarity of the media and is optimal at an NaCl concentration of 0.36 M and

decreases at higher or lower NaCl concentrations (Massey, 1998). If the effect of salt was to stimulate DNA gyrase activity then I would expect there to be no upper salt concentration at which red mutants could form. As there is an optimal salt concentration for the formation of red mutants, this supports the model that the enhanced DNA gyrase activity caused by increased osmolarity, results in an increase in the negative superhelicity of the DNA and this allows the deletion end points to contact each other. Another protein then carries out the deletion reaction. As the osmolarity varies about the optimal NaCl concentration of 0.36 M it becomes more difficult for the deletion end-points to contact each other. At critical NaCl concentrations both above and below the optimal concentration it is no longer possible for the deletion end points to come in contact and the deletion cannot form. Novobiocin counteracts the effect of NaCl and so inhibits deletion formation by preventing the deletion end-points contacting each other. The third piece of support for the indirect model of DNA gyrase involvement in the deletion comes from the experiments carried out with CJD661. This strain can form deletion mutants in L-lac broth alone i.e. in the absence of osmotic stress. The deletion end-points are similar in CJD661 and CJD671. If gyrase was acting directly on the end-points in both strains then I would expect the conditions for deletion formation to be similar in both strains. The evidence therefore supports a model in which the deletion end-points are able to contact each other in CJD661 without the need for increased osmolarity and so the deletion can occur. Novobiocin affects the ability of the deletion end-points to contact each other in CJD661 by altering the negative superhelicity of the DNA and so inhibits deletion formation. Such a model could be tested by the insertion of pieces of DNA of varying length between the deletion end-points and examining their effect on the ability of the deletion end-points to contact each other and form a mutant. It has been previously reported that osmotic stress can induce genetic rearrangements in *E. coli* (Jolivet-Gougeon *et al.*, 2000). They proposed that a combination of DNA supercoiling and DNA binding proteins played a role in these genome rearrangements.

The deletion event in CJD671 appears to occur via a non-gyrase mediated DNA breakage and rejoining mechanism similar to the model for the formation of λ*bio* transducing phage by SHDIR. There are however a number of key differences between the two systems. As mentioned previously the deletion does not appear to require any homology between the sequences being recombined. Also the deletion

event in CJD671 occurs in stationary phase when the cells are presumed to have ceased dividing. SHDIR in the λ*bio* system requires DNA replication (Hanada *et al.*, 2001) and it is thought that the stalling of the replication fork is the source of double-stranded breaks required for illegitimate recombination in the λ*bio* system. H-NS is known to suppress the formation of λ*bio* transducing phage by SHDIR however the effect of H-NS on deletion formation in CJD671 could not be tested due to the role of H-NS is *spv* gene regulation. Similarities also exist between the two systems. A role for the Fis protein has been demonstrated in both systems (Shanado *et al.*, 1997; section 3.3). Fis could modulate the deletion event by regulating DNA gyrase, by constraining supercoils in the vicinity of the deletion end-points thereby allowing them to contact each other or by contributing to the formation a nucleoprotein complex, similar to that described for the Hin system, that allows the deletion to occur. However it must be noted that the deletion occurs at a time when the Fis levels in the cell are known to be very low (Ball *et al.*, 1992; section 6.2.2). This mitigates against Fis forming a nucleoprotein complex required for deletion formation and would support the model that the role of Fis is in regulating DNA gyrase activity. Low levels of Fis would relieve repression of DNA gyrase and this may facilitate deletion formation. Both systems are also RecA independent (Ikeda *et al.*, 1995; section 3.1). The deletion does not seem to occur by a mechanism such as the slipping of the replication fork during DNA replication, as DNA replication is presumably not taking place when the deletion occurs. Also it is unlikely a 5 kb deletion could be generated by such a mechanism.

Transposon mutagenesis was carried out to try and identify the protein factor that was responsible for the deletion event. No mutant was found that resulted in instantaneous deletion of the 5 kb of DNA between *rlgA* and *spvR*. This may have been for a number of reasons. It is possible that not enough colonies were screened as the transposon appeared to insert preferentially into the large virulence plasmid. Alternatively it may not be possible to inactivate the enzyme necessary for deletion formation as it may be essential for cell growth and survival. Another possible explanation is that deletion formation may not be able to be accelerated even if the enzyme factor responsible is overexpressed or a factor retarding the deletion rate is removed.

A number of regulators of the *spv* system were found in the transposon mutant screen however. Inactivation of *rssB* resulted in increased *spvB-lacZ* transcription. This is probably due to the effect of *rssB* on RpoS protein levels. Inactivation of *rssB* results in higher RpoS levels due to inefficient proteolytic degradation of RpoS by ClpXP (Zhou *et al.*, 2001). As RpoS is the σ factor required for *spv* gene transcription, this leads to higher levels of the *spvB-lacZ* fusion. Overexpressing CspC also increases *spvB-lacZ* levels due to the fact that increased levels of CspC increases the *rpoS* mRNA half-life and hence increases RpoS protein levels (Phadtare and Inouye, 2001). Transposon insertions in the *sucA*, *sdhA* and *sdhD* genes were also isolated. These genes encode enzymes that function in the Krebs cycle. *sdhA* and *sdhD* encode subunits of succinate dehydrogenase, the enzyme that converts succinate to fumarate with the transfer of the reducing equivalents to ubiquinone. *sucA* encodes a subunit of 2-ketoglutarate dehydrogenase, the enzyme that converts 2-ketoglutarate to succinyl-CoA (Sanderson *et al.*, 1996). A previous reports has shown that metabolism of succinate by stationary phase *E. coli* or *Salmonella* protects the cells from killing by antimicrobial peptides such as bactericidal/permeability increasing protein BPI (Barker *et al.*, 2000). These antimicrobial peptides are proposed to act by affecting the respiratory chain. Growth in succinate with its subsequent metabolism circumvents this inhibition. A *sdhO* mutant is not protected from killing by BPI. Therefore there exists a link between succinate metabolism, virulence and stationary phase. The precise nature of the link remains to be elucidated however this work has shown that it may involve the *spv* genes in *S. typhimurium*. It is known that it does not require RpoS (Barker *et al.*, 2000).

In order to adapt better to a new lactose-containing environment the bacterium must delete 5 kb of DNA, which it cannot recover. This terminal differentiation initially does not appear an advantageous event. However such terminal differentiation is known to occur in other bacterial species when subjected to stress. In the filamentous cyanobacteria *Anabaena*, terminally differentiated non-reproducing specialised cells called heterocysts are produced at regular intervals along the filament when cells become starved of nitrogen. These heterocysts fix nitrogen while the vegetative cells continue to carry out oxygenic photosynthesis. The heterocyst is therefore a permissive anaerobic compartment for redox enzymes such as nitrogenase that are poisoned by oxygen (Adams, 2000). Two different DNA rearrangements occur near

the nitrogen fixation (*nif)* genes during heterocyst differentiation: the deletion of a 55 kb element from within the *fdx*N gene and the deletion of an 11 kb element from within the *nifD* gene. Both rearrangements are the result of site-specific recombination between short directly repeated sequences at the ends of the elements (Golden and Wiest, 1988). Sporulation of *Bacillus subtilis* occurs when the bacteria are subjected to stress. This involves the formation of a sporangium consisting of two cell types the terminally differentiated mother cell and the forespore. One of the genes involved in the regulation of sporulation in *B. subtilis* is the *sigK* gene. This gene encodes the σ^K sigma factor. The appearance of σ^K is a complex point of control during sporulation and is regulated at many levels. The primary level of regulation is the creation of the gene. In the vegetative chromosome the *sigK* open reading frame is interrupted by 48 kb of DNA. During sporulation a site-specific recombinase is activated that catalyses the precise excision of the 48 kb resulting in the truncated *sigK* coding elements being fused in frame. This terminal differentiation event is specific to the mother cell (Stragier, *et al.*, 1989). Both of these examples of terminal differentiation result in phenotypes that are beneficial to the population as a whole. The deletion event in CJD671 is also beneficial to the entire population as once the deletion mutant becomes common in the population it can cross-feed the wild-type strain. Therefore the formation of deletion mutants is beneficial to the entire population as well as the mutant cell. The ability of the deleted region to be mobilised is also not so surprising as a number of transposase genes are encoded in this region and it has been hypothesised that this region has been recently horizontally acquired after speciation of the *Salmonellae* (Boyd and Hartl, 1998). Also this region of DNA is not found on the genome of *S. typhi* showing that some *Salmonella* strains evolved without it.

A similar type of deletion event to that described for CJD671 has also been previously shown to assist bacteria adapt to new antibiotic containing environments. *S. enteritidis* that were resistant to the aminoglycoside antibiotics were isolated from a patient. This resistance was due to a 60 kb deletion event that resulted in a transcriptional fusion between an upstream gene *nmpC* and the cryptic *acc(6')-Iy* gene. This results in expression of *acc(6')-Iy* and hence aminoglycoside resistance (Magnet *et al.*, 1999). Although the mechanism of deletion was not elucidated it may be similar to the mechanism employed by CJD671 to delete the 5 kb of DNA between *rlgA* and *spvR*.

This demonstrates the potential usefulness of such a deletion mechanism to the bacteria.

Darwinian evolution is based on the theory that variation randomly accumulates in the population due to a basal rate of DNA polymerase replication errors. The best adapted individuals thrive and survive. An alternative to this gradual evolutionary process has also been postulated. It involves a highly variable rate of mutation which gives rise to punctuated evolution between periods of equilibrium, characterised by low mutation rates (Gould and Eldredge, 1993). Such punctuated evolution could be caused by moveable segments of DNA such as transposons. Adaptive mutation events such as that described in this work represent examples of punctuated evolution. It has been proposed that transfer of DNA from dead (non-colony forming) cells to living cells may be important in allowing bacteria adapt to stress (Higgins, 1992). Such DNA transfer could occur by transformation, transduction or conjugation. The naturally transformable bacteria *Neisseria gonorrhoeae* is known to take up DNA from neighbouring lysed cells. This allows *N. gonorrhoeae* change its cell surface antigens and escape the host immune system (Seifert, 1996). The precise mechanism of adaptation in the case of CJD671 has not been fully elucidated however adaptation has only been detected on the large virulence plasmid. Strains with chromosomal MudJ insertions do not appear to have the ability to upregulate *lacZ* expression. The deletion event described in this work does not appear to involve either hypermutation or amplification (as described in sections 1.5.3 and 1.5.4 respectively for *lacZ* frameshift adaptive mutation). While these two mechanisms have not been directly tested for (e.g. no other genetic locus was examined for mutation rate in order to see if a subpopulation of cells was undergoing hypermutation) they both require the function of the RecA protein. The adaptive mutation described in this work does not require RecA and so is unlikely to be mechanistically similar to either mechanism. It is more likely that this event occurs via a mechanism similar to that hypothesised by Barbara Wright for directed mutation and evolution (section 1.5.5). This mechanism is represented schematically in Fig. 3.8. It involves the contact of the deletion end-points. Negative supercoiling is required for this contact. Double stranded breaks are introduced in the DNA. Whether this occurs spontaneously or is the concerted action of some protein that brings about the reaction is not known. The *rlgA* and *spvR* end-

points are then ligated together by an unknown protein and the intervening sequence deleted.

The study of adaptive mutation events is important for a number of aspects. The environment encountered by the bacterium is vastly different depending on whether the bacterium is free-living or associated with a host. The ability of bacteria to adapt to new environments is of critical importance if they are to exploit new environmental niches and infect new hosts. Adaptive mutation also provides a model for how evolution can proceed on a larger scale. Last but not least stationary phase mutations are being studied in an attempt to understand how non-growing cells mutate. This has implications for somatic cell mutations that allow the cells to become cancerous (Cairns, 1998).

Future work in this area should focus on elucidating the molecular mechanism of deletion formation. As mentioned previously the insertion of pieces of DNA of varying length between *rlgA* and *spvR* could be used to test whether gyrase is involved directly or indirectly in the deletion event. Alternatively sequences between *rlgA* and *spvR* could be removed. This should allow the *rlgA* and *spvR* end-points come in contact and form the deletion in the absence of increased osmolarity. The role of the putative integrases, resolvases and transposases in the *spv* region which have been revealed by the genome sequencing project should also be examined. Overexpression of these proteins may allow the deletion occur at an accelerated rate. Alternatively inactivation of the proteins could result in a strain unable to form deletions. However care must be taken in any attempt to knockout these genes as altering the DNA structure in this region may affect the ability of the deletion end-points to contact each other. The ability of a smaller multicopy plasmid containing the *rlgA* to *spvB-lacZ* region to form deletion mutants in a pSLT free background should also be examined as this will indicate if the proteins required for deletion formation are plasmid or chromosomally encoded.

As well as stochastic genetic changes in the cell such as that described above, bacteria can adapt to new environments by modulating gene expression. This can be done for each gene individually. Alternatively a number of global regulators also exist that can co-ordinately regulate the expression of a large number of unlinked genes around the

genome in response to stress (Hengge-Aronis, 1999). Many of these global regulators are proteins such as LRP, IHF, H-NS and Fis. DNA supercoiling also has the ability to function as a global regulator as it modulates the transcription of many genes (Gmuender *et al.*, 2001; Sheridan *et al.*, 2001; Dorman, 2002), in addition to its role in compaction of the DNA in the nucleoid.

Many of the studies on the effect of DNA supercoiling on gene expression and the effect of environmental parameters on DNA gyrase gene expression have been carried out in *E. coli* and *S. typhimurium*. In general both species responded similarly to each environmental stimulus with the exception of temperature. In *E. coli* negative superhelicity increases as temperature increases while in *S. typhimurium* negative superhelicity decreases as temperature increases (effect of environment on supercoiling reviewed in section 1.3.7). Previous results from our laboratory, generated by Dr. David Marshall indicated that the *S. typhimurium gyrA* promoter sequence differed from that of *E. coli*. The *Salmonella gyrB* promoter region does not differ significantly from its *E. coli* counterpart (Marshall *et al.*, 2000). In this work the *Salmonella gyrA* promoter was cloned and sequenced. It was found to differ significantly in sequence from that of *E. coli*. The *Salmonella* and *E. coli gyrA* promoters retain homology until upstream of the –10 hexamer (Fig. 5.6). The region of homology, between the translation start codon and the –10 box is known to be required for the relaxation-stimulated transcription response (RST response) (Menzel and Gellert, 1987b). Therefore both species are under selective pressure to retain this sequence due to the importance of the homeostatic regulation of DNA gyrase activity. The transcription start site for the *Salmonella gyrA* promoter was mapped by primer extension and found to be the same as that for *E. coli*. Both the *E. coli* and *Salmonella gyrA* genes belong to a family of genes with so-called "extended –10 promoters." This family of genes contains a TG motif immediately 5' to the –10 Pribnow box and increase promoter strength in the absence of a good –35 sequence (Burr *et al.*, 2000). Both promoters lack a –35 sequence with good homology to the consensus sequence.

The reason for this divergence between the *Salmonella* and *E. coli gyrA* promoter sequences is not known. This sequence divergence could potentially allow differential regulation of *gyrA* between the two species by encoding protein binding sites upstream of one of the promoters that are not present upstream of the other. However unless

gyrB is co-ordinately expressed with *gyrA* a functional enzyme will not be formed. There are a number of possible reasons why the *Salmonella gyrA* promoter sequence may differ from that of *E. coli* but not the *gyrB* sequence. Firstly it has previously been reported that *gyrA* is more sensitive to DNA supercoiling than *gyrB* (Neuman and Quinones, 1997). Therefore this gene may require an extra level of regulation over and above that of *gyrB*. Secondly the expression from the pGyrB-lacZ plasmid used in this study is higher that that from the pGyrA-lacZ plasmid implying that *gyrB* is expressed more highly in *Salmonella* than *gyrA* (Fig. 6..1 and Fig. 6.2). Similar results have also been obtained in *E. coli* by Schneider *et al.* (1999). They found that *lacZ* expression from pGYRB1 (*E. coli gyrB-lacZ* fusion plasmid) was more than double that from pGYRA1 (*E. coli gyrA-lacZ* fusion plasmid). If the cell contains more GyrB than GyrA then GyrA is limiting for the formation of a functional DNA gyrase holoenzyme. This would make the regulation of *gyrA* of critical importance in determining the amount of functional DNA gyrase in the cell.

Fis is an important regulator of the *E. coli gyrA* and *gyrB* genes. It functions to fine-tune the homeostatic control of DNA gyrase activity (described in section 1.4.3 Schneider *et al.*, 1997, 1999, 2000). The sequence to which Fis binds upstream of the *E. coli gyrA* promoter is not present upstream of *Salmonella gyrA*. Fis is known to bind DNA in a sequence specific manner although Fis has a very weak consensus binding site (Hubner and Arber, 1989). The results presented in Chapter 6 show that Fis represses *gyrA* and *gyrB* in *S. typhimurium,* much as it does in *E. coli*. Fis can bind to the *Salmonella gyrA* and *gyrB* promoter regions at multiple sites. The site closest to the transcription start site was mapped by DNase I footprinting for both *gyrA* and *gyrB*. The site to which Fis bound upstream of the *Salmonella gyrB* promoter was the same site to which Fis bound upstream of *the E. coli gyrB* promoter. In contrast Fis binds to a site immediately upstream of the *E. coli gyrA* transcription start site, closely approaching the –10 hexamer. In *S. typhimurium* it was found that the closest Fis binding site to the *gyrA* transcription start site was centered approximately 56 bp upstream. Therefore the mechanism of repression of *gyrA* by Fis may differ in *E. coli* and *Salmonella*. In *E. coli* Fis represses *gyrA* by preventing binding of the promoter by RNA polymerase (Schneider *et al.*, 1999). Whether this is the case for *Salmonella* remains to be determined. Fis may repress the *Salmonella gyrA* promoter by preventing isomerisation from a closed complex to an open complex. Alternatively Fis

may constrain negative supercoils in the vicinity of the *gyrA* promoter. This would result in repression of *gyrA* as it is known that this promoter is supercoiling sensitive and is repressed by negative supercoiling. Another possible mechanism of repression is that Fis binding upstream of the *gyrA* promoter could prevent another as yet unidentified protein from binding and activating *gyrA* transcription. Further work is required to determine which, if any, of these models are correct.

These results highlight the importance of Fis in the homeostatic regulation of DNA gyrase activity. Although the *E. coli* and *Salmonella gyrA* promoter sequences have diverged through the insertion of 4 kb of DNA upstream of the *Salmonella gyrA* gene, they both retain the ability to be repressed by the Fis protein. Both species must therefore be under selection pressure to do so. The fact that the sequences recognised by Fis show weak conservation may have allowed the *Salmonella* and *E. coli gyrA* promoter sequences diverge while retaining Fis binding.

Upon uptake of bacteria by macrophages their DNA becomes very relaxed. This results in the induction of the *gyrB* gene (Marshall *et al.*, 2000). This work has shown that *gyrA* is also induced thus confirming previous results. Upon uptake of the bacteria by macrophages, many genes are induced including those on *Salmonella* pathogenicity island-2 (Ochman *et al.*, 1996). These gene products allow the bacteria to survive and grow inside macrophage. Modulating the level of DNA supercoiling from highly relaxed upon uptake of the bacteria by macrophage to progressively more negative supercoiled as DNA gyrase is induced may allow subsets of supercoiling sensitive genes to be temporally regulated. This may facilitate bacterial adaptation to the intracellular environment. The role of Fis in the regulation of DNA gyrase activity intracellularly was examined. The mouse macrophage-like cell line J774A.1 was infected with wild-type *Salmonella* SL1344 containing pGyrA-lacZ or pGyrB-lacZ, and SL1344*fis*$^-$ containing pGyrA-lacZ or pGyrB-lacZ. Bacteria recovered from the intracellular milieu were assayed for β-galactosidase activity. Both *gyrA* and *gyrB* have lower levels of expression in a *fis* mutant inside macrophage although expression is only marginally lower (Fig. 6.13). This was a little surprising as *in vitro* Fis has been shown to repress *gyrA* and *gyrB*. It is known that Fis is expressed in macrophage engulfed bacteria at a constant level for the duration of the experiment (Carroll *et al.*, 2002). Why does Fis function as a repressor *in vitro* but not *in vivo*? Upon nutrient

upshift Fis is induced *in vitro* as is DNA gyrase (Balke and Gralla, 1987; Schneider *et al.*, 1999). Fis functions to prevent the appearance of hypernegatively supercoiled DNA by binding directly to the DNA and stabilising topoisomers of intermediate superhelical density (Schneider *et al.*, 1997) and by repressing *gyrA* and *gyrB* (Schneider *et al.*, 1999). *In vivo* the induction of DNA gyrase may be crucial to the survival of the cell inside the macrophage. Therefore repression of DNA gyrase by Fis may not be beneficial to the cell. The bacteria appear to have adapted to utilise Fis to repress gyrase genes *in vitro* but not *in vivo*. Such a phenomenon has been observed previously. Fis has been shown to regulate SPI-2 virulence genes *in vivo* but not *in vitro* (Carroll *et al.*, 2002). How repression of *gyrA* and *gyrB in vivo* is avoided is unclear but it may be by the titration of Fis by higher affinity binding sites *in vivo*. Alternatively the binding of an activator protein to the *gyrA* and *gyrB* promoters *in vivo* could prevent binding by Fis.

Future work in this area should examine the roles of H-NS and LRP in regulating DNA gyrase gene expression, if any. Single copy *gyrA-lacZ* and *gyrB-lacZ* fusions should be constructed to eliminate the potential pitfalls of working with multicopy plasmid, such as variation in plasmid copy number. The role of cAMP-CRP in regulating *gyrA* and *gyrB* of *Salmonella* should also be examined, as this protein is known to regulate the DNA gyrase genes of *E. coli*. Whether CRP acts directly or indirectly should also be determined. Finally the role of DNA gyrase in facilitating *Salmonella* adaption to an intracellular environment should be studied. The abililty of strains with different levels of DNA negative superhelicity, such as *topA* mutants to invade macrophages should be examined. Regulation of bacterial gene expression in wild-type and *topA* mutant bacteria isolated from macrophage could be examined on a global level by microarray analysis.

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