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Expression of *hns* and *stpA* in *Salmonella enterica* servoar Typhimurium

By

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

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Declarations

I, Stephen Fitzgerald, am the sole author of this thesis. The work presented within 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.

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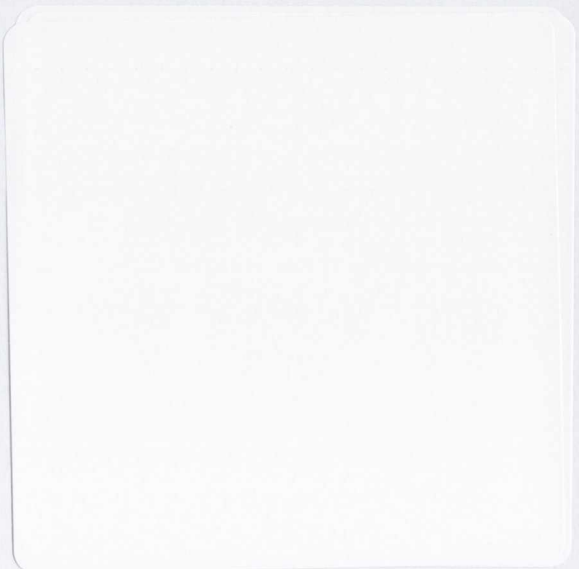

H-NS-like proteins are widespread amongst *Enterobacteriaceae* and other Gram-negative bacteria. They are small (15 kDa) nucleoid associated proteins involved in many cellular processes such as replication, transposition, chromosome compaction and global gene regulation. *Salmonella enterica* serovar Typhimurium possesses two such proteins, H-NS and StpA, encoded chromosomally by the *hns* and *stpA* genes, respectively. Experimental evidence from both *S.* Typhimurium and *E. coli* has shown H-NS and StpA to be involved in many of the same regulatory interactions and indeed to bind at many of the same loci across the genome. These functional similarities probably reflect their structural similarity, with each having a C-terminal nucleic acid binding domain, a flexible linker region at mid-protein and an N-terminal oligomerization domain which is used for both self- and hetero-associations.

Despite such similarities in terms of structure and function, each protein exhibits a highly individual pattern of expression. H-NS protein levels are constant throughout growth while transcription of *hns* mRNA is coupled with chromosome replication and is maximal throughout exponential phase. StpA protein and *stpA* mRNA on the other hand peak in early exponential phase before declining to a steady state level thereafter. By interchanging the open reading frames (ORFs) of *hns* and *stpA* we aimed to assess the contributions made by the regulatory region, ORF and chromosomal location of each gene to these individual expression patterns. In making these exchanges an *hns*-like pattern of expression was conferred on *stpA* and *vice versa*. Subtle alterations in *hns* and *stpA* expression, however, were observed at both the mRNA and protein levels compared to wild-type cells. Interestingly, irrespective of which ORF was under its control, transcriptional activity via the *stpA* promoter was significantly greater than that observed for the *hns* promoter. This, however, is not reflected at the protein level and may reflect differences in mRNA stability. Finally, in making these regulatory changes, although subtle, the observed variations in *hns* and *stpA* expression at the mRNA and protein levels places the bacterium at a competitive advantage relative to wild-type at 37°C and 42°C. The mutants with exchanged *stpA* and *hns* ORFs also exhibit alterations in gene expression throughout their respective regulons that are accompanied by a number of new phenotypes.

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And finally to my parents, I can't thank you enough! You've been amazing throughout the last PhD years and the last 28 years in general! Thanks for your constant support, encouragement, advice and a great night in Chapter 'wan'!!

For My Parents,

With Thanks.

Contents

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These results have demonstrated that the genetic changes made in SL1344^{2Xhns}, SL1344^{2XstpA} and SL1344^{swap} have incurred a strain specific fitness phenotype with respect to temperature. This has been largely attributed to the significant alterations in the H-NS and StpA populations in each strain. However, each strain has also attained an antibiotic resistance gene(s) cassette by the process of their construction. Furthermore, each strain has a different complement of resistance cassettes with SL1344^{2Xhns} encoding the *kan* gene, SL1344^{2XstpA} encoding the *tetRA* genes and SL1344^{swap} encoding both the *kan* and *tetRA* genes. For each competition experiment no antibiotic selection was maintained therefore it is unlikely that the tetRA genes were expressed. It cannot be ruled out, however, that some 'leaky' expression of

List of abbreviations

AUC	analytical ultracentrifugation
bp	base pair
cAMP	cyclic adenosine monophosphate
Da	Dalton
DRE	down-stream regulatory region
f.i.	fitness index
HA	Haemagglutinin
HGT	horizontal gene transfer
IgA	Immunoglobulin A
LB	Luria-Bertani
LPS	lipopolysaccharide

ml	millilitre
µl	microlitre
M-cells	Microfold cells
mRNA	messenger RNA
NAP	nucleoid-associated protein
NB	nutrient broth
ng	nanogram
NMR	nuclear magnetic resonance
OD	optical density
ONPG	o-nitrophenol-β-D-galactoside
ORF	open-reading frame
PCR	polymerase chain reaction
PHB	polyhydroxybutyrate
PmB	polymyxin B
PMN	polymorphonuclear lymphocytes
RBS	ribosome binding site
RES	reticuloendothelial system
rRNA	ribosomal RNA
RT-qPCR	reverse transcription quantitative polymerase chain reaction
SCV	*Salmonella* containing vacuole
SD	shine-delgarno sequence

SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SFM	Scanning force microscopy
SIDD	supercoiling-induced duplex destabilisation
Sif	*Salmonella* induced filaments
SPI	*Salmonella* pathogenicity island
sRNA	small non-coding regulatory RNA
Tm	melting temperature
tRNA	transfer RNA
TTSS	Type-three Secretion System
URE	upstream regulatory element

Chapter 1 General Introduction

1.1 Introduction

Enterobacteriaceae inhabit numerous environmental niches and are capable of adapting to a diverse range of environmental conditions. Adaptation to changes in environmental conditions requires both sensing environmental signals and then integrating these signals into the gene regulatory network in order to elicit an appropriate transcriptional response (Orr, 2005a). To fulfil these requirements *Enterobacteriaceae* have evolved complex genetic regulatory systems. These systems are manipulated by a combination of alterations in DNA supercoiling and global and local transcription factors to orchestrate an appropriate transcriptional output to adapt to the prevailing environment (Dorman, 2006). Such responses are swift and highly co-ordinated in order to facilitate transitions between fundamentally different habitats such as soil and water in the external environment to that of the host intestinal gut (Dorman, 2006).

The aim of this chapter is to provide some information on the bacterial genus *Salmonella* and its process of infection. This chapter will also introduce the bacterial nucleoid and discuss the roles played by DNA supercoiling and nucleoid-associated proteins (NAPs) in maintaining and organising the structure of the nucleoid and gene expression. Finally, the primary focus of this chapter is to provide a detailed summary of one particular family of NAPs, the H-NS-like proteins.

1.2 *Salmonella*

*Salmonella*e are members of the *Enterobacteriaceae* family of bacteria (Rhen & Dorman, 2005, Ibarra & Steele-Mortimer, 2009). They are Gram-negative, facultative intracellular pathogens thought to have diverged from a common ancestor shared with *Escherichia coli* between 100 and 150 million years ago (Sabbagh *et al.*, 2010, Fookes *et al.*, 2011). The genus is comprised of two species: *Salmonella bongori* and *Salmonella enterica* (Sabbagh *et al.*, 2010, Fookes *et al.*, 2011). *Salmonella bongori* is predominantly a commensal of cold-blooded animals, although a limited number of human infections have been reported (Fookes *et al.*, 2011, Sabbagh *et al.*, 2010).

Conversely, *Salmonella enterica* infects a wide-variety of warm blooded hosts including cattle, pigs, chickens and importantly humans (Ibarra & Steele-Mortimer, 2009, Rhen & Dorman, 2005). Owing to the human health risk, much of the current knowledge regarding *Salmonella* is derived from studies undertaken with *Salmonella enterica*. The species *Salmonella enterica* is divided into six sub-species: *enterica*, *salamae*, *arizonae*, *diarizonae*, *houtenae* and *indica* (Fookes *et al.*, 2011, Sabbagh *et al.*, 2010). These subspecies are further classified into over 50 serogroups based upon the O antigen and divided into > 2,500 serovars based on the H (flagellar) antigen (Sabbagh *et al.*, 2010, Fookes *et al.*, 2011). Of the vast number of serovars only a small proportion are capable of human infection, the majority of which belong to *Salmonella enterica* subspecies *enterica* (Sabbagh *et al.*, 2010).

The clinical manifestations of this infection, termed salmonellosis, include gastroenteritis and the more serious illness typhoid fever (Sabbagh *et al.*, 2010, Ohl & Miller, 2001). *Salmonella enterica* subspecies *enterica* serovars Typhi and Paratyphi are exclusively host restricted to humans and are the causative agents of the life-threatening typhoid fever (Ohl & Miller, 2001, Sabbagh *et al.*, 2010, Thompson *et al.*, 2006). They are normally transmitted to the human host via contaminated food or water sources, and are estimated to cause 16 million cases of systemic typhoid disease resulting in approximately 600,000 deaths annually (Thompson *et al.*, 2006). Typhoid fever occurs when *Salmonella* establish a systemic infection as a result of their ability to survive and proliferate within mononuclear phagocytes and symptoms include fever, abdominal pain and transient diarrhea or constipation (Ohl & Miller, 2001). Gastroenteritis is primarily caused by *Salmonella enterica* subspecies *enterica* serovar Enteritidis and *Salmonella enterica* subspecies *enterica* serovar Typhimurium (hereafter referred to as *S.* Typhimurium) (Ohl & Miller, 2001, Sabbagh *et al.*, 2010). The incidence of non-typhoidal *Salmonella* infections is estimated to be between 200 million and 1.3 billion cases, leading to 3 million deaths annually (Coburn *et al.*, 2007). Unlike typhoidal serovars, non-typhoidal *S.* Typhimurium is not host restricted and can infect a broad spectrum of warm- and cold-blooded hosts (Sabbagh *et al.*, 2010, Rhen

& Dorman, 2005). In humans *S.* Typhimurium causes self-limiting cases of gastroenteritis. However, in mice, infection by *S.* Typhimurium results in a typhoid-like systemic fever. Importantly, due to host restriction of the natural human pathogens *Salmonella enterica* subspecies *enterica* serovar Typhi and serovar Paratyphi, murine systemic infection by *S.* Typhimurium has been used for many years for the study of typhoid fever pathogenesis (Coburn *et al.*, 2007, Sabbagh *et al.*, 2010).

1.2.1 Infective process of *S. enterica*

The primary route of infection for *Salmonella enterica* is by oral ingestion of contaminated food or water (Thompson *et al.*, 2006, Ohl & Miller, 2001). Upon ingestion, *S. enterica* passes through the stomach to reach the small intestine. Passage through the stomach is thought to be facilitated by the adaptive acid-tolerance response (ATR) which promotes survival during the low pH challenge of the stomach (Ohl & Miller, 2001, Haraga *et al.*, 2008). After reaching the small intestine, *S. enterica* then traverses the intestinal mucosal layer, resisting inactivation by bile salts, anti-microbial peptides, secretory IgA and digestive enzymes in order to access and adhere to the underlying epithelium (Haraga *et al.*, 2008). Adherence to the apical surface of epithelial cells is facilitated by the presence of fimbriae on the bacterial outer surface which make contact with receptors on the epithelial cell surface (Haraga *et al.*, 2008). *S.* Typhimurium and *S.* Typhi share a total of eight putative fimbral operons (*csg* (*agf*), *fim*, *saf*, *stb*, *stc*, *std*, *sth* and *bcf*) with each having an additional five and six unique fimbrial sequences respectively (Sabbagh *et al.*, 2010). For the majority of these fimbriae, little is known about the role they play in establishing infection or the conditions under which they are expressed, however the AgfA and Lpf fimbriae have been shown to be involved in adherence of *S.* Typhimurium to the intestinal epithelial cells of mice *in vitro* (Sabbagh *et al.*, 2010, Rhen & Dorman, 2005). In addition, two high molecular weight non-fimbrial surface proteins, BapA and SiiE, have also been implicated in adherence (Haraga *et al.*, 2008, Sabbagh *et al.*, 2010). In particular SiiE has been shown to mediate interactions with microvilli present on the apical side of

epithelial cells (Sabbagh *et al.*, 2010). Once adhered *Salmonellae* can invade both phagocytic and non-phagocytic epithelial cells but show a preference for microfold (M) cells (Fig. 1.1) (Haraga *et al.*, 2008, Ohl & Miller, 2001). M cells are specialised epithelial cells which by the process of pinocytosis sample antigens present in the small intestine and present them to lymphoid cells present in the underlying Peyer's patches (Ohl & Miller, 2001). Using a type-three secretion system (TTSS) encoded by the genes of SPI-1, *Salmonella* translocates effector proteins into the cytosol of these non-phagocytic cells (Srikanth *et al.*, 2011). Once inside the cytosol, the translocated effector proteins mediate host cytoskeletal rearrangements to induce membrane ruffling and uptake of *Salmonella* in a vacuole by a process termed bacterial mediated endocytosis (Srikanth *et al.*, 2011, Haraga *et al.*, 2008). From inside the epithelial, cell non-typhoidal *Salmonellae* begin to infect enterocytes adjacent to M cells via their apical and basolateral surfaces. Further secretion of effector proteins into the cytosol of enterocytes results in early inflammation and the migration of polymorphonuclear leukocytes (PMNs) into the intestinal lumen. Cytotoxic granules released by PMNs coupled with activation of the innate immune system by bacterial effectors results in inflammation of the gut, fluid secretion and the symptoms of gastroenteritis. Conversely, typhoid-causing *Salmonella* serovars are transcytosed to the basolateral surface of the intestinal epithelium where they are engulfed by intestinal macrophage cells (Haraga *et al.*, 2008, Ohl & Miller, 2001). Once inside the macrophage, *Salmonella* remains within a modified phagosome known as a *Salmonella* containing vacuole (SCV) (Haraga *et al.*, 2008). Within the SCV, *Salmonella* sense the prevailing environment and utilise a second TTSS and effector proteins, encoded by the genes of SPI-2, to resist the antimicrobial properties of the macrophage in order to survive and replicate (Haraga *et al.*, 2008). Inside the macrophage *Salmonella* disseminate throughout the reticuloendothelial system (RES) to cause systemic infection (Haraga *et al.*, 2008).

1.2.2 *Salmonella* Pathogenicity Islands (SPIs)

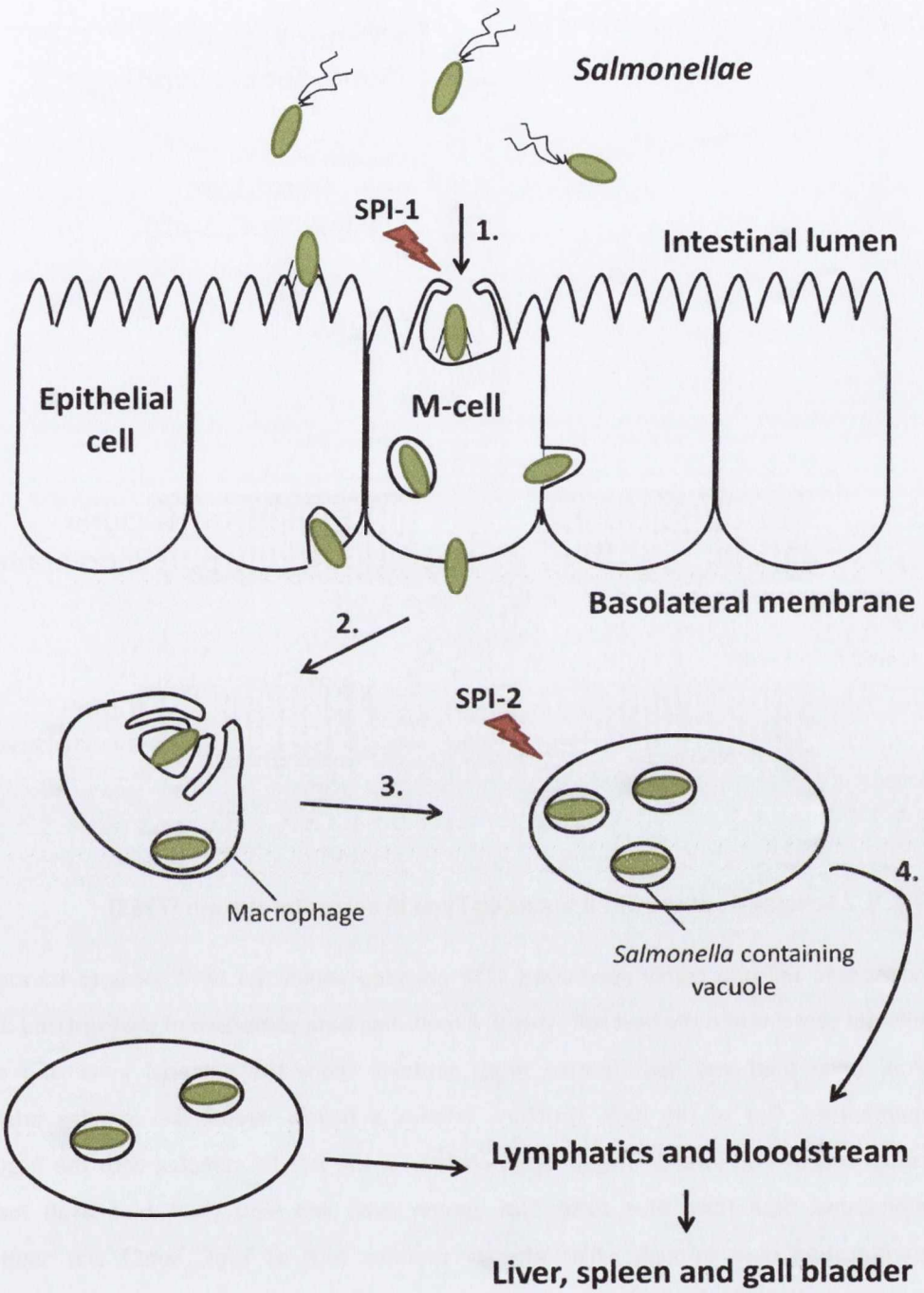
Much of the ability of *Salmonella* to cause disease can be attributed to clusters of genes encoding virulence factors which can be mapped to several large regions of the chromosome termed pathogenicity islands (Groisman & Ochman, 1996). Pathogenicity islands are characterised by having a lower G + C content and different codon usage to the rest of the chromosome, are not found in closely related non-pathogenic species and the genes encoded within a given island often determine a specific disease condition (Groisman & Ochman, 1996, Ochman *et al.*, 1996). Pathogenicity islands are acquired by horizontal gene transfer and are frequently found integrated at tRNA loci, which are thought to facilitate the integration of pathogenicity islands into the chromosome due to their high degree of conservation (Jacobsen *et al.*, 2011). To date a total of twenty-one pathogenicity islands have been identified in *Salmonella* (Sabbagh *et al.*, 2010). The genomes of *S.* Typhimurium and *S.* Typhi have 11 SPIs in common: SPIs-1 to 6, 9, 11, 12, 13 and 16 (Sabbagh *et al.*, 2010). Of particular importance for infection by both serovars are SPI-1 and SPI-2 which are required for invasion of epithelial cells and intracellular survival within macrophages, respectively (Haraga *et al.*, 2008).

1.2.3 *Salmonella* pathogenicity island-1 (SPI-1)

Appropriate expression of the genes of SPI-1 is a pre-requisite for symptoms of both typhoidal and non-typhoidal infection (Ellermeier & Slauch, 2007). The SPI-1 gene cluster is a 40 kb locus located at centisome 63 on the *S.* Typhimurium chromosome (Ellermeier & Slauch, 2007). The island encodes a TTSS and effector-proteins (Fig. 1.2) that allow *S.* Typhimurium to invade non-phagocytic endothelial cells (Ellermeier & Slauch, 2007, Sabbagh *et al.*, 2010). Genes encoding the structural components of the TTSS and effector proteins are organised into three operons: *prg/org*, *inv/spa* and *sic/sip* (Ellermeier & Slauch, 2007). The *prg/org* and *inv/spa* operons encode the needle complex while the *sic/sip* operon encodes the translocon which embeds in the host cell membrane (Ellermeier & Slauch, 2007, Srikanth *et al.*, 2011). The TTSS is comprised of a multi-ring base which spans the inner and outer membranes of the bacterial envelope and is composed of the SPI-1 encoded PrgK, PrgH and InvG proteins

Fig. 1.1 Schematic model of the principle steps of the *Salmonella* infection process.

The primary route of *Salmonella* infection is via ingestion of contaminated food or water sources. (1) Once ingested the bacteria must traverse through the host gut to reach the small intestine. In the small intestine, *Salmonella* adheres to both M-cells and enterocytes of the gut lumen and mediates its own uptake by translocating SPI-1 effector proteins into the host cell cytosol via the SPI-1 encoded TTSS (Haraga *et al.*, 2008). (2) After internalization, non-typhoidal *Salmonella* strains begin to invade adjacent enterocytes via their apical and basolateral surfaces causing gastroenteritis. Typhoid-causing *Salmonella* strains are transcytosed to the basolateral surface where they are engulfed by host macrophage cells. (3) Once inside the macrophage, *Salmonella* survive and replicate inside a specialized phagosome termed the *Salmonella* containing vacuole (SCV). Genes encoded within the SPI-2 locus are essential for survival and proliferation within the macrophage. (4) *Salmonella* disseminate throughout the reticuloendothelial system via migration of infected macrophages to infect organs such as the spleen and liver eventually to cause typhoid.


Salmonellae
SPI-1
1.
Intestinal lumen
Epithelial cell
M-cell
Basolateral membrane
2.
SPI-2
3.
4.
Macrophage
Salmonella containing vacuole
Lymphatics and bloodstream
Liver, spleen and gall bladder

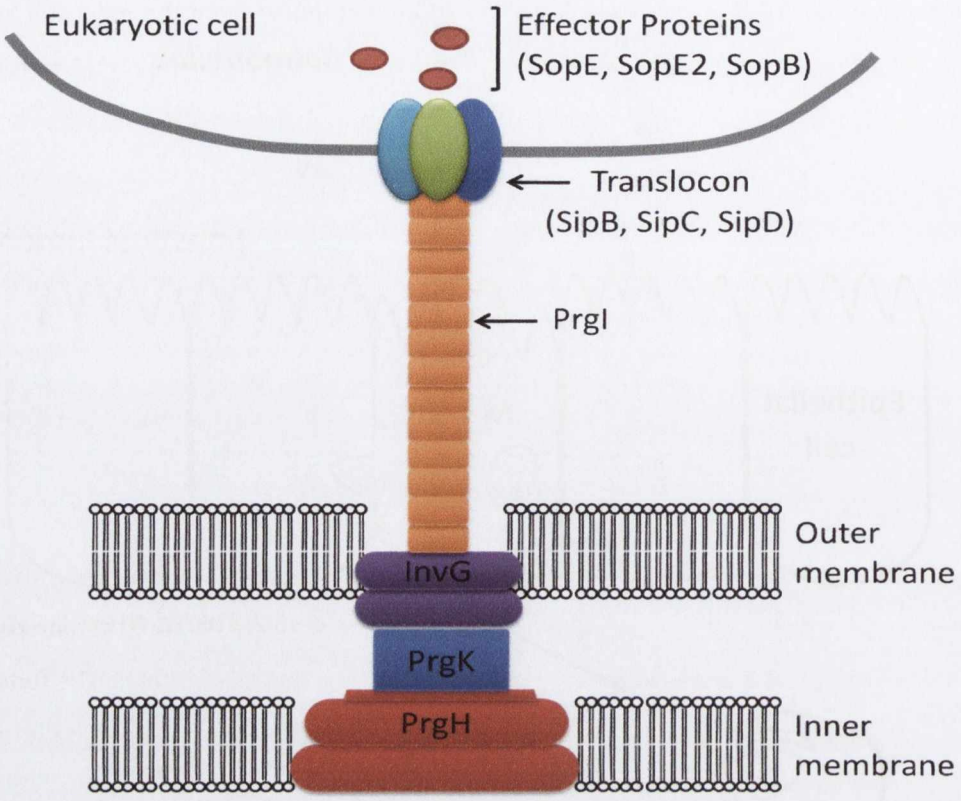


Fig. 1.2 Schematic of the SPI-1 encoded Type III secretion system (TTSS).

Salmonella utilize a highly specialized TTSS encoded within the SPI-1 locus to translocate effector proteins into the host cell cytosol. A multi-ring base comprised of the PrgH (red rings), PrgK (blue ring) and InvG (purple rings) proteins spans the bacterial inner and outer membranes. Out of the base structure extends a hollow needle-like complex primarily comprised of PrgI (orange rings). Upon contact of the needle complex with the host cell membrane, SipB (pale blue oval), SipC (green oval) and SipD (dark blue oval) form a translocation pore through which effector proteins such as SopE, SopE2 and SopB are translocated into the host cell cytosol to mediate engulfment of the bacterium.

(Haraga *et al.*, 2008, Kimbrough & Miller, 2000). Extending from the base is a hollow needle-like structure which protrudes out from the bacterial envelope and consists of the PrgI and PrgJ proteins (Kimbrough & Miller, 2000). Upon contact with the host cell membrane the SipB, SipC and SipD proteins insert into the membrane forming the translocon which is thought to facilitate the translocation of bacterial effector proteins into the host cell cytosol (Haraga *et al.*, 2008).

Upon contact with epithelial cells, *S.* Typhimurium initiates the translocation of several effector proteins in a highly co-ordinated fashion in order to mediate its uptake by endocytosis (Srikanth *et al.*, 2011). The effectors SopE, SopE2 and SopB are amongst the first translocated proteins and together they co-operatively activate the Rho GTPases Cdc42, Rac1 and RhoG (Srikanth *et al.*, 2011). Activation of Cdc42, Rac1 and RhoG drives actin cytoskeleton rearrangements leading to massive membrane ruffling and uptake of the bacterium by macropinocytosis (Srikanth *et al.*, 2011). The functions of SopE, SopE2 and SopB are essential for invasion of *S.* Typhimurium as mutants defective in all three are incapable of inducing actin cytoskeleton rearrangements (Srikanth *et al.*, 2011, Haraga *et al.*, 2008). Membrane ruffling and engulfment are further facilitated by the activities of SipA and SipC (Srikanth *et al.*, 2011). SipC is part of the translocon and its C terminal plays a key role in both actin nucleation and translocation (Srikanth *et al.*, 2011). The SipA protein is involved in actin polymerization and helps reduce the monomer threshold required for polymerization (Srikanth *et al.*, 2011, Haraga *et al.*, 2008). In addition SipA plays a role in preventing de-polymerisation of assembled actin filaments (Haraga *et al.*, 2008). Interestingly, once internalisation is complete the host actin cytoskeleton regains its normal architecture (Srikanth *et al.*, 2011, Haraga *et al.*, 2008). This has in part been attributed to the activity of a sixth translocated effector, SptP, which opposes the actions of SopE and SopE2 on Cdc42 and Rac1 (Srikanth *et al.*, 2011, Haraga *et al.*, 2008).

The regulation of SPI-1 gene expression is complex and multifaceted (Ellermeier & Slauch, 2007). Ultimately, all forms of regulation act via modulating the expression of

the SPI-1 encoded master regulator *hilA* (Ellermeier & Slauch, 2007). HilA, binds to and activates the promoters of the *prg/org* and *inv/spa* operons (Ellermeier & Slauch, 2007). As HilA controls expression of the structural components of the TTSS, *hilA* mutants are incapable of invasion and are phenotypically equivalent to SPI-1 mutants (Ellermeier & Slauch, 2007). Expression of *hilA* is controlled by a combination of three positive regulators, HilC and HilD, also encoded by SPI-1 and RtsA (Ellermeier & Slauch, 2007). Together these act in a feed forward loop to regulate the expression of *hilA* (Ellermeier & Slauch, 2007). In addition, whether by direct regulation of *hilA* or indirect regulation via regulation of *hilC*, *hilD* or *rtsA*, SPI-1 expression is controlled by numerous environmental cues which are sensed and integrated by several two component regulatory systems such as BarA/SirA, OmpR/EnvZ and PhoP/PhoQ (Ellermeier & Slauch, 2007). Furthermore, SPI-1 is also known to be regulated by changes in DNA supercoiling and by several global regulators including the nucleoid-associated proteins FIS, H-NS, IHF, Hha and HU (Cameron *et al.*, 2011, Ono *et al.*, 2005, Troxell *et al.*, 2011, Mangan *et al.*, 2011, Mangan *et al.*, 2006, Banos *et al.*, 2009).

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

SPI-2 is a 40 kb locus located adjacent to the *valV* tRNA at centisome 30 of the *Salmonella* chromosome (Sabbagh *et al.*, 2010). Like SPI-1, the SPI-2 locus also encodes genes for a TTSS, effector protein, specific effector protein chaperones and a regulatory two-component system (Sabbagh *et al.*, 2010, Haraga *et al.*, 2008). SPI-2 is essential for intracellular survival and replication within both epithelial cells and macrophages and for systemic infection (Haraga *et al.*, 2008). Consistent with this, SPI-2 defective strains are severely attenuated for virulence in the murine infection model and fail to colonise organs such as the liver and spleen (Haraga *et al.*, 2008). The SPI-2 island is absent from the phylogenetically older species *S.* bongori and is therefore thought to have been acquired more recently than SPI-1 (Fookes *et al.*, 2011). The genes encoding the structural components of the TTSS (*ssa* genes), secreted effectors (*sse* genes) and their specific chaperones are all clustered within a 25 kb region of SPI-

2 and are organised into four operons termed regulatory, structural I, structural II and effector/chaperone.

Once *Salmonella* is internalised into either epithelial cells or macrophages and the actin cytoskeleton has been restored, a series of modifications to the phagosome occurs which serve to shrink the vacuole to an adherent membrane containing one or more bacteria (Haraga *et al.*, 2008). This specialised vacuole is termed the *Salmonella* containing vacuole (SCV) where the bacteria persist ranging from a few hours to days (Haraga *et al.*, 2008). Upon formation, the SCV micro-environment undergoes drastic changes to contain reactive nitrogen and oxygen species, antimicrobial peptides, decreased abundance of divalent cations and acidification of the vacuole occurs (Haraga *et al.*, 2008). *Salmonella* senses and integrates these environmental cues into its gene regulatory network to bring about activation of SPI-2 genes and subsequent formation of the SPI-2 TTSS (Haraga *et al.*, 2008). Once formed, the SPI-2 TTSS translocates at least 20 effector proteins across the SCV membrane into the host cell cytosol (Haraga *et al.*, 2008, Srikanth *et al.*, 2011). The function and specific roles of all SPI-2 effectors is not yet fully understood. Those most important for intracellular survival are SifA, SseJ, SseF, SseG, SopD2 and PipB2 (Haraga *et al.*, 2008, Srikanth *et al.*, 2011). After activation of SPI-2 the SCV migrates to the Golgi apparatus in order to acquire nutrients and membrane constituents required to maintain the SCV (Haraga *et al.*, 2008). SifA, SseG and SseF have been implicated in targeting the SCV to the Golgi and mutants deficient for either SseG or SseF are incapable of Golgi localisation and replication (Haraga *et al.*, 2008, Srikanth *et al.*, 2011). The hallmark of maturation of the SCV is the formation of *Salmonella*-induced filaments (Sifs) (Haraga *et al.*, 2008, Srikanth *et al.*, 2011). Sifs are long, filamentous, membrane structures extending from the membrane of the SCV and are required for correct positioning of the SCV in close proximity to the Golgi apparatus (Haraga *et al.*, 2008, Srikanth *et al.*, 2011). Formation of Sifs requires the activity of the SifA and PipB2 effector proteins (Haraga *et al.*, 2008, Srikanth *et al.*, 2011). Together these proteins interact with the microtubule network and microtubule motor kinesin-1 to facilitate correct localization of the SCV (Haraga *et*

al., 2008, Srikanth *et al.*, 2011). Sif formation coincides with bacterial replication within the SCV. The exact biological function of Sifs however remains unknown (Srikanth *et al.*, 2011). It is speculated that they are involved in increasing the size of the SCV to accommodate the increase in bacterial numbers upon replication (Haraga *et al.*, 2008). After several hours of infection F-actin filaments condense around the SCV which are required for replication as inhibition of filament formation results in decreased replication (Haraga *et al.*, 2008, Srikanth *et al.*, 2011). The exact effectors involved in actin recruitment and polymerization are yet to be identified although SspH2 and SseI have been shown to co-localise with the actin cytoskeleton (Srikanth *et al.*, 2011).

Regulation of expression of SPI-2 genes is complex, responding to numerous environmental conditions and multiple transcription factors (Fass & Groisman, 2009). Analogous to SPI-1 and HilA, expression of SPI-2 genes is primarily regulated by a master regulatory protein, SsrB (Fass & Groisman, 2009). SsrB is the response regulator of a SPI-2 encoded two-component regulatory system SpiR/SsrB and is essential for expression of the TTSS and effector proteins (Fass & Groisman, 2009). SsrB has been shown to bind the promoters of all functional gene clusters in SPI-2 in addition to its own promoter where it exerts a positive effect on transcription (Fass & Groisman, 2009). The environmental signals which stimulate the SpiR sensor kinase to phosphorylate and activate SsrB remain unknown although two ancestral two-component systems, OmpR/EnvZ and PhoP/PhoP, have been shown to activate transcription of *spiR* and *ssrB* in response to low pH and low Mg^{2+} concentrations, respectively (Fass & Groisman, 2009). Furthermore, OmpR has been demonstrated to bind to the promoter of *ssrB* and activate transcription in response to changes in DNA supercoiling (Cameron & Dorman, 2012). In addition the H-NS, Hha and YdgT nucleoid-associated proteins have all been demonstrated to repress SPI-2 gene expression while the FIS and IHF nucleoid-associated proteins serve to stimulate expression with each being required for full expression of SPI-2 genes (Fass & Groisman, 2009).

1.3 The nucleoid

Like most organisms, bacteria are challenged with the task of packaging their genetic material within the confines of the cell (Dame, 2005, Dorman, 2006). For example, *E. coli* must successfully package its chromosome of 4.7 Mbp with a length of 1.5 mm, into a cell measuring 1 μm in width and 2 μm in length (Dorman, 2006, Dame, 2005). Furthermore, this must be achieved in such a way as to facilitate the processes of DNA replication, chromosome segregation and gene transcription (Mercier *et al.*, 2008). As such the bacterial chromosome is condensed into a highly compacted structure termed the nucleoid (Pavitt & Higgins, 1993). Packaging of the chromosome in such a manner is achieved by the actions of DNA supercoiling and Nucleoid-associated proteins (NAPs) (Dorman, 2006).

1.3.1 DNA supercoiling

DNA supercoiling refers to the over- or under-winding of the DNA molecule. It is a pervasive property capable of organising the morphology of the chromosome and affecting global gene expression (Rimsky & Travers, 2011). DNA isolated from bacterial nucleoids is negatively supercoiled, with approximately half of the negative supercoils being constrained by NAPs (Pavitt & Higgins, 1993). The remaining negative supercoils are naturally occurring unconstrained supercoils (Pavitt & Higgins, 1993). Thus, the chromosome contains net negative supercoils (Pavitt & Higgins, 1993). When first imaged by electron microscopy, isolated bacterial chromosomes were visualised as rosettes comprised of a central core out of which multiple topologically distinct loop-domains radiated (Fig. 1.3) (Toro & Shapiro, 2010). Each individual domain was subsequently found to be negatively supercoiled owing to a deficit in the number of DNA helical turns (Dorman, 2006). To compensate for this, writhes of the helical axis are introduced giving rise to the observed plectonemic interwinding of each loop domain (Dorman, 2006, Dame, 2005).

As a result of supercoiling, free energy is transferred to the DNA duplex that can be used to drive the processes of transcription, recombination and replication (Dorman,

2006). The available free energy is a function of the change in linking number (number of helical turns) squared (Dorman, 2006). Changes in linking number are facilitated by specific enzymes known as topoisomerases which catalyse the breaking, crossing-over and reunion of DNA strands. In *E. coli* these reactions are achieved by the activities of the DNA gyrase and DNA topoisomerase I enzymes which act antagonistically by reducing or increasing the linking number, respectively (Dorman, 2006, Pavitt & Higgins, 1993). The resultant fluctuations in free energy influence gene transcription in a number of ways. At promoters, superhelical energy can positively influence transcription by aiding the breaking of hydrogen bonds in promoter regions to facilitate open complex formation (Travers & Muskhelishvili, 2005, Dorman, 2006). In particular, superhelical energy is utilised to aid open complex formation at numerous promoters which contain GC-rich discriminator sequences (Travers & Muskhelishvili, 2005). Superhelical energy can also positively and negatively affect transcription as a result of repositioning the – 10 and – 35 sigma factor recognition sequences whose relative positioning about the DNA helix can either facilitate or hinder transcription (Travers & Muskhelishvili, 2005, Dorman, 2006). Naturally occurring fluctuations in DNA superhelicity also have implications for growth phase control of gene expression (Travers & Muskhelishvili, 2005). During exponential growth, DNA is more negatively supercoiled which favours transcription by the housekeeping sigma factor RpoD (Travers & Muskhelishvili, 2005). Upon entry into stationary phase however, the DNA template becomes more positively supercoiled and the stationary phase sigma factor RpoS competes for binding of holoenzyme, shifting the preference of the holoenzyme to the less supercoiled template (Travers & Muskhelishvili, 2005). Consequently genes within the RpoS regulon are up-regulated in preference to RpoD-dependent genes (Travers & Muskhelishvili, 2005). In addition to growth phase transitions, supercoiling levels and thus gene transcription are also known to be affected by a number of environmental fluctuations such as temperature, pH, anaerobiosis and osmolarity (Ní Bhriain *et al.*, 1989, Dorman *et al.*, 1990, Dorman, 2006).

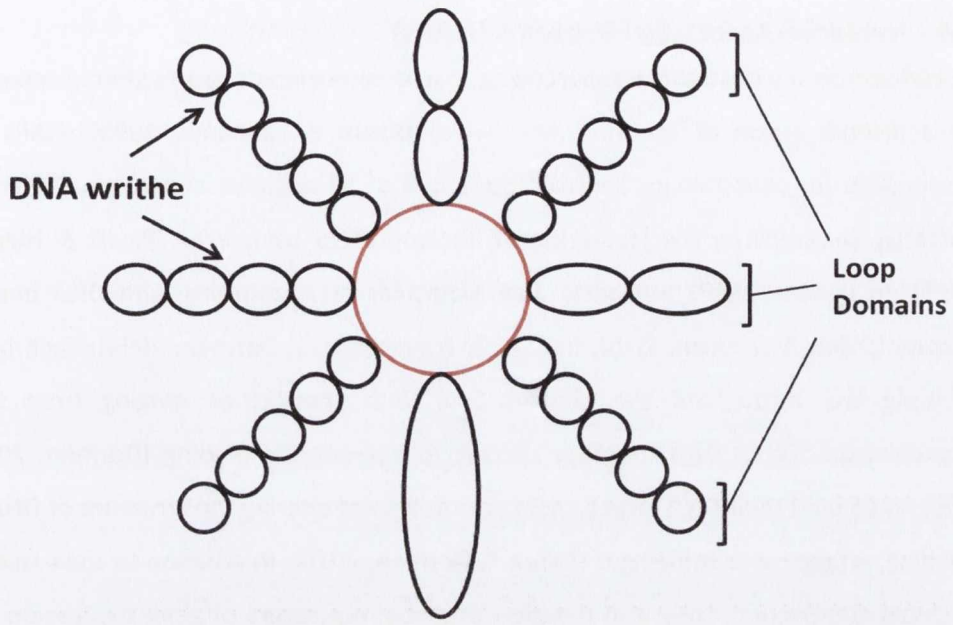


Fig. 1.3 Diagram representing the loop domain structure of the bacterial chromosome.

To facilitate packaging into the bacterial cell, the chromosome is condensed into distinct topological loop domains by the action of DNA supercoiling and nucleoid-associated proteins. Loop domains emanate from a central core (represented as a red circle) to form a rosette-like structure. Each domain is negatively supercoiled which is compensated for by the incorporation of DNA writhes. The number of writhes introduced is proportional to the negative superhelicity of each loop domain.

1.4 Nucleoid-Associated Proteins (NAPs)

In addition to unconstrained supercoiling, bacterial nucleoids are further compacted by a distinct group of proteins (the NAPs) (Azam & Ishihama, 1999). NAPs are responsible for constraining approximately half of all negative supercoils present in bacterial nucleoids by the formation of nucleoprotein complexes (Pavitt & Higgins, 1993). In general, NAPs are basic, low molecular mass proteins with DNA binding activity (Dillon & Dorman, 2010, Rimsky & Travers, 2011, Dorman, 2004). Each binds multiple loci throughout the chromosome with specificities ranging from DNA sequence-specific to DNA topology-specific to non-specific binding (Dorman, 2006). Once NAPS bind their DNA target, most are capable of altering the structure of DNA by bending, wrapping or bridging it (Dillon & Dorman, 2010). In addition to their roles in nucleoid compaction, they also function as global regulators of gene expression and are involved in all major DNA transactions such as replication and recombination (Dillon & Dorman, 2010, Dorman, 2004). The cellular population of NAPs is dynamic as each exhibits a unique pattern of expression as a function of growth (Dorman, 2004, Dame, 2005). In Gram-negative bacteria 12 species of NAPs have been characterised, the most studied and well characterised of which are: factor for inversion stimulation (FIS), integration host factor (IHF), heat unstable protein (HU), histone-like nucleoid structuring protein (H-NS) and its paralogue suppressor of td $^{-}$ phenotype (StpA) (Rimsky & Travers, 2011, Azam & Ishihama, 1999).

1.4.1 Factor for inversion stimulation (FIS)

Factor for inversion stimulation (FIS) was first identified for its role in site-specific recombination reactions in bacteria (Dorman, 2006). It is a homodimeric DNA binding protein consisting of two identical sub-units each ≈ 11 kDa in size and is thought to be restricted to the Gammaproteobacteria (Dorman, 2006, Azam & Ishihama, 1999, Ohniwa *et al.*, 2011). FIS recognizes a 17 bp consensus DNA binding sequence that is predominantly AT rich with the exception of positions 2 and 16 where G or C residues are often located (Dillon & Dorman, 2010). DNA binding is facilitated by the presence

of a conserved helix-turn-helix motif in each monomer that inserts into the major groove of the DNA helix (Dillon & Dorman, 2010, Dame, 2005). Insertion of FIS between two adjacent major grooves has been demonstrated to induce a DNA bend of between 50^{o} and 90^{o} (Dame, 2005, Dorman, 2006). Such bends constrain DNA supercoils and can conserve local levels of DNA supercoiling (Dame, 2005).

In addition to its role in site-specific recombination FIS is involved in DNA inversion, phage integration/excision, transposition and global gene regulation (Schneider *et al.*, 1999). FIS binding sites are distributed globally throughout the chromosome accounting for its observed scattered distribution about the nucleoid (Grainger *et al.*, 2006, Wang *et al.*, 2011). Importantly, many FIS binding sites are located in the promoter regions of numerous genes where, upon binding, FIS can exert a positive or negative effect on transcription depending on the position of the binding site relative to that of RNA polymerase (Grainger *et al.*, 2006). FIS can exert its effect on gene expression either directly or indirectly (Dillon & Dorman, 2010). Indirectly, FIS affects gene expression by modulating global levels of DNA supercoiling (Dillon & Dorman, 2010). FIS binds to and represses the promoters of genes encoding the A and B sub-units of DNA gyrase, an enzyme responsible for introducing negative supercoils (Dillon & Dorman, 2010). Depending on its cellular concentration, FIS is also capable of activating or repressing the gene encoding Topoisomerase I which produces positive supercoils (Dillon & Dorman, 2010, Dorman, 2006). Thus, by modulating the relative abundance of these two enzymes, FIS can indirectly affect expression of multiple supercoiling-sensitive genes (Dillon & Dorman, 2010). FIS also acts directly as a conventional transcription factor, making physical contact with RNA polymerase poised at promoters to enhance transcription (Dillon & Dorman, 2010). Conversely, FIS binding close to the sequence recognised by RNA polymerase can act as a blockade and repress transcription (Dillon & Dorman, 2010). When more distally bound within promoter regions, FIS-mediated DNA bending can serve to transfer superhelical energy downstream to facilitate open-complex formation (Grainger *et al.*, 2008, Dillon & Dorman, 2010). FIS regulation of gene expression plays a key role in growth phase

transitions, as FIS is known to regulate many genes belonging to the stringent response regulon of which the *fis* gene is a member (Dillon & Dorman, 2010). Such genes contain a GC discriminator sequence downstream of the – 10 position and are supercoiling-sensitive (Paul *et al.*, 2004). FIS activates transcription of these genes both directly, by binding within their promoter regions and indirectly, by regulating global levels of supercoiling (Paul *et al.*, 2004). Many stringently controlled genes encode ribosomal RNA (rRNA) and transfer RNA (tRNA) molecules which are massively expressed during exponential growth (Paul *et al.*, 2004). This coincides with maximal FIS expression which peaks during early exponential growth activating transcription of rRNA, tRNA and numerous other genes throughout the chromosome (Ball *et al.*, 1992). During early exponential growth FIS is one of the most abundant NAPs present in the cell with an estimated 60,000 molecules present (Ball *et al.*, 1992, Azam & Ishihama, 1999). Thereafter, however, *fis* transcription shuts off and FIS protein levels decline to almost undetectable levels by the time stationary phase is reached (Ball *et al.*, 1992). One exception to this is in *S.* Typhimurium where expression of *fis* can be maintained at significant levels well into stationary phase in microaerobic conditions (Ó. Cróinín & Dorman, 2007).

1.4.2 Heat-unstable (HU) protein.

The HU protein is one of the most abundant NAPs and is present at ≈55,000 copies per cell (Maurer *et al.*, 2009). It is a dimeric protein comprised of two sub-units, HUα and HUβ, which share 70% amino acid sequence identity (Dillon & Dorman, 2010, Mangan *et al.*, 2011). The genes encoding each sub-unit, *hupA* and *hupB*, respectively, are highly conserved amongst Gammaproteobacteria and the *hupB* gene has also been identified in the Gram-positive bacteria *Bacillus subtilis* and *Staphylococcus aureus* (Ohniwa *et al.*, 2011). In addition, plasmid encoded HU homologs have been identified in multiple Gram-positive and Gram-negative bacteria species (Takeda *et al.*, 2011).

The composition of HU varies as a function of growth phase and can exist as a homodimer comprised of either two HUα or two HUβ sub-units or as heterodimer made from both sub-units (Dillon & Dorman, 2010, Mangan *et al.*, 2011). Structurally, it consists of a compact body of α-helices out of which extends β-ribbon arms which wrap around the minor groove upon DNA binding (Dame, 2005). Conserved proline residues at the tip of each arm intercalate between base pairs to facilitate DNA binding (Dame, 2005). HU shows no sequence preference for DNA binding but instead favours binding to regions of distortion such as bends or four-way junctions (Dillon & Dorman, 2010). Upon binding HU can introduce bends into the DNA polymer of up to 140^{o} which have been shown *in vitro* to constrain toroidal DNA supercoils (Maurer *et al.*, 2009).

Like FIS, HU is found evenly dispersed throughout the chromosome and it also influences many DNA-based transactions including replication, DNA repair, recombination and global gene transcription (Wang *et al.*, 2011, Mangan *et al.*, 2011). Consistent with its role as a NAP the loss of HU has pleiotropic effects on global gene transcription with expression of genes involved in anaerobic respiration, osmotic and acid stress resistance, DNA damage repair and central metabolism influenced by its loss in both *S.* Typhimurium and *E. coli* (Mangan *et al.*, 2011, Dillon & Dorman, 2010). The ability of HU to affect global gene expression appears to be based on its ability to alter DNA topology (Berger *et al.*, 2010). Microarray based studies in *E. coli* demonstrated that the majority of genes up-regulated in HU deficient strains clustered within a macrodomain encompassing the OriC region (Berger *et al.*, 2010). Further investigation revealed that this was due to an impaired ability to form transcription foci which contain the heavily transcribed rRNA genes in the absence of HU (Berger *et al.*, 2010). As DNA gyrase binding sites are 10-fold more abundant in the OriC macrodomain than elsewhere on the chromosome, this led the authors to hypothesise that HU constrained free DNA supercoils generated from the actively transcribing rRNA promoters, so as to produce metastable structures which maintain focus integrity (Berger *et al.*, 2010). Conversely, the loss of HU is compensated for by

DNA gyrase activity which now has more accessibility to binding sites in the absence of transcription foci (Berger *et al.*, 2010).

Interestingly, the composition of the HU dimer has varying effects on DNA supercoiling (Berger *et al.*, 2010, Mangan *et al.*, 2011). Both the HUαβ heterodimer and the $HU\alpha_2$ homodimer are capable of introducing negative supercoils in the presence of topoisomerase I. The $HU\beta_2$ homodimer, however, is incapable of introducing negative supercoils. Furthermore, an HU octamer is thought to constrain toroidal left-handed supercoils whilst a hyperactive $HU\alpha_2$ dimer can constrain right-handed supercoils (Berger *et al.*, 2010, Mangan *et al.*, 2011). Throughout growth the composition of HU naturally varies due to differential regulation of the *hupA* and *hupB* genes (Mangan *et al.*, 2011). The HUαβ heterodimer is the dominant form during growth with $HU\alpha_2$ and $HU\beta_2$ homodimers only detectable during early exponential phase and late stationary phase, respectively (Mangan *et al.*, 2011). It thus follows that the dynamic composition of HU helps mediate growth phase transitions in supercoiling with possible implications for global gene expression. This indeed has been observed in *S.* Typhimurium where distinct but overlapping growth phase dependent patterns of gene expression were observed for mutants deficient in *hupA*, *hupB* or *hupA/hupB* (Mangan *et al.*, 2011).

1.4.2 Integration-host factor (IHF)

The IHF protein was first identified for its role as a cofactor in the integration and excision process of bacteriophage λ (Dillon & Dorman, 2010, Dorman, 2006). It is widespread throughout the Gammaproteobacteria and homologs have been identified on plasmids isolated from numerous Gram-negative bacteria and plasmids isolated from the Gram-positive bacteria *Alicyclobacillus* and *Arthrobacter* (Takeda *et al.*, 2011, Ohniwa *et al.*, 2011). IHF is an abundant NAP with cellular levels observed to increase from an estimated 12,000 copies present during exponential phase to ≈55,000 at the onset of stationary phase (Maurer *et al.*, 2009). It is a dimeric protein with each monomer encoded separately by the *himA* and *himB* genes (Mangan *et al.*, 2006). Like

HU, differential regulation of these genes as a function of growth results in homodimeric and heterodimeric forms of IHF (Mangan *et al.*, 2006). IHF also has a similar structure to HU consisting of a compact body of α-helices out of which β-ribbon arms extend to contact DNA via conserved proline residues at their tip (Dame, 2005). As a consequence of these arms inserting into the minor groove of DNA, two kinks are introduced at the points of contact allowing IHF to introduce a U-turn bend of up to 180^0 (Rice, 1997, Dame, 2005). Unlike FIS and HU, IHF binds DNA in a sequence specific manner showing a preference for the degenerate consensus site WATCAANNNNTTR (where W is A or T and R is A or G) (Goosen & van de Putte, 1995).

IHF plays an integral role in the timing of replication initiation and affects several site-specific-recombination systems and transposition (Dillon & Dorman, 2010). Close to 1000 IHF binding sites have been identified in the *E. coli* chromosome, many of which are located within promoter regions where, upon binding, IHF can act as a conventional transcription factor by recruiting RNA polymerase containing the alternative sigma factor RpoN to promoters (Goosen & van de Putte, 1995) (Dame, 2005). As a result of IHF-induced DNA bending it can also bring about close contact between normally distal transcription factors and RNA polymerase (Goosen & van de Putte, 1995). In addition, IHF has been shown to activate transcription of a number of genes by facilitating the transfer of superhelical energy (Dorman, 2006). Certain regions of DNA are prone to supercoiling-induced duplex destabilisation (SIDD) whereby double-stranded DNA opens to form a single-stranded DNA bubble due to the torsional stress of supercoiling (Dillon & Dorman, 2010, Dorman, 2006). At certain promoters, IHF binding sites and SIDD sites overlap (Dillon & Dorman, 2010). Where overlap occurs, binding of IHF to the DNA quenches the tendency of the SIDD site to become single stranded and the torsional energy gets transferred to the next feasible SIDD site. Frequently, the next labile site occurs within promoters and the free energy is used to drive open-complex formation (Dillon & Dorman, 2010, Dorman, 2006).

1.5 H-NS: structure and function

1.5.1 Histone-like Nucleoid structuring protein (H-NS).

H-NS is one of the most abundant nucleoid-associated proteins present in Enterobacteria under standard laboratory growth conditions along with HU (Dame, 2005). It was first identified over 40 years ago in *E. coli* when it was isolated during a protocol for the isolation of RNA polymerase and was designated the H1 protein (Jacquet *et al.*, 1971). Subsequently, it was shown to bind DNA with high affinity and could stimulate *in vitro* transcription from phage templates at low concentrations but inhibit transcription at high concentrations (Cukier-Kahn *et al.*, 1972). After its discovery work on H-NS ceased for a number of years until in 1977 and again during the 1980s it was repeatedly isolated as part of screens searching for bacterial 'histone-like' proteins thus earning it the title of H-NS (heat-stable nucleoid structuring protein) (Varshavsky *et al.*, 1977, Bakaev, 1981). Upon discovery that H-NS could condense DNA *in vitro* and *in vivo* in a similar manner to eukaryotic histones, it was once again renamed as histone-like nucleoid structuring protein a title which it has retained to this day despite sharing no homology with any histones (Spassky *et al.*, 1984, Spurio *et al.*, 1992). H-NS is a dimeric, heat-stable, basic protein of about 15.6 kDa and is present in the cell more or less constantly at about 20,000 copies per genome equivalent (Spassky *et al.*, 1984, Dorman, 2004). It is neutral under native conditions migrating with a pI of ≈ 7.5 but acidic under denaturing conditions and migrates with a pI ≈5.6 (Spassky *et al.*, 1984). The *hns* gene is located at centisome 27.8 on the *E. coli* chromosome and centisome 38.4 in *S.* Typhimurium and encodes the 134 amino acid protein product H-NS (Falconi *et al.*, 1988, Sanderson *et al.*, 1995). Mutations mapping to the *hns* gene are highly pleiotropic affecting the transcription of numerous unrelated genes in both *S.* Typhmurium and *E. coli.* This is thought to be due to the DNA binding activity of H-NS within promoter regions. In addition, *hns* mutations and the resulting H-NS protein alterations were shown to affect global supercoiling levels in both species (Dorman *et al.*, 1989, Hinton *et al.*, 1992).

1.5.2 Structure of H-NS

Initial studies concerning H-NS were focused on its activity within the cell and highlighted its role in global gene regulation and modulation of supercoiling. How H-NS carried out these functions however was unknown and prompted studies aimed at determining the structure-function relationship for H-NS. Early genetic studies indicated that H-NS was comprised of two distinct biologically-active domains (Dorman *et al.*, 1999) (Fig. 1.4). Using trypsin digestion, Shindo *et al.*, 1995, successfully isolated a C-terminal portion of H-NS from amino acid residues A91 to Q137 and demonstrated that this region of H-NS retained the ability to bind DNA, albeit with a reduced affinity to full length H-NS (Shindo *et al.*, 1995). The structure of this region was then determined by NMR and found to consist of a two-stranded β-sheet, an α-helix and a 3_{10} helix each joined by small loops (Shindo *et al.*, 1995). The DNA binding activity of the C-terminal region of H-NS was again confirmed in a follow up study by the same group using a series missense and nonsense *hns* mutant derivatives (Ueguchi *et al.*, 1996). The ability of 26 such mutants to repress the two characterised H-NS repressed loci, *proU* and *bgl*, was assessed for each mutant. It was found that each mutation could be categorised into two groups, those that affected expression of *proU* and *bgl* (Group I) and those that only affected expression of *proU* (Group II) (Ueguchi *et al.*, 1996). Further characterisation of these mutations revealed that those mapping between C-terminal residues 91 – 137 (Group I) impaired or eliminated the DNA binding capacity of H-NS whereas those mapping in the N-terminal region between residues 1 – 89 (Group II) affected the ability of H-NS to dimerise (Ueguchi *et al.*, 1996). This led the authors to conclude that H-NS was comprised of an N-terminal dimerisation domain and a C-terminal nucleic acid binding domain (Ueguchi *et al.*, 1996). This two domain structure was again confirmed in a separate study using dominant negative derivatives (Williams *et al.*, 1996).

In a more recent study by Smyth *et al.*, 2000, a series of biochemical and biophysical techniques were used to further characterise the structure of H-NS from *S.* Typhimurium. Using nuclear magnetic resonance (NMR) spectroscopy they demonstrated that while the N-terminal domain appeared to be rigid, the C-terminal

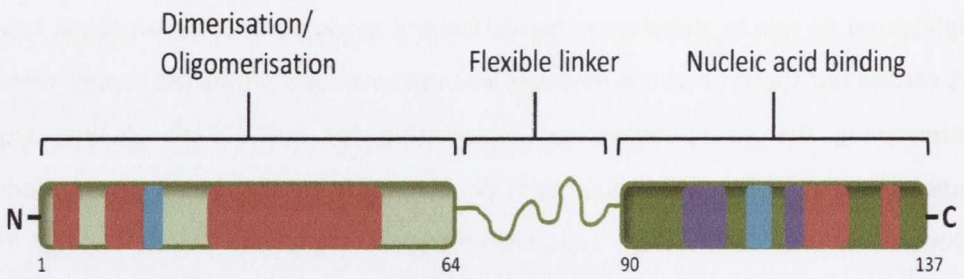


Fig. 1.4 Two-domain structure of H-NS.

H-NS is a 137 amino acid protein comprised of two functionally distinct domains. The N-terminal domain (pale green) spans amino acid residues 1 – 64 and is responsible for dimer and higher-order oligomer formation. The C-terminal domain (dark green) is responsible for nucleic acid binding and spans amino residues 91 – 137. Both domains are connected by a flexible linker region. Protein secondary structures and their relative positions are represented by maroon blocks (α-helices), blue blocks (turns) and purple blocks (β-sheets). Secondary structure predictions and their relative positions were obtained from the Uniprot database (www.uniprot.org).

domain was structurally independent and highly mobile, suggesting that the two domains were connected by a flexible linker region (Smyth *et al.*, 2000). Further investigation, employing gel filtration and analytical ultracentrifugation (AUC) techniques, revealed that the N-terminal amino acids from 1 – 64 were necessary and sufficient for dimerisation of H-NS while the addition of residues 65 – 89 conferred the ability of H-NS to form multimeric complexes (Smyth *et al.*, 2000).

1.5.2.1 N-terminal dimerisation and higher order H-NS oligomers.

Early investigations into the structure of H-NS revealed that it primarily existed as a homodimer when in solution (Fig. 1.5) and self-associated even when present at low concentrations (Falconi *et al.*, 1988). At higher protein concentrations, however, H-NS formed tetrameric complexes (Falconi *et al.*, 1988). Both dimerisation and tetramerisation were thought to involve hydrophobic interactions between monomers. Dimerisation was once again observed by Ueguchi *et al* whilst trying to solve the primary structure of H-NS using gel filtration (Ueguchi *et al.*, 1996). This study demonstrated that H-NS did indeed form dimers and that this was dependent on the presence of the N-terminal domain (Ueguchi & Mizuno, 1996). Interestingly however it was also noticed that H-NS could form higher-order multimeric complexes of up to ≈20mers in a concentration dependent manner and that the H-NS dimer appeared to be the basic building block of these oligomeric complexes (Ueguchi *et al.*, 1996). Since this study, much controversy has surrounded the basic building block of H-NS oligomers and their formation. Using a combination of gel filtration and analytical ultracentrifugation (AUC) (Smyth *et al.*, 2000) demonstrated that H-NS 1 - 64 N-terminal truncates formed trimeric structures in solution the formation of which was dependent on a coiled-coil structure present within the N-terminal domain. Thus it was proposed that an H-NS trimer was the basic building block for oligomer formation (Smyth *et al.*, 2000). In support of this proposal, the same group later demonstrated this secondary trimeric structure using NMR (Renzoni *et al.*, 2001). This trimeric form, however, did not agree with the proposed structures of H-NS in both *E.*

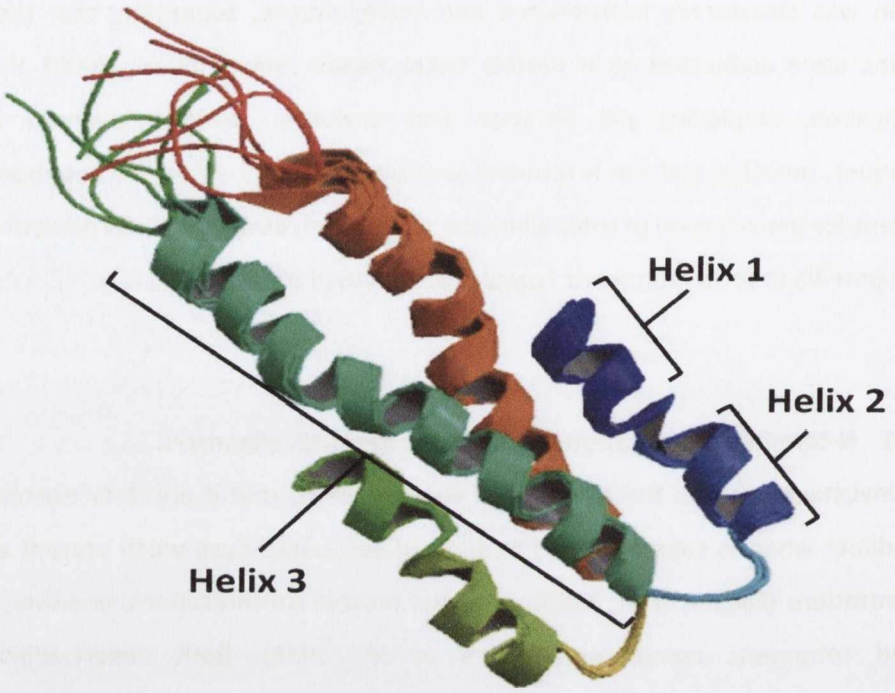


Fig. 1.5 3-D structure of the H-NS N-terminal domain.

3-D ribbon diagram depicting the H-NS N-terminal from residues 2 – 57 as solved using NMR by Esposito *et al.*, 2002. The H-NS N-terminal is shown in dimeric form with each sub-unit comprised of two short α-helices (helix 1 and helix 2) which lie anti-parallel to the longer helix 3 which spans amino acid residues 24 – 57.

coli and *S.* Typhimurium from other studies (Stella *et al.*, 2005). As part of further studies, the basic building block was again proposed to exist as a dimer with each partner aligning in a head to tail (N-terminal to C-terminal) manner upon oligomerisation (Fig. 1.6) (Esposito *et al.*, 2002). Subsequently, using a series of chimeric proteins H-NS was again demonstrated to form both dimers and tetramers, but the wild-type H-NS molecule was thought to be tetrameric (Stella *et al.*, 2005). A more recent model suggests that the basic building block is indeed the H-NS dimer but both tetramerisation and oligomerisaton are heavily influenced by prevailing environmental conditions (Leonard *et al.*, 2009, Arold *et al.*, 2010). Increases in temperature, salt concentrations and the presence of the divalent cation Mg^{2+} have all been observed to influence the oligomeric state of H-NS *in vivo* and *in vitro* (Leonard *et al.*, 2009, Stella *et al.*, 2006, Liu *et al.*, 2010). Intriguingly, the latest study concerning the oligomeric state of H-NS has shown by crystallization that H-NS dimers self-associate to form a multimeric superhelical protein scaffold about which DNA is wrapped and condensed (Arold *et al.*, 2010). In agreement with previous studies, this H-NS protein scaffold is perturbed by increases in temperature allowing flexibility for both DNA transactions such as transcription while at the same time maintaining nucleoid condensation (Arold *et al.*, 2010).

1.5.2.2 C-terminal nucleic acid binding.

The C-terminal domain of H-NS is responsible for nucleic acid interactions and is thought to span from residues 91 – 137 (Dorman *et al.*, 1999). It consists of a two anti-parallel β-sheets spanning residues 97 – 100 and 105 – 109, one α-helix (residues 117 – 126) and one 3_{10} helix (residues 130 – 133) (Fig. 1.7.) (Gordon *et al.*, 2011). Recently the exact residues responsible for H-NS binding to DNA in *S.* Typhimurium were identified and were found to be conserved in a number of Gram-negative bacteria and in the *Mycobacterium tuberculosis* homolog Lsr2 (Gordon *et al.*, 2011). The residues responsible are part of a newly identified DNA binding motif (QGRTPA) and were

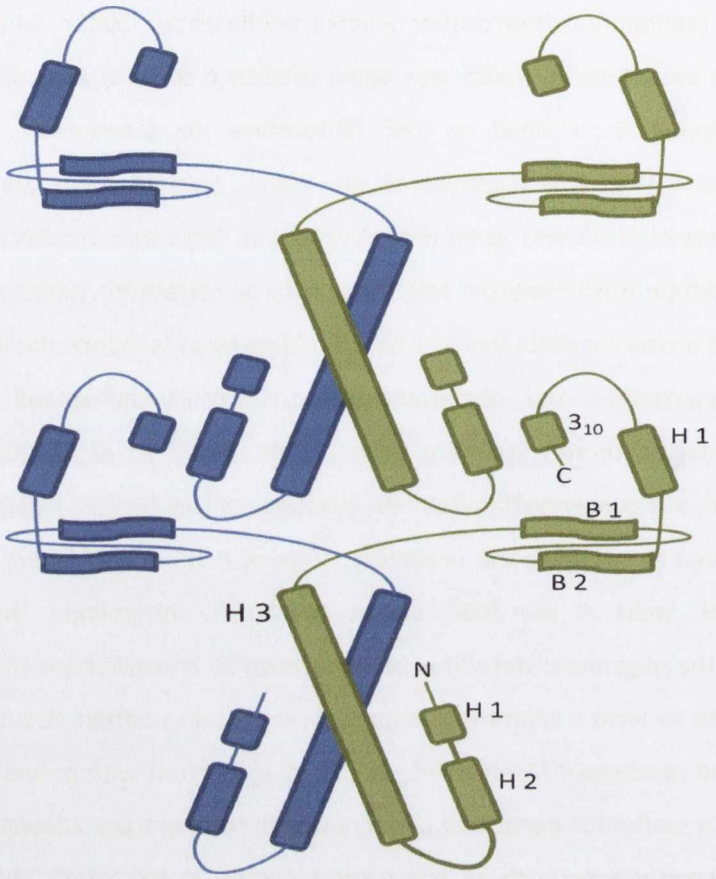


Fig. 1.6 Schematic representation of an H-NS tetramer.

Tetramer and higher order oligomer formation of H-NS occurs by the polymerization of H-NS dimers in a head-to-tail manner (N-terminal to C-terminal). H-NS monomers are here represented as green and blue sub-units. Interactions between residues in helix 3 (H 3) from each sub-unit facilitate dimer formation (Esposito *et al.*, 2002). The C-terminal domain, comprising β-sheets, B1 and B2, α-helix (H1) and a 3_{10} helix, is freely connected to the N-Terminal domain via a flexible linker region. Interactions between N-terminal residues 65 – 89 of one dimer with residues located within the loop structure linking helix 2 (H2) and helix 3 (H 3) of a second dimer, mediates tetramer and higher order oligomer formation.

located within the loop region that connects the β2 strand and the α-helix (Gordon *et al.*, 2011). Most importantly the Q112-G113-R114 motif was demonstrated to be essential and mutant combinations of these three residues were found to either impair or completely abolish DNA binding (Gordon *et al.*, 2011). Computer modelling predicted that these three residues insert into the DNA minor groove where the side chains of Gln112 and Arg114 orientated parallel to the DNA strand and away from each other to span a distance of ≈5bp in a conformation known as an AT-hook (Gordon *et al.*, 2011). Binding within the minor groove was confirmed using netropsin, an antibiotic that targets the minor groove of A/T rich sequences, which could successfully out-compete H-NS for binding to DNA (Gordon *et al.*, 2011).

Much controversy exists about the exact DNA binding preference of H-NS. For many years it was thought that H-NS had no sequence preference for DNA binding but instead favoured regions of DNA with intrinsic planar curvature (Dorman, 2004). Such regions are frequently found within gene promoters thereby facilitating the role of H-NS in global gene repression (Dorman, 2004). In recent years, genome-wide studies using CHIP-chip analysis revealed a stronger correlation between H-NS binding and AT-content with H-NS displaying a binding preference for AT rich regions (Dillon *et al.*, 2010, Lucchini *et al.*, 2006). Subsequent analysis of several CHIP-chip datasets from *S.* Typhimurium, however, led to the proposal that a degenerate consensus sequence motif (tcgATAAAtt) constituted an H-NS binding site (Lang *et al.*, 2007). This sequence was also found within the promoter regions of several known H-NS-regulated genes in *E. coli* and is thought to serve as a high affinity binding site for H-NS which, when bound, polymerises along AT-rich DNA tracts (Lang *et al.*, 2007, Sette *et al.*, 2009). The most current information regarding a potential binding preference for H-NS is derived from NMR and CHIP-chip data and suggests that H-NS prefers contiguous AT sequences devoid of A-tracts (Gordon *et al.*, 2011).

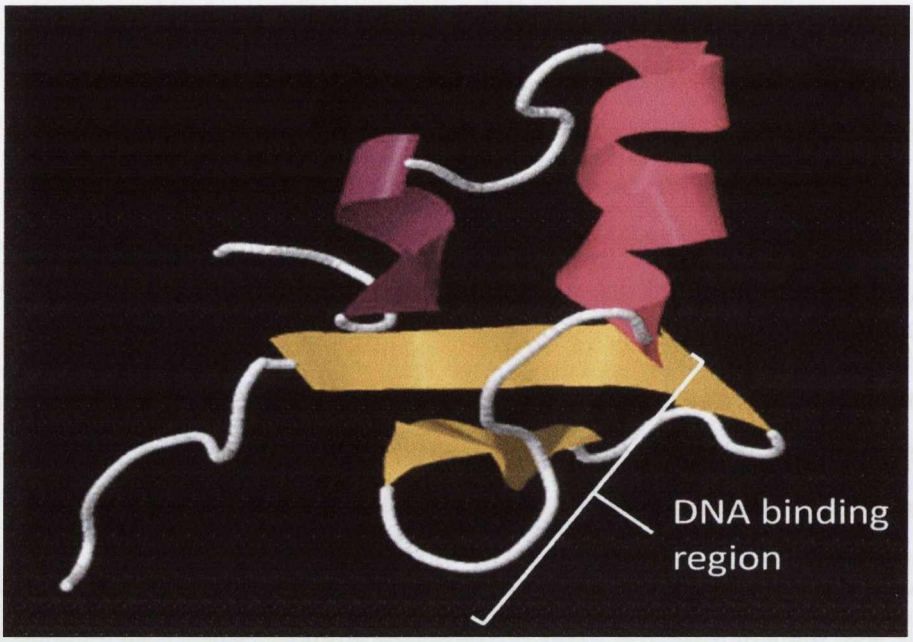


Fig. 1.7 3-D structure of the H-NS C-terminal nucleic acid binding domain.

3-Dimensional ribbon diagram depicting amino acid residues 91 – 137 of the H-NS C-terminal domain as solved by NMR (Shindo *et al.*, 1995). The domain is comprised of two β-sheet regions, β 1 and β 2 (yellow arrows), which lie anti-parallel to each other, one α-helix (pink coil) and a 3_{10} helix (purple coil). Amino acid residues 112 – 117 thought to be responsible for DNA binding are located within the loop region connecting β-sheet β 2 and α-helix α 1.

1.5.2.3 Structure-function relationship.

Despite the N- and C-terminal domains being described separately and having individual functions they co-exist within in the cell as a singular protein and influence the activity of one another. In recent years several advanced biophysical techniques have been used to analyse the interactions of H-NS with DNA. Dimerisation and higher order oligomerisation of H-NS in a parallel fashion potentiates the availability of multiple free DNA binding domains on either side of the multimer (Dame *et al.*, 2005). Each binding domain has the capability to interact with DNA on either side of the multimer to form a bridge between the two strands (Dame *et al.*, 2005, Dorman & Kane, 2009). Such H-NS mediated bridging was indeed observed using Scanning force microscopy (SFM) whereby H-NS was observed to bridge two adjacent strands of λ DNA *in vitro* (Dame *et al.*, 2005). The discovery of H-NS 'nucleation' sites offers the prospect of a second type of nucleoprotein complex forming upon H-NS oligomerisation (Rimsky *et al.*, 2001). By this method upon recognition of the high affinity binding site and subsequent polymerisation of H-NS along the DNA polymer results in more extended stiffer structures (Rimsky *et al.*, 2001, Amit *et al.*, 2003). This 'stiffened' DNA structure has also been observed in experiments using magnetic tweezers (Amit *et al.*, 2003). Furthermore, the newly characterised formation of an H-NS superhelical-like structure about which DNA is wrapped offers a new prospect about the mechanism of action of H-NS (Arold *et al.*, 2010). Importantly, each of the structures discussed here can be disrupted and/or altered depending on the prevailing environmental conditions and as H-NS oligomerises in a concentration dependent manner their formation is subject to the availability of free H-NS (Liu *et al.*, 2010, Leonard *et al.*, 2009, Arold *et al.*, 2010). This intimate relationship between H-NS structure and function in the formation of nucleoprotein complexes has important implications for role of H-NS in chromosome compaction and global gene expression.

1.5.3 Biological roles of H-NS

1.5.3.1 Global gene regulation.

The regulation of gene expression by H-NS has been the subject of much investigation since its discovery. Predominantly H-NS has a repressive effect on transcription. However a few rare examples of transcription activation have been documented (Dorman, 2004). Early research labelled H-NS as a global regulator of gene expression owing to the vast number of unrelated genes whose expression was affected by the deletion or mutation of the *hns* gene (Hulton *et al.*, 1990). At present it is estimated that H-NS is directly or indirectly involved in the regulation of 5% and 13% of all chromosomally-encoded genes in *E. coli* and *S.* Typhimurium, respectively (Navarre *et al.*, 2006). This ability to regulate such a vast number of genes is in part due to the binding preference of H-NS for AT-tracts of DNA which are commonly found within promoter regions. Indeed several CHIP-chip studies have identified H-NS binding sites located within promoter regions throughout the chromosome (Dillon *et al.*, 2010, Lucchini *et al.*, 2006, Navarre *et al.*, 2006). H-NS binding sites, however, are also frequently located within open-reading frames (ORFs) and non-coding regions of the chromosome (Grainger *et al.*, 2006, Fang & Rimsky, 2008). While H-NS binding within ORFs often functions in the formation of a repressive complex the binding of H-NS within non-coding regions is thought to play an architectural role (Grainger *et al.*, 2006).

Unlike conventional transcription factors H-NS does not affect gene transcription via interactions with RNA polymerase (Shin *et al.*, 2005). Instead, H-NS establishes repression at gene promoters by the formation of nucleoprotein complexes (Shin *et al.*, 2005, Dorman & Deighan, 2003, Dorman & Kane, 2009, Kane & Dorman, 2011). Nucleoprotein-mediated repression by H-NS has been demonstrated experimentally at a number of gene promoters in *E. coli* such as *proU*, *bgl*, *rrnB* P1 and *hdeAB* (Fang & Rimsky, 2008, Dorman & Deighan, 2003, Shin *et al.*, 2005). At each of these promoters repression is dependent upon H-NS binding to two so called regulatory elements, one located within the upstream promoter region (URE) and the other located

downstream within the ORF (DRE) (Dorman & Deighan, 2003, Fang & Rimsky, 2008). Repression first involves recruitment of H-NS to the promoter regions of genes such as *proU*, *bgl*, *rrnB* P1 and *hdeAB* and is likely achieved by the presence of a nucleation site (Bouffartigues *et al.*, 2007, Dorman & Deighan, 2003). In the case of *proU*, both the URE and DRE have been shown to act as high affinity nucleation sites for H-NS (Bouffartigues *et al.*, 2007) (Fig. 1.8). Once H-NS has bound to these sites, a loop structure is formed via protein-protein interactions between H-NS bound at both sites. Subsequent oligomerisation of H-NS bound between DNA strands serves to 'zipper' the promoter region thus either trapping DNA bound RNA polymerase or preventing its initial binding to inhibit transcription (Dorman & Deighan, 2003).

1.5.3.2 H-NS as a defence system.

Bacterial genomes are dynamic entities over evolutionary, constantly being shaped by mutation, gene duplication, gene loss and chromosomal rearrangements. In addition, new genes can be acquired by means of horizontal gene transfer (HGT) and stably integrated into the genome (Serres *et al.*, 2009). Genes acquired by HGT normally enter bacteria by means of conjugative-plasmids or infection by bacteriophage before being integrated into the bacterial genome where they are frequently found located adjacent to tRNA genes (Groisman & Ochman, 1996). Such sequences are identified by aberrant codon usage when compared to the host and, in general, have a higher AT content than the native host (Ochman et al., 1996). The acquisition of genes by HGT is thought to be the primary driving force behind genetic diversity amongst microbes. However, more often than not, genes acquired by HGT impose a fitness deficit upon the bacterium until properly integrated into the gene regulatory network (Navarre *et al.*, 2007). Recent CHIP-chip and microarray studies have identified a role for H-NS as a genome guardian that acts to selectively silence genes acquired by HGT (Lucchini *et al.*, 2006, Navarre *et al.*, 2007). Data from two separate microarray experiments undertaken in *S.* Typhimurium revealed that of the >400 genes whose expression was altered in a *hns* mutant strain 90% showed evidence of being acquired by HGT

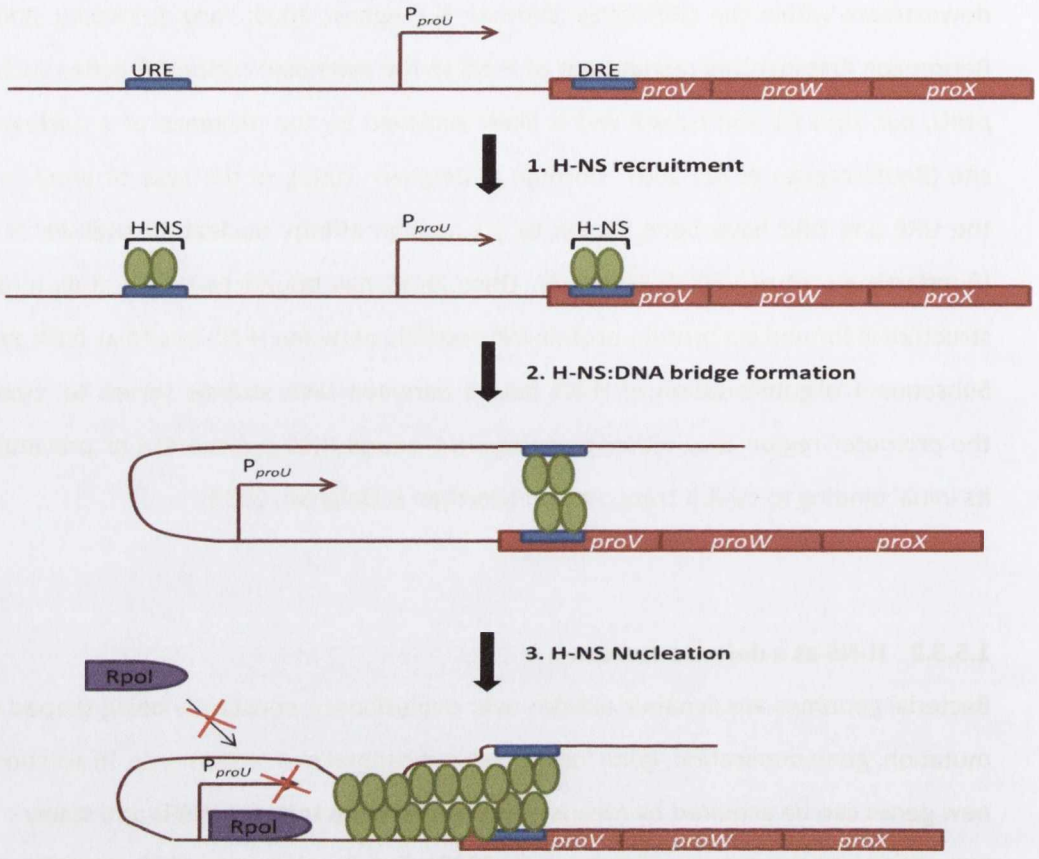


Fig. 1.8 Model for H-NS mediated repression at the *proU* promoter.

Repression by H-NS is achieved by the formation of nucleoprotein filaments. (1) At the *proU* promoter H-NS is first recruited to two high affinity nucleation sites (URE and DRE). (2) Protein-protein interactions between H-NS bound at the URE and DRE facilitates the formation of an H-NS-DNA bridge. (3) Further H-NS oligomerisation serves to 'zipper' the promoter region preventing binding of RNA polymerase or elongation of bound RNA polymerase. Figure was adapted from Kane and Dorman (2009).

(Navarre *et al.*, 2006, Lucchini *et al.*, 2006). These genes had, on average, a 5% higher AT content than that of the host *S.* Typhimurium genome (Lucchini *et al.*, 2006, Navarre *et al.*, 2006). The majority of these genes were found to be repressed by H-NS. This led to the deduction that AT-rich genes were 20 times more likely to be repressed by H-NS (Lucchini *et al.*, 2006). Subsequent CHIP-chip analyses showed that all regions with enhanced AT content relative to the core genome were also bound by H-NS including several SPI regions known to have been acquired by HGT (Lucchini *et al.*, 2006). These results led to the hypothesis that H-NS acts as a 'xenogenic silencer' which is capable of discriminating between genes acquired by HGT and those of the core genome on the basis of AT-content (Navarre *et al.*, 2007).

1.5.3.3 Removal of H-NS repression.

Expression of genes regulated by H-NS, whether part of the core genome or acquired by HGT, requires removal or disruption of the H-NS repression complex. This can be achieved in a number of ways. Changes in certain environmental conditions are known to relieve H-NS repression in a number of instances (Fig. 1.9) (Stoebel *et al.*, 2008). For example, upon osmotic up-shifts increased environmental salt concentrations have been shown to alter the DNA topology of the *proU* promoter affecting binding of H-NS within the regulatory region and resulting in de-repression of the locus (Stoebel *et al.*, 2008). Similarly, expression of the *Shigella flexneri* virulence gene master regulator VirF is induced upon temperature up-shifts due to removal of H-NS repression by local changes in the DNA topology of the *virF* promoter (Stoebel *et al.*, 2008). Furthermore, in *S.* Typhimurium H-NS mediated repression of *phoP* and several PhoP regulated genes is sensitive to the environmental concentration of Mg^{2+}, with repression relieved upon a decrease in environmental levels (Kong *et al.*, 2008). A number of proteins which act as non-conventional transcriptional regulators can serve to antagonise H-NS mediated repression by re-organising the DNA within the H-NS-DNA complex (Stoebel *et al.*, 2008). The VirB protein of *Shigella flexneri* is such a protein which acts by remodelling H-NS-DNA complexes at virulence gene promoters (Fig. 1.9)

(Stoebel *et al.*, 2008). Furthermore, judicious placement of a VirB binding site and subsequent introduction of VirB into *S.* flexneri was shown to relieve repression of *proU* (Kane & Dorman, 2011). A number of AraC-like proteins, such as the SPI-1 gene regulators HilC and HilD, are capable of both removing H-NS from promoter regions and simultaneously act as transcriptional enhancers (Fig. 1.9) (Stoebel *et al.*, 2008). In a unique case, the LeuO protein acts to contain the nucleation of H-NS (Fig. 1.9) (Stoebel *et al.*, 2008). By binding between the promoter and the upstream regulatory element within the *leuABCD* operon, LeuO blocks the propagation of the H-NS nucleoprotein complex and preserves the operon in a transcriptionally active state (Stoebel *et al.*, 2008). Other NAPs play a key role in the removal of H-NS mediated repression (Fig. 1.9). In particular there is a distinct correlation between both FIS and H-NS binding sites at numerous promoters in *E. coli* and *S.* Typhimurium where FIS has been shown to relieve repression by out-competing H-NS for binding (Stoebel *et al.*, 2008). This is particularly evident at several ribosomal RNA (rRNA) gene promoters where FIS is required to relieve H-NS repression and ensure appropriate expression of rRNA genes (Hillebrand *et al.*, 2005). Overlap also exists between HU and H-NS binding sites where, upon binding, HU is thought to oppose the supercoiling constraint imposed by H-NS by forming more open toroidal supercoiled complexes which facilitate transcription (Dame, 2005). Controlling the availability of H-NS to form nucleoprotein complexes is yet another method by which the bacterium has evolved to modulate H-NS regulated gene transcription (Stoebel *et al.*, 2008). This has been elegantly demonstrated by the recent discovery that the *hns* gene is repressed by the ferric uptake regulator (FUR) protein under conditions of iron starvation in *S.* Typhimurium (Troxell *et al.*, 2011). Binding of FUR within the *hns* promoter region imposes repression and thus reduces the levels of H-NS resulting in the de-repression of the *hilA* master regulator of the SPI-1 virulence genes (Troxell *et al.*, 2011). Finally, a number of truncated paralogues and orthologues of H-NS are known to effect H-NS mediated repression. In general, they resemble the N-terminal domain of H-NS and have no DNA binding activity (Stoebel *et al.*, 2008). Their mode of action therefore is to interfere with or alter the oligomeric properties of H-NS (Stoebel *et al.*, 2008). For

example the *HNS*-T family of proteins have been shown to affect *proU* expression in *E. coli* by interacting with H-NS (Stoebel *et al.*, 2008). Truncated H-NS paralogues or orthologues however do not always have an anti-repressive effect upon H-NS as the Hha and Hha-like proteins in *S.* Typhimurium are thought to confer some specificity upon H-NS for binding to virulence gene promoters (Banos *et al.*, 2009).

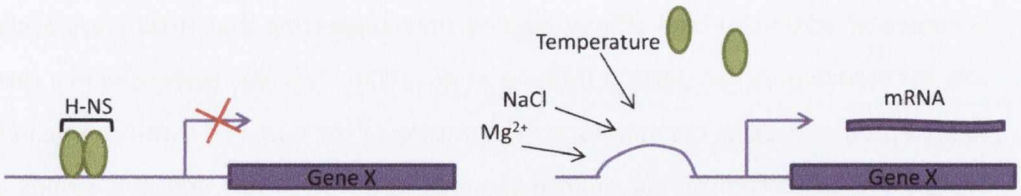
1.5.3.4 Supercoiling and chromosome organisation.

Early research into the pleiotropic effects of *hns* mutations identified a role for H-NS in the modulation of supercoiling. Using reporter plasmids as a means of identifying changes in the negative superhelicity of the chromosome, Higgins *et al* (1988) showed that *E. coli hns* mutants had an increased linking number indicative of relaxation of DNA supercoils. The opposite effect, however, was observed in *S.* Typhimurium where an *hns* mutant resulted in a linking number deficit. These species differences were attributed to differences in the nature of the *hns* mutations but importantly suggested that H-NS played a role in the modulation of global supercoiling levels in both species (Higgins *et al.*, 1988, Hulton *et al.*, 1990). Work in *S.* Typhimurium then demonstrated using a series of truncated *hns* mutants that depending on the nature of the mutation a range of superhelicities were achievable (Hinton *et al.*, 1992). Thus the overall structural composition of H-NS appeared to be important for the modulation of global DNA supercoiling. Two subsequent studies demonstrated that the mode of action of H-NS was to constrain negative DNA supercoils within plasmid and chromosomal DNA (Fig. 1.10 A) (Mojica & Higgins, 1997, Tupper *et al.*, 1994). As a result of the ubiquitous nature of H-NS binding throughout the chromosome and its ability to constrain DNA supercoils, much research has focused on the role of H-NS in chromosome organisation. The over-expression of *hns* in *E. coli* is known to produce highly condensed chromosomes (Spurio *et al.*, 1992). In agreement with this finding, it was recently shown using λ DNA that upon binding, H-NS pre-dominantly induced right handed plectonemic supercoils which served to condense the DNA polymer

Fig. 1.9 Mechanisms for removal of H-NS mediated repression.

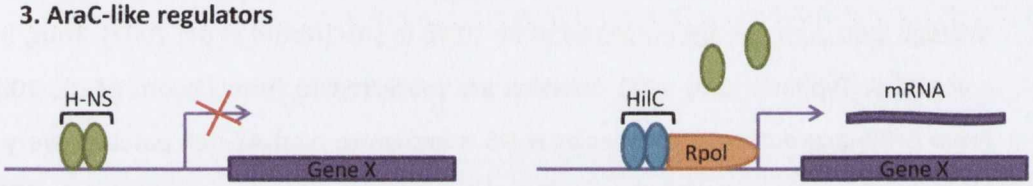
Diagram summarizing the primary methods evolved by *Enterobacteria* to alleviate H-NS gene repression (Stoebel *et al.*, 2008). (1) Changes in prevailing environmental conditions, such as temperature, can alter promoter DNA topology so that H-NS can no longer bind. (2) Transcriptional regulators such as VirB antagonize H-NS repression by remodeling DNA within the H-NS-DNA complex. (3) Certain AraC-like transcriptional regulators both remove H-NS bound at promoter regions and contact RNA polymerase to act as transcriptional enhancers. (4) The LeuO protein acts as a boundary protein to prevent H-NS nucleation. (5) Competition for overlapping binding sites with other NAPs, when present in the cell, can remove H-NS repression.

1. Environmental alterations



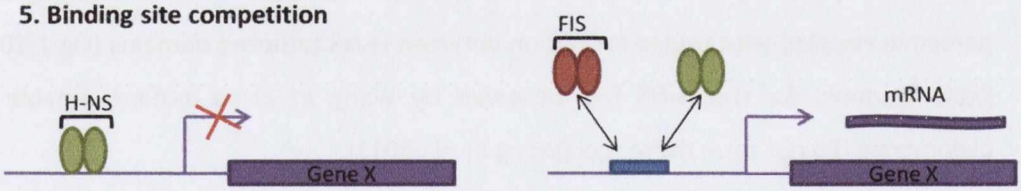
2. DNA remodelling

3. AraC-like regulators



4. Prevention of nucleation

5. Binding site competition



(Maurer *et al.*, 2009). Furthermore, the level of condensation was enhanced by the presence of additional high-affinity binding sites suggesting that H-NS plays a major role in chromosome compaction (Maurer *et al.*, 2009). This was evidenced in a recent study aimed at imaging the structural organisation of the bacterial chromosome in live bacteria. Major chromosomal re-arrangements occurred in *hns* deficient strains and gave rise to less compacted nucleoids (Wang *et al.*, 2011). Interestingly, Wang *et al* also observed that all H-NS appeared to localise within two clusters in live cells despite having multiple binding sites throughout the chromosome (Wang *et al.*, 2011). As mentioned in Section 1.3.1 the bacterial nucleoid is organised into loop domains which emanate from a central core in a rosette like fashion. Several studies have implicated a role for H-NS in tethering these loop domains (Dorman, 2006). On average loop domains are predicted to be 10 kb in size (Noom *et al.*, 2007). Thus, in *E. coli* and *S.* Typhimurium, ≈400 domains are predicted to form (Noom *et al.*, 2007). From CHIP-chip data in both species H-NS is known to bind AT-rich patches every 11 kb in good agreement with the predicted size of loop domains (Noom *et al.*, 2007). Assuming bridge formation between H-NS bound regions to form a loop, it was thus predicted that H-NS may serve as a so called 'domainin' protein (Noom *et al.*, 2007). H-NS also fulfils several criteria thought to be expected of a domainin protein as the loss of *hns* results in relaxation and de-condensation of the chromosome, renders the bacterium sensitive to DNA damaging agents and certain cell cycle parameters such as replication synchrony are affected (Hardy & Cozzarelli, 2005). H-NS functioning as a domainin coupled with bridge formation between H-NS tethered domains (Fig 1.10 B) likely accounts for the H-NS foci observed by Wang *et al* as mutants unable to oligomerise did not form these foci (Wang *et al.*, 2011).

1.5.4 Expression of *hns* in *E. coli* and *S.* Typhimurium

The expression pattern of *hns* has been well characterised in both *E. coli* and *S.* Typhimurium. In both organisms expression of *hns* mRNA is maximal during exponential growth when cells are rapidly dividing before levels decline rapidly upon

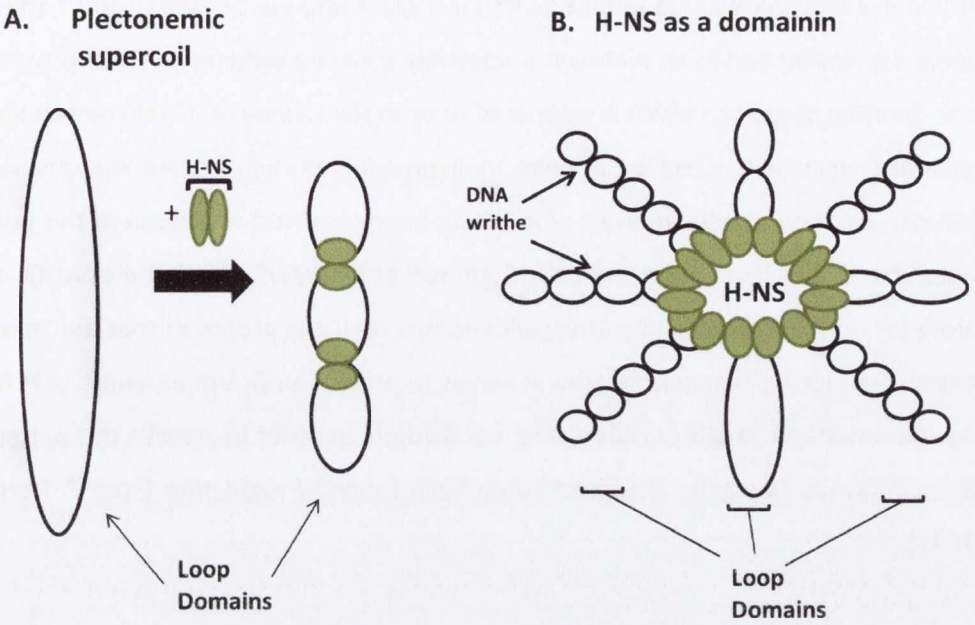


Fig. 1.10 Roles played by H-NS in bacterial chromosome organization.

H-NS is involved in both local and global chromosome organization. (A) Binding of H-NS within loop domain regions introduces right-handed plectonemic supercoils which condense the DNA polymer. (B) H-NS has also been proposed to act as a domainin protein, tethering each loop domain to comprise the central core of the observed rosette-like chromosome structure.

entry into stationary phase (Free & Dorman, 1995, Hinton *et al.*, 1992). This pattern of gene expression serves to maintain a relatively constant amount of H-NS protein for the duration of growth which is estimated to be in the vicinity of 20,000 molecules per genome equivalent. Despite a peak in expression during exponential phase, no obvious increase in cellular levels of H-NS has been observed as a result of this peak of gene expression (Free & Dorman, 1995, Hinton *et al.*, 1992). Given the essentiality of H-NS for normal chromosome structure and function, it is proposed that the 'spike' in expression during exponential growth serves to provide sufficient amounts of H-NS to both mother and daughter cells during cell division. In order to achieve this pattern of expression the *hns* gene is subject to multiple forms of regulation (Free & Dorman, 1995).

1.5.4.1 Growth phase regulation.

The characteristic peak of *hns* expression during exponential growth has been attributed to the transcriptional coupling of *hns* to DNA synthesis (Free & Dorman, 1995). In *E. coli* the temperature sensitive *dnaC*^{ts} mutant, which grows normally at 30°C, is incapable of DNA replication at the non-permissive temperature of 42°C (Free & Dorman, 1995). Using this mutant strain, Free and Dorman (1995) showed that upon shifting exponentially growing cultures from 30°C to 42°C, transcription of the *hns* gene progressively declined to about 30% that of the starting level within 60 min (Free & Dorman, 1995). Upon returning cultures to 30°C both DNA replication and expression of *hns* re-initiated and *hns* transcript levels progressively returned to levels comparable with a wild-type control (Free & Dorman, 1995). This coupling of expression with DNA replication was again validated by a second method whereby DNA replication was inhibited by the addition of nalidixic acid (Free & Dorman, 1995). When both protein synthesis and DNA synthesis were simultaneously inhibited no 'shutting down' of *hns* transcription was observed, suggesting the involvement of a protein factor (Free & Dorman, 1995). This protein factor was H-NS itself which was

inhibiting transcription of its own promoter as had been seen in other studies (See section 1.5.4.3).

1.5.4.2 Environmental regulation.

Under standard laboratory growth conditions bacterial cultures are routinely inoculated from nutrient starved stationary phase cultures. Inoculation into fresh nutrient rich media thus constitutes a nutritional up-shift which was shown to result in a brief spike in cellular levels of H-NS (Fig. 1.11) (Laurent-Winter *et al.*, 1995). Using 2-D gel electrophoresis to analyse S^{35}-labelled extracts from wild-type *E. coli* total protein extracts Laurent-Winter and co-workers observed an increase in the cellular levels of H-NS as soon as 10 min after a nutritional up-shift (Laurent-Winter *et al.*, 1995). H-NS levels then continued to rise, reaching peak protein levels after 60 min before decreasing to normal cellular levels for the remainder of growth (Laurent-Winter *et al.*, 1995).

A drop in growth temperature from 37°C to 10°C temporarily shuts down transcription, inhibit growth and induce the cold-shock response (Fig. 1.11) (Phadtare & Severinov, 2010). As part of this response, bacteria such as *E. coli* undergo an acclimation period during which time a limited number of genes are up-regulated, cell membrane structures are altered and the cellular translational apparatus is modified to translate cold-shock induced mRNA molecules preferentially (Phadtare & Severinov, 2010, Giuliodori *et al.*, 2004). The *hns* gene was identified as one of 13 genes in *E. coli* whose expression was induced during cold-shock conditions (La Teana *et al.*, 1991). Upon shifting cultures of *E. coli* from 37°C to 10°C, La Teana and co-workers observed a ≈4-fold increase in the cellular levels of both *hns* mRNA and H-NS protein after 2 – 4 h at 10°C during the cold acclimation period (La Teana *et al.*, 1991). This increase in *hns* expression was dependent on a 110-bp fragment of the *hns* promoter region which was demonstrated to bind the cold-shock transcriptional master regulator, CspA (La Teana *et al.*, 1991). In addition, a subsequent study showed that the *hns* mRNA

molecule, along with other cold-shock induced mRNAs, was more stable and was preferentially translated at 10°C. The reasons accounting for the increased stability of cold-shock mRNA molecules remain unknown (Phadtare & Severinov, 2010). It is thought however to involve elements of the 5' untranslated region (Giuliodori *et al.*, 2004). Preferential translation of cold-shock mRNA at 10°C involves modification of the translational apparatus of which an increased amount of the IF3 constituent was shown to have the greatest affect (Giuliodori *et al.*, 2004).

1.5.4.3 Regulation by transcription factors.

The *hns* gene is subject to negative auto-regulation in both *E. coli* and *S.* Typhimurium (Fig. 1.11). In both species Falconi *et al* demonstrated that a promoterless *cat* gene when fused with a 400 bp (-313 bp - +87 bp) region of the *hns* gene was significantly repressed in both species (Falconi *et al.*, 1993). No repression of the *cat* gene fusion however was observed in *hns* mutant derivatives (Falconi *et al.*, 1993). Shortening the fusion construct to contain only a 110 bp fragment of *hns* effectively abolished any repressive effect and indicated that H-NS mediated auto-repression involved sequences located between -313 bp and -13 bp of the *hns* promoter (Falconi *et al.*, 1993). Further investigation revealed that H-NS bound extensively at two sites located either side of a DNA bend centred at -150 bp (Falconi *et al.*, 1993). Once bound to these sites a third site located between -20 bp and -65 bp was then occupied which was speculated to be the primary cause of repression due to its overlap with the -35 element (Falconi *et al.*, 1993).

One of the major contributing factors to the peak of H-NS expression during exponential growth is binding of the FIS protein within the *hns* promoter region (Fig. 1.11) (Falconi *et al.*, 1996). A total of seven FIS binding sites (F1 – F7) were identified within the *hns* regulatory region spanning the region from -296 bp to +50 bp (Falconi *et al.*, 1996). Importantly, the F4 and F5 sites centred at -118 bp and -174 bp, respectively, overlap with the two known H-NS binding sites located either side of the

DNA bend at -150 (Falconi *et al.*, 1996). Falconi *et al* showed that FIS could compete for binding to these sites with H-NS and, in a concentration-dependent manner, could displace H-NS (Falconi *et al.*, 1996). *In vitro* transcription assays demonstrated that FIS binding to these sites relieved H-NS repression and resulted in a 2- to 3-fold increase in *hns* expression (Falconi *et al.*, 1996). Similar assays showed that the remaining FIS binding sites were either not involved in or were insufficient for transcriptional stimulation (Falconi *et al.*, 1996).

As mentioned above (Section 1.5.4.2) *hns* is part of the cold-shock regulon and becomes up-regulated upon temperature down-shifts (La Teana *et al.*, 1991). La Teana *et al* identified a 110 bp region of the *hns* promoter spanning the region between -55 bp and +55 bp and the cold-shock regulatory protein CspA as being responsible for the observed up-regulation of *hns* during cold-shock but could not identify a specific binding site for CspA (La Teana *et al.*, 1991). Several possibly regulatory features of the 110 bp fragment have since been identified including two 4 bp GC clamps located between -5 bp and +13 bp thought to be involved in cold-shock regulation (Giangrossi *et al.*, 2001). Giangrossi *et al* have proposed that CspA binding within the *hns* promoter region serves to weaken these GC clamps which under normal circumstances would be inhibitory to transcription (Giangrossi *et al.*, 2001).

In *S.* Typhimurium the *hns* promoter has acquired a new species-specific regulatory input. Troxell *et al* identified *hns* as part of a set of genes up-regulated in the absence of the Ferric Uptake Regulator (FUR) protein (Troxell *et al.*, 2011). For a number of years strains lacking the *fur* gene were known to be avirulent due to significant repression of the SPI-1 master regulator *hilA* in the absence of functional FUR protein (Troxell *et al.*, 2011). The exact mechanism by which FUR was involved in the regulation of *hilA*, however, was unknown (Troxell *et al.*, 2011). As the *hilA* gene is known to be regulated by H-NS, Troxell *et al* postulated that FUR may act indirectly by controlling H-NS levels to repress virulence gene activation. Indeed, *hns* expression was >14-fold higher in the absence of FUR (Troxell *et al.*, 2011). A putative 28-bp FUR binding site was then identified within the *hns* promoter region between -385 bp and -

357 bp which when removed resulted in repression of *hilA* (Troxell *et al.*, 2011). Thus, under conditions which favour FUR activation (presence of iron, anaerobiosis), FUR binds to and represses the *hns* promoter. The resultant decrease in H-NS protein levels then allows transcription of *hilA* and activation of the SPI-1 gene locus (Troxell *et al.*, 2011).

1.5.4.4 Post-transcriptional regulation.

Riboregulation by small non-coding regulatory RNA (sRNA) molecules has been intensively studied in recent years (Papenfort & Vogel, 2009). sRNA molecules are short RNA sequences which interact with mRNA molecules to stabilise and enhance their translation or target them for degradation (Gottesman *et al.*, 2006). They are normally transcribed antisense to the mRNA sequence which they regulate or are located within intergenic regions (Gottesman *et al.*, 2006). The DsrA sRNA targets both the *hns* and *rpoS* mRNA molecules in *S.* Typhimurium and *E. coli* (Lease *et al.*, 1998). The DsrA molecule forms a structure consisting of three stem loops of which loop 1 is required for interaction with *rpoS* while loop 2 interacts with *hns* mRNA (Lease *et al.*, 1998). DsrA interacts with *hns* mRNA via complimentary RNA-RNA base pairing and upon binding DsrA enhances degradation of *hns* mRNA (Fig. 1.11) (Lease *et al.*, 1998). Over-expression of DsrA results in a 2-fold decrease in the cellular levels of *hns* mRNA and in the absence of continued *hns* expression DsrA can decrease the half-life of *hns* mRNA 8-fold (Lease & Belfort, 2000a, Lease *et al.*, 1998).

Translation of *hns* mRNA has also been shown to be influenced by the presence of polyamines (Terui *et al.*, 2007). Polyamines such as putrescine, spermidine and spermine are ubiquitous in prokaryotes and eukaryotes where they play regulatory roles in cell growth. In *E. coli* they have been shown to enhance the translation of numerous mRNA molecules under defined sets of growth conditions (Terui *et al.*, 2009). For example in response to heat-shock, translation of the *fis*, *rpoS*, *oppA* and *fecI* mRNA molecules was enhanced in the presence of putrescine (Terui *et al.*, 2009). In general polyamines target mRNA molecules with sub-optimal translation initiation

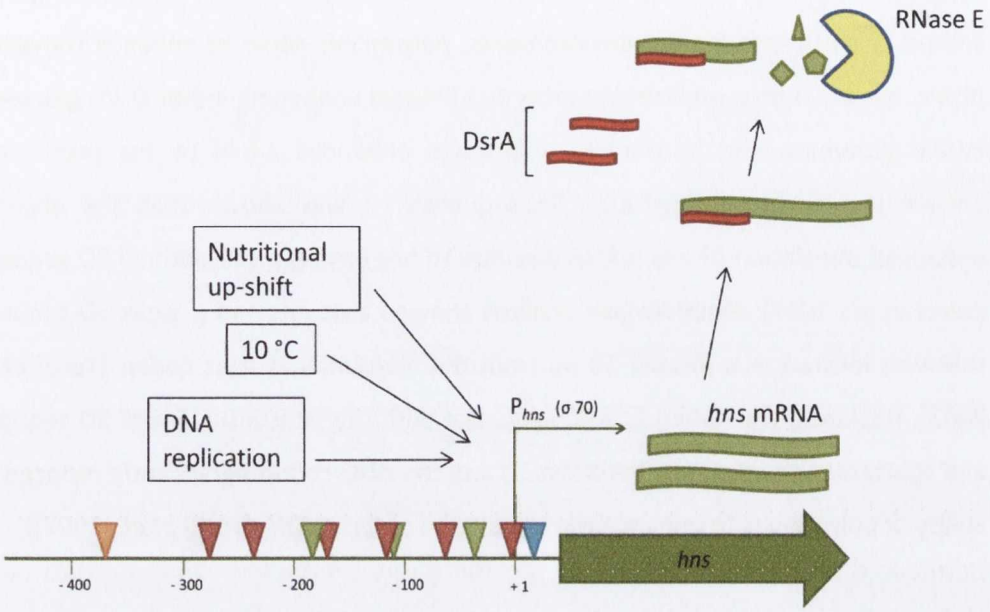


Fig. 1.11 Regulation of *hns* expression.

Diagram summarizing the factors involved in regulating *hns* gene expression. Environmental cues such as cold-shock and nutritional up-shifts stimulate *hns* expression. Expression is also proportional to the rate of DNA replication. Several transcription factors (coloured triangles) bind within the *hns* promoter region to influence transcription. FIS (maroon triangle) and CspA (blue triangle) act as transcriptional enhancers while H-NS (green triangle) and FUR (orange triangle) repress *hns* transcription. The *hns* mRNA molecule (green ribbon) is also post-transcriptionally regulated by the DsrA sRNA (red ribbon). Complementary base pairing between *hns* mRNA and DsrA serves to target *hns* mRNA for degradation.

signals such as distal spacing between the Shine-Delgarno sequence and the AUG start codon or mRNAs containing inefficient start codons (Terui *et al.*, 2007). In glucose limited or glutamate limited environments, polyamines serve to enhance translation of *hns* mRNA. During exponential growth, in media containing either 0.1% glucose or 0.02% glutamate, the translation of *hns* was enhanced 2-fold by the presence of polyamines (Terui *et al.*, 2007). Subsequently, it was shown that the observed enhanced translation of *hns* mRNA was due to *hns* having a sub-optimal SD sequence (Terui *et al.*, 2007). Bioinformatic analysis showed that *hns* had a weak SD sequence that was located at a distally 10 bp from the translational start codon (Terui *et al.*, 2007). Replacing the native *hns* SD sequence with the consensus *E. coli* SD sequence and shortening the distance between SD and the AUG codon significantly reduced the ability of polyamines to enhance the translation of *hns* mRNA (Terui *et al.*, 2007).

1.6 H-NS-like proteins

1.6.1 Suppressor of mutant *td*⁻ phenotype (StpA).

Soon after the discovery of H-NS, the sequence of a gene located at 58 min on the *E. coli* chromosome was published that encoded a protein predicted to share 58% amino acid sequence identity with H-NS (Fig. 1.12) (Zhang & Belfort, 1992). The sequence was identified from a restriction digest library based on its ability to suppress the splicing defective phenotype of the plasmid-borne *td*⁻ gene from bacteriophage T4 (Zhang & Belfort, 1992). A subsequent plasmid-based screen looking for genes whose protein product could complement *hns* mutations by maintaining repression of the *adi* locus then identified *stpA* as one of two genes whose product could compensate for the *hns* deficit (Shi & Bennett, 1994). Over-expression of *stpA* in the *hns* mutant background decreased expression of the *adi* gene locus 10-fold but could not fully compensate for the loss of *hns* as plasmids bearing a copy of *hns* repressed *adi* more efficiently (Shi & Bennett, 1994). Further analysis revealed that the *stpA* locus encoded a 134 amino acid protein, now called StpA, of 15.4 kDa in size. StpA was again predicted to have ≈ 58% amino acid sequence identity with H-NS and like H-NS, was

```
H-NS
MSEALKILNNIRTLRAQARECTLETLEEMLEKLEVVVNERREEESAAAAEVEERTRKLQQYREMLIAD
StpA
MNLMLQNLNNIRTLRAMAREFSIDVLEEMLEKFRVVTKERREEELQQRQLAEKQEKINAFLELMKAD
*.  *: ********* *** :::.*******:.**.:******.    :: *: .*:: : *:: **

H-NS
GIDPNELLNSMAAAKSGTKAKRAARPAKYSYVDENGETKTWTGQGRTPAVIKKAMEEQGKQLEDFLIKE
StpA
GINPEELF-AMDSAMPRSAKKRQPRPAKYRFTDFNGEEKTWTGQGRTPKPIAQALA-AGKSLDDFL--I
**:*:**: :* :* . :  ** .***** :.* *** **********  * :*:   **.*:***
```

Fig. 1.12 Alignment of the H-NS and StpA amino acid sequences.

H-NS and StpA share 58% amino acid sequence conservation. The 137 amino acid residues comprising H-NS were aligned with the 133 amino acids of StpA in a pairwise fashion using the T-Coffee alignment tool (Di Tommaso *et al.*, 2011). Residues common to both proteins are highlighted in red and are marked with an asterisk. Colons indicate conservation between residues of strongly similar properties. Periods indicate conservation between residues of weakly similar properties.

also a basic protein (pI ≈ 9) (Shi & Bennett, 1994). Coupled with its functional overlap with H-NS in repressing *adi*, StpA was termed an H-NS-like protein (Shi & Bennett, 1994).

1.6.1.1 Structure and functional overlap with H-NS.

In light of the identified similarities between H-NS and StpA, much work ensued to determine the extent of their structural and functional overlap. An intricate relationship governing expression of these two genes was first revealed. Using truncated mutants Zhang *et al* showed that *stpA* transcript levels were increased in *hns* mutant strains indicating that, in addition to repressing its own gene expression, H-NS also repressed expression of *stpA* (Zhang *et al.*, 1996). Furthermore, in an *hns/stpA* double mutant both *hns* and *stpA* transcript levels were found to increase. Thus, both the *hns* and *stpA* genes were subject to negative auto-regulation and cross-regulation by H-NS and StpA, respectively (Zhang *et al.*, 1996). Zhang *et al* also showed that StpA mimicked the effect H-NS had on transcription from a synthetic *gal* promoter *in vitro* (Zhang *et al.*, 1996). Since then numerous examples of cross-regulation of similar genes have been published. Sonden and Uhlin showed that over-expression of StpA in an *hns* mutant background decreased the expression of the *hns*, *pap*, *proU* and *bgl* loci, all of which are normally repressed in the presence of H-NS (Sonden & Uhlin, 1996). Two-dimensional electrophoresis experiments conducted in *hns*, *stpA* and *hns/stpA* mutant backgrounds also demonstrated significant overlap in the regulatory capacities of H-NS and StpA as each could substitute for each other in the regulation of a total of 16 identified genes (Zhang *et al.*, 1996).

1.6.1.2 Conserved domain structures.

Taking cross-regulation of *proU* and the synthetic *5A6AgalP1* promoter as a read-out of H-NS and StpA activity, Williams *et al* generated a series of dominant negative derivatives to probe for further structural and functional similarities (Williams *et al.*,

1996). At both promoters the presence of a dominant negative StpA derivative reduced the effectiveness of multicopy StpA repression (Williams *et al.*, 1996). This indicated that, similar to H-NS, StpA was likely to form homodimeric complexes *in vivo*, the formation of which was impaired by the dominant negative derivative (Williams *et al.*, 1996). Furthermore, both H-NS and StpA dominant negative derivatives could interfere with the activity of either full length protein to reduce their repressive effect on *proU*. This suggested that both H-NS and StpA could form both homodimeric and heterodimeric complexes (Williams *et al.*, 1996). The formation of hybrid H-NS-StpA complexes was confirmed *in vitro* by chemical cross-linking. Coupled with mapping of the mutations leading to the dominant negative phenotypes, Williams *et al* proposed that StpA was also comprised of a two domain structure (Williams *et al.*, 1996). Like H-NS, StpA was thought to have an N-terminal oligomerisation domain responsible for both self- and hetero- interactions and a C-terminal nucleic acid binding domain (Williams *et al.*, 1996).

Two follow-up studies confirmed this proposed two domain structure of StpA genetically and biochemically. Firstly, during the characterisation of H-NS it was observed that truncated forms of H-NS which lacked the DNA binding domain were still capable of repressing expression of the *bgl* locus in *E. coli* (Ueguchi *et al.*, 1996). Free *et al* showed that this was due to the presence of StpA as in a *stpA* deletion mutant the *bgl* locus was de-repressed. It was thus proposed by Free *et al* that StpA acted as a molecular adaptor to target H-NS to the *bgl* promoter via its C-terminal DNA binding domain (Free *et al.*, 2001). Using partial proteolysis coupled with mass spectrometry Cusick and Belfort conclusively showed that StpA was comprised of two distinct domains connected by a flexible linker region (Cusick & Belfort, 1998). Both StpA and H-NS had equivalent patterns of proteolysis leading to the isolation of a 6 kDa C-terminal domain containing the amino acid residues 91 – 134 of StpA and 91 – 137 of H-NS (Cusick & Belfort, 1998). Each domain was linked by a protease sensitive linker region to an N-terminal domain which was unable to be purified (Cusick & Belfort, 1998). Furthermore, Cusick and Belfort also showed that the isolated C-

terminal domain of StpA was responsible for interactions with nucleic acids (Cusick & Belfort, 1998).

1.6.1.3 Protein-protein interactions.

Heteromeric interactions between H-NS and StpA were observed by Johansson and Uhlin who identified a spontaneous mutation in the *stpA* coding sequence which could restore long-term stationary phase viability in cultures of *E. coli* strains lacking the *hns* and *rpoS* genes (Johansson & Uhlin, 1999). The mutation which mapped to codon 21 gave rise to increased cellular levels of StpA without a concurrent increase in *stpA* expression suggesting that it served to stabilise the StpA protein (Johansson & Uhlin, 1999). Subsequent investigation revealed that the wild-type StpA protein was subject to proteolytic degradation by the Lon protease and that the isolated mutation abolished this sensitivity (Johansson & Uhlin, 1999). Furthermore, the sensitivity of wild-type StpA to Lon degradation was significantly increased in the absence of functional H-NS leading the authors to hypothesis that StpA primarily existed as a heterodimer with H-NS which served to protect it from proteolysis (Johansson & Uhlin, 1999). This was confirmed by Johansson *et al* who showed that in the absence of H-NS, StpA was predominantly found as an oligomer which was sensitive to degradation by Lon whilst the insensitive StpA mutant existed solely in dimeric form (Johansson *et al.*, 2001). In the presence of H-NS, however, wild-type StpA was protected from degradation by the formation of heteromeric complexes with H-NS (Johansson *et al.*, 2001). Interestingly, complex formation required interactions between residues in the N-terminal domain (between 39 and 60) as expected but also between residues 80 and the end of the C-terminal domain suggesting that either the linker or nucleic acid binding domain was also involved in heteromeric interactions (Johansson *et al.*, 2001). As part of a recent comprehensive *in vitro* study Leonard *et al* proved that amino acid residues from 1 – 77 of H-NS and StpA were necessary and sufficient for both self- and hetero-associations (Leonard *et al.*, 2009). Truncated mutant derivatives comprised of the first 77 amino acids of H-NS and StpA were

analysed by size exclusion chromatography and found to form both homo- and hetero-dimers and multimers in a concentration dependent manner. Residues required for multimeric complex formation however were isolated to a proposed α-helical structure between residues 72 and 81 (Leonard *et al.*, 2009). Previous work investigating oligomerisation of H-NS proposed that dimers aligned in a head-to-tail conformation (Smyth *et al.*, 2000). This was also confirmed by Leonard *et al* who showed that upon oligomerisation both homo- and hetero-associated forms were found to align in the proposed head-to-tail format (Leonard *et al.*, 2009).

1.6.1.4 DNA interactions.

Like H-NS, StpA shows a preference for AT rich DNA with intrinsic curvature. Zhang *et al* showed that StpA bound to a similar AT rich sequence with intrinsic planar curvature within the synthetic *5A6AgalP1* promoter as H-NS (Zhang *et al.*, 1996). A preference for similar sequence binding with H-NS was further enhanced by a recent CHIP-chip study which showed StpA co-localised with H-NS throughout the chromosome of wild-type *E. coli* (Uyar *et al.*, 2009). Zhang *et al* showed that StpA could constrain DNA supercoils in negatively supercoiled plasmid DNA in the presence of the DNA relaxing enzyme topoisomerase I and could also introduce negative supercoils into relaxed plasmid DNA in a manner similar to H-NS (Zhang *et al.*, 1996). StpA was also found to share the property of DNA bridging with H-NS (Dame *et al.*, 2005). To examine if StpA could function as a DNA bridging molecule, Dame *et al* pre-incubated pUC19 plasmid DNA with various concentrations of purified StpA protein before visualising complex formation by scanning force microscopy (SFM) (Dame *et al.*, 2005). As a control, similar quantities of H-NS were also incubated with the same plasmid DNA and visualised (Dame *et al.*, 2005). Dame *et al* observed significant bridging of the plasmid DNA by both proteins and estimated that StpA could bridge DNA at a concentration of ≈ one dimer/200 bp (Dame *et al.*, 2005).

1.6.1.5 StpA, a molecular backup?

Studies have shown that StpA can functionally compensate for the loss of *hns* in the regulation of several genes and *hns* related phenotypes (Sonden & Uhlin, 1996, Zhang *et al.*, 1996). Structurally, it is highly similar to H-NS and indeed exhibits similar DNA binding preferences (Zhang *et al.*, 1996, Cusick & Belfort, 1998). Like H-NS it facilitates compaction of DNA by forming protein-DNA bridges and CHIP-chip experiments have shown that it co-localises with H-NS throughout the chromosome (Uyar *et al.*, 2009, Dame *et al.*, 2005). In each case that StpA could compensate for the lack of H-NS, StpA was over-expressed from plasmids (Shi & Bennett, 1994, Sonden & Uhlin, 1996). However, in wild-type *E. coli* cells the *stpA* transcript and StpA protein are almost undetectable but for a transient burst of expression during mid-exponential growth and both are only present at significant levels in *hns* (Free & Dorman, 1997, Zhang *et al.*, 1996, Sonnenfield *et al.*, 2001). From 2D-gel electrophoresis experiments several genes were identified as differentially regulated in an *hns/stpA* double mutant strain but no genes were identified as differentially expressed in mutants deficient for StpA alone (Zhang *et al.*, 1996). In the absence of H-NS, the number of chromosomal sites bound by StpA is also significantly reduced to approximately one-third of those occupied in the presence of H-NS (Uyar *et al.*, 2009). StpA is also partially dependent on having functional H-NS present in the cell to protect it from degradation by the Lon protease (Johansson & Uhlin, 1999). These observations have led to the hypothesis that StpA merely serves as a molecular back-up for H-NS which only properly functions in the absence of H-NS (Zhang *et al.*, 1996, Johansson & Uhlin, 1999). StpA, however, exhibits several unique characteristics suggesting that it and H-NS may have distinct but overlapping biological roles.

1.6.1.6 Unique pattern of expression.

The expression pattern of H-NS has been well characterised and is conserved in both *S.* Typhimurium and *E. coli.* Conversely, StpA displays a markedly different pattern of expression. Initial studies concluded that *stpA* mRNA was undetectable in wild-type *E.*

coli cells under the conditions tested (Zhang *et al.*, 1996, Sonden & Uhlin, 1996). Free and Dorman, however, discovered that the *stpA* gene is transiently expressed during exponential growth of wild-type cultures in nutrient-rich L broth (Free & Dorman, 1997). Probing total RNA samples by Northern blotting, Free and Dorman found that the *stpA* transcript could be detected at significant levels in *hns* mutant strains at all times during growth (Free & Dorman, 1997). In wild-type *E. coli* strain CSH50 , however, detection of *stpA* mRNA was restricted to a transient burst found in samples taken 150 min after inoculation, a time corresponding to early exponential growth phase (Free & Dorman, 1997). The levels of *stpA* transcript detected, however, were only one tenth that present in an *hns* mutant (Free & Dorman, 1997). This unique pattern of expression was also conserved in a second wild-type *E. coli* strain, MC4100 (Free & Dorman, 1997). The StpA protein profile during growth was found to complement this growth-phase-specific pattern of gene expression. Azam and Ishihama showed that in concurrence with *stpA* gene expression, StpA protein levels were maximal during early-exponential growth before steadily declining to an almost undetectable level during stationary phase (Ali Azam *et al.*, 1999).

Whilst the regulation of *stpA* and *hns* expression overlapped in terms of negative auto- and cross-repression by each of their protein products, *stpA* also has its own unique set of regulatory inputs (Zhang *et al.*, 1996, Free & Dorman, 1997). Expression of *stpA* was found to be very sensitive to environmental fluctuations (Free & Dorman, 1997). Sonden and Uhlin showed that the *stpA* transcript could be detected at low levels in a wild-type background at 37°C but no transcript could be detected at 26°C (Sonden & Uhlin, 1996). A 332 bp fragment of the upstream regulatory region was found to confer temperature sensitivity (Sonden & Uhlin, 1996). These findings were further supported by Free and Dorman who showed that upon shifting exponentially growing cultures growing at 30°C to 37°C a 4-fold induction of *stpA* transcript levels was achieved after 30 min at 37°C (Free & Dorman, 1997). Thus, in opposition to the cold-shock induction of *hns* transcription, *stpA* expression is induced by thermal up-shifts. Free and Dorman also observed a 50-fold induction of *stpA* expression when

exponential phase cultures growing in media lacking NaCl were osmotically shocked by transfer to media containing 0.4 M NaCl (Free & Dorman, 1997). A 4-fold induction of *stpA* expression was also observed during carbon starvation (Free & Dorman, 1997). Both of the above studies identified a role for the global regulatory protein LRP in the regulation of *stpA* expression. Sonden and Uhlin found expression of *stpA* to be reduced 2-fold in an *lrp* mutant strain grown at 37°C (Sonden & Uhlin, 1996). Free and Dorman identified a role for LRP in achieving maximal *stpA* expression grown under glucose limiting conditions (Free & Dorman, 1997). Under conditions of glucose starvation the *stpA* transcript could be detected at all growth phases (Free & Dorman, 1997). In an *lrp* mutant strain grown under the same conditions, however, the levels of *stpA* transcript was significantly reduced at every time point sampled (Free & Dorman, 1997).

1.6.1.7 Specific DNA interactions.

Zhang *et al* demonstrated that both H-NS and StpA possessed the ability to constrain negative DNA supercoils on plasmid DNA (Zhang *et al.*, 1996). In addition they also showed that both proteins could introduce negative supercoils into relaxed plasmid DNA (Zhang *et al.*, 1996). StpA, however, was more efficient at introducing negative supercoils into DNA as 2.88 μM of StpA was required to achieve maximal DNA supercoiling compared to 3.84 μM of H-NS (Zhang *et al.*, 1996). Studies investigating the DNA binding properties of StpA have shown that like H-NS it too has a preference for regions with intrinsic planar curvature (Zhang *et al.*, 1996). This was also observed by Sonnenfield *et al* by *in vitro* methods using a restriction digested DNA fragment containing an AT-planar curve (Sonnenfield *et al.*, 2001). Both H-NS and StpA selectively bound to this fragment of DNA but 6-fold more H-NS was required for complete binding than that of StpA (5.9 μM versus 1.0 μM) (Sonnenfield *et al.*, 2001). StpA also bound to the same fragment with a higher affinity than H-NS with a K_d of 0.7 μM compared to 2.8 μM for H-NS (Sonnenfield *et al.*, 2001). Similar observations were made by Dame *et al* whilst determining the DNA bridging capacity of StpA (Dame *et*

al., 2005). The formation of DNA bridges by StpA occurred at significantly lower protein/DNA concentrations for StpA (one dimer/200 bp) than for H-NS (one dimer/24 bp) which was thought to be a reflection of the increased affinity of StpA for DNA (Dame *et al.*, 2005). In a more recent investigation into the DNA bridging properties of StpA, Lim *et al* observed the formation of nucleoprotein filaments in a concentration-dependent manner upon incubation of purified StpA with naked DNA as has been observed for H-NS (Lim *et al.*, 2011, Dame *et al.*, 2005). In the H-NS model of bridge formation two H-NS coated DNA filaments interact by protein-protein interaction to form a bridge between the strands (Maurer *et al.*, 2009). Lim *et al*, however, showed that StpA nucleoprotein filaments did not have this property but instead bridge formation required only one strand of DNA to be coated with StpA which could then interact with an adjacent naked DNA strand to form a bridge (Lim *et al.*, 2011).

1.6.1.8 RNA interactions of StpA.

StpA was identified during a screening process searching for proteins that could compensate for the splicing defective mutant thymidylate synthase (*td*) gene derived from bacteriophage T4 (Zhang & Belfort, 1992). The *td* gene is a group I intron which *in vivo* and *in vitro* is capable of self-splicing (Doetsch *et al.*, 2010). Upon introducing the splicing defective *td*$^{-}$ mutant pre-mRNA complex into *E. coli* cells over-expressing StpA, an overall 3-fold increase in the level of *td*$^{-}$ pre-mRNA and a 7.4-fold increase in the level of *td*$^{-}$ mRNA was observed. Thus, the over-expression of StpA resulted in a net 2.5-fold increase in the conversion of *td*$^{-}$ pre-mRNA to *td*$^{-}$ mRNA, indicative of RNA chaperone activity (Zhang *et al.*, 1995). This splicing function was not unique to StpA as H-NS was also observed to facilitate splicing when over-expressed (Zhang *et al.*, 1995). H-NS, however, was 8-fold less efficient than StpA at promoting splicing at low concentrations and was unable to achieve equivalent splicing of introns even at 10-fold higher concentrations (Zhang *et al.*, 1995). Furthermore, the rate of splicing enhancement was at least 100-fold higher for StpA than H-NS at equivalent concentrations. Thus, StpA appeared to have a unique ability to act as an RNA

chaperone when compared to H-NS (Zhang *et al.*, 1995). The two basic functions an RNA chaperone should perform are strand-annealing and strand-displacement (Doetsch *et al.*, 2010). In a series of subsequent investigations StpA was found to exhibit both of these properties. StpA was shown to bind preferentially to unstructured regions of two synthetic 21-mer RNA molecules capable of self-annealing (Mayer *et al.*, 2007). In the presence of StpA the rate at which these two molecules annealed increased 4-fold (Mayer *et al.*, 2007). StpA was also demonstrated to enhance the displacement of strands between two pre-annealed 21-mer RNA molecules in the presence of a competitor molecule (Mayer *et al.*, 2007). Similar activity was demonstrated for StpA in the annealing and displacement of strands during splicing of the *td* gene pre-mRNA. Several screens however failed to identify any specific mRNA molecules in *E. coli* in which StpA could perform similar functions (Doetsch *et al.*, 2010). StpA has been shown interact with mRNA molecules in *E. coli* although not in an RNA chaperone capacity as described above (Mayer *et al.*, 2007). The *micF* small regulatory RNA is a known regulator of the major outer membrane porin OmpF. The *micF* sRNA was shown to bind antisense to the *ompF* mRNA and target it for degradation (Deighan *et al.*, 2000). In the absence of StpA, however, this regulatory function was diminished (Deighan *et al.*, 2000). Deighan *et al* showed that this was due to an increased level of *micF* in the absence of StpA (Deighan *et al.*, 2000). Further investigation revealed that StpA acted post-transcriptionally to de-stabiliise the *micF* sRNA in wild-type cells. Thus in its absence *micF* levels were observed to increase (Deighan *et al.*, 2000).

Although thought of as a molecular back-up for H-NS the unique activity of StpA as a possible RNA chaperone hints that StpA may have distinct biological functions independent of H-NS. Its unique pattern of expression means that StpA is only present in the cell in significant amounts to interact with H-NS at distinct times and under certain environmental conditions. Due to its distinct DNA binding properties, however, when present it has the capacity to influence DNA topology and alter global levels of supercoiling with implications for global gene expression.

1.6.1.9 Species specific functions.

The best example of StpA and H-NS having distinct but biologically overlapping roles comes from a recent study conducted in *S.* Typhimurium. In *S.* Typhimurium, StpA was found to have a pattern of expression distinct from that observed in *E. coli* (Lucchini *et al.*, 2009). Similar to *E. coli* a transient peak in expression was observed during early exponential growth which corresponded with maximal StpA protein levels (Lucchini *et al.*, 2009). At every stage of growth, however, both *stpA* mRNA and StpA protein were detected in significant amounts (Lucchini *et al.*, 2009). As higher levels of StpA were present throughout growth this suggested that StpA may have a more defined physiological role in *S.* Typhimurium. Microarray analysis revealed that StpA was indeed more biologically active in *S.* Typhimurium as in a *stpA* deletion strain 5% of the genome was differentially regulated (Lucchini *et al.*, 2009). Most of these genes were also known H-NS regulated genes. However, a distinct sub-set of genes were found to be affected specifically by StpA (Lucchini *et al.*, 2009). Several genes up-regulated during exponential growth in the absence of StpA were genes involved in stress adaptation (Lucchini *et al.*, 2009). This led to the discovery of the first StpA specific phenotypes as the absence of StpA increased the resistance of mid-exponential phase cultures of *S.* Typhimurium when exposed to high salt, low pH, oxidative stress and when challenged with the anti-microbial peptide polymyxin B (Lucchini *et al.*, 2009). Further analysis found that the genes responsible for these phenotypes were all regulated by the stationary phase and stress sigma factor RpoS (Lucchini *et al.*, 2009). Subsequently, it was shown that in the absence of StpA, RpoS was detected during exponential growth and that StpA was responsible for modulating its levels by interacting with the anti-adaptor molecule RssC which binds to and targets RpoS for degradation (Lucchini *et al.*, 2009). StpA was found to have a more direct role in the repression of several *phoP* regulated genes as CHIP-chip data confirmed binding of StpA within the promoter regions of several *phoP* regulated genes found to be differentially expressed in the in the absence of StpA (Lucchini *et*

al., 2009). In addition, StpA was found to indirectly regulate the CRP-cAMP regulon which is responsible for regulating genes required for the uptake and utilisation of non-preferred sugar sources (Lucchini *et al.*, 2009).

1.6.2 Diversity of H-NS-like proteins.

Whilst StpA is the best characterised H-NS-like protein thus far, the increasing availability of complete genome sequences has significantly aided the search for H-NS-like proteins present in other species. To date sequence-based searches have identified numerous H-NS-like proteins in the α, β and γ subdivisions of proteobacteria and in the Gram-positive species *Mycobacterium* (Tendeng & Bertin, 2003, Gordon *et al.*, 2008). H-NS-like proteins identified within the γ-proteobacteria, in general, display a high level of sequence conservation and have been identified on the basis of homology. For example, StpA has been identified in *S.* Typhimurium, *S.* Typhi, *E. coli*, *S. flexneri* and *Citrobacter rodentium*. A number of less well conserved H-NS-like proteins, however, have been identified on the basis of complementation of *hns* phenotypes or a conservation of function with H-NS. In *Bordetella pertussis*, a member of the β proteobacteria, the BpH3 protein was the first H-NS-like protein to be identified outside of the *Enterobacteriaceae* (Goyard & Bertin, 1997). Expression of BpH3 in an *E. coli hns* mutant background was found to restore cellular motility in a concentration dependent manner and activate expression of the flagella gene operons (Goyard & Bertin, 1997). BpH3 can also restore repression of the *bgl* locus and displays a distinct DNA binding preference for curved DNA such as that found at the *flhDC* promoter region (Goyard & Bertin, 1997, Bertin *et al.*, 2001, Bertin *et al.*, 1999). In a sequence based search Bertin *et al* identified a less well conserved H-NS-like protein in the α-proteobacteria species *Rhodobacter capsulatus* (Bertin *et al.*, 1999). The HvrA protein shares only 30% amino acid sequence homology with H-NS yet, like BpH3, HvrA could complement for the loss of *hns* in *E. coli* by restoring motility, maintaining repression of the *bgl* locus and rendering the bacterium susceptible to serine (Bertin *et al.*, 1999). Bertin *et al* then demonstrated that, like H-NS, both HvrA and BpH3 had

global effects on the proteome of *E. coli*. By comparing the proteomes of wild-type *E. coli*, a *hns* mutant derivative and *hns* mutants expressing either HvrA or BpH3 it was observed that both HvrA and BpH3 could partially restore the wild-type proteome (Bertin *et al.*, 1999).

Having shown a high degree of functional overlap between BpH3, HvrA and H-NS, Bertin *et al* then examined the structural overlap of these proteins (Bertin *et al.*, 1999). Results from protein cross-linking experiments demonstrated that both BpH3 and HvrA could crosslink to form protein species which had a two fold increase in molecular mass compared with that of the BpH3 or HvrA monomer (Bertin *et al.*, 1999). This indicated that, like H-NS and StpA, BpH3 and HvrA were also capable of dimer formation (Bertin *et al.*, 1999). Amino acid sequence analysis of the C-terminal domains showed that H-NS and StpA had 70% conservation of residues within this region while BpH3 and HvrA shared only 40% conservation with H-NS (Bertin *et al.*, 1999). *In silico* predications of the C-terminal domain, however, revealed that each had a common core of β-turns and helices (α or 3_{10}) (Bertin *et al.*, 1999). Subsequent gel shift experiments using the *bla* promoter, a region known to be bound by H-NS, showed that at low protein concentrations H-NS, StpA, BpH3 and HvrA were all capable of shifting the *bla* DNA probe (Bertin *et al.*, 1999). Upon increasing protein concentration further shifting was also apparent in agreement with the co-operative binding nature of H-NS and StpA (Bertin *et al.*, 1999). Thus, despite significant sequence divergence, both BpH3 and HvrA still maintain the functionality and structural features of H-NS. Analysis of the C-teminal domain from this study identified a 9 amino acid sequence pattern centred at Gln at position 112 of H-NS which was conserved in all four proteins (Bertin *et al.*, 1999). Using this sequence to search genome databases several more H-NS-like proteins were identified such as VicH from *Vibrio cholera*, the N-terminal domain of which cross-reacted with antibodies raised against H-NS. Also deletion of *vicH* resulted pleiotropic effects on gene expression in *Vibrio cholera* including a loss of motility and mucoid colony morphology (Bertin *et al.*, 1999, Tendeng *et al.*, 2000). Furthermore, like H-NS, VicH was also cold shock

inducible (Tendeng *et al.*, 2000). The DNA bridging capabilities of H-NS-like proteins was investigated by Dame *et al* using the MvaT H-NS-like protein discovered in *Pseudomonas aeruginosa* again owing to its ability to complement *hns* phenotypes (Tendeng *et al.*, 2003, Dame *et al.*, 2005). Scanning-force microscopy showed that MvaT was capable of forming protein-DNA bridges and did so at a ratio of one dimer/160 bp and could also form extended higher order nucleoprotein filaments at a ratio of one dimer/80 bp (Dame *et al.*, 2005). A paralog of MvaT has also been identified in *P. aeruginosa* known as MvaU (Castang *et al.*, 2008). Analogous to H-NS and StpA, both MvaT and MvaU co-localise throughout the chromosome and bind preferentially to AT rich regions (Castang *et al.*, 2008, Lucchini *et al.*, 2009, Uyar *et al.*, 2009). Furthermore, microarray analysis revealed extensive overlap between the MvaT and MvaU regulons which were determined to consist of ≈ 150 genes (Castang *et al.*, 2008).

1.6.3 Plamid encoded H-NS-like proteins.

A recent study aimed at investigating the distribution of plasmid encoded NAPs identified a total of 21 plasmid encoded homologs of H-NS (Takeda *et al.*, 2011). All of the plasmids identified as encoding an H-NS-like protein were restricted to Gram-negative bacteria with the majority belonging to closely related species of the *Enterobacteriaceae* family (Takeda *et al.*, 2011). Whilst many of the plasmids were only recently sequenced or were identified in a particular species whose genome was recently sequenced, several were well characterised plasmids isolated from virulent *Salmonella* strains (Takeda *et al.*, 2011, Wain & Kidgell, 2004). Since the 1970s the presence of a large self-transmissible plasmid belonging to incompatibility group H has been associated with outbreaks of *S.* Typhi, particularly in south and south-east Asia (Wain & Kidgell, 2004, Phan *et al.*, 2009, Phan & Wain, 2008). A defining feature of these plasmids was the presence of a gene encoding an H-NS-like protein (Phan *et al.*, 2009). Such was the propensity for these plasmids to contain an *hns*-like gene that the *hns* gene sequence became an identifier for multi-locus sequence typing of other

plasmids associated with virulence found in strains of *S.* Typhi isolated from different geographical regions (Phan *et al.*, 2009). Of these plasmids the best characterised is R27 whose H-NS-like protein was shown to be required for conjugation of the plasmid between bacterial cells (Forns *et al.*, 2005). R27, like most IncHI plasmids, is temperature-sensitive for conjugation with conjugation optimally occurring at 27^{o}C (Forns *et al.*, 2005). The genes required for conjugation are encoded in two separate operons whose promoters are bound and repressed by the plasmid-encoded H-NS at non-permissive temperatures (Alonso *et al.*, 2005). In terms of structure and function, however, the best characterised of the plasmid encoded H-NS-like proteins comes from a plasmid 99% identical at the DNA sequence level to R27 which was isolated from *Shigella flexneri* 2a strain 2457T (Beloin *et al.*, 2003).

The protein, termed Sfh (*Shigella flexneri* H-NS-like protein) was found to be biologically active and could interact with the host genome by restoring motility, abolishing mucoid colony morphology and maintaining repression of the *proU* and *bgl* loci in an *hns* mutant background (Beloin *et al.*, 2003). Sfh also showed a DNA binding preference for curved DNA and was found to bind within the known H-NS binding regions of the *virF* and *virB* genes which regulate *Shigella* virulence gene expression (Beloin *et al.*, 2003). Like *hns* and *stpA*, the *sfh* gene is subject to negative auto-repression and, despite not being chromosomally located, is negatively cross-regulated by H-NS and StpA (Deighan *et al.*, 2003). Similarly, Sfh was shown to repress both the *hns* and *stpA* gene promoters (Deighan *et al.*, 2003). Using a yeast two hybrid system Deighan *et al* demonstrated that Sfh was capable of N-terminal mediated protein-protein interactions with both H-NS and StpA (Deighan *et al.*, 2003). Furthermore, Sfh exhibited its own unique pattern of expression suggesting the possibility that Sfh may also have distinct but overlapping biological roles (Doyle & Dorman, 2006). Similar to *hns* and *stpA* peak expression of *sfh* occurs during early exponential growth (Doyle & Dorman, 2006). No Sfh protein however was observed until the onset of stationary phase (Doyle & Dorman, 2006). Owing to its properties as an H-NS-like protein, Doyle *et al* examined the effect Sfh had on global gene

expression by conjugating the *sfh*-containing plasmid, pSf-R27 into *S.* Typhimurium and monitoring global gene expression (Doyle *et al.*, 2007). As a control the pSf-R27 plasmid with the *sfh* gene deleted, was also conjugated into *S.* Typhimurium and global gene expression was monitored (Doyle *et al.*, 2007). Surprisingly, the pSf-R27 plasmid alone had little effect on global gene expression but the plasmid devoid of *sfh* resulted in differential regulation of over 400 genes (Doyle *et al.*, 2007). Conjugation of this plasmid also decreased the fitness of *S.* Typhimurium and significantly reduced intracellular survival (Doyle *et al.*, 2007). Analysis of the pSf-R27 plasmid revealed it to have a high AT content which could potentially be attracting binding of H-NS, removing it from its chromosomal location and thus resulting in the observed changes in global gene expression and fitness (Doyle *et al.*, 2007). This was indeed the case as similar fitness results were observed upon introduction of a high copy number plasmid containing a preferred H-NS binding site (Doyle *et al.*, 2007). This led Doyle *et al* to hypothesise that Sfh acted as a stealth factor to selectively silence the AT rich genes of the plasmid and thus enhance conjugation (Doyle *et al.*, 2007). In a later study, Sfh was also shown to have a role in the regulation of chromosomal gene expression (Dillon *et al.*, 2010). Sfh was found to bind at a distinct sub-set of H-NS binding sites throughout the *S.* Typhimurium chromosome and pre-dominantly affected genes encoding chaperones, transcription factors, DNA recombination and repair factors and central metabolism enzymes (Dillon *et al.*, 2010). Importantly, many of the regions bound by Sfh were regions thought to have been acquired by HGT (Dillon *et al.*, 2010). Further sequence binding analysis demonstrated that Sfh preferentially bound to regions with a slightly higher AT content than those preferred by H-NS (Dillon *et al.*, 2010). Thus, like StpA, Sfh appears to have its own distinct regulon within the greater H-NS regulon (Dillon *et al.*, 2010).

Chapter 2 Materials and Methods

2.1 Chemicals and Growth Media

2.1.1 Chemicals and reagents.

All chemicals or reagents used in this study were obtained from one of the following suppliers: Sigma, Fermentas, New England Biolabs, Fischer, Promega, Invitrogen, Oxoid and Qiagen. Specific suppliers are named in parenthesis after the product supplied. Several molecular biological kits were also used in this study, the supplier of which and a brief summary of the principle of each kit are described in the relevant sections below. Custom automated DNA sequencing was performed by GATC-Biotech (Konstanz, Germany).

2.1.2 Growth media

Materials used for the preparation of growth media were sourced from Difco or Oxoid. Prior to use or storage, all media was sterilized by autoclaving at 120 ^{o}C for at least 20 min. Any solutions unsuitable for autoclaving were sterilized by filtration through 0.22-μm Acrodisc Filters (Millipore) or through Stericup Filters (Millipore) for sterilization of volumes up to 250 ml. Growth media was routinely made in 1 litre volumes in analytical grade, deionised water (Analar™, BDH) and supplemented with appropriate antibiotics as required. Media containing agar was allowed to cool to 55 ^{o}C before being supplemented with antibiotics. All quantities listed below are for the preparation of 1 litre volumes unless otherwise stated.

2.1.2.1 Luria-Bertani broth and Luria-Bertani agar

Bacterial strains were routinely grown in nutrient rich Luria-Bertani (LB) broth adjusted to pH 7 unless otherwise stated. Throughout this study LB agar plates were used for culturing strains, reviving strains from freezer stocks, colony counting, and the selection of transductants, transconjugants and recombineering mutants.

LB broth: 10 g Oxoid Tryptone, 5 g Yeast Extract, 10 g NaCl

LB agar: 10 g Oxoid Tryptone, 5 g Yeast Extract, 10 g NaCl, 15 g agar

2.1.2.2 Green agar plates

Green agar plates were used to distinguish between phage-cured transductants and pseudolysogens after infection of *Salmonella* by bacteriophage P22 (See Section 2.4.1). Bacteriophage P22 HT 105/1 *int-201* used in this study is incapable of forming true lysogens but can form pseudolysogens (Schmieger, 1972). Green agar plates contain excess glucose which is fermented by *Salmonella* growing on the plate to produce abnormal amounts of acid. In colonies of pseudolysogens some cells will lyse causing a pH drop in the surrounding media. Due to the presence of the pH indicators Alizarin Yellow (Sigma) and Aniline Blue (Sigma) in the media, pseudolysogens appear dark blue-green in colour while phage-cured colonies are light green.

Green Agar Base: 8 g Oxoid tryptone, 1 g yeast extract, 5 g NaCl, 15 g agar

Prior to autoclaving 62.5 mg of Alizarin yellow was added to the Green Agar Base. After autoclaving the media was allowed to cool to 55 ^{o}C before 0.33 ml of 2 % (w/v) aniline blue, 4.2 ml of 20 % (v/v) glucose solution and appropriate antibiotics were added.

2.1.2.3 Motility agar plates

The chemotaxis and motility of bacterial strains was measured on motility agar (swarm) plates. Motility agar contains a low concentration of agar which allows bacteria to migrate or swarm outwards from an innoculum focus. The distance migrated from the focus per unit time is calculated and used as a measure of the chemotaxis and motility of the bacterial strain (Macnab, 1986).

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

2.1.2.4 MacConkey maltose agar plates

MacConkey maltose plates are a variation of the MacConkey lactose plates commonly used for assaying the production of the enzyme β-galactosidase. In this study, MacConkey maltose plates were used to assay the capability of bacterial strains with altered H-NS and H-NS-like protein populations to metabolism maltose. The pH indicator phenol red is a constituent of MacConkey base and turns red under acid conditions. As such colonies capable of metabolising the sugar appear red due to the production of acidic metabolic by-products. Colonies incapable of metabolising maltose or colonies with a low level of maltose metabolism appear white or pale pink.

MacConkey maltose agar: 40 g Difco MacConkey agar base, 10 g maltose, 15 g agar

2.1.2.5 L top agar

L top agar contains a low concentration of agar and was used to evenly distribute bacterial cells over the surface of LB agar plates. L top agar was used for all fitness assays and enumeration of colony forming units in this study where colony counting was employed. Diluted suspensions of bacterial cells were routinely added to 3 ml of L top agar, mixed and then evenly dispersed over the surface of LB agar plates.

L top agar: 10 g Difco tryptone, 5 g yeast extract, 5 g NaCl, 7 g agar

2.1.2.6 Minimal agar

Minimal agar is a nutrient poor medium which was used to minimise the risk of growth of contaminant organisms on LB plates. For all fitness assays and assays where colony counting was employed, 3 ml of minimal agar was layered on top of the bacteria containing L top layer (See Section 2.1.2.5). Any contaminant organisms that made contact with the plates were then less likely to grow on the nutrient poor upper layer.

Minimal agar: 12 g Difco Bacto-agar

2.1.3 Antibiotics

Stocks of antibiotic solutions were maintained in aliquots and stored at – 20 °C. All stocks were made at 1000 x concentration. Those made in water were filter sterilized using 0.22 µm Acrodisc Filters (Millipore). Kanamycin, carbenicillin, nalidixic acid and spectinomycin were prepared as 50 mg ml^{-1} stock solutions in Analar™ analytical grade water and used at a final concentration of 50 µg ml^{-1}. Gentamicin was prepared as 15 mg ml^{-1} stock in Analar™ analytical grade water and used at a final concentration of 15 µg ml^{-1}. Tetracycline and chloramphenicol were prepared as 12 mg ml^{-1} and 20 mg ml^{-1} stocks in 100 % ethanol and used at a final concentration of 12 µg ml^{-1} and 20 µg ml^{-1} respectively. Rifampicin was prepared no longer than one hour prior to use as a 50 mg ml^{-1} stock in 100 % methanol and was used at a final concentration of 250 µg ml^{-1}.

2.2 Bacterial strains and culture conditions

2.2.1 Bacterial strains

All bacterial strains used in this study were derivatives of *Salmonella enterica* serovar Typhimurium strain SL1344 (Hoiseth & Stocker, 1981). The name, genotype and source of each strain are listed in Table (Table 2.1). Bacterial strains were maintained as permanent stocks in 20 % (v/v) glycerol in LB - broth and stored at – 80 °C.

2.2.2 Bacterial culture conditions

Bacterial cultures were typically grown at 30 °C or 37 °C in a shaking incubator (200 r.p.m) under aerobic conditions unless otherwise stated. For overnight cultures, single colonies were inoculated into 3 ml of LB-broth in sterile test-tubes and grown for 12 – 16 h at the appropriate temperature. Sub-cultures for sampling throughout growth were grown by inoculating ≈ 1 x 10^{-7} bacterial cells from overnight cultures into 25 ml of fresh LB-broth in a 250 ml conical flask. Growth of bacterial cultures was monitored by measuring the optical density (OD_{600}) of the culture by spectrophotometry (See Section 2.5.1). For all bacterial fitness experiments, overnight cultures were grown by

Table 2.1 Table of bacterial strains.

Strain or Plasmid	Relevant characteristic	Resistance	Source/reference
***Salmonella enterica* sv. Typhimurium SL1344**			
SL1344	*rpsL hisG46*		Hoiseth and Stocker(1981)
hns-flag	*hns::*3xFLAG tag	Km^R	This work
stpA-HA	*stpA*::HA tag	Cm^R	This work
Δ*hns*	Δ*hns* transduced from JH4000	Km^R	Ono *et al,* (2005)
Δ*stpA*	Δ*stpA*::*kan*	Km^R	This work
$SL1344^{2xhns}$	P_{stpA}-*hns*	Km^R	This work
$SL1344^{2xstpA}$	P_{hns}-*stpA*	Tc^R	This work
$SL1344^{swap}$	P_{stpA}-*hns* P_{hns}-*stpA*	Km^R Tc^R	This work
$SL1344^{2xhns\ 3xFLAG}$	P_{stpA}-*hns-3xFLAG*	Km^R	This work
$SL1344^{2xstpA\ 3xFLAG}$	P_{hns}-*stpA-3xFLAG*	Tc^R	This work
$SL1344^{swap}$ *hns*-FLAG	P_{stpA}-*hns-3xFLAG* P_{hns}-*stpA*	Tc^R	This work
proU-lacZ	*lacZYΔlacA::cat* fused with *proV* at + 610 bp	Cm^R	This work
$SL1344^{2xhns}$*proU-lacZ*	*proU-lacZ* fusion P22 tranduced into $SL1344^{2xhns}$	Km^R Cm^R	This work
$SL1344^{2xstpA}$*proU-lacZ*	*proU-lacZ* fusion P22 tranduced into $SL1344^{2xstpA}$	Tc^R Cm^R	This work
$SL1344^{swap}$ proU-lacZ	*proU-lacZ* fusion P22 transduced into $SL1344^{swap}$	Km^R Tc^R Cm^R	This work
Δ*lon*	Δ*lon::cat*	Cm^R	This work
Δ*lon stpA*-HA	Δ*lon*::*cat stpA*-HA tag	Cm^R	This work
Δ*lon* Δ*hns*	Δ*hns* transduced from JH4000 Δ*lon::cat*	Cm^R Km^R	This work
Δ*cspA*	Δ*cspA*::*kan*	Km^R	Dillon and Dorman, unpublished
Δ*fis*	Δ*fis*::*kan*	Km^R	Cameron and Dorman (2011)
Eschericia coli K-12			
DH5ά	*supE44 ΔlacU169*(*Φ80lacZΔM15*) *hsdR17* *recA1 endA1 gyrA96 thi-1relA1*		Lab Stocks
***Shigella flexneri* 2A 2457T**			
BS185	BS184 *hns*::Tn10	TcR	

Table 2.2 Table of bacteriophage and plasmids.

Bacteriophage or Plasmid	Relevant Characteristics	Resistance	Source or Reference
Bacteriophage			
P22	HT105/1 *int-201*		Lab Stocks
Plasmids			
pKD46	λ genes *gam*, *bet* and *exo* under the control of the arabinose inducible pBAD promoter	Amp^R	Datsenko and Wanner (2000)
pSUB11	pGP704 derivative containing the Km^R cassette with flanking FRT sites from pKD4 and upstream 3xFLAG-tag sequence		Uzzau *et al* (2001)
pSUB14	pGP704 derivative containing the Cm^R cassette with flanking FRT sites from pKD3 and upstream HA-tag sequence		Uzzau *et al* (2001)
pCP20	Plasmid with yeast Flp recombinase gene, FLP, Cm^R and Amp^R resistant genes, and temperature sensitive replication.		Cherepenov and Wackernagel(1995)

inoculating 25 ml of LB-broth in a 250 ml conical flask directly from freezer stocks of strains using a sterile wire loop.

2.3 Plasmids, bacteriophage and oligonucleotides

2.3.1 Plasmids

The names of all plasmids used in this study together with their genotype and source are listed in Table 2.2. Stocks of plasmids were maintained as aliquots in Analar™ analytical grade water at – 20 °C.

2.3.2 Bacteriophage

Bacteriophage P22 HT 105/1 *int-201* was used in this study for all genetic transductions. Stocks of bacteriophage P22 were stored in 3 – 5 ml volumes supplemented with chloroform at 4 °C in the dark.

2.3.3 Oligonucleotides

Oligonucletides used in this study were purchased from MWG-Biotech (Germany) or IDT Technologies (United Kingdom). The names and sequences of all oligonucleotides used are listed in Table 2.3.

2.4 Genetic Techniques

2.4.1 Transduction with bacteriophage P22 HT 105/1 *int-201*

Bacteriophage P22 was used to transfer genetic material between *Salmonella* strains throughout this study. P22 is a temperate phage that infects *Salmonella* by binding to the O-antigen component of lipopolysaccharide on the outer membrane (Susskind & Botstein, 1978). During the lytic cycle, the P22 genome is first replicated by θ-replication (Susskind & Botstein, 1978). Rolling circle replication then takes over to

Table 2.3 Table of oligonucleotide sequences

stpA.HA.fw	5'-gcgctggcggcggggaaatctctggatgatttcttaatctatgatgttcctgat-3'
stpA.HA.rev	5'-atagagacaggaaacgaagcgccatctgttaaaagctatccatatgaatatcctccttag-3'
lon.K.O.fw	5'-atc tga tta cct gcc gga cac taa act aag aga gag ctc tca tat gaa tat cct cct tag-3'
lon.K.O.rev	5'-tgc cag ccc tgt ttt tat tag cgc tat ttg cgc gag gtc agt gta ggc tgg agc tgc ttc-3'
kan.stpA.int.rev	5'-gac agg aaa cga agc gcc atc tgt taa aag cta tcc gtg aca tat gaa tat cct cct tag-3'
stpA.rev	5'-ttc cct gag tcc cgg tta at-3'
stpA.K.O.fw	5'-aat agt ttt ttg ttt tct gcg tta aaa ggt ttt tat tga tga cta caa aga cca tga cgg-3'
stpA.K.O.rev	5'-gac agg aaa cga agc gcc atc tgt taa aag cta tcc gtg aca tat gaa tat cct cct tag-3'
hns.flag.fw	5'-aga aca agg taa gca act gga aga ttt cct gat caa gga aga cta caa aga cca tga cgg-3'
hns.flag.rev	5'-aaa aat ccc gcc agc ggc ggg att tta agc atc cag gaa gca tat gaa tat cct cct tag-3'
stpA.flag.fw	5'-ggc gct ggc ggc ggg gaa atc tct gga tga ttt ctt aat cga cta caa aga cca tga cgg-3'
stpA.flag.rev	5'-cgg att aga aaa aca act taa atg tga aag tgg gtc tta aca tat gaa tat cct cct tag-3'
proV.fw	5'-agg gtt gtc tca gat tct ga-3'
proU.lacZ.fw	5'-cgt ttt ccg ccc tcg atc cat taa ttc gta ccg aaa tgc tga gcg gat aac aat ttc aca-3'
proU.lacZ.rev	5'-aat ggt gcg ctg atg ttt cgc ctg taa ttt cac cag ctc aca tat gaa tat cct cct tag-3'
hns.pst1.fw	5'-cgt act gca gac cca aaa taa ttc gcg ttg-3'
hns.pst1.rev	5'-cgt act gca ggg gat ttt aag cat cca gga a-3'
hns.fw	5'-cgt gtg ctt aat aaa gcg ta-3'
tetR.rev	5'-tca cca agg tgc aga gcc ag-3'
tet.*hns*.rev	5'-atc ccg cca gcg gcg gga ttt taa gca tcc agg aag taa act aag cac ttg tct cct g-3'
stpA.tet.fw	5'-gga tag ctt tta aca gat ggc gct tcg ttt cct gtc tta aga ccc act ttc aca tt-3'
stpA.tet.rev	5'-atg agc atg gcg caa cgt tcc ctg agt ccc ggt taa cta agc act tgt ctc ctg-3'
hns.kan.rev	5'-gcc tgg ggt cgt cag cgg aga act cag gca aaa aaa gac tac aaa gac cat gac gg-3'
kan.*hns*.fw	5'-ctt cct gga tgc tta aaa tcc cgc cgc tgg cgg gat cat atg aat atc ctc ctt ag-3'
kan.*hns*.rev	5'-gac tac aaa gac cat gac ggt tat gcc tgg ggt cgt cag cgg aga act cag gca a-3'
kan.rev	5'-gac tac aaa gac cat gac gg-3'
hns.stpA.int.fw	5'- aat agt ttt ttg ttt tct gcg tta aaa ggt ttt tat tga tat gag cga agc act taa aat-3'
hns.stpA.int.rev	5'-gac agg aaa cga agc gcc atc tgt taa aag cta tcc gtg att acg ccc cgc cct gcc act-3'
stpA.*hns*.int.fw	5'-gct caa caa acc acc cca ata taa gtt tga gat tac tac aat gaa ttt gat gtt aca gaa-3'
stpA.*hns*.int.rev	5'-atc ccg cca gcg gcg gga ttt taa gca tcc agg aag taa att acg ccc cgc cct gcc act-3'
stpA.pst1.fw	5'-cgt act gca ggg cga gat ttt cgt tgt tgt-3'
stpA.pst1.rev	5'-cgt act gca gtt ccc tga gtc ccg gtt aat-3'
stpA.f21c.fw	5'-cta tgg ctc gcg aat gct cca ttg acg ttc t-3'
stpA.f21c.rev	5'-aga acg tca atg gag cat tcg cga gcc ata g-3'
hns.rt.fw	5'-gca caa ctg aat tta tcg ct-3'
hns.RT.rev	5'-cat tct ctt gcc tgc gca-3'
stpA.RT.fw	5'-aat cgc ata caa tac cgc-3'
stpA.RT.rev	5'-ggc tcg cga att ctc cat tg-3'

produce long concatemers of double stranded P22 DNA (Susskind & Botstein, 1978). Concatemers are subsequently packaged into new phage heads. Packaging is initiated at a specific *pac* site after which a nuclease cleaves the concatemer every 48 kilobase (kb) pairs (Susskind & Botstein, 1978, Casjens & Hayden, 1988). This serves to package the 44 kb genome of P22 into each new phage particle. Upon lysis 50-100 phage particles are released from the cell which can then infect neighbouring uninfected cells (Susskind & Botstein, 1978). The *Salmonella* chromosome contains sites that are homologous to the P22 *pac* site (Ebel-Tsipis *et al.*, 1972). Occasionally during infection by P22, packaging of a 48 kb fragment of chromosomal DNA occurs (Ebel-Tsipis *et al.*, 1972). Subsequent infection of uninfected cells releases this 48 kb fragment of chromosomal DNA which can then recombine into the chromosome by homologous recombination (Ebel-Tsipis *et al.*, 1972). By this method desired fragments of DNA can be moved between *Salmonella* strains.

2.4.1.1 Preparation of high titre bacteriophage P22 lysates

A derivative of bacteriophage P22, P22 HT 105/1 *int-201*, was used in this study for all transductions. This derivative has a high transducing (HT) frequency due to a nuclease with less specificity for *pac* sites and is unable to form true lysogens due to the *int-201* mutation (Schmieger, 1972). For the preparation of high titre lysates, cells were pelleted from 1 ml of an overnight culture by centrifugation at 13,200 r.p.m. for 5 min. Cells were then washed with 1 ml of sterile phosphate-buffered saline (PBS) (Oxoid) and re-pelleted. After this wash step, cells were re-suspended in 5 ml of LB broth in sterile test-tubes to which 50 µl of high titre P22 HT 105/1 *int-201* stock solution was added. Cells were cultured in the presence of the phage particles for 2 h at 37 ^{o}C to allow for P22 phage adsorption and infection. After 2 h, 250 µl of chloroform was added to kill any remaining uninfected cells and cultures were allowed to stand at room temperature for 10 min. Dead cells and cellular debris were removed by centrifugation at 4000 r.p.m for 20 min leaving a high titre of phage particles

containing chromosomal DNA in the supernatant. The phage containing supernatant was decanted into sterile 15 ml falcon tubes (Sterilin) to which 50 µl of chloroform was added and kept as phage stocks at 4 ^{o}C in the dark. Typical phage titres by this method were $10^{9} - 10^{10}$ plaque forming units (p.f.u) per ml of lysate.

2.4.1.2 Generalized transduction by bacteriophage P22

High titre bacteriophage P22 lysates were routinely used to transfer genetic markers between donor and recipient strains of *Salmonella*. All mutant or fusion strains constructed in this study were linked with an antibiotic resistance cassette as a means of selection for the mutation or fusion in question. P22 lysates were made for each mutant and fusion strain for marker rescue and combining mutations or fusions into single strains. To transduce marker linked mutations and fusions, 100 µl of a high titre donor lysate was diluted serially to 10^{-2} in sterile 1.5 ml eppendorf tubes. To each dilution, 100 µl of overnight recipient culture was added and the mixtures were incubated for 1 h without shaking at 37 ^{o}C. No phage and no recipient controls were also included to ensure that there were no mutants in the recipient population and that the lysate was not contaminated with bacteria. After 1 h the entire mixtures were spread over LB agar plates with appropriate antibiotic selection and allowed to grow for 12 – 16 h. To reduce the possibility of working with a double transductant, colonies from the plate containing the lowest dilution of phage P22 were picked for phage clearance.

2.4.1.3 Clearance of bacteriophage P22

Although the *int-201* mutation in bacteriophage P22 prevents the formation of lysogens, P22 is still capable of forming pseudolysogens. To ensure that strains were phage free and contained no pseudolysogens, transductant colonies were twice single colony purified on green agar plates with antibiotic selection (See section 2.1.2.2). Dark blue colonies were indicative of pseudolysogens on green agar plates and were

avoided while pale green phage free colonies were re-streaked and used to make freezer stocks of the transductant strain.

2.4.2 Transformation of *Salmonella* with plasmid DNA

Plasmid DNA can be transformed into *Salmonella* either by chemical induction of competence followed by heat shock uptake of DNA or by electroporation. The efficiency of transformation is higher by electroporation and so this method was used for the introduction of plasmid DNA into *Salmonella* strains in this study (Dower *et al.*, 1988). Briefly, electroporation uses a high voltage electrical pulse to induce transient pores in the bacterial membranes (Sheng *et al.*, 1995). Plasmid DNA is then driven through these pores by the electrical field (Sheng *et al.*, 1995). Transformed cells are then allowed to recover in nutrient rich medium and the plasmid DNA is maintained within the bacterium.

2.4.2.1 Preparation of electrocompetent cells

To prepare electrocompetent cells 100 µl of an overnight recipient culture was added to 10 ml of LB broth in a 250 ml conical flask. Cells were grown at 37 °C with shaking to an $OD_{600} \approx 0.5$ before harvesting by centrifugation (4000 r.p.m, 10 min, 4 °C). The supernatant was then removed and cells were re-suspended in 10 ml of pre-chilled sterile Analar™ water and held on ice for 20 min. After 20 min cells were again harvested and washed in 1 ml of pre-chilled sterile Analar™ water to remove salts and facilitate efficient transmission of an electrical pulse. This wash step was repeated twice before a final re-suspension of the cells in 200 µl of pre-chilled sterile Analar™ water. Cells were used immediately for electroporation.

2.4.2.2 Electroporation of electrocompetent cells

On ice, 100 – 500 ng of plamid DNA was added to a pre-chilled electroporation cuvette (Cell Projects) with a gap-width of 2 mm. 50 µl of electrocompetent cells were then added to the plasmid DNA and the cuvette was wiped dry of any moisture before being placed in the Gene Pulser chamber. An electrical pulse was administered for 4 – 5 milliseconds with the Gene Pulser set to deliver 2.5 kV from a 25 µF capacitor with a Pulse Controller resistance of 200 Ω. After electroporation, 1 ml of pre-warmed (37 ^{o}C) LB-broth was added to the cuvette and the cells were allowed to recover for 1 h at 37 ^{o}C with shaking (200 r.p.m). 100 µl of culture was then spread on LB-agar plates with appropriate antibiotic selection for plasmid transformation and allowed to grow for 12 – 16 h. Control samples in which no plasmid was added to the cuvette were also used to ensure that no mutants were present in the recipient culture.

2.4.3 λ-red mediated homologous recombination

All chromosomal mutations, genetic fusions and epitope tags made during this study were created using λ-red mediated recombination as outlined by (Karlinsey, 2007). This method utilizes plasmid pKD46 which harbours the *γ*, *β* and *exo* genes from phage λ under the control of an arabinose inducible promoter (Datsenko & Wanner, 2000). The products of these genes, Gam, Bet and Exo respectively, suppress endogenous exonuclease attack of exogenous linear DNA, a function assigned to Gam, while Bet and Exo work on DNA ends to promote recombination (Datsenko & Wanner, 2000). As such, when pKD46 is present in *Salmonella* strains, expression of these genes in the presence of linear DNA fragments with regions of homology to the *Salmonella* chromosome can mediate the specific recombination of the linear DNA fragments onto the chromosome.

2.4.3.1 Induction of *γ*, *β* and *exo* genes

Plasmid pKD46 has a temperature sensitive replicon, overnight cultures were therefore grown at 30 °C in the presence of carbenicillin to maintain selection for the pKD46 plasmid. Sub-cultures were started using a 1/70 inoculum in 25 ml of LB-broth. Cultures were grown at 30 °C with shaking and selection to an $OD_{600} \approx 0.4 - 0.6$. To induce transcription of the λ genes, 250 µl of a 20 % (w/v) L-arabinose solution was then added and cultures were grown for a further hour to allow for induction. Cells were then made electrocompetent (See Section 2.4.2.1) in preparation for integration of linear DNA.

2.4.3.2 Integration of linear exogenous DNA

Linear DNA to be incorporated onto the *Salmonella* chromosome was made by polymerase Chain Reaction (PCR) (See Section 2.7.2). Oligonucleotide primers were designed to amplify a region of DNA to be integrated onto the chromosome, usually an antibiotic resistance cassette or gene coupled with an antibiotic resistance cassette. Primers were designed such that PCR products contained 40 bp regions of homology at the 3' and 5' ends to the region of the chromosome where it was to be integrated. In a similar manner to transformation of plasmid DNA, 100 – 200 ng of linear DNA PCR product was combined with electrocompetent cells expressing the λ genes and subjected to electroporation (See section 2.4.2.2). During the recovery period after electroporation Bet and Exo act on the open ends of the linear DNA to recombine the fragment at the target site on the chromosome by homologous recombination. After a 1 h recovery period, cultures were plated onto LB-agar plates with appropriate antibiotic selection for the incoming integrant DNA. Successful DNA integrations were subsequently moved into the wild-type SL1344 genetic background by P22 transduction (See section 2.4.1.2).

2.4.3.3 Epitope tagging of chromosomal genes

Epitope tagging of genes as part of this study was performed as described by Uzzau., *et al* (2001). Genes were epitope tagged such that the resultant protein product contained three copies of the FLAG-tag epitope (D Y K D D D D K), 3xFLAG, or the HA-tag epitope (Y P Y D V P D Y A). Taking the process of tagging H-NS with the 3xFLAG tag as an example, the 3x FLAG-tag nucleotide sequence was first PCR amplified along with the kanamycin resistance cassette (*kan*) from the template plasmid pSUB11 (Uzzau *et al.*, 2001) (Fig. 2.1). PCR primers were designed such that the resultant PCR products contained 40 bp overhangs complementary to the 40 bp immediately upstream of the *hns* stop codon at the 5' end and the first 40 bp immediately downstream of the *hns* stop codon at the 3' end. PCR products were electroporated (See section 2.4.2.2) into *S.* Typhimurium strains expressing the λ genes via plasmid pKD46 and introduced onto the chromosome by homologous recombination (See section 2.4.3.2). This resulted in the removal of the *hns* stop codon which was replaced by the Flag-tag nucleotide sequence and stop codon and the downstream *kan* cassette. The *kan* resistance cassette was subsequently removed by the introduction of plasmid pCP20 which encodes the FLP recombinase (Datsenko & Wanner, 2000). FLP mediated recombination between two FRT (FLP recognition target) sites located at either side of the *kan* cassette resulted in the removal of the resistance marker and strains were then cured of plasmid pCP20 according to the methods used by Datsenko and Wanner, 2000. Expression of the *hns* gene then gave rise to an H-NS protein which contained a 3xFLAG-tag at its C-terminal end. In a similar manner StpA was HA tagged using plasmid pSUB14 as a template for PCR (Uzzau *et al.*, 2001). While tagging proteins in such a manner has the potential to affect their activity much evidence exists to suggest that tagging has no impact. Epitope tagging of the LepB protein in *S.* Typhimurium by this method was shown to have no effect on the growth of *S.* Typhimurium or the activity of LepB (Uzzau *et al.*, 2001). Similarly, 3xFLAG tagging of the Cat protein had no effect on chloramphenicol resistance and more relevant to this study, tagging of H-NS with the 3xFLAG-tag by this method had no effect on the global DNA binding profile of H-NS (Uzzau *et al.*, 2001, Dillon *et al.*,

2010). Furthermore, no change in global patterns of transcription were observed, when StpA was 3xFLAG-tagged (Lucchini *et al.*, 2009). Strains which contained FLAG-tagged copies of H-NS or StpA were used only when observing their respective protein profiles during growth. For all other experiments non-epitope tagged strains were used.

2.5 Assays based on spectrophotometry

2.5.1 Monitoring bacterial growth

Bacterial growth was monitored by measuring the optical density (OD) of cultures during growth. Measurements were taken at a wavelength of 600 nm (OD_{600}) using a spectrophotometer. For each measurement 1 ml of culture grown in LB-broth was placed in a disposable plastic cuvette (Sarstedt) and the OD_{600} value was measured relative to a cuvette containing just LB-broth as a blank. Values were in the linear range between 0.1 – 0.8. Cultures in which the optical density lay outside the linear range were diluted 1/10 in LB-broth in a final volume of 1 ml before measuring the OD_{600} value.

2.5.2 Assay of β-galactosidase activity

The ability of the β-galactosidase enzyme, encoded by *lacZ*, to breakdown o-nitrophenol-β-D-galactoside (ONPG) with a resultant colorometric change was utilized to measure transcription from strains containing *lacZ* transcriptional fusions. Upon cleavage of ONPG by β-galactosidase the chromogenic o-nitrophenol turns yellow. By assaying the concentration of o-nitrophenol the cellular levels of β-galactosidase can be inferred which are directly correlated with transcriptional activity of the target promoter.

β-galactosidase activity was assayed by an adaptation of the method used by (Miller, 1972). Briefly, overnight cultures containing a *lacZ* transcriptional fusion were held on ice for 20 min before the OD_{600nm} was measured. 500 µl of culture was then combined

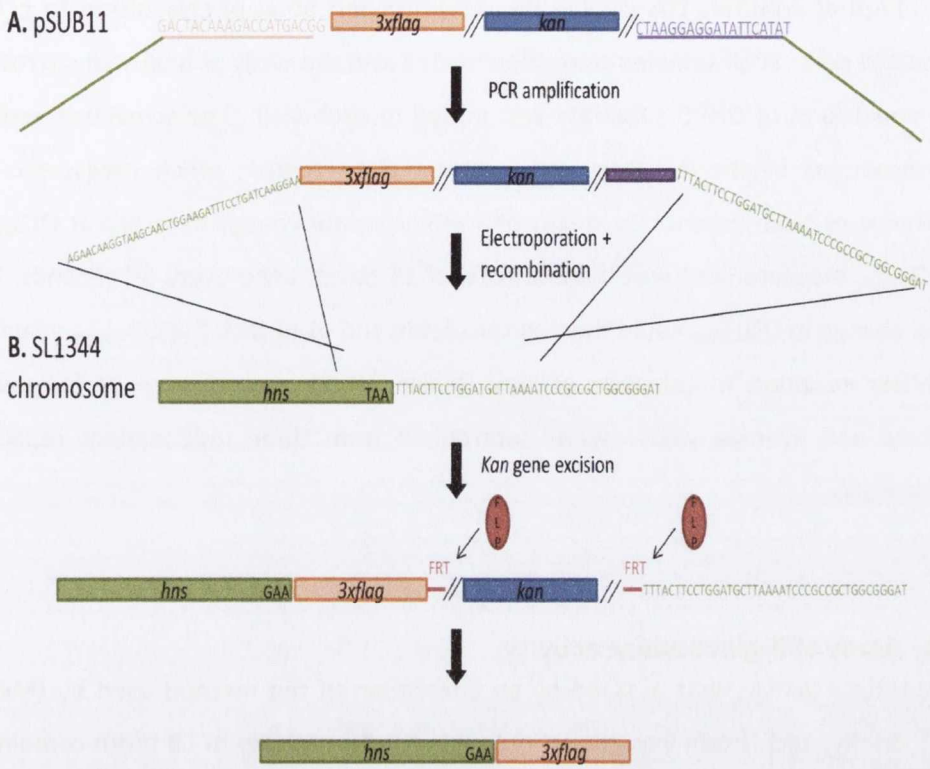


Fig. 2.1 Overview of the epitope tagging process.

Plasmid pSUB11 which contains the 3x FLAG-tag epitope nucleotide sequence and the *kan* resistance cassette was used as template DNA for PCR (A). The sequences of the primers used for PCR amplification (orange sequence (forward) and purple sequence (reverse)) are highlighted. The 3xFLAG-tag sequence and *kan* gene are represented as orange and blue boxes, respectively. Double lines between the 3xFLAG sequence and *kan* represent a 430 bp which contains an FRT site and the *kan* promoter while double lines after the *kan* gene represent 190 bp between the end of the *kan* gene and a second FRT site. Amplification yields a product with 40 bp overhangs (green sequence) at the 3' and 5' ends with homology to the *hns* (green box) 3' end and flanking sequence which is recombined onto the chromosome by λ-red mediated recombination (B). After recombination the FLP recombinase (red ovals) mediates recombination between FRT sites located at either side of the *kan* gene and the *kan* gene is removed.

with 500 µl of Z-buffer, 100 µl of 0.1% (w/v) SDS and 50 µl of chloroform to gently disrupt the cells. 20 µl samples were then loaded into the wells of a 96 well microtitre plate and 180 µl of ONPG substrate was added to each well. β-galactosidase activity was measured kinetically using a microtitre plate reader which measured the occurrence of o-nitrophenol by means of a yellow colour change detected at OD_{420nm}. An OD_{420nm} measurement was made a total of 15 times, once every 30 seconds. The rate of change in OD_{420nm} could then be calculated and used with the OD_{600nm} values in the Miller equation to calculate activity (Miller, 1972). Assays were performed in duplicate and average values were determined from three independent replicate experiments.

2.5.3 Assay of β-glucosidase activity

β-glucosidase activity was assayed by an adaptation of the method used by (Miller, 1972). Briefly, each strain was grown in 3 ml test-tube cultures of LB broth containing 1% (v/v) salicin for 12 h. Also added to the culture was 10 mM of the chromogenic β-glucoside substrate p-nitrophenyl-β-D-glucopyranoside. Cleavage of this artificial substrate by the *bgl*-encoded enzyme phospho-β-glucosidase results in a yellow colorimetric change, the intensity of which can be measured spectrophotometrically at OD_{420} and used to calculate the enzyme activity of phospho-β-glucosidase according to the Miller method (Miller, 1972). Assays were performed in duplicate and average values were determined from three independent replicate experiments.

2.5.4 Determination of nucleic acid concentration

The concentration of DNA or RNA in solution was measured using a Nanodrop ND-1000 spectrophotometer (Thermo Scientific). For each measurement, 2 µl of either DNA or RNA in solution with sterile nuclease free water (Promega) was applied to the optical arm of the Nanodrop. The upper lever arm was then lowered to contact the nucleic acid solution and the DNA or RNA concentration was measured at an absorbance wavelength of 260 nm (A_{260nm}). At this wavelength a value of $A_{260nm} = 1$

corresponds to a concentration of 50 μg ml^{-1} of double-stranded DNA or 40 μg ml^{-1} of single-stranded DNA or RNA. The Nanodrop simultaneously measures the purity of DNA and RNA solutions by measuring absorbance at A_{280nm}. For pure uncontaminated DNA the ratio of A_{260nm} to A_{280nm} is ≈ 1.8 while the ideal ratio for uncontaminated RNA is ≈ 2. These values were checked during all measurements to ensure purity of nucleic acid solutions.

2.6 Isolation of chromosomal DNA, plasmid DNA and RNA

2.6.1 Isolation of chromosomal DNA

Chromosomal DNA for PCR, RT-qPCR and standard input DNA for microarrays was isolated using a Puregene DNA purification system kit (Qiagen). A 0.5 ml sample of overnight culture was used for DNA purification as per the manufacturers' guidelines. This procedure entailed lysis of the bacterial cells with an anionic detergent in the presence of a DNA stabilizer which limits the activity of any DNase enzymes present. RNA and proteins were then removed by the addition of RNase enzymes and precipitation by salts, respectively. Genomic DNA was then precipitated using alcohol and dissolved in a buffered solution containing DNA stabilizer. Expected yields using this method are 10 – 35 μg DNA.

2.6.2 Isolation of plasmid DNA

Plasmid DNA was routinely extracted using a GeneJet™ plasmid miniprep kit (Fermentas). For high copy number plasmids (300 – 700 copies per cell) 2 ml of an overnight culture was typically processed according to the manufacturers' guidelines while up to 10 ml of overnight culture was processed for low copy number plasmids (10 – 50 copies). Briefly, the procedure involved lysis of the plasmid containing cells by the addition of Lysis solution containing lysozyme, SDS and NaOH (Birnboim & Doly, 1979). The pH of the lysate solution was then neutralized by the addition of Neutralization solution containing sodium acetate (Birnboim & Doly, 1979). The

addition of sodium acetate serves to re-nature and aggregate chromosomal DNA and simultaneously precipitate proteins and high-molecular weight RNA. Cell debris was then pelleted by centrifugation and the plasmid containing supernatant was applied to a silica membrane spin column supplied with the kit. After two wash steps to remove contaminants, purified plasmid DNA was then eluted from the spin column in 50 µl of elution buffer. By this method up to 20 µg of plasmid DNA could be isolated.

2.6.3 Isolation of RNA

RNA is a chemically unstable molecule that is readily digested by RNase enzymes which are pervasive in nature (Sambrook, 1989). RNases are extremely stable and can remain active even after autoclaving and other sterilization methods (Sambrook, 1989). As such, several precautions were taken when isolating RNA from bacterial cells. These included cleaning all work surfaces with 100 % ethanol prior to RNA isolation, using RNase-free pipette tips with filters and RNase-free tubes (Sarstedt), regularly changing protective latex gloves and keeping tubes and reagents in ice as much as possible.

RNA was isolated from bacterial cultures and used for RT-qPCR and transcriptional microarray analysis. Total RNA extracts were prepared by harvesting 2 OD_{600nm} units of bacteria for microarray analysis or 0.2 OD_{600nm} units of bacteria for RT-qPCR. The appropriate volume of culture was then added to 50 ml Falcon tubes containing 40 % of the culture volume of ice-cold 5 % (v/v) phenol (pH 4.3): ethanol solution and held on ice for at least 30 min. Cells were then pelleted by centrifugation at 4000 r.p.m for 10 min and the supernatant was decanted. The bacterial pellet was re-suspended in any residual supernatant and transferred to a 1.5 ml microcentrifuge tube. Bacteria were pelleted once more, this time in a microfuge (13,000 r.p.m, 5 min) and any remaining liquid was discarded. Pellets were re-suspended in 100 µl of Tris-EDTA buffer (pH 8) containing 50 mg/ml of lysozyme and incubated at room temperature for 5 min to help break down the bacterial cell wall and aid lysis. From this point total RNA was extracted using an SV Total RNA Isolation kit (Promega) according to the

manufacturers' guidelines. Briefly, a second lysis step was performed using the provided lysis solution which contains guanidine thiocyanate (GTC), β-mercaptoethanol and SDS. This step inactivates any ribonucleases present in the cell extracts and disrupts nucleoprotein complexes releasing protein free RNA into solution. Subsequent dilution of extracts with RNA dilution buffer causes precipitation of cellular proteins which were then removed along with cellular debris by centrifugation. The RNA containing supernatant was transferred to an RNase-free 2 ml microcentrifuge tube and 200 µl of ethanol was added to selectively precipitate the RNA. The RNA solution was added to spin columns and the RNA was absorbed to a silica fibre disc. RNA was then treated with DNase to remove any contaminating DNA and subjected to several wash steps before final elution in 100 µl of nuclease free water. A further DNase step was then undertaken to ensure the purity of the total RNA using the DNA-free™ kit (Ambion Inc.). RNA concentration and purity were assessed by spectrophotometry (See Section 2.5.3).

2.7 Manipulation of DNA *in vitro*

2.7.1 Purification of linear DNA from agarose gels

Linear DNA fragments were purified from agarose gels for use with λ-red recombination (See Section 2.4.4) or to be sent for commercial sequencing (GATC-Biotech, Germany). Linear DNA fragments, usually PCR products, were first size-separated by electrophoresis (See Section 2.9.1) in a 1 x TAE agarose gel containing 1 µg/ml of ethidium bromide. Gels were subjected to a brief exposure of ultraviolet (UV) light and simultaneously visualised using an AlphaImager imaging system (Alpha Innotech). The desired DNA products were identified, excised from the gel using a surgical scalpel blade and the DNA-containing fragment was placed in a sterile 1.5 ml Eppendorf tube. DNA was purified using a HiYield Gel/PCR Fragments extraction kit (RBC Bioscience) according to the manufacturers' guidelines. DNA was finally re-suspended in 50 µl of either elution buffer supplied with the kit or nuclease free water (Promega)

2.7.2 Amplification of DNA by polymerase chain reaction (PCR)

PCR was used for *in vitro* amplification of specific DNA fragments used in the construction of mutant, fusion and epitope-tagged *Salmonella* strains by λ-red recombination and for the genetic confirmation of strains by sequencing. The method used in this study requires a thermostable DNA polymerase enzyme capable of amplifying denatured single stranded DNA template primed with specifically annealed oligonucleotides (Saiki *et al.*, 1988). Amplification involves heat denaturation of a double-stranded DNA template followed by annealing of specific oligonucleotides to sites flanking the region to be amplified. A thermostable polymerase recognises these primed sites and in the presence of deoxy-nucleotide triphosphates (dNTPs) carries out second-strand synthesis (Saiki *et al.*, 1988). Cycles of denaturation, primer-annealing and second-strand-synthesis are repeated 25 – 35 times, during which the specific DNA fragment is amplified. Newly synthesised DNA fragments are also complementary to the specific nucleotides and serve as templates for subsequent reactions. Amplification occurs exponentially with 2^n new fragments synthesised per cycle where n is the number of cycles.

2.7.2.1 Preparation of DNA template from bacterial colonies

For PCR reactions where purified DNA was unavailable for use as a template, bacterial colonies were used. Single bacterial colonies were suspended in 50-100 µl of sterile Analar™ water and samples were boiled at 98 ^{o}C for 5 min. Boiling of samples facilitates cell lysis allowing access of DNA polymerase to DNA.

2.7.2.2 Choice of DNA polymerase

PCR reactions throughout this study were routinely performed using Phusion™ High-Fidelity DNA polymerase (Finnzymes). This polymerase is a novel *Pyrococcus*-like enzyme with a greatly reduced error rate compared with the commercially available *Thermus aquaticus* (*Taq*) DNA polymerase (50-fold lower error rate) and *Pyrococcus*

furiosus (*pfu*) DNA polymerase (6-fold lower error rate). In addition to DNA polymerase activity Phusion™ High-Fidelity DNA polymerase also has 3' – 5' exonuclease activity. Due to the high-fidelty of Phusion™ DNA polymerase occasionally PCR amplification occurred of non-specific DNA fragments as a result of primer annealing to regions of DNA with low levels of homology to the primer sequence. In these instances *Taq* DNA polymerase (New England Biolabs) was used to achieve PCR amplification of specific DNA fragments.

2.7.2.3 Amplification of DNA using Phusion™ High-Fidelity DNA polymerase

PCR reactions were carried out according to the manufacturers' guidelines. For each reaction 2 µl of dilute purified DNA or lysed colony template was added to a 0.5 ml PCR tube (Sarstedt). A PCR master mix was then prepared per reaction as follows: 10 µl Phusion HF buffer, 1 µl 10 mM dNTP mix, 0.25 µl each of 100 pmol oligonucleotide primers, 0.5 µl Phusion™ High-Fidelity DNA polymerase and 36 µl of nuclease free water (Promega). 48 µl of master mix was added to each reaction being undertaken and amplification was carried out using a Peltier Thermal Cycler PTC-200 (MJ Research). Unless otherwise stated reactions were cycled as follows:

1. 98 °C, 30 s (Initial Denaturtation)
2. 98 °C, 10 s (Denaturation)
3. Oligonucleotide annealing temperature[1], 30 s
4. 72 °C, 30 s/ kilobase of DNA being amplified (Extension)
5. Steps 2 – 4 repeated, 34 cycles
6. 72 °C, 10 min (Final Extension)

[1] Annealing temperatures of oligonucleotide primers were calculated using an online calculation tool on the Finnzymes website: www.finnzymes.fi/tm_determination.html[1]

7. 4 °C, hold

2.7.2.4 Amplification of DNA using *taq* DNA polymerase

Amplification of DNA using *taq* DNA polymerase was performed as in Section 2.7.2.3 with the following exceptions: 5 µl of 10 x *taq* DNA polymerase buffer, 33 pmol of each oligonucleotide primer, 1 U *taq* DNA polymerase, 2.5 mM $MgSO_4$ and nuclease free water were mixed to a final volume of 48 µl/ reaction. Furthermore, annealing temperatures were typically 5 °C less than that of the melting temperature (Tm) of the primers. Tm was calculated using the formula Tm = 2° x (A +T) + 4° x (G + C) where A, T, G, C, correspond to the base pair composition of the oligonucleotides. Where the Tm for oligonucleotide pairs did not match the lower value was used.

2.7.3 Determination of DNA quantity by quantitative PCR (qPCR)

Quantitative PCR was used in this study for the quantification of specific chromosomal genes or gene transcription levels from cDNA pools (See Section 2.8.2). qPCR makes use of the fact that during a PCR reaction, template DNA is amplified exponentially once reagents are not limiting (Yuan *et al.*, 2006). As such at any point during exponential amplification the amount of DNA present in a reaction is directly proportional to the starting quantity of template. The dynamics of a PCR reaction can be visualised using DNA incorporating dyes which fluoresce upon incorporation with DNA (Yuan *et al.*, 2006). There is a direct positive association between the incorporation of a dye, and thus fluorescence, with the number of amplicons present in a reaction (Yuan *et al.*, 2006). qPCR works by measuring fluorescence due to dye incorporation throughout a PCR reaction. By plotting the resulting log 2-based transformed fluorescence versus cycle number a linear range in which the log of fluorescence signal is a function of the initial amount of template can be defined (Yuan *et al.*, 2006). A threshold fluorescence value can then be defined manually. The cycle number (Ct) at which this threshold is achieved can then be used for quantification against a calibration curve for absolute quantification or quantification relative to a control gene. By either method the same threshold value is maintained.

Throughout this study qPCR was performed using GoTaq qPCR master mix (Promega) which contains the DNA incorporating dye Syber™ Green. Reactions were set up in 20 µl volumes in a Framestar 96 well PCR plate (Rainin). For each reaction, 8 µl of DNA or cDNA template of known concentration was added to relevant wells in the qPCR plate before 12 µl of qPCR master mix was added to each. The qPCR master mix consisted of the following per reaction: 10 µl 2 x GoTaq qPCR master mix, 0.6 µl of 10 µM oligonucleotide primers and 1.4 µl of nuclease free water. The qPCR reaction and fluorescence measurement was performed using a StepOnePlus™ Real-time PCR system. Cycling conditions for all reactions were as follows:

1. 95 ^{o}C, 2 min (Hot-start activation of GoTaq polymerase)
2. 95 ^{o}C, 15 s (Denaturation of template)
3. 60 ^{o}C, 60 s (Annealing and extension)
4. Steps 2 – 3 repeated 40 times

Fluorescence data collection occurred during the 60 s annealing extension step. All data analysis, threshold settings and determination of Ct values was performed using the StepOnePlus™ software provided.

2.7.3.1 Determination of *hns* and *stpA* gene abundance by qPCR

The relative abundance of the *hns* and *stpA* genes in *Salmonella* Typhimurium was determined for exponential phase and stationary phase cultures by qPCR. Total chromosomal DNA was isolated from cultures (See Section 2.6.1) at OD_{600nm} = 0.3 for exponential phase samples or OD_{600nm} = 3.0 for stationary phase samples. Samples were then analysed by qPCR using primer pair *hns*.RT.fw, *hns*.RT.rev for determination of *hns* gene abundance and stpA.RT.fw, stpA.RT.rev for determination of *stpA* gene abundance. Primer pairs were chosen such that the forward primers annealed within the 5' untranslated region of *hns* or *stpA* mRNA and reverse primers chosen within the first 100 bp of the *hns* or *stpA* translated region (Fig. 2.2). As such no amplification can

occur on transcripts partially degraded by RNase E which targets sequences within the 5 ' untranslated region (Carpousis, 2007, Burger *et al.*, 2011). Primer pairs chosen for amplification of the 3' ends of mRNA may amplify partially degraded mRNA molecules giving a false estimation of transcript abundance. The abundance of each gene was quantified relative to an internal calibration curve whereby a known quantity of isolated genomic DNA was serially-diluted and each dilution was also analysed by qPCR alongside the test samples. This produced a linear calibration curve with 3.3 cycle differences between dilutions under ideal conditions against which gene abundance was calculated. qPCR experiments were carried out in duplicate to determine the abundance of *hns* and *stpA* and average values were calculated from independent experiments (n = 3).

2.8 Manipulation of RNA *in vitro*

2.8.1 Reverse transcription (RT) of RNA

Reverse transcription was used to convert mRNA molecules into complementary DNA (cDNA) molecules. This procedure utilizes the reverse transcriptase enzyme which is capable of synthesizing a complementary DNA strand using RNA as a template. Reverse transcription was routinely used in this study to convert mRNA transcripts from total RNA extracts into cDNA pools. These cDNA pools could then be analysed by qPCR (See Section 2.7.3.1) to determine the mRNA abundance and thus the level of transcription of any given gene using gene specific primers. Reverse transcription was carried out using the GoScript™ reverse transcription system (Promega) according to the manufacturers' guidelines. In brief, 200 ng of total RNA extracts (See Section 2.6.3) in solution was combined with 1 µl of random primer mixture (0.5 µg / reaction) and nuclease free water in a final volume of 5 µl in a nuclease free 0.5 ml PCR tube (Sarstedt). The tubes were placed in a heat block poised at 70 ^{o}C for 5 min to thermally denature the template/primer mix before being chilled in ice-water for 5 min. During this time the reverse transcription reaction mix was made on ice which contained per reaction: 6 µl of nuclease free water, 4 µl of

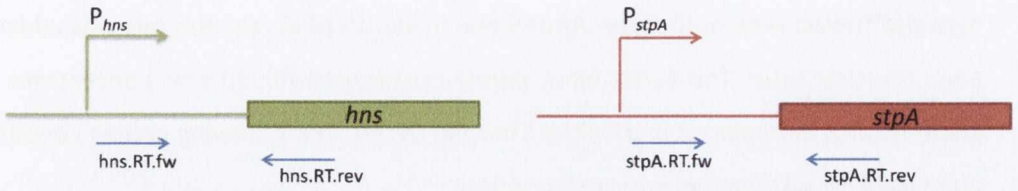


Fig. 2.2 RT-PCR promer annealing sites.

Diagram depicting the relative annealing sites of primers (blue arrows) *hns*.RT.fw, *hns*.RT.rev, stpA.RT.fw and stpA.RT.rev within *hns* (green) and *stpA* (red) promoter and ORF regions. Combinations of these primers were used for RT-qPCR with promoter-ORF fusion strains.

GoScript™ 5x reaction buffer, 3 µl of $MgCl_2$, 1 µl of PCR nucleotide mix and 1 µl of GoScript™ reverse transcriptase. After 5 min in ice, 15 µl of reaction mix was added to each reaction tube. The tubes were gently centrifuged for 30 s to concentrate the contents and then placed in a Peltier Thermal Cycler. The following cycling conditions were used for efficient reverse transcription:

1. 25 °C, 5 min (Annealing of random primers)
2. 42 °C, 1 h (Extension)
3. 4 °C, hold.

2.8.2 Quantification of mRNA levels

The abundance of *hns* and *stpA* mRNA transcripts was determined in this study at various points during the growth of wild-type *Salmonella* Typhimurium SL1344 and several mutant derivatives. Determination of transcript abundance for these genes was achieved by the two step process of RT-qPCR. From total RNA extracts (See Section 2.6.3) mRNA was converted to cDNA pools by RT (See Section 2.8.1). cDNA pools were then used as template DNA for qPCR using primer pairs specific for either the *hns* or *stpA* transcript (See Section 2.7.3). Quantification of transcripts was achieved using an internal calibration curve whereby a known quantity of isolated genomic DNA was serially diluted and each dilution was also analysed by qPCR alongside the test samples. This produced a linear calibration curve with 3.3 cycle differences between dilutions under ideal conditions against which *hns* and *stpA* transcript abundance was calculated.

2.8.3 RNA stabililty assay

The stability of the *hns*, *stpA* and *hns/stpA* hybrid mRNA molecules was assessed using a similar method to that used by Deighan *et al.*, (2000). For this, cultures of wild-type *S*.Typhimurium SL1344 or mutant derivatives were grown to exponential phase

(OD_{600nm} = 0.2) before transcription was inhibited by the addition of the antibiotic rifampicin (250 μg ml^{-1}). Total RNA was isolated upon addition of rifampicin and again every 5 min thereafter for a total of 25 min. The stability of the mRNA molecule in question was then determined by RT-qPCR (See Section 2.8.2). Experiments were performed in duplicate on three separate occasions.

2.9 Gel electrophoresis

2.9.1 Agarose gel electrophoresis

Agarose gel electrophoresis was used for the separation of DNA and RNA molecules on the basis of size for analysis of abundance and stability, the preliminary confirmation of knock-out mutations and purification of both linear and plasmid DNA molecules. Typically 300 ml of 1 % (w/v) agarose gel stock was made in 1x TAE buffer (40 mM Tris, 1 mM EDTA, 0.114 % (v/v) glacial acetic acid) using electrophoresis grade agarose (Invitrogen) and heated to 100 °C. Gels used in this study were mini-gels (5.9 x 8.3 cm) and required 20 ml stock gel. The stock gel mixture was melted at 100 °C and allowed to cool to 55 °C before the addition of ethidium bromide to a final concentration of 1 μg ml^{-1}. Ethidium bromide is a chemical which intercalates with nucleic acids and fluoresces strongly when exposed to long wavelength UV light. Samples of DNA or RNA were mixed with 6 x DNA loading dye (Promega) and loaded into gel wells alongside a 1 kb DNA size ladder (Promega). Gels were run at 100 V for 35 – 50 min before visualisation of the DNA or RNA under UV light using an AlphaImager system (Alpha Innotech).

2.9.2 SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was used for the routine separation of denatured proteins based on their molecular weight. Proteins were separated by discontinuous 12 % polyacrylamide gel electrophoresis as described by (Sambrook, 1989). This method employs a discontinuous buffering system whereby the running buffer reservoirs are of a

different pH and ionic strength to that of the stacking and resolving gels (Ornstein, 1964). For this method, denatured proteins are loaded into the wells of the upper stacking gel and an electrical current is applied. Proteins migrate quickly through the porous stacking gel before being deposited in a thin layer (stack) on the surface of the resolving gel. These protein stacks are then electrophoresed through the resolving gel in a zone of uniform voltage and pH. Separation of proteins based on size takes place in the resolving gel and relies on the binding of the anionic detergent SDS to the denatured proteins. The amount of SDS binding is directly proportional to molecular weight. Larger proteins will bind more SDS and migrate slower than smaller proteins whilst smaller proteins bind less SDS and migrate quickly through the gel. The molecular weight of specific protein bands can be estimated using a protein molecular weight standard loaded alongside samples being analysed.

2.9.2.1 Preparation of protein samples for SDS-PAGE

Total protein samples were prepared from bacterial cultures according to the method used by Laemmli, (1970). 0.2 OD_{600nm} units of bacterial cultures were harvested and cells were pelleted by centrifugation (13,000 r.p.m, 5 min). The supernatant was then removed and the cell pellet was re-suspended in 200 µl of 1 x Laemmli buffer (Sigma) (Laemmli, 1970). Laemmli sample buffer contains β-mercaptoethanol, which breaks disulphide bonds within proteins, and the anionic detergent SDS, which denatures proteins and gives them a negative charge. Samples were then placed in heating block set at 95 °C for 5 min and used immediately for SDS-PAGE or stored at – 20 °C.

2.9.2.2 Preparation of SDS-PAGE gels

Glass gel plates were cleaned before use and 5 ml of 12 % (v/v) acrylamide resolving gel solution was made per gel. Plates were assembled, sealed and clamped and the gel solution was poured between the plates until 1 cm below the position of the comb tip when inserted. The gel was immediately overlaid with 1 ml of isopropanol to allow for

anoxic polymerisation and gels were allowed to set for 20 min. The isopropanol layer was then poured off and 2 ml of 5 % (v/v) acrylamide stacking gel was made and layered on top of the resolving gel. Plastic combs containing 15 wells were then immersed into the stacking gel and the gels were allowed to set before being used for SDS-PAGE. Recipes for the resolving and stacking gels are as follows:

Resolving gel (5 ml): 1.7 ml water, 1.25 ml of 1.5 M Tris-HCl (pH 8.8), 50 µl 10 % (v/v) SDS, 2 ml of Protogel (National Diagnostics), 50 µl 10 % (w/v) ammonium persulfate, 2 µl TEMED.

Stacking gel (5 ml): 2.8 ml water, 1.25 ml of 0.5 M Tris-HCl (pH 6.8), 50 µl 10 % (w/v) SDS, 833 µl of Protogel, 50 µl ammonium persulfate, 5 µl TEMED.

2.10 Western immunoblot analysis

Western immunoblotting analysis is a technique whereby denatured proteins separated by their molecular weight by SDS-PAGE (See Section 2.9.3) are irreversibly transferred to a nitrocellulose membrane. The membrane bound proteins can then be probed with antigen specific mono- or polyclonal primary antibodies. Secondary horseradish-peroxidase-conjugated antibodies raised against mouse IgG in a rabbit host were then used to detect antigen-primary antibody complexes which can be visualised by chemiluminescent photography. All antibodies used in this study were monoclonal and were raised against their epitope in mice. Antibodies used and the dilution at which they were used are as follows: Flag-tag antibody (1/10,000) (Sigma), HA-tag antibody (1/2,000) (Sigma), RpoS antibody (1/5,000) (Neoclone) and DnaK antibody (1/100,000) (Neoclone). In addition, H-NS and StpA polyclonal anti-sera were also used at dilutions of 1/5000 and 1/1000, respectively.

2.10.1 Transfer of proteins to nitrocellulose membranes

SDS-PAGE gels were electroblotted to 0.22 µm PROTRAN nitrocellulose membranes (Whatman) using a mini-Trans-blot electrophoretic transfer cell (Bio-Rad) according to the manufacturers' guidelines. Transfer of proteins was performed in transfer buffer (25 mM Tris, 192 mM glycine, 20 % (v/v) methanol) at 150 V for 35 min at 4 ^{o}C.

2.10.2 Detection of protein antigens

Protein-bound nitrocellulose membranes were blocked for 1 h in blocking solution (5 % (w/v) Marvel non-fat dry milk in PBS). Primary antibodies were then diluted appropriately in 20 ml of blocking solution and incubated with the membranes for 12 – 16 h at 4 ^{o}C. Membranes were subjected to 4 x 15 min washes in PBS. Secondary HRP-conjugated antibodies (Millipore) were diluted 1:10,000 in blocking buffer and incubated with the membrane for 1 h. The nitrocellulose membranes were washed in PBS as before. Bound antigens were then detected using a Supersignal detection kit (Pierce) according to the manufacturers' guidlines. In brief, the membranes were incubated with a 1:1 mixture of the H_2O_2 and luminol solutions provided for 5 min. Luminol is a substrate for HRP which becomes oxidised to form an excited state substrate which emits light. Detection of the emitted light and thus membrane-bound antigens was achieved by photography using X-ray film (See Section 2.11). The more antigen present the greater the signal detected.

2.10.3 Protein stability assay

The stability of the StpA protein in wild-type *S.*Typhimurium SL1344 and several mutant derivatives was determined by the method described by Johannson and Uhlin (Johansson & Uhlin, 1999). Cultures were grown to exponential phase OD_{600nm}= 0.3 before protein translation was inhibited by the addition of spectinomycin (200 µg ml^{-1}). Total protein samples were taken (See Section 2.9.3.1) upon the addition of spectinomycin and every 30 min thereafter for 3 h. The stability of StpA was then determined by SDS-PAGE followed by Western Immunoblotting.

2.11 Chemiluminescent Photography

Photography was used for the visualisation and quantification of membrane bound antigens on western immunoblots. In a red-light photography room, X-ray film (Amersham) was placed over immunoblots in an enclosed chamber. The X-ray film was exposed to the chemiluminescent photon emissions from the cleavage of luminol by HRP which caused the silver halide crystals coating the X-ray film to absorb energy and emit an electron. This electron is attracted to the positively charged silver ion to form an atom of metallic silver. After an appropriate exposure time the film was washed in developer solution (Kodak) for 30 – 60 s which amplified the signal by reducing exposed silver halide crystals to metallic silver. The film was washed briefly in water and then the image was fixed in using fixer solution (Kodak) which reduces unexposed silver halide to soluble silver thiosulphate. This process results in images containing dark black bands where exposure has occurred against an otherwise clear background. The intensity of these bands is a measure of how much light emission occurred and thus protein abundance.

2.11.1 Quantification of protein on Western immunoblot images.

All protein bands on photographically developed Western immunoblots were routinely visualised and quantified using an AlphaImager imaging system. The pixel density of protein bands was estimated using densitometric software supplied with the imaging system to give an integrated density value (IDV). The IDV value represents the pixel density of each band relative to an average of twelve reference points chosen automatically within the unpixelated background surrounding the protein band in question. To compensate for unequal loading of protein samples, all blots were probed for the presence of DnaK protein in each sample lane. Differences in the relative levels of DnaK between protein samples were indicative of unequal sample loading as the cellular concentration of DnaK does not change appreciably over-time (Blum *et al.*, 1992). Protein samples of interest were therefore first normalised to the average of the three maximum IDV values obtained for DnaK within any given blot

image. For quantification of proteins containing the FLAG-tag or HA-tag epitopes, samples of the carboxy terminal FLAG-BAP™ protein (Sigma) or purified HA-tagged protein, respectively, of a known concentration were also subjected to Western immunoblot analysis. After normalisation to DnaK, protein samples of interest were then expressed relative to the IDV of this internal standard and expressed in ng/µl. Every effort was made to enhance the method of quantification including the use of an imaging system instead of an office scanner for image capture, background correction, transillumination to obtain shading free images and measuring the integral value of bands (Gassmann *et al.*, 2009). Quantification by densitometry of X-ray film Western blots, however, allows for quantification of proteins within a limited dynamic range when compared with more modern methods of image capture such as digital or fluorescent imaging systems which increase the linear range of detection 2 – 4 orders of magnitude (Mathews *et al.*, 2009) . The abundance of protein samples herein may therefore have been underestimated due to the nature of detection used. This, however, was the only method of detection available for use at the time this study was undertaken.

2.12 Relative fitness assay

The fitness of a bacterium can be described as the capacity of a particular genotype to survive and proliferate in a given set of environmental conditions. Genotype alterations and the resultant phenotypic changes can incur a fitness advantage or disadvantage to one bacterium relative to another in a mixed population. In this study, alterations to the expression patterns of *hns* and *stpA* were made to create three novel strains of *S.* Typhimurium each having distinct populations of H-NS and H-NS-like proteins throughout growth. To assess if the genotypic and phenotypic changes made in these strains conferred any fitness advantage or disadvantage, competitive fitness assays were performed in varying temperature and osmotic conditions.

For each fitness assay wild-type *S.*Typhimurium SL1344 and a competitor strain were grown for 24 h in 25 ml of LB-broth[2] in the test environment. This served to pre-condition each strain to that environment before competition. Mixed cultures were founded with 10^5 cells each of wild-type and competitor strains and allowed to compete for 24 h in the test environment. Populations of competing strains were enumerated at time zero (t = 0) and after 24 h growth by selective plating. The fitness of each competitor relative to wild-type was defined as the ratio of the number of doublings of a competitor strain to the number of doublings of wild-type during the 24 h period of competition (Lenski, 1991). This ratio was calculated using the formula:

Fitness index (f.i.) = LN (N_j (1)/ N_i (0)) / LN (N_j (1)/ N_j (0))

N_i (0) and N_j (1) = initial and final counts of competitor strain, respectively

N_j (0) and N_j (1) = initial and final counts of wild-type strain, respectively

A competitor fitness of 1 indicates an identical fitness to that of wild-type. A fitness > 1 indicated a fitness advantage to the competitor in the test environment while a fitness < 1 indicated that the competitor was disadvantaged relative to wild-type. Mean fitness values were determined from 3 independent experiments.

2.13 Microarray analysis

Transcription microarray analysis was used to determine the total changes in the transcriptional output of strain SL1344^{swap} relative to wild-type SL1344 as a result of

[2] LB-broth was used for fitness assays where temperature was varied. For fitness assays where osmotic conditions were altered the normal concentration of NaCl (172 mM) was varied.

the regulatory alterations made to *hns* and *stpA* in SL1344^{swap}. Analysis was performed on both exponential and stationary phase samples of each strain. The process entails isolation of total RNA from cells in each growth phase. Isolated RNA is used as a template for double-stranded cDNA synthesis which is then fluorescently labelled with Cy3 dye. Cy3 labelled cDNA samples are combined with an equal volume Cy5 labelled reference genomic DNA and the mixture is hybridized to a high density oligonucleotide microarray. After hybridization, microarrays are scanned and fluorescence was detected using a GenePix scanner. The microarrays used in this study contain 44,000 probes specific for the genome of *Salmonella* Typhimurium SL1344 (Oxford Gene Technologies).

2.13.1 RNA isolation and double-stranded cDNA synthesis

Total RNA for microarray analysis was isolated from bacterial cultures at OD_{600nm} = 0.3 and OD_{600nm} = 3.0 for exponential and stationary phase samples, respectively (See Section 2.6.3). Isolated RNA was then converted to double-stranded cDNA in a two-step process using a SuperScript™ Double-Stranded cDNA Synthesis kit (Invitrogen) according to manufacturers' guidelines. First-strand synthesis was achieved by step one of this procedure whereby 1 µg of total RNA was converted to single stranded cDNA. Briefly, RNA (1 µg RNA in 10 µl nuclease free water) and 1 µl random primers (100 pmol μl^{-1}) were combined and heated to 70 ^{o}C for 10 min to thermally denature the primer/RNA mix. To each reaction 7 µl of reaction mix (5xfirst-strand reaction buffer, 0.1 M DTT and 10 mM dNTP mix) was added and the tubes were heated to 45 ^{o}C for 2 min to equilibrate the contents. 1 µl of SuperScript™ II RT was added to each and reactions were held at 45 ^{o}C for 1 h during which time the SuperScript™ II reverse transcription enzyme synthesized pools of single-stranded cDNA from the RNA template. Reactions were terminated by placing them in ice.

For second strand synthesis, whilst on ice the following contents were added to each reaction tube: 91 µl nuclease free water, 30 µl 5x second-strand synthesis buffer, 3µl 10 mM dNTP mix, 1 µl *E. coli* DNA ligase (10 U/µl), 4 µl *E. coli* DNA polymerase I (10

U/µl) and 1 µl *E. coli* RNase H (2 U/µl). Reactions were mixed by vortexing and incubated at 16 °C for 2 h. During this time synthesis of double-stranded cDNA was achieved using the single-stranded cDNA as a template by the DNA polymerase enzyme. After 2 h, 2 µl (10 units) of T4 DNA polymerase was added to fill in ends and reactions were held at 16 °C for a further 5 min. Double-stranded cDNA was then purified by phenol:chloroform extraction according to manufacturers' guidelines.

2.13.2 Fluorescent labelling of DNA and cDNA

Once double-stranded cDNA had been made it was fluorescently labelled with Cy3™ dCTP using a BioPrime®Array CGH Genomic Labelling System (Invitrogen). Labelling of the cDNA was carried out as described below.

1. 60 µl of 2 x random primer solution, x µl cDNA[3] and 70.5 – x µl cDNA of nuclease free water were mixed in a 1.5 ml micrfuge tube on ice.
2. The mixture was heated to 100 °C for 10 min to denature the DNA and then snap-chilled on ice.
3. 15 µl 10 x dNTP mix (2 mM dATP, dTTP,dGTP and 1 mM dCTP), 1.5 µl 1 mM Cy3™ dCTP and 3 µl of exo-Klenow polymerase (40 U/µl) were added to each reaction. 150 µl final volume.
4. Reagents were mixed thoroughly and incubated for 12 – 16 h at 37 °C.
5. 15 µl of stop buffer was added to terminate the reaction.

[3] The volume of cDNA solution added to each reaction was adjusted such that 1 µg of double-stranded cDNA was used as template for labelling each time.

Exo-Klenow is a derivative of Klenow polymerase that lacks both 3' – 5' and 5' – 3' exonuclease activity to give higher yields of labelling than that of standard Klenow. In the same manner as above 200 ng of sonicated genomic wild-type SL1344 DNA was labelled with Cy5™ dCTP. Labelled genomic DNA was used as reference input DNA on all microarrays against which comparisons between microarrays could be made.

2.13.3 Purification of labelled double-stranded cDNA

Cy3™ and Cy5™ labelled cDNA was purified using Micro-spin G50 columns (GE Healthcare) to remove unlabelled nucleotides. Three spin columns were used for each 150 µl reaction. The resin within the columns was re-suspended by vortexing, columns were placed in 2.0 ml nuclease free microfuge tubes and centrifuged at 4000 r.p.m for 1 min. 50 µl of Analar™ water was applied to the resin bed and columns were centrifuged for a further 1 min at 4000 r.p.m. Columns were placed in 1.5 ml nuclease free microfuge collection tubes and 50 µl of labelled cDNA was applied to each before a third centrifugation step at 4000 r.p.m for 2 min. Purified labelled cDNA was collected in the microfuge tubes while unlabelled nucleotides remained trapped in the resin column. Samples from the same labelling reaction were pooled together in a final volume of 180 µl. 5 µl of each was analysed by agarose gel electrophoresis for purity (See Section 2.9.1).

2.13.4 Hybridisation and washing of microarray slides

For hybridisation samples of Cy3™ labelled cDNA and Cy5™ labelled reference genomic DNA were mixed in a 1:1 ratio (180 µl of each). DNA from these mixed samples was then ethanol precipitated as follows:

1. 36 µl of 3 M sodium acetate (pH 5.2) and 1 ml of ethanol added to each.

2. Samples were held at – 80 ^{o}C for 1 h

3. Samples were centrifuged at 13,200 r.p.m for 20 min at 4 °C
4. The supernatant was decanted and DNA pellets were washed with 80 % (v/v) ethanol.
5. Samples were re-centrifuged for 5 min at 13,200 r.p.m, the supernatant was removed and DNA pellets were allowed to dry at room temperature for 15 min the dark.

Once dried, the precipitated DNA pellets were re-suspended in 100 µl of hybridisation buffer (4 ml 5 M NaCl, 1 ml MES (pH 6.5), 4 ml 0.1 M EDTA, 4 ml formamide, 2 ml Triton X-100, 5 ml Analar™ water) at 70 °C for 15 min. DNA was then denatured at 100 °C for 10 min before being snap-chilled on ice for 5 min. Total sample volumes were applied to chambers of microarray gasket slides (Agilent Technologies) taking care to remove any air bubbles and the *Salmonella* Typhimurium SL1344 44 k probe microarray slide (Oxford Gene Technologies) was laid on top. Slides were assembled in a hybridisation chamber (Agilent Technologies) which was tightly sealed and placed in a hybridisation oven at 55 °C for 48 h.

When hybridisation was complete microarray slides were carefully removed from the hybridisation chamber and placed in a clean glass wash chamber (Agilent Technologies). Slides were twice washed for 5 min in hybridisation wash buffer 1 (75 ml 20x SSPE (Sigma), 175 ml Analar™ water and 125 µl 10 % (v/v) Tween) and twice washed for 5 min in hybridisation wash buffer 2 (750 µl 20x SSPE, 450 µl PEG 200 (Fluka), 248.2 ml Analar™ water) before being scanned.

2.13.5 Scanning of microarray slides and data handling

Hybridised microarray slides were scanned using a GenePix 4000B scanner (Axon Instruments, Inc.). Quantification of fluorescent spot intensities and local background data was performed using the GenePix 3.0 software supplied. The Cy3/Cy5 ratio and median of the Cy3/Cy5 ratio for each probe was then calculated on excel spreadsheets

for each array. Cy3/Cy5 values were median-normalised and the intensities of the multiple probes across each gene were averaged. Expression of genes from SL1344^{swap} relative to wild-type SL1344 was analysed and only genes that showed a 2-fold Log_2 ratio difference in expression relative to wild-type SL1344 were deemed to be statistically significant. Microarray data was averaged from three independent experimental replicates.

Chapter 3 Expression of *hns* and *stpA* in *S.* Typhimurium

3.1 Introduction

3.1.1 H-NS-like proteins: H-NS and StpA

H-NS and H-NS-like proteins are widespread in Gram-negative bacteria and are particularly prevalent in *Enterobacteriaceae* (Bertin *et al.*, 2001). They are involved in many cellular functions such as chromosome organization, transposition, recombination, plasmid conjugation and global gene regulation (Dorman, 2007, Maurer *et al.*, 2009, Beloin *et al.*, 2003, Tendeng & Bertin, 2003, Lucchini *et al.*, 2006). *Salmonella enterica* serovar Typhimurium and the closely related bacterium *Escherichia coli* each possess H-NS and the H-NS-like protein, StpA, which are chromosomally encoded by the *hns* and *stpA* genes respectively (Lucchini *et al.*, 2009, Hinton *et al.*, 1992).

At the amino acid level, StpA has 58% sequence identity with H-NS and both share a common two domain structure comprised of an N-terminal dimerization/oligomerization domain (residues 1 – 83) and a C-terminal nucleic acid binding domain (residues 91 – 137) with a flexible linker region between (Bertin *et al.*, 2001, Dorman *et al.*, 1999, Ono *et al.*, 2005, Ueguchi *et al.*, 1996, Rimsky, 2004, Arold *et al.*, 2010, Zhang & Belfort, 1992). The C-terminal domain facilitates binding to DNA and RNA with each protein showing a DNA binding preference for A/T-rich regions of the chromosome with intrinsic planar curvature (Dorman, 2006, Dame *et al.*, 2001, Mayer *et al.*, 2007, Sonnenfield *et al.*, 2001). Such regions are often located in the promoters of many genes where binding of H-NS and/or StpA predominantly has a repressive effect on gene transcription (Dillon *et al.*, 2010). This preference for binding to A/T-rich DNA is of particular importance to the role of H-NS in xenogeneic silencing whereby H-NS has been shown to bind to and repress horizontally acquired genes of higher A/T content than the core genome (Navarre *et al.*, 2006, Navarre *et al.*, 2007, Lucchini *et al.*, 2006, Banos *et al.*, 2009). Both H-NS and StpA are capable of self- and hetero-association to form homo-dimers, hetero-dimers and higher-order oligomers via protein-protein interactions between N-terminal domains (Johansson *et al.*, 2001, Deighan *et al.*, 2003, Leonard *et al.*, 2009). Oligomerization of H-NS and StpA occurs in

a concentration-dependent manner (Badaut *et al.*, 2002, Johansson *et al.*, 2001). Unlike StpA, oligomerization of H-NS is affected by several environmental conditions such as temperature, osmotic pressure and pH (Leonard *et al.*, 2009, Amit *et al.*, 2003, Stella *et al.*, 2006). The StpA protein, however, is subject to proteolytic degradation by the Lon protease and thus is reliant on its ability to form hetero-dimers and hetero-oligomers with H-NS to avoid degradation (Johansson & Uhlin, 1999).

As a consequence of their structural similarities, H-NS and StpA also exhibit a high degree of functional overlap. Both proteins can constrain DNA supercoils, exhibit similar binding profiles throughout the chromosome and regulate many of the same genes (Lucchini *et al.*, 2009, Mojica & Higgins, 1997, Sonnenfield *et al.*, 2001, Uyar *et al.*, 2009). The structural and functional overlap of StpA with H-NS coupled with the fact that the StpA protein is only detected at significant levels in *hns* mutants and that no obvious phenotypes arise from the loss of StpA has led to the hypothesis that StpA served as a molecular back up for H-NS (Free & Dorman, 1997, Sonden & Uhlin, 1996). Indeed over-expression of StpA in *hns* mutant strains has been shown to compensate functionally for the loss *hns* by maintaining repression of the *proU*, *bgl*, *adi* and *hns* genes (Johansson *et al.*, 2001, Zhang *et al.*, 1996, Shi & Bennett, 1994). A great body of work now exists, however, to support the theory that H-NS and StpA have distinct yet overlapping biological roles.

Although structurally very similar, recent biophysical evidence suggests that H-NS and StpA bind DNA in different manners with H-NS observed to form a superhelical structure upon oligomerization, capable of condensing DNA and mediating transcriptional repression (Arold *et al.*, 2010). StpA on the other hand forms a rigid protein:DNA filament structure upon oligomerization which blocks DNA access (Lim *et al.*, 2011). This filamentous structure can also interact with naked DNA to form bridges, a function not observed for H-NS which requires interaction between two H-NS bound regions of DNA for bridge formation (Lim *et al.*, 2011). Furthermore, StpA binds DNA with greater affinity than H-NS and has been shown to act as an RNA chaperone to destabilise the *micF* antisense RNA and compensate for the splicing

defect of the T4 phage *td* mRNA (Deighan *et al.*, 2000, Sonnenfield *et al.*, 2001, Zhang *et al.*, 1995). H-NS, however, acts as a transcriptional repressor of the *micF* RNA and has been shown to enhance transcription of several mRNAs containing suboptimal ribosome binding sites (*malT, lrhA, dpiA*) (Suzuki *et al.*, 1996, Park *et al.*, 2010). In addition, several phenotypes are associated with a loss of *hns* from the cell such as de-repression of the cryptic *bgl* locus, mucoid colony morphology and cells are rendered non-motile (Wolf *et al.*, 2006, Free *et al.*, 2001, Paul *et al.*, 2011). At least in *E. coli*, no phenotypes have been identified due to a loss of *stpA*.

The highly individual expression patterns of *hns* and *stpA* further supports the idea of H-NS and StpA having distinct but overlapping biological roles. Expression of the *hns* gene is coupled with DNA replication (Free & Dorman, 1995). As such *hns* mRNA levels are maximal during exponential growth when cells are dividing rapidly (Free & Dorman, 1995). This has the net effect of maintaining relatively constant levels of H-NS protein at all stages of growth and a constant H-NS:DNA ratio (Free & Dorman, 1995). Expression of *stpA*, however, is somewhat transient with *stpA* mRNA and StpA protein levels being maximal in early exponential growth phase before declining to almost undetectable levels in stationary phase (Free & Dorman, 1997, Sonden & Uhlin, 1996). This allows for various populations of homodimers, hetero-dimers and higher order homo- and hetero-oligomers of these H-NS-like proteins to exist throughout growth, each possibly having differential nucleic acid binding and gene regulatory properties. Much of the structural and functional information regarding H-NS and StpA discussed above is derived from studies conducted in *S.* Typhimurium and *E. coli* and for the most part what was observed in one organism is true for the other. Data regarding the regulation of expression of *hns* and *stpA*, however, is predominantly derived from studies in *E. coli*.

3.1.2 Environmental control of expression

The role of H-NS in temperature mediated gene regulation has been extensively studied (Ono *et al.*, 2005, Atlung & Ingmer, 1997, White-Ziegler & Davis, 2009).

Expression of the *hns* gene is itself subject to temperature regulation, with an increase in expression observed under cold-shock conditions (La Teana *et al.*, 1991). The *hns* gene is part of the cold shock regulon and *hns* mRNA expression was observed to increase 3- to 4-fold upon a temperature downshift from 37 ^{o}C to 10 ^{o}C (La Teana *et al.*, 1991). This increase in transcription is mediated by another cold shock protein, CspA, which binds to a CCAAT motif located within a 110-base-pair region of DNA containing the *hns* promoter (La Teana *et al.*, 1991, Giangrossi *et al.*, 2001). Conversely, *stpA* expression was observed to increase upon a temperature up-shift from 30 ^{o}C to 37 ^{o}C (Free & Dorman, 1997). Nutrient availability is another key factor in the expression of *hns* and *stpA*. Repression of *stpA* was observed upon carbon starvation while expression increased in cultures grown in minimal medium in an LRP dependent manner (Free & Dorman, 1997, Sonden & Uhlin, 1996). H-NS, however, was identified as one of the first proteins to be synthesized upon a nutritional up-shift (Laurent-Winter *et al.*, 1995). In addition, *stpA* is significantly up-regulated by osmotic shock with up to 50-fold more expression resulting from an osmotic shock equivalent to exposure to 0.6 M sucrose or 0.4 M NaCl (Free & Dorman, 1997). A similar up-regulation of *hns* has not been observed (Ono *et al.*, 2005).

3.1.3 Transcription factor regulation of *hns* and *stpA*.

A key overlapping feature of H-NS and StpA is that each protein represses both its own gene and that of the other. H-NS has been extensively shown to repress transcription of the *hns* gene by directly binding to two regions displaying intrinsic DNA curvature, one is centred at –150 base pairs relative to the transcriptional start site and the other is a region which overlaps the –35 element within the *hns* promoter (Ueguchi *et al.*, 1993, Falconi *et al.*, 1993) (Fig. 3.1.) H-NS also exerts a repressive effect on *stpA* transcription again by binding within the *stpA* regulatory region (Wolf *et al.*, 2006). Such is the repressive effect of H-NS that, apart from the transient exponential phase expression of *stpA*, both *stpA* mRNA and StpA protein are only detected in significant amounts in *hns* mutant strains (Sonnenfield *et al.*, 2001, Wolf *et al.*, 2006, Sonden &

Uhlin, 1996). Similarily, experiments using *hns* and *stpA* mutants demonstrated the auto- and cross-repressive effect of the StpA protein. In mutant strains deficient in *stpA*, expression of both *hns* and *stpA* mRNA increases (Sonden & Uhlin, 1996). This increase is enhanced in an *hns stpA* double mutant strain where levels of *hns* and *stpA* mRNA were greater than that observed in either single *stpA* or *hns* mutants, respectively (Sonden & Uhlin, 1996, Free & Dorman, 1997).

The peak of *hns* expression during exponential phase of growth is, in part, due to the activity of the transcriptional activator protein, FIS. Analysis of the *hns* promoter region revealed the presence of seven FIS binding sites (F1 – F7) spanning the region between - 296 bp and + 50 bp relative to the transcriptional start site (Falconi *et al.*, 1996) (Fig 3.1.). Two of these sites (F4 and F5) partially overlap the H-NS binding sites centred at – 150 bp that are necessary for repression (Falconi *et al.*, 1996). *In vitro* transcription assays demonstrated that FIS binding to one or more of the sites between F4 and F7, which span the H-NS-bound region, stimulates activity from the *hns* promoter (Falconi *et al.*, 1996). This is supported by *in vivo* data whereby 2- to 4-fold higher levels of *hns* mRNA were observed in wild-type *E. coli* compared to a *fis* mutant derivative strain during exponential growth (Falconi *et al.*, 1996). Electrophoretic mobility shift assays showed that binding of FIS within this region could displace and remodel H-NS bound DNA within the – 150 bp region suggesting a possible mechanism of transcriptional activation (Falconi *et al.*, 1996). As mentioned in Section 3.1.2, the *hns* gene is also up-regulated by the CspA protein in response to cold shock. Outside of repression by H-NS and StpA, the only other identified regulator of *stpA* expression is the leucine-responsive regulatory protein, LRP (Free & Dorman, 1997, Sonden & Uhlin, 1996). LRP binds within the *stpA* regulatory region most likely at the degenerate LRP consensus sequence centred at – 107 bp relative to the transcriptional start (Free & Dorman, 1997). In cultures grown in minimal medium LRP

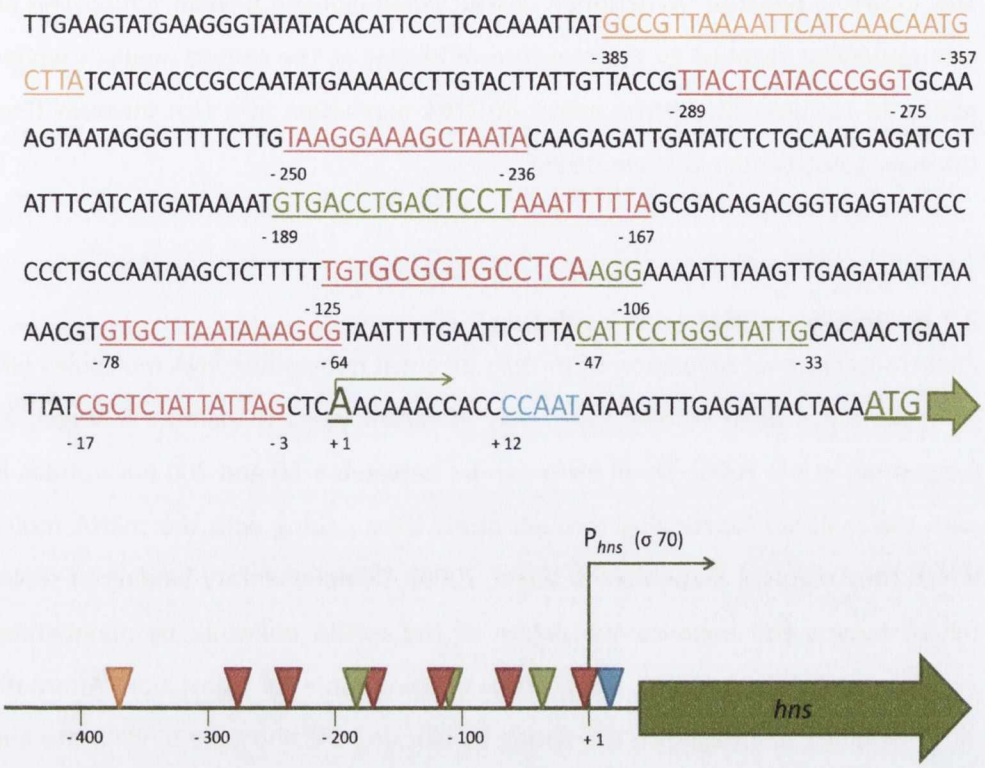


Fig. 3.1 Representation of the *hns* regulatory region in *S.* Typhimurium.

The sequence (A) of the first translated codon (ATG) and 400 bp upstream of the *hns* gene regulatory region is depicted. Maroon, green and orange highlighted base pairs depict FIS, H-NS and FUR binding sites/regions within the promoter, respectively, while blue highlighted base pairs represent the 'cold-box' required for cold-shock regulation of *hns*. Numbers indicate the base pair positions upstream (-) or downstream (+) from the transcriptional start site (+1). Larger size font indicates regions of overlap between two binding sites. The promoter region and *hns* ORF (B) are also represented diagrammatically. Coloured arrow heads point to the centre of each transcriptional regulator binding site. Maroon, green and orange arrows represent FIS, H-NS and FUR binding sites/regions, respectively. The 'cold-box' is indicated with a blue arrow.

was shown to enhance transcription of *stpA* 2-fold (Sonden & Uhlin, 1996). This effect was somewhat relieved by the presence of leucine in the culture medium while LRP appeared to have little or no effect on *stpA* expression in a rich medium (Free & Dorman, 1997, Sonden & Uhlin, 1996).

3.1.4 Post-transcriptional regulation

Post-transcriptional regulation of mRNAs by small non-coding RNA molecules (sRNA) in bacteria has been studied intensively in recent years (Papenfort & Vogel, 2009, Gottesman *et al.*, 2006). Small RNAs can be between ≈ 50 and 200 nucleotides long, with the majority functioning through direct base pairing with the mRNA molecule which they regulate (Papenfort & Vogel, 2009). Complementary binding of sRNAs to mRNA targets can enhance translation of the mRNA molecule by maintaining its structure or by altering it to a conformation favourable for translation. Alternatively sRNA binding can antagonize translation by blocking the ribosome binding site and/or by maintaining the mRNA in a conformation favourable for degradation (Gottesman *et al.*, 2006, Gottesman *et al.*, 2001, Papenfort & Vogel, 2009). Often, sRNAs are transcribed antisense to the mRNA which they regulate. However, many have been found to be encoded within intergenic regions (Papenfort & Vogel, 2009, Gottesman *et al.*, 2006).

One such intergenic sRNA is DsrA which is known to regulate the *hns* mRNA post-transcriptionally (Sledjeski & Gottesman, 1995, Sledjeski *et al.*, 1996, Lease *et al.*, 1998). DsrA is ≈85 nucleotides in length and exhibits duality of function in that it can both antagonize translation of *hns* mRNA and stimulate translation of the stress σ-factor, *rpoS* mRNA (Lease & Belfort, 2000a, Lease & Belfort, 2000b). The DsrA molecule adopts a structure comprised of three stem loops of which stem loop II is essential for *hns* regulation (Lease *et al.*, 1998). Bioinformatic analysis has identified a 13 nucleotide region of stem loop II that is capable of forming a double-stranded RNA complex with the *hns* mRNA in the region of the *hns* translation initiation start codon (Lease *et al.*, 1998). Binding of DsrA to the *hns* transcript prevents ribosome binding

and thus translation of the *hns* mRNA and decreases the half-life of the *hns* mRNA by 8-fold (Lease & Belfort, 2000b). The interaction of DsrA with *hns* mRNA requires the RNA chaperone Hfq (Lease & Woodson, 2004). Hfq is a multimeric protein which binds single stranded RNA molecules with a preference for AU-rich regions followed by a step loop (Schumacher *et al.*, 2002, Moller *et al.*, 2002). Hfq binds to such a region in DsrA and is required for the activity of DsrA in regulating both *hns* and *rpoS* by preventing degradation of DsrA and enhancing the interactions between DsrA and the *hns* and *rpoS* mRNAs (Brescia *et al.*, 2003, Gottesman *et al.*, 2006).

3.1.5 Translational control by polyamines.

Polyamines (putrescine, spermidine and spermine) have recently been shown to enhance the synthesis of several proteins at the translational level, including FIS, RpoS, OppA and adenylate cyclase (Terui *et al.*, 2007, Terui *et al.*, 2009). Polyamines are present in millimolar quantities in most living organisms where they stimulate the translation of mRNA molecules in a number of ways (Terui *et al.*, 2007, Terui *et al.*, 2009). Firstly, they enhance translation of those mRNAs that have an aberrant or distantly positioned Shine-Delgarno (SD) sequence relative to the translation initiation start codon (Terui *et al.*, 2007, Terui *et al.*, 2009). Secondly, they stimulate read-through of Gln-tRNA^{supE} on ribosome-associated mRNA molecules and thirdly they can enhance translation from the inefficient start codon UUG (Terui *et al.*, 2007, Terui *et al.*, 2009). Two studies by Terui *et al.* demonstrated that polyamines can enhance the translation of both *hns* mRNA and *stpA* mRNA (Terui *et al.*, 2007, Terui *et al.*, 2009). The consensus SD sequence is 5'-AGGAGG-3' which ideally should be located 6-7 base pairs upstream of the start codon. The SD sequences of *hns* mRNA and *stpA* mRNA fall into the first class of those that are regulated by polyamines in that each has a degenerate consensus sequence located distally from the translation start codon (Terui *et al.*, 2007, Terui *et al.*, 2009). In exponential-phase cultures, Terui *et al.* showed that in the presence of the polyamine putrescine, H-NS and StpA protein levels increased while mRNA levels were unchanged, showing that regulation was at

the level of translation (Terui *et al.*, 2007, Terui *et al.*, 2009). This translational stimulation by putrescine could be abolished by replacing the native *hns* and *stpA* SD sequences with an optimally spaced SD sequence (Terui *et al.*, 2007, Terui *et al.*, 2009). While regulation by polyamines was the key focus of these studies, two new environmental conditions contributing to the expression of *hns* and *stpA* were also identified (Terui *et al.*, 2007, Terui *et al.*, 2009). Polyamines stimulated translation of *hns* mRNA when cells were cultured in the presence of 0.1 % glucose or 0.02 % glutamate but not in the presence of 0.4 % glucose, while stimulation of *stpA* mRNA only occurred at 42 °C and not at 37 °C (Terui *et al.*, 2007, Terui *et al.*, 2009).

3.1.6 Proteolysis and post-translational modification.

In addition to control of *stpA* gene expression, StpA protein levels are also modulated. Control of StpA was first investigated as a result of a spontaneous point mutation which arose in the *stpA* coding sequence in long-term stationary phase cultures of *E. coli* lacking both the *hns* and *rpoS* genes (Johansson & Uhlin, 1999). The mutation resulted in a change in the amino acid sequence of StpA from phenylalanine to cysteine at position 21 ($StpA_{F21C}$) (Johansson & Uhlin, 1999). This change restored viability of cells in long-term stationary phase *E. coli* cultures and resulted in increased levels of StpA protein (Johansson & Uhlin, 1999). As no increase in *stpA* expression was observed, the stability of the wildtype StpA protein ($StpA_{wt}$) was compared to that $StpA_{F21C}$ (Johansson & Uhlin, 1999). Only in the absence of functional H-NS, $StpA_{wt}$ was subject to proteolytic degradation and had a half life of about 35 min (Johansson & Uhlin, 1999). Under the same conditions, however, no degradation of $StpA_{F21C}$ was observed (Johansson & Uhlin, 1999). Subsequently, the Lon protease was shown to be responsible for the degradation of StpA, as StpA protein levels were unaffected in *lon* mutant strains (Johansson & Uhlin, 1999). The implications of these findings are twofold: (i) StpA most likely exists coupled with H-NS as a heterodimer so any idiosyncratic function of the StpA:StpA homodimer is unlikely to occur; (ii) the spontaneously-occurring $StpA_{F21C}$ mutation which renders StpA insensitive to Lon gives

insights into how the cell can evolve to overcome the loss of H-NS and provides an opportunity to study gene regulation mediated by StpA alone.

Although not subject to proteolysis,the H-NS protein is possibly modified post-translationally by binding of poly-(R)-3-hydroxybutyrate (PHB) (Reusch *et al.*, 2002). PHB is produced in all prokaryotes and was shown by Western blotting and chemical degradation assays to form complexes with H-NS with ≈21 units of PHB present per H-NS molecule (Reusch *et al.*, 2002). Due to its protein- and DNA-binding properties, it was hypothesised that PHB may bind and modify H-NS and thus aid in the H-NS:DNA interaction (Reusch *et al.*, 2002). This model is at best speculative as demonstrations of precise modifications of H-NS by PHB are thus far lacking and PHB has only been shown to complex with H-NS.

3.1.7 Specific expression and regulation in *S.* Typimurium.

As stated above in Section 3.1.1, much of the data regarding the regulation of expression of *hns* and *stpA* has been derived from studies in *E. coli* and due to their close relatedness these findings are assumed to apply equally to *S.* Typhimurium. However, many differences from the *E. coli* paradigm are emerging from *Salmonella*-Typhimurium-specific studies, particularly regarding the role of StpA. In *S.* Typhimurium, *stpA* mRNA and StpA protein levels have been observed to be maximal in the early stages of exponential growth (Lucchini *et al.*, 2009). Lucchini *et al.* showed that the transient nature of this expression, as seen in *E. coli,* did not hold true in *S.* Typhimurium where *stpA* mRNA and StpA protein remained detectable at significant levels throughout the exponential and the stationary phases of growth (Lucchini *et al.*, 2009). In the same study, Lucchini *et al.* identified the members of the StpA regulon in *S.* Typhimurium and found that StpA represses the RpoS regulon during exponential growth phase by modulating the stability of RpoS. It was also discovered that StpA activated the cAMP-CRP regulon in late exponential phase and influenced expression of several PhoP-dependent genes (Lucchini *et al.*, 2009). Furthermore, in contrast to *E. coli* where a loss of StpA resulted in no obvious phenotypes, in *S.* Typhimurium loss of

StpA resulted in an increased resistance of mid-exponential phase cultures to several stresses including low pH, 3 M NaCl, hydrogen peroxide and the antimicrobial agent polymyxin B (Lucchini *et al.*, 2009). In a separate study, *stpA* was identified as being part of the SOS regulon as expression of *stpA* mRNA increased in response to DNA damage mediated by mitomycin C (Benson *et al.*, 2000). This increase in *stpA* expression is dependent on RecA which induces the SOS response (Benson *et al.*, 2000).

The expression profile of *hns* mRNA and H-NS protein is equivalent in both *E. coli* and *S.* Typhimurium and is subject to cold-shock induction in both species (Hinton *et al.*, 1992). In *S.* Typhimurium, the *hns* gene has an additional regulatory input in the form of the Ferric Uptake Regulator (Fur) which has been identified as a negative regulator of *hns* expression (Troxell *et al.*, 2011). Fur binds to a 28-base-pair region of the *hns* promoter and strongly represses *hns* expression as expression was found to be 14-fold higher in *fur* mutants compared to wildtype *S.* Typhimurium (Troxell *et al.*, 2011). Again an environmental aspect is involved in regulation by Fur as its binding to the *hns* promoter and thus repression of *hns* transcription required the presence iron (Troxell *et al.*, 2011).

Thus, while the general structure and function of H-NS and StpA has been maintained in both organisms since the divergence of *S.* Typhimurium and *E. coli* from their last common ancestor, the expression of their genes and the factors governing their expression have diversified. In *S.* Typhimurium, StpA has evolved to exhibit more sustained expression throughout growth and as a result StpA plays a more integral role in the transcriptional regulatory network than it does in *E. coli* (Lucchini *et al.*, 2009). Expression of *hns* and *stpA* has also been co-opted to respond to new environmental conditions in *S.* Typhimurium, with *hns* now influenced by iron availability mediated by Fur and *stpA* responding to DNA damage (Troxell *et al.*, 2011, Benson *et al.*, 2000). These changes in expression of two pleiotropic global regulators may have helped contribute to the divergence of *Salmonella* from *E. coli* and perhaps allowed *Salmonella* to adapt to new ecological environments such as the intracellular

environment of the macrophage. This study aimed to characterise further the expression patterns of *hns* and *stpA* in *S*. Typhimurium. It also aimed to investigate the possible regulatory reasons responsible for the sustained *Salmonella*-specific expression pattern of *stpA*.

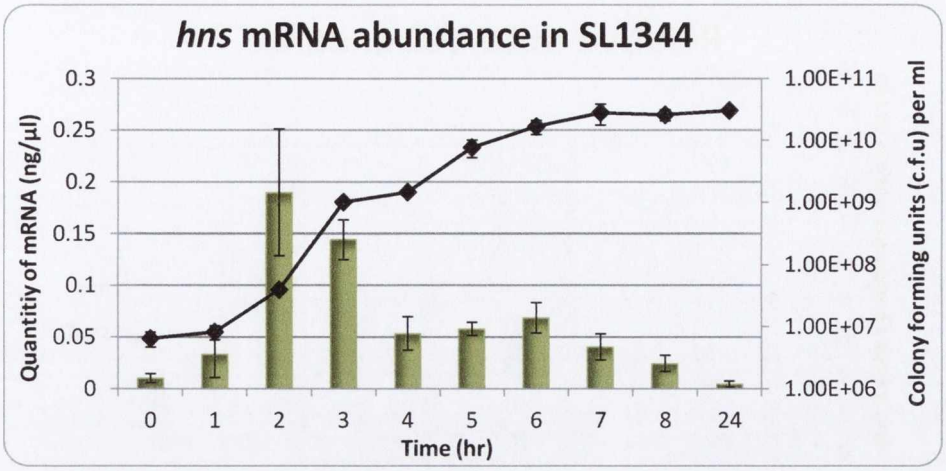
3.2 Results

3.2.1 Transcription profiles of *hns* and *stpA* in *Salmonella* Typhimurium

In previous studies, transcription of *hns* or *stpA* was monitored by means of transcriptional-fusions to reporter genes such as *gfp* or *lacZ*, or by direct visualization and quantification of mRNA levels via Northern blotting analysis (Dersch *et al.*, 1993, Free & Dorman, 1995, Free & Dorman, 1997, Lucchini *et al.*, 2009). In this study, Reverse Transcription-quantitative Polymerase Chain Reaction (RT-qPCR) was used to monitor the abundance of both *hns* and *stpA* mRNA throughout the normal growth of *S.* Typhimurium strain SL1344 at 37 ^{o}C in Luria-Bertani (LB) broth. Total RNA was extracted at 1 h time-points at a fixed OD_{600} value ($OD_{600} \approx 0.2$) throughout growth of SL1344 and used to create total cDNA pools by Reverse Transcription using random hexamer primers. From these cDNA pools the abundance of *hns* mRNA and *stpA* mRNA was quantified for each time-point by qPCR using either *hns* specific (*hns*.RT.fw, *hns*.RT.rev) or *stpA* specific (*stpA*.RT.fw, *stpA*.RT.rev) primer pairs. Both *hns* and *stpA* mRNA levels were quantified relative to known quantities of SL1344 chromosomal DNA which were also subjected to qPCR.

Expression of *hns* mRNA in *S.* Typhimurium was found to be maximal during the early- to mid-exponential phase of growth 2 – 3 h post-innoculation (Fig. 3.2 A). After this peak in expression, *hns* mRNA levels declined to a steady-state level for the remainder of growth before reaching a final baseline level after 24 h (Fig. 3.2 A). This pattern of *hns* expression has been observed previously in both *E. coli* and *S.* Typhimurium (Free & Dorman, 1995, Hinton *et al.*, 1992) and is consistent with the cell maintaining a relatively constant H-NS:DNA ratio by expressing *hns* maximally during exponential growth while chromosome replication is occurring (Free & Dorman, 1995, Hinton *et al.*, 1992). Consistent with the recently published transcription profile of *stpA* in *S.* Typhimurium, expression of *stpA* was also maximal 2 – 3 h post-innoculation (Fig. 3.2 B) during the early- to mid-exponential phase of growth (Lucchini *et al.*, 2009). In contrast to *E. coli*, where levels of *stpA* mRNA steadily decline after this burst of expression (Free & Dorman, 1997, Sonden & Uhlin, 1996), in *S.* Typhimurium an initial

A.



B.

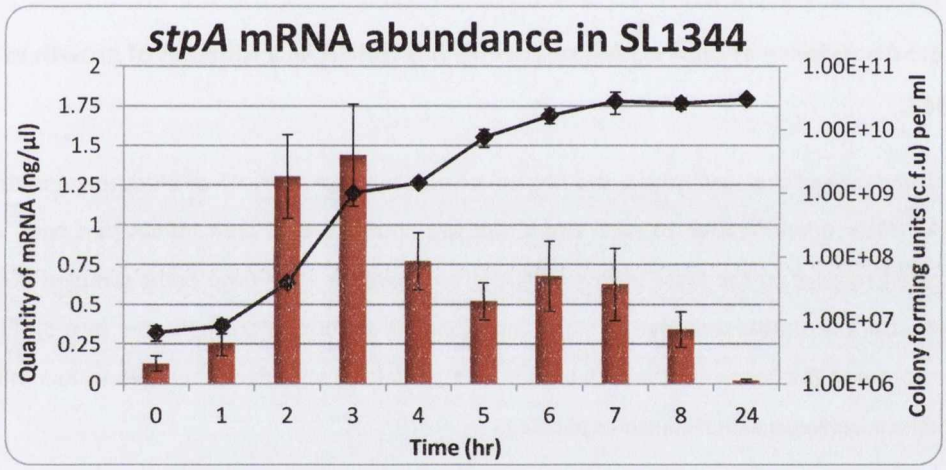


Fig. 3.2 Expression of *hns* and *stpA* in *S*. Typhumurium as a function of growth.

The expression patterns of *hns* (green bars) (A) and *stpA* (red bars) (B) were monitored during growth of wild-type strain SL1344 by RT-qPCR. Transcript abundance (primary y-axis) was quantified relative to a standard curve generated from serially diluted genomic DNA. Transcript levels are depicted as a function of growth (secondary y-axis) which was monitored by enumeration of viable colonies at equivalent time points. Data for transcript abundance and colony forming units was obtained from three independent experimental replicates (n = 3). Error bars represent the standard deviation between experimental replicates.

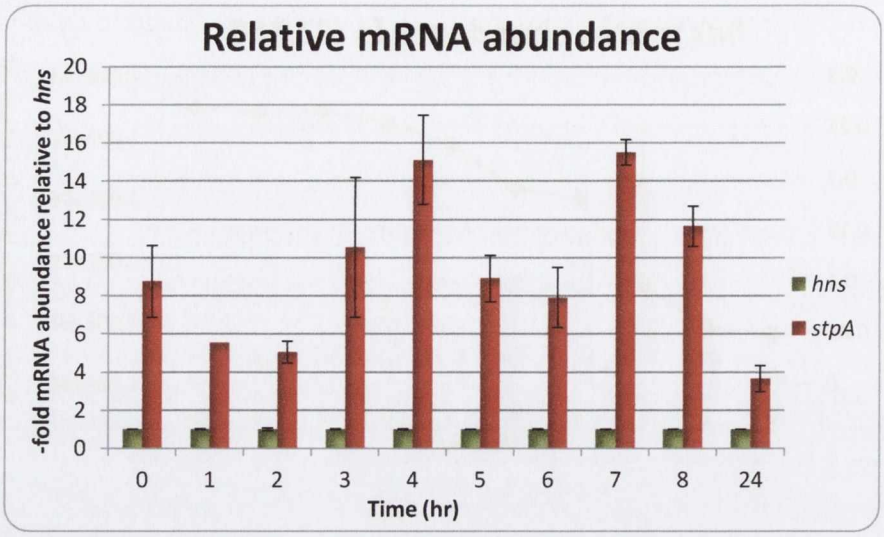


Fig. 3.3 Abundance of *stpA* mRNA relative to *hns* mRNA as a function of growth in SL1344.

The abundance of the *stpA* mRNA and *hns* mRNA was monitored by RT-qPCR during growth of SL1344. After quantification of each transcript, the abundance of *stpA* mRNA (red bars) was expressed relative to the level of *hns* mRNA (green bars) at each time point sampled during growth. Data are representative of three independent experimental replicates (n = 3). Error bars represent the mean standard deviation of *stpA* mRNA abundance relative to *hns* mRNA abundance between experimental replicates.

decline in expression was still observed but *stpA* mRNA continued to be detectable at relatively constant levels well into stationary phase of growth before declining to an almost undetectable level after 24 h of growth (Fig. 3.2 B). This was in good agreement with the findings of Lucchini *et al.* where a similar pattern of expression was observed using a chromosomal *stpA::gfp*$^{+}$ transcriptional fusion (Lucchini *et al.*, 2009).

Interestingly, quantification of the levels of *hns* and *stpA* mRNA in *S.* Typhimurium revealed that at all points throughout growth significantly more *stpA* mRNA than *hns* mRNA was present. In each sample analysed by RT-qPCR, *stpA* mRNA levels were 7- to 15-fold higher than that of *hns* with the exception of the 24-h time point where *stpA* mRNA was only 4.5-fold higher (Fig. 3.3). This is in stark contrast to the pattern of expression of *stpA* in *E. coli* where apart from a transient burst of expression at the mid-exponential growth, *stpA* mRNA is largely undetectable and is only detected at significant levels in strains devoid of *hns* (Sonnenfield *et al.*, 2001, Sonden & Uhlin, 1996, Free & Dorman, 1997). This high level of *stpA* transcription further indicates that the pattern of *stpA* expression in *S.* Typhimurium is distinct from that of the *stpA* gene in *E. coli* and provides a mechanism by which *S.* Typhimurium can maintain significant levels of StpA protein throughout its growth.

3.2.2 Epitope tagging and protein abundance of H-NS and StpA

To assay the abundance of H-NS and StpA it was decided to tag the H-NS and StpA proteins with distinct immunologically-reactive epitopes in wild-type *S.* Typhimurium. This was achieved using the method outlined by Uzzau *et al.*, 2001 whereby the DNA sequences encoding the relevant epitope tag are chromosomally fused with the 3' end of the gene of interest (Fig. 3.4). When the gene is expressed the resultant protein is epitope-tagged at its C-terminal end and can be detected using commercially available antibodies (Uzzau *et al.*, 2001). By this method H-NS and StpA were epitope-tagged with a 3xFLAG-tag and a Haemagglutinin (HA) tag, respectively, to give strains SL1344 *hns-flag* and SL1344 *stpA-HA* (Uzzau *et al.*, 2001). Chromosomally tagging *hns* and

Fig. 3.4 Diagramatic representation of chromosomal epitope tagging process using λ-red mediated recombination.

The epitope tag of choice is firstly PCR amplified in conjunction with a resistance marker cassette using primers designed such that the resultant PCR fragment contains 40 bp of homology at the 3' and 5' ends with the region of the chromosome where it is to be integrated. Cells expressing the λ-red recombination genes (*γ*, *β* and *exo*) are made electrocompetent and the epitope tag containing PCR product is introduced by electroporation. The PCR product homologous overhangs are acted upon by the recombinase and the entire product is integrated into the chromosome by homologous recombination. In the example outlined a 3 x Flag-tag was PCR amplified with a kanamycin resistance marker gene and was integrated at the 3' end of the chromosomal *hns* gene. When expressed the resultant H-NS protein contained a C-terminal 3 x Flag epitope which could be detected by Western immunoblotting.

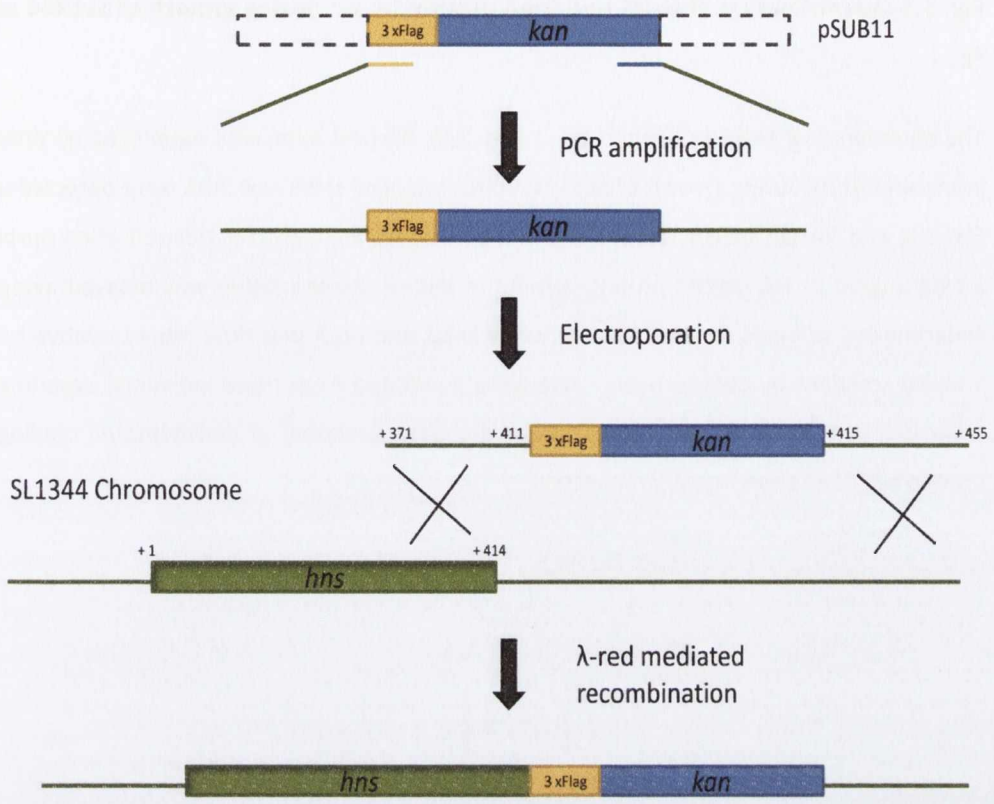
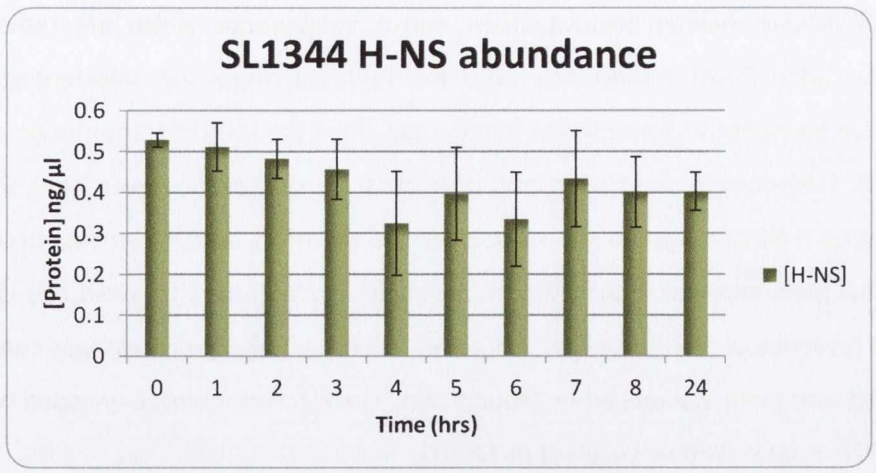
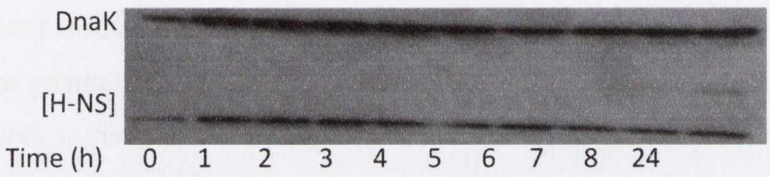

3 xFlag
kan
pSUB11
PCR amplification
3 xFlag
kan
Electroporation
+371
+411
3 xFlag
kan
+415
+455
SL1344 Chromosome
+1
+414
hns
λ-red mediated
recombination
hns
3 xFlag
kan

Fig. 3.5 Quantification of H-NS and StpA protein levels during growth of SL1344 at 37 ^{o}C.

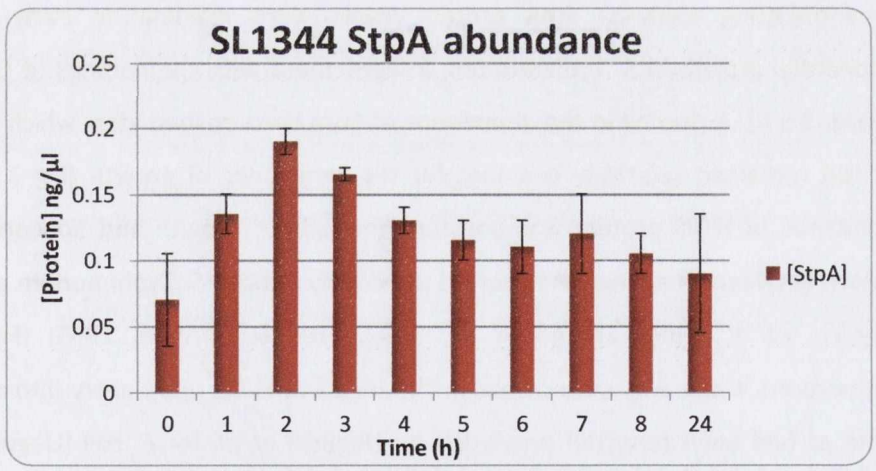
The abundance of H-NS (A) (green bars) and StpA (B) (red bars) was monitored by Western immunoblotting during growth of SL1344. Epitope-tagged H-NS and StpA were detected using Flag-tag and HA-tag specific monoclonal antibodies, respectively. For quantification purposes a Flag-tagged or HA-tagged protein sample of known concentration was assayed alongside experimental samples and the abundance of H-NS and StpA was determined relative to this internal standard by densitometry. Data were generated from three individual experimental replicates (n = 3). Error bars represent the standard deviation of densitometric readings of protein between experimental replicates.

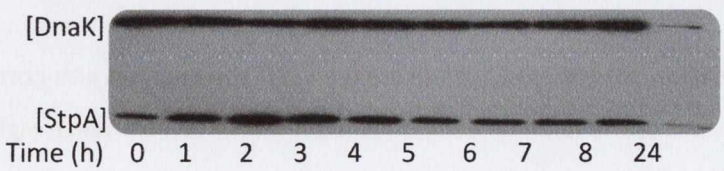
A.


SL1344 H-NS abundance
[Protein] ng/µl
0.6
0.5
0.4
0.3
0.2
0.1
0
0 1 2 3 4 5 6 7 8 24
Time (hrs)
[H-NS]


DnaK
[H-NS]
Time (h) 0 1 2 3 4 5 6 7 8 24

B.


SL1344 StpA abundance
[Protein] ng/µl
0.25
0.2
0.15
0.1
0.05
0
0 1 2 3 4 5 6 7 8 24
Time (h)
[StpA]


[DnaK]
[StpA]
Time (h) 0 1 2 3 4 5 6 7 8 24

stpA in this manner removes their native stop-codons which are replaced by a stop=codon (TAA) immediately after the FLAG-tag sequence. Interfereing with the native transcription termination signals may affect the level of transcription of *hns* and *stpA*. ChIP-on-chip and transcriptional microarray studies, however, have shown that tagging H-NS and StpA in such a manner had no affect on DNA binding occupancy or global gene expression patterns, respectively. Furthermore, to avoid any issues with the presence of resistance markers genes, once the tagging process was complete the resistance gene markers were 'flipped'using the FLP recombinase encoded on plasmid pCP20 as described by Uzzau *et al.* (2001).

Once H-NS and StpA had been epitope tagged, overnight cultures of SL1344 *hns-flag* and SL1344 *stpA-HA* were sub-cultured into 25 ml of LB broth and samples were taken over a 24-h time course ($OD_{600} \approx 0.2$). Samples were taken hourly for the first 8 h after sub-culturing and a final sample was taken after 24 h of growth. The abundance of H-NS and StpA throughout the growth was then quantified relative to a known concentration of a FLAG-tagged or HA-tagged protein, respectively, by Western immunoblotting analysis. StpA protein levels were maximal in early- to mid-exponential growth in *S.* Typhimurium, in agreement with the findings of Lucchini *et al.* (Fig. 3.5 B). A decline in the abundance of StpA then ensued after which the levels of StpA remained relatively constant for the remainder of growth (Fig 3.5 B). The abundance of H-NS protein was constant throughout growth and appeared not to deviate between samplings as reported previously in both *S.* Typhimurium and *E. coli* (Ueguchi *et al.*, 1993, Hinton *et al.*, 1992, Free & Dorman, 1995) (Fig 3.5 A). Furthermore, there was no increase in the levels of H-NS upon entry into stationary phase as had been reported previously by Ueguchi *et al.* for *E. coli* (Ueguchi *et al.*, 1993).

Upon quantification of H-NS and StpA protein levels, however, an anti-correlation was detected with that of the abundance *hns* and *stpA* mRNA levels. While higher levels of *stpA* mRNA relative to *hns* mRNA were detected at every time point sampled during growth, the opposite was observed for H-NS and StpA protein levels. Here the

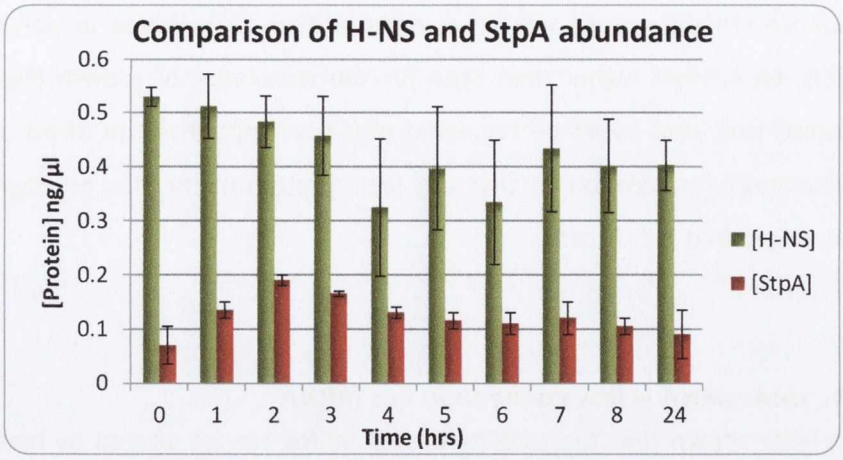


Fig. 3.6 Comparison of the levels of H-NS and StpA proteins during growth of SL1344.

The levels of the H-NS (green bars) and StpA (red bars) proteins were monitored by Western immunoblotting during growth of SL1344. The abundance of each protein was quantified by densitometry relative to a FLAG-tagged or HA-tagged protein of known quantity and plotted for comparison. Data were obtained from three independent experimental replicates (n = 3). Error bars represent the standard deviation of protein abundance between experimental replicates.

abundance of H-NS protein was 7.5-fold higher than that of StpA in starting cultures and 2.5- to 4.5-fold higher than StpA for the remainder of growth (Fig. 3.6). This suggested that *stpA* could be regulated post-transcriptionally to affect *stpA* mRNA stability and/or translation or that the StpA protein might also be degraded in *S.* Typhimurium as it is in *E. coli.*

3.2.3 *stpA* mRNA is less stable than *hns* mRNA

It has been shown that the stability of *hns* mRNA can be altered by binding of the small RNA, DsrA, immediately downstream of the translational start codon (Sledjeski *et al.*, 1996, Sledjeski & Gottesman, 1995). When over-expressed, the binding of DsrA to *hns* mRNA reduces the stability of the *hns* message. To date no similar form of regulation has been shown for *stpA.* However, a high rate of turnover of *stpA* mRNA may help to explain the anti-correlation between the high levels of *stpA* mRNA and the relatively low levels of StpA protein in comparison to *hns* mRNA and H-NS protein. To test this hypothesis the stability of *stpA* mRNA was compared with that of *hns* mRNA in wild-type SL1344. From overnight cultures, SL1344 was sub-cultured into 25 ml of LB-broth and grown until early-exponential phase of growth (OD_{600} 0.13-0.15). Transcription was then inhibited by the addition of the antibiotic rifampicin to prevent further accumulation of *stpA* or *hns* mRNA. Total RNA was extracted in a time-course experiment and samples were then analysed by RT-qPCR to estimate the relative abundance of *stpA* and *hns* mRNA.

Upon the addition of rifampicin to the culture a sample total RNA was immediately taken to determine the starting levels (t=0) of *stpA* and *hns* mRNA and all subsequent mRNA levels were expressed relative to these values. Five minutes after the inhibition of transcription, the amount of *hns* transcript was reduced to 60% of the starting level (Fig. 3.7). The level of *hns* mRNA remained at 60 % of that of the starting quantity for 15 min post-transcription inhibition before dropping to 40 % after 20 min. Thus, the *hns* mRNA molecule appeared to be relatively stable over time and was maintained at readily detectable levels. A more dramatic effect was observed with the stability of

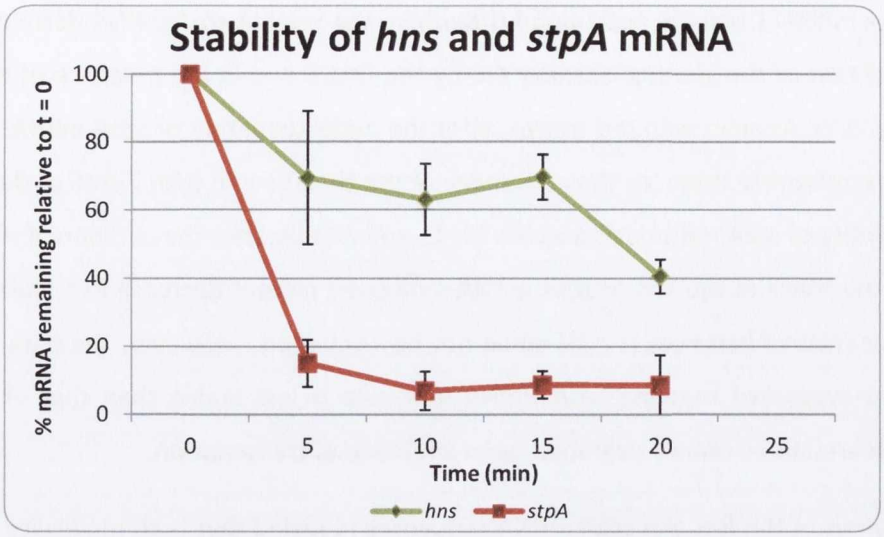


Fig. 3.7 Comparison of stability of the *hns* and *stpA* mRNA molecules.

The stability of the *hns* (green diamonds) and *stpA* (red squares) mRNA molecules over time was assessed by RT-qPCR. Following inhibition of transcription using rifampicin at time T = 0, the abundance of each mRNA was measured every 5 min over a 20 min time period. Abundance is expressed as a percentage of the starting quantity of either *hns* or *stpA* mRNA at time T = 0. Data represent three independent experimental replicates (n = 3). Error bars represent the standard deviation of the percentage transcript remaining between experimental replicates.

stpA mRNA. Upon the addition of rifampicin, the level of *stpA* mRNA decreased to 15 % of that of the starting quantity during the first 5 min in the presence of rifampicin (Fig. 3.7). As seen with *hns* mRNA, after the initial depletion of *stpA* mRNA, its levels were relatively static for the remainder of the time course with 7-8 % of the starting quantity of *stpA* mRNA still present 10, 15 and 20 min after the addition of rifampicin. As the levels of the *hns* or *stpA* mRNA molecules did not decrease in a linear fashion their relative half-lives (t = ½) could not be calculated. However, the data obtained here suggested that the *stpA* mRNA molecule is less stable than that of *hns* and appeared to be rapidly degraded upon inhibition of transcription.

Analysis of the *hns* and *stpA* mRNA sequences revealed that both molecules had sub-optimal ribosome binding sites (RBS). In *E. coli* the consensus RBS sequence is AGGAGG which is optimally positioned 7/8 base pairs upstream of the translation initiation codon ATG (Delvillani *et al.*, 2011, Vimberg *et al.*, 2007). The RBS binding site of *hns*, although optimally positioned 8 bps upstream of the ATG triplet, was a degenerate and shortened sequence consisting of GAGA (Fig. 3.8A). Similarly, the *stpA* mRNA molecule had a shortened RBS (AGG) which was also distally located 11 bp upstream from the ATG triplet (Fig. 3.8 B). The presence of shortened RBS sequences and sub-optimal spacing between the RBS and the initiation codon are known to reduce the efficiency of translation (Vimberg *et al.*, 2007). A reduced rate of translation is known to decrease the half-life of mRNA molecules (Delvillani *et al.*, 2011). It is therefore possible that the presence of a shortened RBS positioned distally from ATG is a contributing factor to the apparent instability of the *stpA* mRNA relative to that of *hns* mRNA which, despite also having a shortened RBS, is optimally placed. Efficiency of translation can also be reduced by the presence of secondary structures within the mRNA that block access to the RBS and/or the initiation codon (Kozak, 2005). Secondary structure predications of the *hns* mRNA molecule showed that only two of the four RBS base pairs were complimentary paired within the secondary structure while its initiation codon was readily accessible as part of a single-stranded loop. In contrast, the *stpA* mRNA secondary structure was predicted to have a readily

accessible RBS located within a single-stranded loop, however, its initiation codon was complementary base paired. Furthermore, with the exception of the RBS, much of the *stpA* 5' untranslated region was extensively base paired. Such extensive base pairing coupled with the occlusion of the ATG start codon would greatly reduce the rate of stpA translation and contribute to the observed instability of the molecule. In addition, sequence analysis also showed that the *stpA* mRNA contained an A/U rich region with several poly-U tracts upstream from the RBS in contrast to *hns* which did not. In general, such regions act as translational enhancers and help stabilise mRNA by attracting the binding of ribsomes (Delvillani *et al.*, 2011). The same regions, however, are also preferred cleavage sites for the RNaseE component of the degradosome and may therefore be involved in the rapid turnover of *stpA* mRNA (Carpousis, 2007).

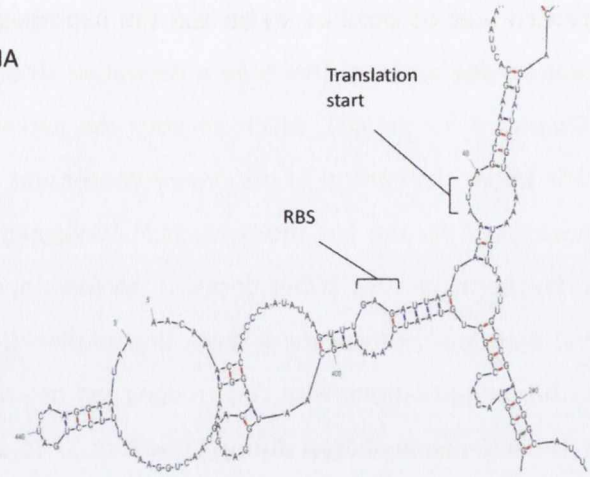
3.2.4 StpA in *S.* Typhimurium is subject to proteolysis by the Lon protease

It is known that in *E. coli* the StpA protein is subject to proteolytic degradation mediated by the Lon protease (Johansson & Uhlin, 1999). At the amino acid sequence level, the StpA protein from *S.* Typhimurium SL1344 shares 84 % identity with StpA from *E. coli*. Importantly, a phenylalanine residue at position 21 that has been deemed essential for cleavage by the Lon protease in *E. coli* is conserved in both species. This suggests that StpA may also be subject to proteolysis by Lon in *S.* Typhimurium. Thus, experiments were undertaken to assess the stability of StpA in SL1344 wildtype, *hns*, *lon* and *hns lon* strains. Cultures of each were grown to exponential phase ($OD_{600} \approx 0.4$) before protein synthesis was inhibited by the addition of the antibiotic spectinomycin. Protein samples were taken upon addition of spectinomycin and at regular time intervals thereafter and the stability of StpA in each genetic background was assessed by quantitative Western immunoblotting. Despite numerous attempts, StpA protein was only detected in wild-type SL1344 and not in any of the other three strains. In spite of repeated attempts at re-constructing the relevant strains *de novo*, the result was the same each time.

Fig. 3.8 Sequences and predicted secondary structures of *hns* and *stpA* mRNA.

The first 90 bps of the *hns* (A) and *stpA* (B) mRNA molecules are shown beneath their predicted secondary structures. Ribosome binding site (RBS) sequences are highlighted in yellow and the translation initiation codons (ATG) are underlined. In the *stpA* mRNA poly-U tracts are shown in blue. Secondary structures show the first 50 - 60 bps of the *hns* and *stpA* mRNA molecules. The positions within predicted secondary structures of the RBS and translation initiation start codons are highlighted. Secondary structure predications were made using full length mRNA sequences. Structures shown were present in all ten of the lowest energy structures predicted.

A. *hns* mRNA

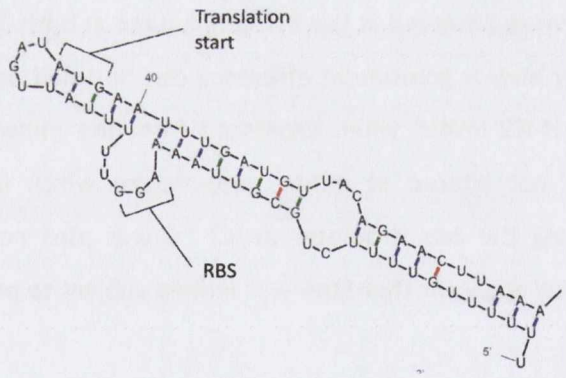


AACAAACCACCCCAATATAAGTTTGAGATTACTACAATGAGCGAAGCACTTAAAATTCTGAACAACATCCGTACTCTTCGTGCGCAGGCAA

RBS

Translation
start

B. *hns* mRNA



Poly-U tracts

TTTTTTGTTTTCTGCGTTAAAAGGTTTTTATTGATATGAATTTGATGTTACAGAACTTAAATAATATCCGCACGCTGCGCGCTATGGCTC

RBS

Translation
start

An alternative approach was adopted to try to test the hypothesis that StpA was a substrate for proteolytic degradation. This time a derivative strain of SL1344, strain SL1344$^{2Xstp A}$ (see Chapter 4 for details), which encodes the native *stpA* allele and a copy of the *stpA* ORF under the control of the *hns* promoter was used. In this strain the copy of *stpA* expressed via the *hns* promoter is FLAG-tagged and was used for detection of StpA. Furthermore, this strain contains no functional H-NS which has been shown to bind with StpA, protecting it from degradation (Johansson & Uhlin, 1999). To avoid further complications with constructing *lon* deletion mutants it was decided to look at the abundance of StpA during growth at 37 °C and 42 °C. At 42 °C the expression and activity of Lon and several other proteases is increased (Goff *et al.*, 1984). Therefore, it was reasoned that at this temperature degradation of StpA would be more apparent. As seen in Fig. 3.9, at 37°C the StpA protein was detected after 2 h, 4 h and 6 h of growth in LB medium in strain SL1344$^{2Xstp A}$. Levels of StpA also decreased as a function of time in a manner similar to that observed in wild-type SL1344. At 42 °C, however, while the abundance of StpA after 2 h was equivalent to that observed at 37 °C, thereafter little or no StpA protein was detected. As similar levels of StpA were observed at the 2 h sample time at both 37°C and 42°C differential mRNA stability and/or translation efficiency can be ruled out as causing this effect. Furthermore, H-NS levels, when expressed from the native *hns* gene in wild-type SL1344, were not altered at either temperature which indicated that increased transcription via the *hns* promoter at 42 °C was also not a contributing factor. Therefore it was apparent that StpA was indeed subject to proteolytic degradation at 42 °C.

3.2.5 Gene copy number and *hns* and *stpA* expression levels

A possible contributing factor to the high levels of *stpA* mRNA compared with *hns* mRNA in *S.* Typhimurium is the relative distance of the *hns* and *stpA* genes from the origin of chromosome replication, OriC. In *E. coli* and *S.* Typhimurium, chromosome replication is initiated at OriC and proceeds bi-directionally towards the terminus

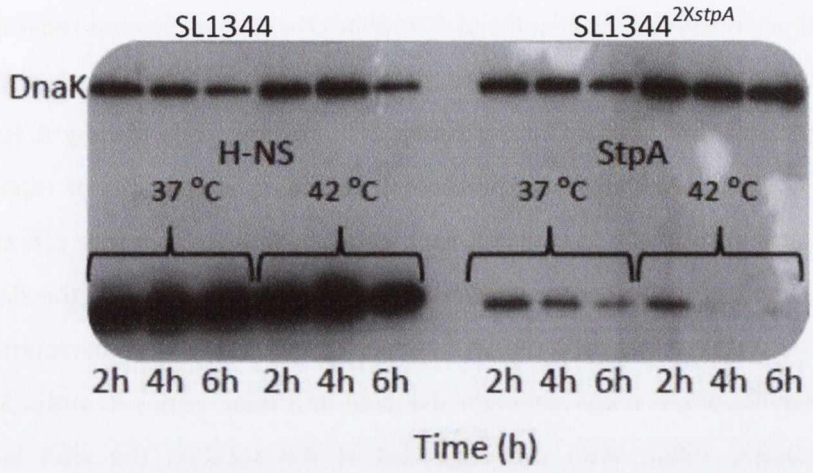


Fig. 3.9 Western immunoblot demonstrating the proteolytic degradation of StpA at 42 °C in *S.* Typhimurium.

The stability of the StpA protein over time was monitored in strain SL1344^{2XstpA} which expresses a Flag-tagged copy of StpA via the *hns* promoter. Samples were taken throughout growth at 37 °C and 42 °C and analysed by Western immunoblot using an FLAG-tag antibody to detect StpA. Blots were also probed with a DnaK antibody for the detection of DnaK which was used as a sample loading control. To ensure no difference in the level of expression via the *hns* promoter was occurring between each experimental temperature, the abundance of Flag-tagged H-NS was also monitored in SL1344 *hns-flag*.

region TerC (Thanbichler, 2010, Haeusser & Levin, 2008). To facilitate the short generation times observed for *E. coli* and *S.* Typhimurium, chromosome replication is initiated synchronously from OriC more than once per cell cycle and results in daughter cells inheriting a partially replicating chromosome copy (Atlung & Hansen, 2002). In any given cell there are therefore 2^n copies of the origin of replication (Atlung & Hansen, 2002). Consequently, each gene on the chromosome can also be present as 2^n copies with gene copy number inversely proportional to the distance from OriC. The origin of replication in *S.* Typhimurium strain LT2 was characterized as a 43 bp sequence which maps between the *gidA* and *mioC* genes in strain SL1344 (Zyskind & Smith, 1980). With the origin set at this location the *stpA* locus is positioned at a distance of 1.1 Mbp while the *hns* locus is positioned much further away at a distance of 2.3 Mbp. Thus, based on proximity to OriC, during the replication process SL1344 can potentially have more copies of the *stpA* gene, and thus template for transcription, than that of the *hns* gene. This may be a contributing factor to the increased levels of *stpA* mRNA relative to *hns* mRNA.

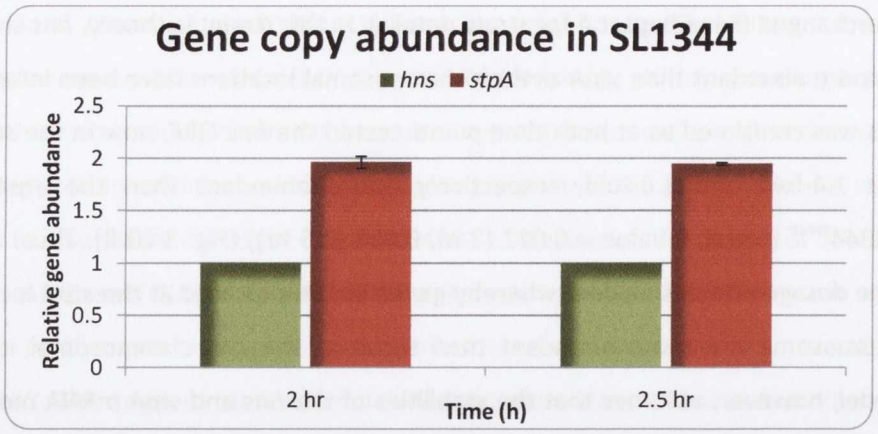
To test this hypothesis, wild-type SL1344 was grown to early-exponential phase of growth when maximal expression of both *stpA* and *hns* was observed. Total cellular DNA was extracted at two time points during this period of maximal expression and qPCR was performed using primers specific for the *stpA* and *hns* loci. Quantification of *stpA* and *hns* by qPCR revealed that at both time points tested the *stpA* locus was 2-fold more abundant than the *hns* locus (t-test, P-values = 0.0012 (2 h), 0.0005 (2.5 h)) (Fig. 3.10 A). This indicated that, at least within the time frame tested, there were twice as many copies of *stpA* available to RNA polymerase for transcription which could contribute to the significantly greater levels of *stpA* mRNA detected in SL1344 relative to *hns* mRNA (Fig. 3.10 A). If the chromosomal location of *stpA* and *hns* was genuinely contributing to gene copy number, and thus to the level of transcription, then in theory any ORF or gene placed at the *stpA* locus should be more abundant than any ORF or gene placed at the *hns* locus. To test this, the experiment was repeated using a strain, SL1344^{swap}, in which the *stpA* and *hns* ORFs have been

interchanged (See Chapter 4 for strain details). In this strain, in theory, *hns* should now be more abundant than *stpA* as their chromosomal locations have been interchanged. This was confirmed as at both time points tested the *hns* ORF, now in the *stpA* locus, was 1.4-fold and 1.6-fold, respectively, more abundant than the *stpA* ORF in SL1344^{swap} (t-test, P-value = 0.012 (2 h), 0.054 (2.5 hr)) (Fig. 3.10 B). Thus, a genuine gene dosage effect is evident whereby genes or ORFs located at the *stpA* locus on the chromosome are more abundant than those at the *hns* chromosomal locus. This model, however, assumes that the stabilities of the *hns* and *stpA* mRNA molecules in *S.* Typhimurium and *E. coli* are equal. Given the apparent instability of the *stpA* mRNA molecule in *S.* Typhimurium the effect of gene dosage may actually have a greater impact on transcription than that assayed by RT-PCR.

3.2.6 Transcription of *hns* but not *stpA* is coupled with DNA replication

The process of chromosome replication, aside from affecting gene dosage, is also known to affect the expression of *hns,* at least in *E. coli*. Transcription of *hns* is tightly coupled with the process of chromosomal DNA replication such that expression is maximal during exponential growth when cells are rapidly dividing (Free & Dorman, 1995). As a result the bacterium maintains a relatively constant H-NS:DNA ratio with *hns* transcription decreasing upon cessation of DNA replication (Free & Dorman, 1995). In *S.* Typhimurium, *hns* transcript levels were most abundant in the exponential phase of growth during peak DNA replication and cell division. Therefore, it was of interest to test if *hns* expression was coupled with DNA replication in *S.* Typhimurium. To do this, cultures of wild-type SL1344 were grown to mid-exponential growth phase ($OD_{600} \approx 0.3$) before replication fork movement was halted by inhibiting DNA gyrase activity with nalidixic acid. Samples of total RNA were extracted upon the addition of nalidixic acid and every 15 min thereafter for a total of 90 min. Cells were then harvested, washed twice in Analar™ water to remove the drug and re-suspended in drug free LB-broth and sampled as before. Samples were analysed by RT-qPCR to assess what effect, if any, the inhibition of replication had on transcription of *hns*.

A.



B.

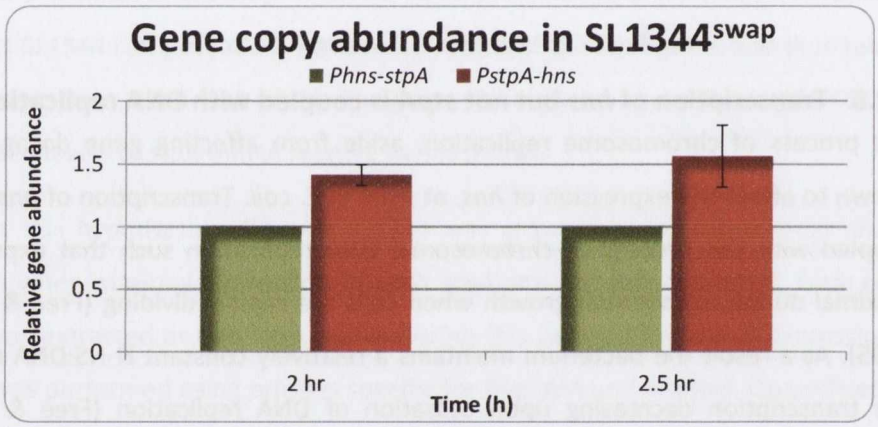


Fig. 3.10 Determination of the *hns* and *stpA* gene copy abundance.

The abundance of the *hns* (green bar) and *stpA* (red bar) genes was quantified in SL1344 (A) and SL1344^{swap} (B). Samples were taken at times corresponding to maximal expression of *hns* and *stpA*. The gene copy abundance is expressed relative to the wild-type *hns* allele in SL1344 and the hybrid P_{hns}-*stpA* allele in SL1344^{swap}. Data presented for SL1344 and SL1344^{swap} was derived from three independent experimental replicates (n = 3). Error bars represent the standard deviation between experimental replicates.

As reported previously for *E. coli,* upon the addition of nalidixic acid a rapid inhibition of *hns* transcription was observed in *S.* Typhimurium (Fig. 3.11). Fifteen minutes after the addition of Nalidixic acid, the expression of *hns* fell to 50% of that of the starting quantity and continued to decline thereafter reaching 11% of the starting quantity after 60 min. After the removal of Nalidixic acid a recovery of *hns* expression over time was observed reaching 60% that of the starting expression after 60 min. This is in contrast with the observed 100% recovery of expression observed by Free and Dorman in *E. coli* but is in good agreement with the data obtained in the same study using a *dnaC*^{ts} mutant to inhibit replication where a 60 – 70 % recovery of expression was observed (Free & Dorman, 1995). Thus, in both *S.* Typhimurium and *E. coli* expression of the *hns* gene is coupled with the process of DNA replication, both of which are maximal during exponential growth. The exact mechanism by which up-regulation of *hns* is mediated by DNA replication, however, remains unknown and it was shown above (Section 3.2.5) that it is not due to an effect of gene dosage.

3.2.7 Transcriptional control by FIS

In *E. coli,* up-regulation of *hns* expression during exponential growth was shown to be, in part, due to the transcriptional regulator FIS binding within the *hns* promoter region (Falconi *et al.*, 1996). A total of seven FIS binding sites were identified within the *hns* regulatory region, two of which overlapped with H-NS binding sites (Falconi *et al.*, 1996). FIS was shown to be capable of out-competing H-NS for binding to these sites in a concentration-dependent manner, relieving H-NS mediated auto-repression and increasing *hns* transcription 2-fold (Falconi *et al.*, 1996). Expression of *fis* and FIS protein levels are maximal in early-exponential growth providing enough FIS protein to relieve H-NS auto-repression (Ó. Cróinín & Dorman, 2007, Ball *et al.*, 1992). As *hns* and *stpA* exhibited a similar peak in expression which coincides with maximal *fis* expression during early- to mid-exponential growth, the effect of FIS on *hns* and *stpA* transcription in *S.* Typhimurium was examined. For this, wild-type SL1344 and a *fis* deletion strain (SL1344 *fis*::*kan*) were grown to early exponential phase. Total RNA

samples were then taken over a 1 h time period during which maximal *hns* and *stpA* expression was found primarily to occur in wild-type cells. Samples were subjected to RT-qPCR to assess the impact of FIS on *hns* and *stpA* transcript levels.

In wild-type SL1334 in the presence of FIS, peak expression of *hns* was observed in early- to mid-exponential growth 2 to 3 h post inoculation, with peak expression occurring after 2.5 h (Fig. 3.12 A). In the absence of FIS, however, the levels of *hns* mRNA were reduced to 58 % of wild-type levels after 2 h and 2.5 h of growth (t-test, P-values = 0.005 and 0.007, respectively) before being restored to wild-type levels after 3 h (Fig. 3.12 A). These results agree with those previously observed in *E. coli* whereby the initial peak in *hns* expression, due to the high levels of FIS, is relieved as cells progress towards stationary phase and the concomitant reduction in *fis* expression (Ball *et al.*, 1992). Previously, it was demonstrated that FIS had no effect on *stpA* transcription in *E. coli* (Free & Dorman, 1997). In *S.* Typhimurium, however, *stpA* has a somewhat different expression profile than the one observed in *E. coli* and *stpA* mRNA was significantly more abundant than that of *hns* mRNA (Fig 3.3). As *hns* and *stpA* have a similar timing of peak expression, FIS may also play a role in growth-phase dependent up-regulation of *stpA* in *S.* Typhimurium. For this reason, *stpA* mRNA levels were monitored in wild-type SL1344 and SL1344 *fis*::*kan*. A similar trend to that observed for *hns* was also seen for expression of *stpA* whereby its expression decreased at both the 2 h and 2.5 h time points in SL1344 *fis*::*kan* compared with that of wild-type SL1344 and expression was again equivalent after 3 h (Fig. 3.12 B). Unfortunately, however, these results were error prone and deemed statistically insignificant.

3.2.8 Temperature regulation of *hns* and *stpA*

Temperature fluctuation is a key environmental factor that affects transcription of *hns* and *stpA* (Free & Dorman, 1997, La Teana *et al.*, 1991). In *E. coli,* expression of *stpA* was found to be induced upon a temperature up-shift from 30 ^{o}C to 37 ^{o}C while in

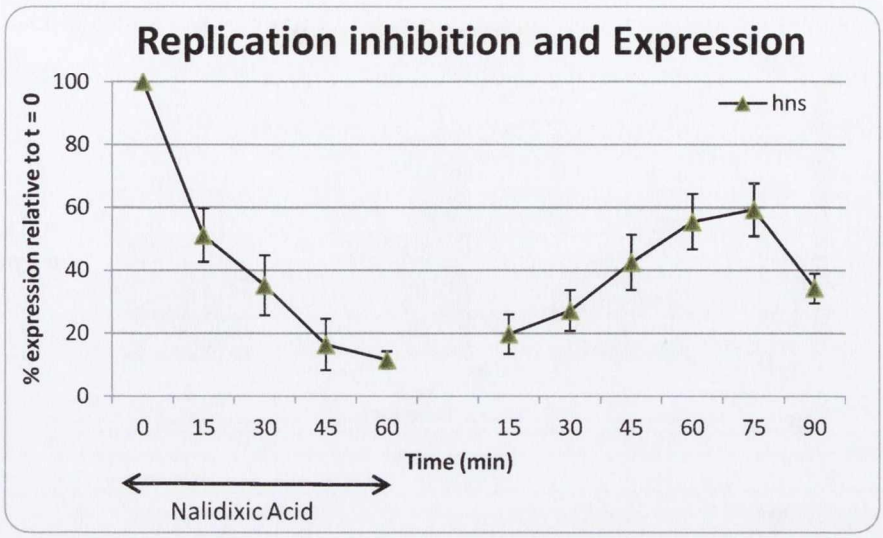


Fig. 3.11 The effect of DNA replication inhibition on *hns* transcription.

Chromosomal DNA replication was inhibited by the addition of Nalidixic acid to exponentially growing cultures of SL1344. Samples were taken upon the addition of Nalidixic acid and every 15 min thereafter for a period of 60 min. Cultures were then harvested and washed to remove the drug and allowed to recover for 90 min in drug free media during which time total RNA samples were again taken every 15 min.The abundance of *hns* mRNA (green diamonds) present at each sample time point is represented as a percentage of *hns* mRNA present at time t = 0. Data were derived from three independent experimental replicates (n = 3). Error bars represent the mean standard deviation of percentage expression relative to t = 0 between experimental replicates.

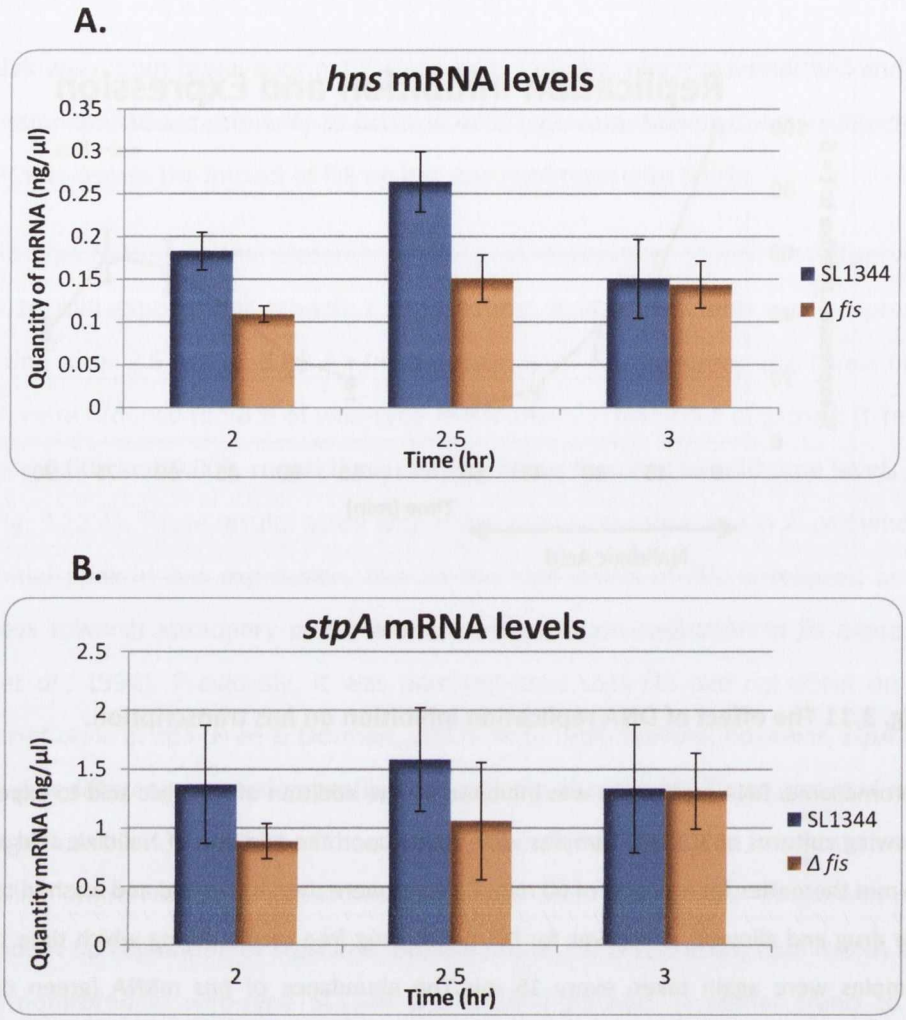


Fig. 3.12 The effect of FIS on *hns* and *stpA* gene expression in *S.* Typhimurium SL1344.

The contribution made by FIS to the peak expression of *hns* (A) and *stpA* (B) was assessed by RT-qPCR in SL1344. Cultures of wild-type SL1344 (Blue bar) and SL1344 *Δ fis* (Orange bar) were grown to early exponential growth phase and total RNA was extracted at regular intervals over a 1-h time period when maximal expression of *hns* and *stpA* is normally observed. The expression level of *hns* and *stpA* in SL1344 *Δ fis* was determined relative to a standard curve generated from genomic DNA of known quantity and plotted against wild-type levels in SL1344 for comparison. Data were derived from three individual experimental replicates (n = 3). Error bars represent the standard deviation in transcript abundance between experimental replicates.

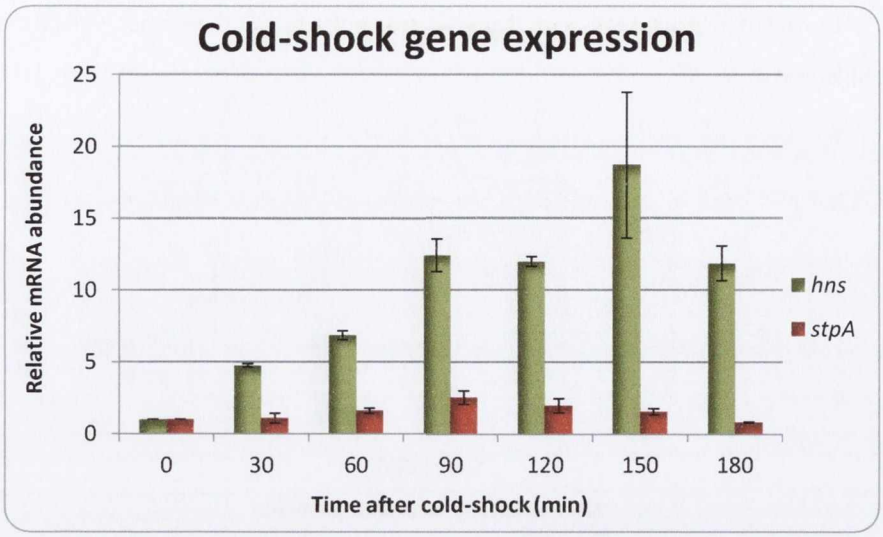
both *S.* Typhimurium and *E. coli* expression of *hns* was found to be up-regulated upon temperature down-shifts from 37 °C to 10 °C (Free & Dorman, 1997, La Teana *et al.*, 1991). In addition to an increase in expression under cold-shock conditions, *hns* mRNA is also more efficiently translated at low temperatures than non-cold shock regulated mRNA molecules (Giuliodori *et al.*, 2004). Coupled with the increase in expression, this has the net effect of increasing the cellular level of H-NS protein. To observe cold-shock regulation of *hns* in *S.* Typhimurium, cultures of wild-type SL1344 were grown to late-exponential phase (OD_{600} = 0.5) at 37 °C before being shifted to 10 °C. Total RNA samples were extracted at the start of the cold-shock (t = 0) and every 30 min thereafter for a total of 180 min. Samples were analysed by RT-qPCR to observe cold-shock induction of *hns* expression.

When subjected to cold-shock conditions *hns* mRNA levels increased in SL1344 while no discernible effect was observed on the levels of *stpA* mRNA (Fig. 3.13 A). When shifted from 37 °C to 10 °C, a steady increase in the levels of *hns* mRNA was observed over the course of the first 90 min of cold-shock. After 30 min at 10°C *hns* mRNA levels increased 5-fold relative to the level observed at time t = 0 and further increased to 12-fold after 90 min. Thereafter, no further increase in *hns* mRNA abundance was observed and levels remained static for the remainder of the experiment. The abundance of a FLAG-tagged copy of the H-NS protein was also observed during cold-shock to monitor any changes to H-NS protein levels in response to cold-shock in *S.* Typhimurium. Protein samples were taken at equivalent time points to those used for sampling mRNA and analysed by Western immunoblotting using a monoclonal FLAG-tag antibody to detect H-NS. To account for unequal loading of protein samples each blot was also probed with a DnaK antibody. As the cellular concentration of DnaK is deemed not to change over-time the levels of H-NS detected were first normalised to DnaK and subsequently quantified relative to a known quantity of a FLAG-tagged protein. By this method the abundance of H-NS was observed to increase steadily as a function of time spent in cold-shock conditions. Similar to *hns* mRNA abundance the abundance of H-NS also reached a peak level after 90 min which was 2-fold greater

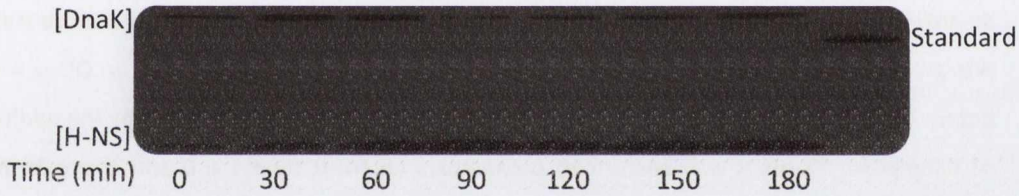
Fig. 3.13 *hns* mRNA and H-NS protein abundance under cold-shock conditions in *S.* Typhimurium.

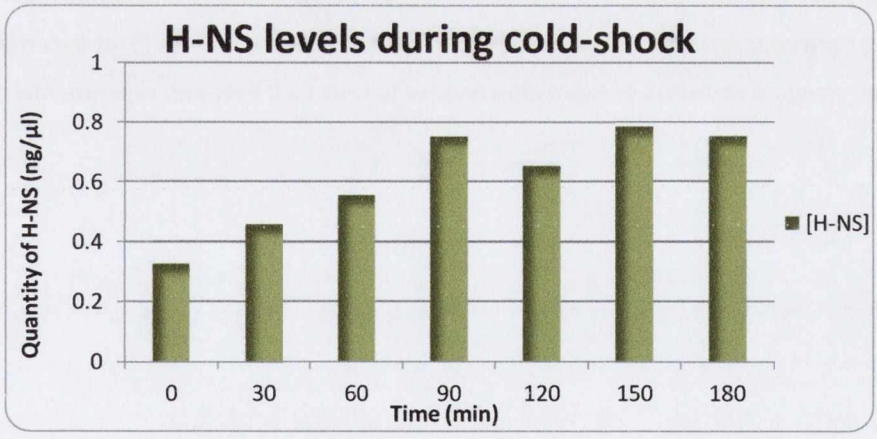
Exponential phase cultures (OD_{600} = 0.5) of wild-type SL1344 grown at 37°C were shifted to 10°C. Samples of total RNA and total protein were extracted at the start of the cold-shock treatment (time, t = 0) and every 30 min thereafter for a total of 180 min. Samples were analysed by RT-qPCR for the abundance of *hns* mRNA (green bars) (A) or by Western immunoblot for H-NS protein abundance (B) at each time-point. The abundance of *hns* mRNA is expressed relative to level present at the start of cold-shock treatment. As a negative control RNA samples were also analysed for the abundance of *stpA* mRNA (red bars), which is not known to be induced by cold-shock, the levels of which did not appreciably change during cold-shock treatment. Flag tagged H-NS protein was detected by Western immunoblotting using a monoclonal Flag-tag antibody. Protein abundance was first normalised to loading control, DnaK, and then quantified relative to a flag-tagged protein of known quantity using densitometry. RT-qPCR data were obtained from three independent experimental replicates (n = 3) while Western immunoblot data are preliminary (n = 1). Error bars represent the standard deviation in *hns* mRNA abundance between experimental replicates.

A.


Cold-shock gene expression
Relative mRNA abundance
25
20
15
10
5
0
0
30
60
90
120
150
180
Time after cold-shock (min)
hns
stpA

B.


[DnaK]
[H-NS]
Standard
Time (min)
0
30
60
90
120
150
180


H-NS levels during cold-shock
Quantity of H-NS (ng/µl)
1
0.8
0.6
0.4
0.2
0
0
30
60
90
120
150
180
Time (min)
[H-NS]

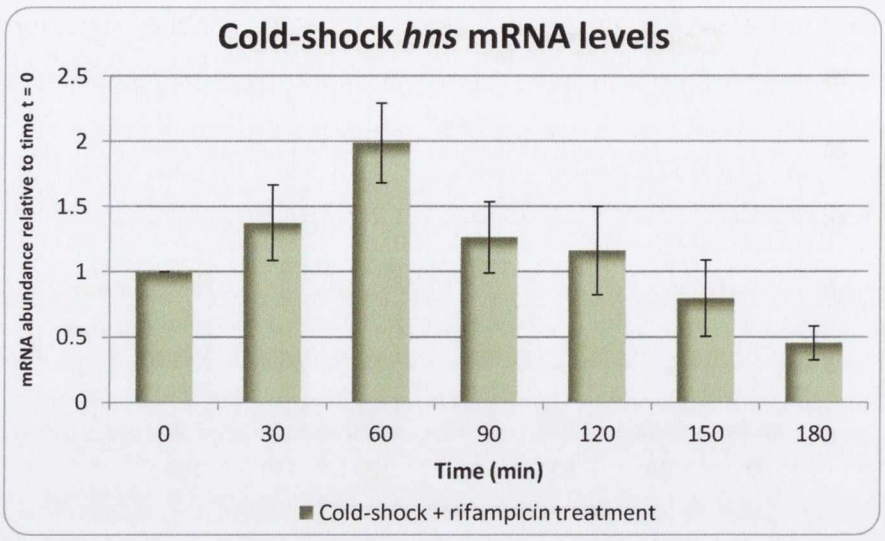


Fig. 3.14 Stabilisation of *hns* mRNA during cold-shock.

To distinguish between up-regulation of *hns* expression and the accumulation of a more stable *hns* mRNA molecule during cold-shock, cultures were again grown at 37°C to an OD_{600} = 0.5 before being cold-shocked at 10°C. Transcription was simultaneously inhibited by the addition of Rifampicin. RT-qPCR was performed on samples taken at time t = 0 and every 30 min thereafter for a period of 180 min. The abundance of *hns* mRNA (pale green bars) for each time point was quantified and expressed relative to the quantity present at time t =0. Data were derived from three independent experimental replicates (n = 3). Error bars represent the mean standard deviation of expression relative to time t = 0 between experimental replicates.

than the level of H-NS at t = 0 (Fig. 3.13). The level of H-NS was then static for the remainder of the experiment. This experiment, however, has only been conducted once and needs to be repeated in order to validate these observations.

The apparent increase in *hns* expression observed under cold-shock conditions may in fact represent normal transcriptional activity from the *hns* promoter coupled with the accumulation of a more stable *hns* mRNA molecule at 10°C. To determine if the above results were a genuine up-regulation of *hns* expression, the experiment was repeated with the addition of rifampicin to inhibit any further transcription at time t = 0. By inhibiting RNA polymerase activity using rifampicin no increase in *hns* expression should be apparent if the effect of cold-shock is at the transcriptional level. Upon inhibiting further transcription, however, the levels of *hns* mRNA again increased over time during cold-shock (Fig. 3.14). Within the first 60 min of cold-shock treatment, the abundance of *hns* mRNA increased 2-fold relative to the amount present at time t = 0. Thereafter the levels of *hns* mRNA then steadily declined. This is in good agreement with results observed in *E. coli* for other cold-shock mRNA molecules such as *cspA* where, after an initial stabilisation during the early stages of cold-shock, their stability was observed to decrease as the acclimation to cold-shock progressed (Gualerzi *et al.*, 2003, Phadtare, 2011). Thus, at least during the early stages of the cold-shock acclimation period, the observed increase in *hns* mRNA abundance is the combinatorial effect of increased transcription coupled with an accumulation of a more stable *hns* mRNA molecule at 10 °C. This is good agreement with results observed in *E. coli* for other cold-shock mRNA molecules such as *cspA* where after an initial stabilisation during the early stages of cold-shock their stability was observed to decrease as the acclimation to cold-shock progressed (Goldenberg *et al.*, 1996, Brandi *et al.*, 1996, Phadtare, 2011).

In contrast to cold-shock regulation of *hns*, expression of *stpA* has been shown in *E. coli* to be up-regulated upon temperature up-shifts. Free and Dorman observed a 4-fold increase in the expression of *stpA* when cultures of *E. coli* were shifted from 30 °C

to 37 °C (Free & Dorman, 1997). To observe if *stpA* was similarly regulated in *S.* Typhimurium, cultures of wild-type SL1344 were grown at 30 °C to an OD_{600} = 0.5 before being shifted to 37 °C. Total RNA samples were extracted and analysed by RT-qPCR for *stpA* mRNA abundance at the start of growth at 37 °C (t = 0) and every 30 min thereafter for a total of 90 min. Akin to the results observed by Free and Dorman, a 3.7-fold increase in *stpA* expression was observed within the first 30 min of growth at 37°C (Fig. 3.15). Expression of *stpA* then steadily decreased before reaching the level observed at time t = 0 after 90 min. As a control the expression of *hns*, which is not known to be regulated by temperature increases, was also monitored and no appreciable increase in expression was observed.

3.2.9 Effect of the cold-shock regulator protein CspA on expression of *hns* and H-NS protein abundance during cold-shock.

Cold-shock induction of *hns* expression in *E. coli* involves the major cold-shock protein CspA. La Teana *et al.* showed that upon a temperature down-shift to 10°C *hns* expression was increased 3- to 4-fold and that this was in part due to binding of CspA within a 110 bp fragment of the *hns* regulatory region (La Teana *et al.*, 1991) (Fig. 3.1). To determine if CspA was also involved in the cold-shock induction of *hns* expression in *S.* Typhimurium, cultures of wild-type SL1344 and SL1344 Δ*cspA* were grown to an OD_{600} = 0.5 at 37°C and were then shifted to 10°C. Total RNA samples were taken and analysed for *hns* mRNA abundance in a time course experiment at the start of cold-shock (t = 0) and every 30 min thereafter for 180 min. Interestingly, when compared with wild-type *hns* expression during cold-shock, in SL1344 Δ*cspA* a steady increase in *hns* mRNA was still observed despite lacking a functional CspA protein (Fig. 3.16 A). Furthermore, although more error was introduced into the experiment than observed for wild-type SL1344, in SL1344 Δ*cspA* expression of *hns* was greater than that observed in wild-type SL1344 at the 120-, 150- and 180-min time points. In addition, in wild-type SL1344 *hns* expression reached a plateau level after 90 min at 10°C. In the absence of CspA, however, a similar plateau was not observed until after 150 min at

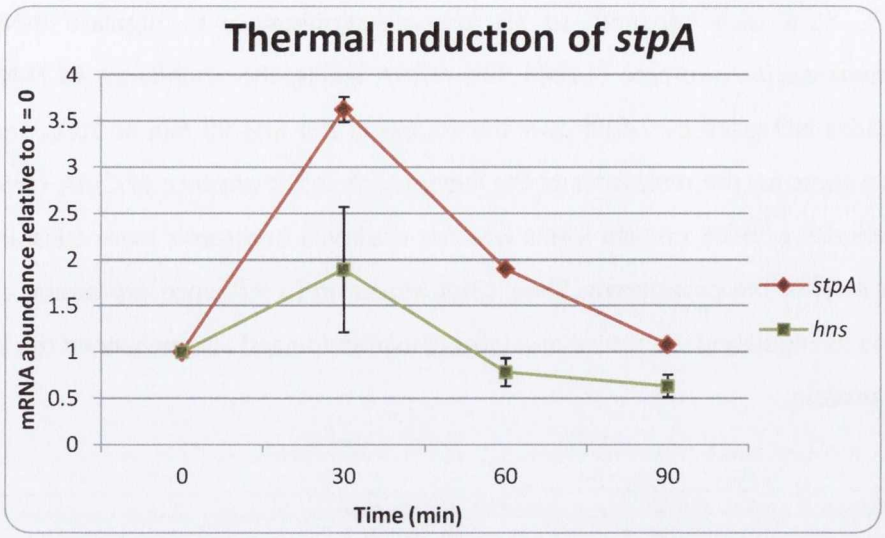


Fig. 3.15 Thermal induction of *stpA* expression.

Cultures of wild-type SL1344 were grown to mid- to late-exponential growth (OD_{600} = 0.5) at 30°C before being shifted to 37°C. Total RNA samples were extracted and analysed by RT-qPCR for the abundance of *stpA* mRNA (red diamonds) upon temperature up-shift (t = 0) and every 30 min thereafter for 90 min. As a control *hns* mRNA abundance (green squares) was also monitored during the experiment. Data represent three independent experimental replicates (n = 3). Error bars represent the mean standard deviation of expression relative to time t = 0 between experimental replicates.

10°C. CspA also appeared to act post-transcriptionally to regulate H-NS protein abundance. In wild-type SL1344, like mRNA levels, the abundance of H-NS protein steadily increased by 2-fold over the course of the first 90 min at 10°C. Levels were then static for the remainder of the experiment. In the absence of CspA, however, the same rise in H-NS protein levels was not observed and levels were constant for the duration of the experiment. Thus, CspA appeared to influence expression of the *hns* gene by regulating the timing and level of expression and also enhanced the level of H-NS protein.

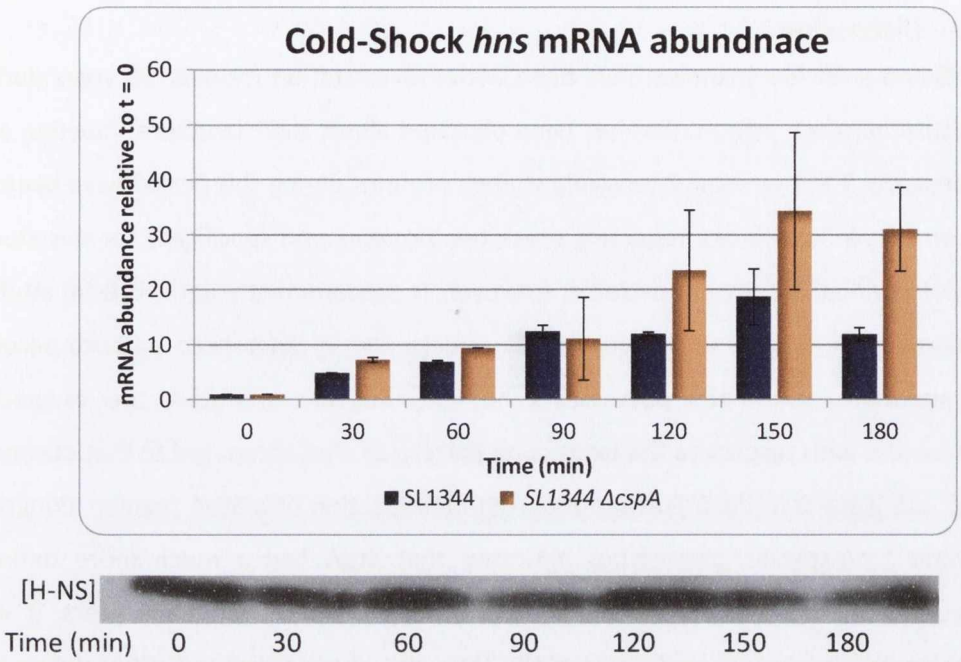


Fig. 3.16 Effect of CspA on *hns* mRNA and H-NS protein abundance during cold-shock conditions.

The abundance of hns mRNA in SL1344 Δ*cspA* was monitored by RT-qPCR during cold-shock treatment (A). For comparison the abundance of *hns* mRNA in SL1344 Δ*cspA* were plotted against wild-type *hns* mRNA levels during cold-shock treatment. The abundance of *hns* mRNA was quantified relative to a standard curve using genomic DNA of known quantity. Results represent three independent experimental replicates (n = 3). The effect of CspA on H-NS protein levels was also monitored at equivalent time-points during cold-shock. Protein samples were analysed by Western immunoblotting using a monoclonal FLAG antibody to detect H-NS (B). Error bars represent the mean standard deviation of expression relative to time t = 0 between experimental replicates.

3.3 Discussion

H-NS and H-NS-like proteins have been under investigation for over 30 years during which time much information has been obtained about their structure, function and expression. The two most intensively studied proteins during this period have been H-NS and StpA. Knowledge regarding these two proteins and specifically for this study, their individual expression patterns, however, is predominantly derived from studies undertaken in *E. coli*. In a recent study undertaken in *Salmonella enterica* serovar Typhimurium, which also possesses genes encoding *hns* and *stpA*, species-specific differences with respect to the expression pattern of *stpA* compared to that observed in *E. coli* were identified. Furthermore, the identification of a StpA regulon alongside several StpA-specific phenotypes indicated that StpA had a much more defined physiological role in *S.* Typhimurium than in *E. coli* where, for many years, it was considered a molecular back-up to H-NS. Thus, this study aimed to further define the expression patterns of *hns* and *stpA* in *S.* Typhimurium and also to investigate possible regulatory reasons accounting for the newly defined *Salmonella*-specific expression pattern of *stpA*. This study also aimed to investigate whether some of the known environmental and cellular factors contributing to expression of *hns* and *stpA* in *E. coli* were also involved in the regulation of their expression in *S.* Typhimurium.

In *E. coli*, expression of *hns* is coupled with DNA replication such that transcription is maximal during exponential growth when cells are rapidly dividing (Free & Dorman, 1995). A similar pattern of expression was also observed in *S.* Typhimurium and was again confirmed in this study (Hinton *et al.*, 1992). Using RT-qPCR maximal levels of the *hns* transcript were observed 2 – 3 hours post inoculation into rich LB medium corresponding to early- to mid-exponential growth of *S.* Typhimurium strain SL1344 (Fig. 3.1 A). Thereafter, *hns* transcript levels declined rapidly to a steady state of approximately 25% that of maximal expression until stationary phase when transcript levels steadily decreased to an almost undetectable level after 24 h. In agreement with studies undertaken by Dorman and Free in *E. coli* and Hinton *et al.* in *S.* Typhimurium, no increase in *hns* transcription was observed upon entry into stationary phase as has been reported in two separate studies undertaken in *E. coli*

(Ueguchi *et al.*, 1993, Dersch *et al.*, 1993, Hinton *et al.*, 1992, Free & Dorman, 1995). In contrast to expression in *E. coli*, where the *stpA* transcript is only detected transiently during early-exponential growth and only at significant levels in the absence of functional H-NS, in *S.* Typhimurium after a similar peak during early- to mid-exponential growth, expression of *stpA* is maintained at significant levels until stationary phase. This species-specific pattern of *stpA* expression was again observed in this study. Akin to *hns* expression, maximal levels of the *stpA* transcript were detected 2 – 3 h post inoculation into rich LB medium (Fig. 3.2 B). Transcript levels then decreased sharply but were sustained at approximately 50 % that of the maximal level observed until late stationary phase.

In accordance with previous work conducted in *E. coli* and *S.* Typhimurium the *hns*-specific pattern of expression served to maintain relatively constant levels of H-NS protein throughout growth (Fig. 3.5 A) of wild-type SL1344. Despite a significant burst in *hns* transcript levels during exponential growth, no concurrent increase in H-NS protein abundance was observed. This is in keeping with the theory that the *hns* expression pattern serves to uphold a constant H-NS:DNA ratio (Free & Dorman, 1995). Thus, the transient burst in *hns* expression serves to provide sufficient amounts of H-NS protein to daughter cells during cell division whilst at the same time sustaining an adequate H-NS:DNA ratio in the mother cell. Again a species-specific pattern of StpA expression was observed and matched that observed in *S.* Typhimurium by Lucchini *et al.* (Lucchini *et al.*, 2009). In conjunction with the *stpA* transcript profile, StpA protein levels were also maximal during exponential growth 2 – 3 post inoculation from overnight cultures. Levels then decreased to ≈ 57% that of the maximal level observed but were maintained at approximately this level until late stationary phase. This is again in contrast to the StpA protein profile previously observed in *E. coli* where, after the exponential phase burst in protein abundance, the levels of StpA steadily decreased to an almost undetectable level (Sonden & Uhlin, 1996).

As a result of the highly individual expression patterns of *hns* and *stpA*, the population of these two H-NS-like proteins is dynamic throughout growth of *S.* Typhimurium and, importantly, likely alters the ratio of homo-dimers, hetero-dimers and homo- and hetero-oligomers present in the cell as a function of growth. As H-NS and StpA have both distinct and overlapping regulons and differ in the manner and affinity with which they bind and constrict DNA, variations in the populations of these two proteins will have important consequences in terms of the gene expression profile and the superhelicity of the nucleoid as *S.* Typhimurium transitions through the various stages of growth (Lim *et al.*, 2011, Arold *et al.*, 2010, Lucchini *et al.*, 2009). Indeed, StpA has been shown to indirectly regulate cellular levels of the stationary phase sigma factor RpoS during exponential growth when StpA levels are maximal but had little effect on RpoS levels throughout stationary phase (Lucchini *et al.*, 2009). This situation is analogous to that observed for the HU protein in *S.* Typhimurium. Mangan *et al.* demonstrated that growth-phase-dependent alterations in the sub-units of the HU protein gave rise to distinct and overlapping transcriptional outputs depending on the dominant protein form (Mangan *et al.*, 2011). The dimeric HU protein is composed of α and β sub-units and depending on the dominant sub-unit present within the cell can exist as homo-dimers comprised of either two α sub-units (HU α_2) or two β sub-units (HU β_2) or as a HU αβ hetero-dimer (Guo & Adhya, 2007). Mangan *et al.* showed using single and double knock-out mutations of the HU α and β sub-units that each resulting altered composition of HU present in the cell gave rise to distinct and overlapping patterns of gene expression which were also growth phase dependent (Mangan *et al.*, 2011). For example, in cultures of wild-type *S.* Typhimurium strain SL1344 the HU αβ hetero-dimer predominates for the majority of growth with the α_2 and β_2 forms more prominent during early-exponential and late-stationary growth, respectively (Claret & Rouviere-Yaniv, 1997). Mutants deficient in *hupB*, encoding the HU β sub-unit, exhibited a repressive effect on several SPI-1 encoded genes during exponential growth. Similar results were also observed for the loss of the *hupA* gene (encoding the HU α sub-unit) or both sub-units. In stationary phase cultures however whilst repression of the same set of genes is maintained in the *hupA* or *hupAhupB* deletion

strains, in the *hupB* single knock-out mutant repression was relieved (Mangan *et al.*, 2011).

Furthermore, the recent observations that H-NS forms superhelical structures upon oligomerization which are capable of condensing DNA and repressing transcription whilst StpA appears to form nucleoprotein filaments that block DNA access to prevent transcription suggests that a third novel nucleoprotein complex may also form upon hetero-oligomerisation (Arold *et al.*, 2010, Lim *et al.*, 2011). Outside of the documented environmental cues, such as temperature and osmolarity, and the numerous proteins capable of alleviating H-NS and StpA mediated repression, toggling between various nucleoprotein complexes as a result of growth phase variations in the levels of H-NS and StpA offers an attractive natural method by which these two proteins manipulate the nucleoid structure in order to co-ordinate expression of their respective and overlapping regulons (Stoebel *et al.*, 2008).

Modulation of gene expression by altering the oligomeric state of H-NS-like proteins is also thought to occur in *S.* Typhimurium upon complex formation with members of the Hha/YmoA family of proteins (Garcia *et al.*, 2005, Madrid *et al.*, 2007). The Hha/YmoA family of proteins are low molecular weight proteins which are widespread in *Enterobacteria* and have been demonstrated to form complexes with H-NS in a number of different species due to having a high level of structural homology with the H-NS N-terminal oligomerization domain (Madrid *et al.*, 2007). Indeed, Hha has been shown to replace functionally the oligomerisation domain of H-NS in *E. coli* (Madrid *et al.*, 2007, Vivero *et al.*, 2008). *S.* Typhimurium possesses two such Hha/YmoA proteins, Hha and YdgT, and loss of either from the cell was shown to de-repress multiple genes encoded within *Salmonella* pathogenicity islands 1 – 5 (Vivero *et al.*, 2008). Of these genes 87 % also contained binding sites for H-NS leading to the hypothesis that H-NS/Hha or H-NS/YdgT complex formation aids the role of H-NS in xenogeneic silencing (Vivero *et al.*, 2008). Although a structural basis for this is yet to be elucidated, Hha is thought to undergo a conformational change upon binding with H-NS perhaps altering

the DNA binding properties of H-NS to target genes of higher A/T content than the chromosome (Garcia *et al.*, 2005).

Intriguingly, as part of this study, analysis of the relative *hns* and *stpA* transcript and protein abundance revealed an anti-correlation. At all time points analysed during growth, the *stpA* transcript was significantly more abundant than that of the *hns* transcript with a maximum of 15-fold more *stpA* transcript present at the 4 h and 7 h time-points (Fig. 3.3). When the abundance of StpA and H-NS protein was estimated, however, H-NS had the more dominant presence within the cell and was at least 2.5-fold more abundant than StpA at all times during growth (Fig. 3.6). This anti-correlation between transcript and protein abundance suggested that differential post-transcriptional regulation of the *hns* and *stpA* transcripts and/or proteins may be at play. Subsequent testing of the stability of the *hns* and *stpA* mRNA molecules revealed the *stpA* mRNA molecule to be significantly unstable relative to *hns* mRNA. Using the antibiotic rifampicin to inhibit transcription, the disappearance of each mRNA molecule was assayed over time by RT-qPCR which showed *stpA* mRNA levels were reduced to 15 % of that of the quantity present at the start of the assay within 5 min (Fig. 3.7). Transcript levels were further reduced to 6 % within the next 5 min time period but remained at his level for the remainder of the assay. The levels of the *hns* transcript also declined within the first 5 min but unlike the *stpA* transcript was only reduced to 70% that of the starting quantity. Thereafter, the *hns* transcript levels remained at this level until after 20 min in the presence of rifampicin when they further decreased to 40% that of the initial quantity. As the disappearance of both transcripts was not linear over time a half-life could not be calculated. The results obtained, however, suggest that the *hns* transcript is significantly more stable than *stpA* which appeared to be rapidly degraded upon transcriptional inhibition. Analysis of the primary and secondary structures of *stpA* mRNA identified several features capable of reducing its stability including a sub-optimal RBS, an A/U rich region upstream of the RBS and an initiation codon which was complementary base paired (Fig. 3.8). The apparent instability of the *stpA* transcript would serve to reduce the

pool of mRNA available for the translation machinery to synthesize the StpA protein and help explain the observed anti-correlation between transcript and protein levels when compared with the *hns* transcript which was more stable and abundant over time.

These results do not exclude the possibility of regulation of either transcript by small regulatory RNA (sRNA) molecules. The observed stabilities of the *hns* and *stpA* transcript could be a consequence of sRNA molecules base-pairing with either transcript to protect *hns* mRNA from degradation on one hand while targeting *stpA* mRNA for degradation on the other. Regulation by a sRNA was previously observed for the *hns* transcript whereby base-pairing with the DsrA sRNA molecule was shown to hinder translation of *hns* mRNA giving rise to a 3-fold reduction in H-NS protein levels (Lease *et al.*, 1998). A sRNA molecule capable of enhancing the stability of *hns* mRNA, however, is yet to be identified while there is no known regulation of *stpA* mRNA by sRNA molecules. The *stpA* mRNA does, however, have one or more poly-U tracts within the region upstream of its RBS. While such tracts often act as translational enhancers they also serve as target sites for RNase E and importantly, Hfq (Panja & Woodson, 2012, Delvillani *et al.*, 2011). Further mRNA stability experiments in strains lacking the Hfq protein, which is required to mediate many sRNA-mRNA interactions in both *S.* Typhimurium and *E. coli*, would help elucidate any involvement of sRNA molecules in regulating *hns* and *stpA* mRNA stability (Fig. 3.17).

In *E. coli*, StpA is subject to proteolysis by the ATP-dependent Lon protease in the absence of H-NS (Goff *et al.*, 1984). When H-NS is present, the formation of H-NS:StpA heterodimers is thought to protect StpA from cleavage by Lon and thus allows StpA to function in the cell (Johansson & Uhlin, 1999). Coupled with the observed instability of *stpA* mRNA, protease turnover of StpA in *S.* Typhimurium could also be a contributing factor to the anti-correlation between the levels of *stpA* mRNA and StpA protein. Proof of StpA degradation by the Lon protease in *S.* Typhimurium, however, was elusive after several attempts. Evidence of proteolysis of StpA was observed at 42 ^{o}C in strain SL1344^{2XstpA} in which a 3X FLAG tagged copy of the *stpA* ORF has replaced the

native *hns* ORF and is expressed via the *hns* promoter. In this strain, despite lacking functional H-NS for protection from proteolysis, at 37°C significant levels of StpA were detected throughout growth. At 42°C, however, StpA was detected at levels comparable to those at 37°C at early exponential phase but rapidly declined thereafter (Fig. 3.9). This observation was not due to alterations at the level of transcription as when *hns* was driven by the same promoter in wild-type SL1344 the levels of H-NS were equivalent at both 37°C and 42°C. This suggests that at 42°C the StpA protein is subject to proteolysis resulting in the observed decrease in protein levels over time (Fig.3.17).

To try to unveil some of the contributing regulatory factors responsible for the high *stpA* expression levels, the effect of gene dosage was examined. Bioinformatic analysis revealed that the gene encoding *stpA* was located 1.1 Mbp away from the origin of replication (OriC) while the *hns* gene was located 2.4 Mbp away and on the opposite side of the chromosome. During the replication process each gene in the chromosome can potentially exist as 2^n copies with genes located close to OriC more abundant than those more distal due to multiple rounds of replication occurring simultaneously in exponentially dividing cells. Using qPCR to examine chromosomal DNA samples taken when maximal expression of *hns* and *stpA* was observed, the *stpA* gene appeared to be 2-fold more abundant than *hns* (Fig. 3.10 A). Having a higher copy number means that there is more *stpA* DNA template available for transcription than there is *hns* which would be a contributing factor to the high levels of *stpA* expression during exponential growth. This was deemed to be a genuine gene dosage effect due to chromosomal location as the *hns* ORF, when placed at the *stpA* locus, was found to be more abundant than the *stpA* ORF placed at the *hns* locus in strain SL1344^{swap} (Fig. 3.10 B). In *E. coli stpA* and *hns* are located at a distance 1.1 Mbp and 2.6 Mbp respectively from OriC and similar to *S.* Typhimurium, a peak in *stpA* expression was observed during early-exponential growth (Free & Dorman, 1997). Thereafter, however, expression of *stpA* declined steadily to almost undetectable levels during stationary phase (Free & Dorman, 1997, Sonnenfield *et al.*, 2001). As the relative

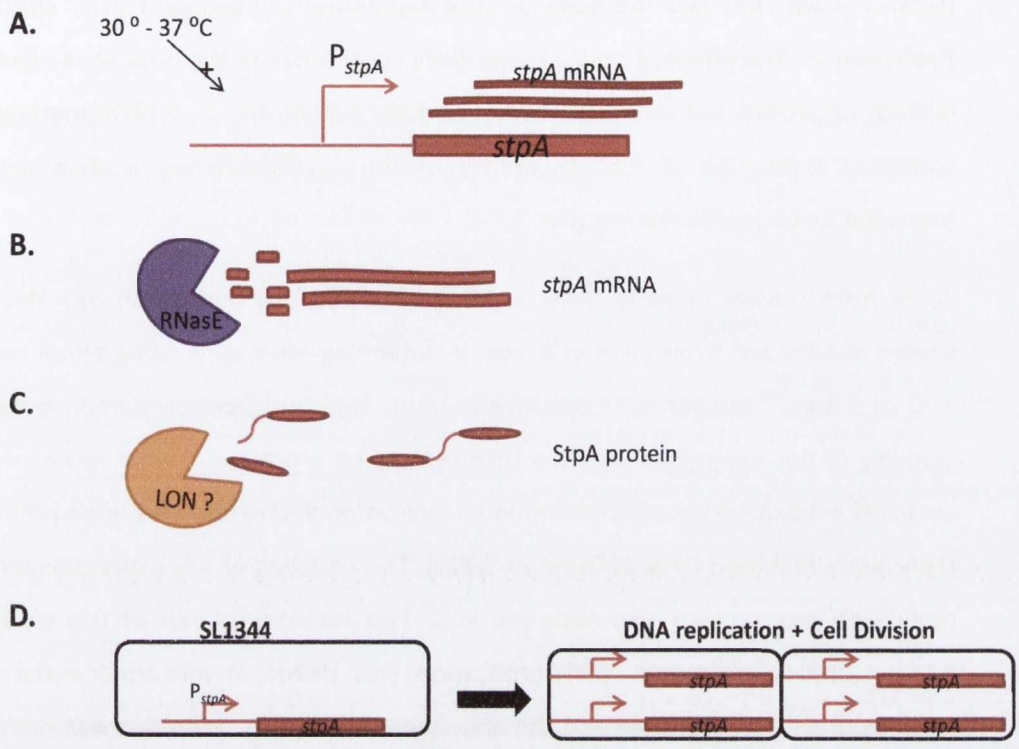


Fig. 3.17 Summary of factors found to influence stpA expression.

Expression of the *stpA* gene (red box) was found to increase upon temperature shifts from 30 ºC to 37 ºC (A). The *stpA* mRNA molecule (red ribbon) was found to be quite unstable and was rapidly degraded (B) compared to that of *hns*. Evidenced from experiments conducted at 42 ºC suggests that StpA may also be subject to proteolysis (C). When DNA replication is occurring the *stpA* gene is resent in higher copy number than *hns* (D).

distance from OriC and the peak of *stpA* expression is equivalent in *E. coli* and *S.* Typhimurium, the effect of gene dosage likely contributes to the peak *stpA* expression in both organisms but does not, however, help explain the *S.* Typhimurium-specific sustained expression of *stpA* thereafter or the significantly more abundant *stpA* transcript levels relative to *hns* (Fig. 3.17).

Aside from control of gene copy number, chromosomal replication has also been shown to alter *hns* expression in *E. coli.* By inhibiting replication using either nalidixic acid or a *dnaC*^{ts} temperature sensitive mutant, Free and Dorman demonstrated the coupling of *hns* expression with the DNA replication process such that *hns* expression shuts off in conjunction with inhibition of replication and re-initiates upon removal of replication inhibition (Free & Dorman, 1995). This coupling of *hns* expression with the replication process was also observed in *S.* Typhimurium as part of this study (Fig. 3.18). Using nalidixic acid, DNA replication was halted in mid-exponential phase cultures of wild-type SL1344 and the abundance of the *hns* transcript was monitored in a time course experiment. Within 15 min after the addition of nalidixic acid the level of *hns* transcript had declined to 50% of that of the starting amount and continued to fall thereafter reaching 11% of that of the starting quantity (Fig. 3.11). Subsequently, upon resuming the replication process in drug free LB media, expression of *hns* re-initiated and reached 60% expression relative to the initial quantity after 75 min in the absence of nalidixic acid. While the progressive shutting down of *hns* expression observed here for *S.* Typhimurium is consistent with the results obtained by Free and Dorman in *E. coli* the recovery of expression is not. Free and Dorman obtained 100% recovery of *hns* expression during an equivalent time frame in drug-free medium (Free & Dorman, 1995). The results obtained in this study, however, are in good agreement with those observed using the temperature-sensitive *dnaC*^{ts} *E. coli* mutant where a similar 60 – 70% recovery of *hns* expression was observed during the same time frame (Free & Dorman, 1995). Differences between experiments using nalidixic acid may have arisen as a result of the transition period where cells were harvested, washed and re-suspended in drug free media in order to remove the nalidixic acid. While every

effort was made to ensure minimal temperature fluctuations, cells would have experienced temperatures below 37 °C during this period which may have effected *hns* expression which is known to be up-regulated upon temperature downshifts and is affected by the sRNA DsrA whose expression is maximal at 25 °C (La Teana *et al.*, 1991, Lease *et al.*, 1998). Nevertheless, a progressive decline in *hns* expression was observed by inhibiting the replication process which was then reversed upon re-commencement of replication. As the method of action of nalidixic acid is to inhibit the type II topoisomerase enzyme, DNA gyrase, which is responsible for relieving the positive superhelical tension ahead of the migrating DNA polymerase during the replication process, the observed changes to *hns* expression may be the result of changes in supercoiling (Free & Dorman, 1995, Gore *et al.*, 2006). Although the *E. coli* *hns* gene is not known to be supercoiling-sensitive (Free, 1994), the same has not been shown for *S.* Typhimurium whose chromosomal supercoiled state is much less variable than that of *E. coli* (Cameron *et al.*, 2011). Further experiments aimed at inhibiting DNA replication by an alternative method are therefore required to demonstrate conclusively the coupling of *hns* expression with DNA replication in *S.* Typhimurium and to rule out any effect by supercoiling. One such method would be to initiate the stringent response using the amino acid serine analogue, serine hydroxamate, which has been shown to inhibit replication at the level of initiation (Ferullo *et al.*, 2009).

A major contributing factor to the peak in *hns* expression during exponential phase in *E. coli* is the FIS nucleoid-associated protein. Expression of FIS is heavily up-regulated during the early stages of exponential growth in response to nutritional up-shift and was shown to bind to multiple locations within the *hns* regulatory region (Falconi *et al.*, 1996, Ball *et al.*, 1992). Binding of FIS alleviates the negative auto-repressive effect H-NS has on *hns* expression by competing for binding to overlapping binding sites with H-NS in the promoter region and increases expression 2- to 4-fold (Falconi *et al.*, 1996). Regulation of *hns* expression by FIS was also observed during this study in *S.* Typhimurium (Fig. 3.18). Comparison of *hns* transcript levels in wild-type SL1344 with

SL1344 *fis*::*kan* throughout the period when maximal *hns* transcription was typically observed showed that the *hns* transcript was ≈ 1.8-fold more abundant when FIS was present in the cell (Fig. 3.12 A). In keeping with observations in *E. coli*, the effect FIS had on *hns* transcription was confined to early-exponential growth as no significant difference in *hns* transcription was observed due to the absence of FIS after 3 h when cells were typically in mid-exponential growth phase. Although FIS has no known effect on *stpA* expression in *E. coli*, as the patterns of expression of *hns* and *stpA* are so similar in *S.* Typhimurium with each showing peak expression after 2 h of growth in LB-broth, the effect of FIS on *stpA* expression was also investigated. Despite using the same samples of total RNA as were used for analysis of *hns* expression in response to FIS, significant amounts of error were introduced upon analysis of *stpA* expression and thus an effect of FIS on *stpA* expression could not be accurately determined (Fig. 3.12 B).

The effect that temperature fluctuations have on *hns* and *stpA* expression has been well documented in *E. coli* with the *hns* and *stpA* genes being up-regulated by thermal down-shifts and up-shifts, respectively. As part of the cold-shock regulon, *hns* was shown to be up-regulated upon temperature down-shifts from 37°C to 10°C due to binding of the cold-shock response regulator, CspA, within the *hns* promoter region. Similarly, in this study *hns* mRNA levels steadily increased with time when subjected to an equivalent cold-shock while levels of *stpA* mRNA were unaffected by the same treatment (Fig. 3.13 A). During the first 90 min of cold-shock treatment *hns* mRNA levels underwent a 12-fold increase but appeared to plateau for the remainder of the experiment. This increase in *hns* mRNA level was reflected in terms of H-NS protein abundance which again was observed to increase steadily within the first 90 min of cold-shock before reaching a plateau level. The increase in *hns* mRNA abundance during cold-shock treatment was subsequently shown to be the result of a combination of increased transcription from the *hns* promoter and the accumulation of a more stable *hns* mRNA at 10 °C. Without continued transcription of the *hns* gene

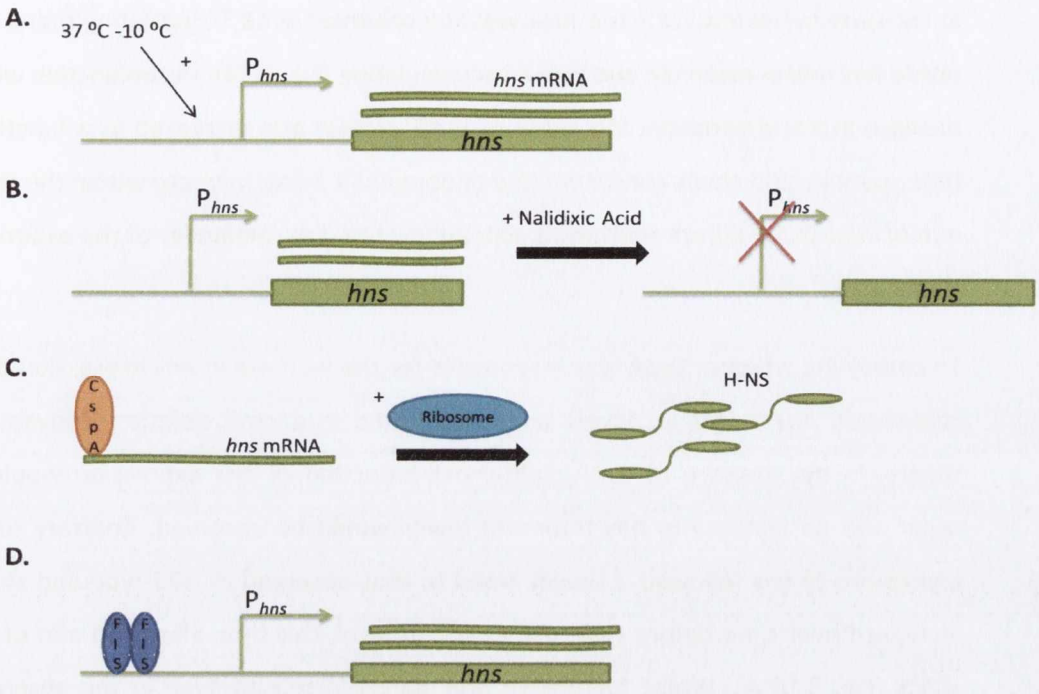


Fig. 3.18 Summary of the factors influencing *hns* expression

As part of this study, expression of *hns* (green box) was found to up-regulated by cold-shock (A) and by the FIS protein (blue oval) during exponential growth (D). Expression of *hns* was repressed by the inhibition of DNA replication using nalidixic acid (B). Up-regulation of *hns* during cold-shock did not require CspA (orange oval), however, the presence of CspA was required for the observed increase in H-NS protein levels during cold-shock (C) suggesting a role for CspA in post-transcriptional regulation of *hns*.

an increase in the level of *hns* mRNA was still observed at 10 ^{o}C indicating that a more stable *hns* mRNA molecule was indeed accumulating (Fig. 3.14). In conjunction with an increase in *hns* expression, the levels of H-NS protein also increased as a function of time spent in cold-shock conditions and underwent a 2-fold increase within the first 90 min of cold-shock before reaching a plateau level for the remainder of the experiment (Fig. 3.13 B).

To determine whether CspA was responsible for the increase in *hns* expression during cold-shock, *hns* transcript levels were monitored in a *cspA* deletion derivative. In theory, in the absence of CspA, cold-shock induction of *hns* expression would not occur and no increase in *hns* transcript levels would be observed. Contrary to this, expression of *hns* followed a similar trend to that observed in wild-type and steadily increased over time before reaching a plateau level, this time after 150 min of cold-shock (Fig. 3.16 A). Whilst *hns* mRNA abundance increased even in the absence of CspA, a longer time was required in order to reach the apparent plateau level in the absence of CspA. This suggested that CspA may play a role in stabilizing *hns* mRNA. In cold-shocked *E. coli* cells, aside from modified translational apparatus, which was shown to translate cold-shock mRNA molecules faster and to a higher level, CspA is capable of increasing translation of cold-shock mRNA molecules up to 2.5-fold. CspA is thought to achieve this by acting as an RNA chaperone to stabilize cold-shock mRNAs for efficient translation. To assess if CspA played a role in enhancing *hns* mRNA translation, H-NS protein abundance was monitored during cold-shock in SL1344 *ΔcspA* which showed that in the absence of CspA no increase in the levels of H-NS occured. Thus, the effects of CspA in regulating *hns* are two-fold, acting both as a transcriptional regulator and post-transcriptionally to enhance the levels of H-NS.

In a similar experiment to that undertaken to show up-regulation of *hns* expression upon cold-shock, when cultures of SL1344 experienced an up-shift in temperature from growth at 30 ^{o}C to 37 ^{o}C a 3.7-fold induction of *stpA* expression was observed during the first 30 min at 37 ^{o}C. This effect was subsequently alleviated when *stpA* mRNA levels returned to a level comparable to that at t = 0 after 90 min at 37^{o}C. Thus,

with respect to thermal fluctuations, up-regulation of *hns* is opposed to the up-regulation of *stpA* in *S.* Typhimurium and *E. coli* suggesting that each plays a particular role in the adaptation of these bacteria to changes in temperature. In support of this, studies conducted in *Salmonella* and *E. coli* demonstrated a 'cold-sensitive' phenotype in strains lacking a functional *hns* gene (Dersch *et al.*, 1994, Muller *et al.*, 2010, Hinton *et al.*, 1992). This phenotype is manifested as an enhanced growth deficit at temperatures below 30°C with respect to the growth handicap observed for *hns* mutants at 37°C. Up-regulation of *hns* upon cold-shock and the presence of a functional *hns* gene appear to be vital for the adaptation and survival of *S.* Typhimurium and *E. coli* at temperatures below human body temperature. Conversely, Muller *et al* demonstrated in *E. coli* that a strain lacking the *hns* gene required *stpA* to be present to maintain growth at temperatures above 37°C (Muller *et al.*, 2010). Coupled with the observed increase in *stpA* expression upon temperature up-shifts it seems that *stpA* has a more essential role at the upper end of the temperature spectrum in which *S.* Typhimurium and *E. coli* can proliferate. Furthermore, *in vitro* studies have demonstrated that the StpA protein is more thermo-stable than H-NS and that at higher temperatures the formation of H-NS:StpA heterodimers is more favourable than the formation of either homodimer (Leonard *et al.*, 2009). As H-NS is thought to undergo a conformational change with temperature increases perhaps heterodimer formation with StpA is a method by which H-NS can remain active at higher temperatures whilst at the same time providing protection to StpA from proteolytic degradation thus allowing StpA to function in the cell (Ono *et al.*, 2005).

The data presented in this study highlight the intricate regulatory control of *hns* and *stpA* expression in *Salmonella enterica* serovar Typhimurium. Modulation of their expression in response to various environmental stimuli gives rise to a dynamic population of H-NS and H-NS-like proteins within the bacterium which, owing to their distinct but over-lapping DNA binding and gene regulatory properties, helps *S.* Typhimurium adapt to and proliferate in the numerous environments which it

encounters. Whilst many of the regulatory mechanisms governing *hns* expression in *E. coli* were, in this study, shown to be true in *S.* Typhimurium, the mechanism by which the observed high level of *stpA* expression, when compared with *hns*, is achieved remains to be elucidated. This high level of expression appears to be a mechanism by which the bacterium produces enough StpA to cope with both mRNA and proteolytic degradation in order to achieve the *Salmonella*-specific StpA protein expression profile. In conjunction with an altered pattern of *stpA* expression, StpA has a more defined physiological role in *S.* Typhimurium than in *E. coli* which appears to be achieved by differential regulation of expression. This is representative of species divergence at the level of transcriptional regulation, a situation analogous to that observed for the PmrA/PmrB two-component system by Winfield and Groisman who observed *pmrA* expression was influenced by low Mg^{2+} in *S.* Typhimurium but not in *E. coli* (Winfield & Groisman, 2004). Similarly, Cameron *et al.* demonstrated that expression of the *topA* gene, encoding topoisomerase I, responded to the FIS transcriptional regulator in both exponential and stationary phase growth in *E. coli* but only during stationary phase growth in *S.* Typhimurium (Cameron *et al.*, 2011). In addition both *hns* and *stpA* have gained new regulatory inputs in *S.* Typhimurium not observed in *E. coli* being regulated by FUR and as part of the SOS response, respectively. This suggests that targeting and altering the expression of *hns* and *stpA* provides the bacterium with genetic flexibility to adapt to and overcome various environmental challenges.

Chapter 4 Varying the expression of *hns* and *stpA* provides adaptive scope for *S.* Typhimurium

4.1 Introduction

Salmonella enterica serovar Typhimurium and the closely related organism *Escherichia coli* regularly encounter environmental fluctuations which these bacteria must adapt to in order to maintain homeostasis. During transitions from the external environment to the human host each experiences fluctuations in temperature, osmolarity, pH, aerobic/anaerobic conditions and nutrient availability (Muller *et al.*, 2009, Shen & Fang, 2012, Hebrard *et al.*, 2011). During these transitions the bacterium aims to optimize its transcriptional output to cope with the environment at hand while at the same time maintaining sufficient nutrient uptake to remain competitive and to proliferate (Babu & Aravind, 2006, Ferenci, 2005). To achieve this goal both *S.* Typhimurium and *E. coli* must first sense the environment at hand and mediate an appropriate transcriptional response using both global and local transcriptional regulators (Shen & Fang, 2012). In this respect, the global regulators H-NS and StpA are uniquely placed within the transcriptional regulatory hierarchy as (i) expression of their own genes is regulated by several environmental cues and (ii) many of the genes regulated by these two proteins are in turn environmentally regulated (Free & Dorman, 1997, Free & Dorman, 1995, Hinton *et al.*, 1992, Lucchini *et al.*, 2009, Ono *et al.*, 2005).

Transcriptomics studies undertaken in *S.* Typhimurium and *E. coli* have repeatedly highlighted the control by H-NS of genes involved in adaptation to temperature, osmotic, pH and aerobic alterations (White-Ziegler & Davis, 2009, Atlung & Ingmer, 1997, Hommais *et al.*, 2001, Ono *et al.*, 2005, Navarre *et al.*, 2006). For example, in *S.* Typhimurium 77% of all genes that were regulated in response to a temperature shift from 25°C to 37°C were also, either directly or indirectly, controlled by H-NS with approximately equal numbers of genes up-regulated and down-regulated (Ono *et al.*, 2005). This situation is surprising as H-NS predominately acts as a repressor and suggests that the down-regulation of some genes may arise as a result of de-repression of genes encoding other repressor proteins. Of those temperature regulated genes that were identified, a large number were also found to be involved in either anaerobic or aerobic respiration (Ono *et al.*, 2005). H-NS mediated

thermoregulation of gene expression also occurs in *E. coli* where 60% of genes regulated by a similar temperature up-shift were also regulated by H-NS (White-Ziegler & Davis, 2009). Furthermore, the same study identified 297 genes regulated by a temperature down-shift from 37°C to 23°C, 72% of which were also regulated by H-NS (White-Ziegler & Davis, 2009). Several genes involved in the adaptation of *S.* Typhimurium and *E. coli* to osmotic pressure are also known to be controlled by H-NS. The most extensively studied of these is the *proU* locus which encodes a glycine/betaine uptake system (Kane & Dorman, 2011, Nagarajavel *et al.*, 2007, Gowrishankar & Manna, 1996). Under low osmotic pressures this locus is directly repressed by H-NS binding within a region of the promoter and a region within the first open-reading frame of the operon (*proV*) (Gowrishankar & Manna, 1996, Fletcher & Csonka, 1995). Upon increasing osmotic pressure H-NS repression is relieved allowing the cell to uptake compatible solutes to cope with the increase in osmotic pressure (Gowrishankar & Manna, 1996, Fletcher & Csonka, 1995).

While many stress regulated genes are known to be directly regulated by H-NS (*proU*, *ompR*, *appY*), an indirect effect of H-NS on some environmentally regulated genes may be attributed to the regulation of the alternate sigma factor RpoS by H-NS (Zhou & Gottesman, 2006). The *rpoS* gene encodes the stationary phase and general stress sigma factor σ^S which is a master regulator of genes required to respond to multiple environmental stresses such as osmotic shock, stationary phase growth, nutrient depletion and temperature fluctuations (Jones *et al.*, 2006). To prevent inappropriate expression of the RpoS regulon, RpoS protein levels are tightly controlled at the level of transcription, post-transcription, translation and protein degradation. In *E. coli* H-NS was observed to influence both *rpoS* mRNA translation and turnover of the RpoS protein in an RssB dependent manner (Zhou & Gottesman, 2006). RssB is a smaller adapter protein that binds to RpoS and presents it to the ClpXP protease for degradation (Zhou & Gottesman, 2006). Zhou and Gottesman showed that in *hns* deficient strains the stability of RpoS is greatly increased despite RssB still being present at significant levels (Zhou & Gottesman, 2006). This led the authors to

hypothesize that H-NS negatively regulated a second protein that could alter the function of or sequester RssB and suggested that this protein might be IraP (Zhou & Gottesman, 2006). Subsequently, IraP was demonstrated to function as an anti-adaptor molecule in *S.* Typhimurium capable of interacting with and sequestering RssB to prevent targeted RpoS degradation (Tu *et al.*, 2006). Interestingly, *iraP* was not regulated by H-NS but was activated by PhoP in response to low Mg^{2+} concentrations (Tu *et al.*, 2006). PhoP is a response regulator of the two component system PhoP/PhoQ which activates expression of genes in response to low Mg^{2+} concentrations and also pH (Kong *et al.*, 2008). Repression of *phoP* in high Mg^{2+} conditions, however, is achieved by binding of H-NS within the *phoP* promoter region. Thus, H-NS indirectly controls RpoS stability in *S.* Typhimurium via PhoP (Kong *et al.*, 2008). This example of H-NS regulation of RpoS in *S.* Typhimurium demonstrates the complexity of mediating a response to environmental changes (Fig. 4.1). On one hand H-NS can mediate a very direct response by activating the PhoP regulon in reaction to low Mg^{2+} conditions while simultaneously altering the general cellular stress response by affecting RpoS stability. Interestingly, PhoP was shown not to regulate *iraP* in *E. coli* and only moderate changes to RpoS stability were observed at low Mg^{2+} concentrations showing divergence in the transcriptional regulatory hierarchy between the two species (Kong *et al.*, 2008). The recent identification of the StpA regulon is *S.* Typhimurium revealed that StpA too is involved in regulating gene expression in response to environmental changes (Lucchini *et al.*, 2009). Lucchini *et al.* showed that StpA represses a variety of genes known to be required for resistance to the antimicrobial peptide polymyxin B (PmB) (Lucchini *et al.*, 2009). Resistance to PmB is known to require modifications to membrane bound Lipopolysaccharide (LPS) (Shi *et al.*, 2004). StpA was shown to directly regulate several genes (*ugtL*, *pagC*, *pagP*) capable of modifying LPS to mediate resistance to PmB (Shi *et al.*, 2004, Lucchini *et al.*, 2009). Interestingly, these genes are part of the PhoP regulon linking both H-NS and StpA to PhoP-mediated stress responses but at different levels of regulation as H-NS

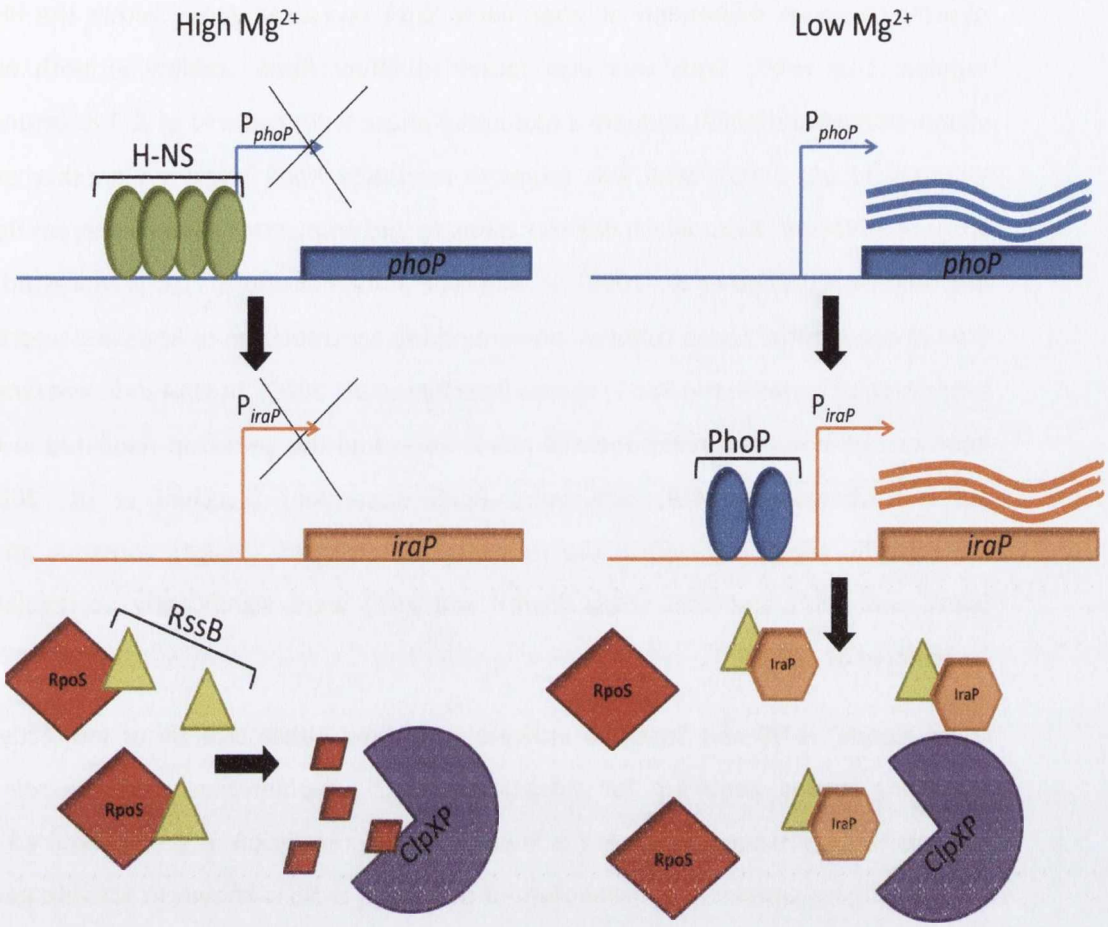


Fig. 4.1 Indirect regulation of RpoS abundance by H-NS in *S.* Typhimurium.

Indirect regulation of RpoS levels by H-NS via repression of *phoP* is represented diagrammatically. In the presence of high levels of Mg^{2+}, H-NS (green ovals) binds within the regulatory region of the *phoP* gene (blue box) and represses transcription. As a result *iraP* (orange box) is not expressed and the RssB protein (yellow triangle) binds to and targets RpoS (red diamond) for degradation by the ClpXP protease (purple circle). When Mg^{2+} levels are low H-NS mediated repression of *phoP* is eliminiated and *phoP* is expressed (blue ribbons). The PhoP protein (blue oval) then binds to the *iraP* promoter to activate transcription (orange ribbons). The resulting anti adaptor IraP protein (orange hexagon) then binds to and sequesters RssB preventing degradation of RpoS.

directly represses expression of *phoP* while StpA represses genes within the PhoP regulon. Like H-NS, StpA was also found to affect RpoS stability in both mid-exponential phase (MEP) and late-exponential phase (LEP) cultures of *S.* Typhimurium (Lucchini *et al.*, 2009). StpA was found to modulate RpoS levels via another anti-adaptor molecule, RssC, which like IraP binds to and sequesters RssB to prevent RpoS degradation (Lucchini *et al.*, 2009). In wild-type *S.* Typhimurium, *rssC* is repressed by StpA in exponential phase cultures, preventing the accumulation of RpoS and wasteful expression of genes in the RpoS regulon (Lucchini *et al.*, 2009). In *stpA*-deficient strains RpoS can be detected in exponential phase and of all the genes up-regulated in the absence of *stpA* in MEP, 39% were RpoS dependent (Lucchini *et al.*, 2009). Importantly, genes involved in the response to salt-shock (*otsBA*), oxidative stress (*katN* and *katE*) and acid stress (*osmY* and *yciE*) were significantly up-regulated (Lucchini *et al.*, 2009).

Quite clearly, H-NS and StpA are intricately involved either directly or indirectly in regulating genes required for adaptation of *S.* Typhimurium and *E. coli* to environmental changes. Each too is involved in the regulation of genes required for the scavenging, uptake and metabolism of nutrients. H-NS is known to activate genes involved in motililty and chemotaxis (Navarre *et al.*, 2006, Ono *et al.*, 2005). Regulation of flagellar biosynthesis by H-NS has been extensively studied in both *S.* Typhimurium and *E. coli* where H-NS acts to repress the *hdfR* gene, the product of which represses the flagellar gene operon master regulator *flhDC* (Soutourina & Bertin, 2003, Paul *et al.*, 2011). Thus indirectly H-NS is positively regulating cellular motility. A more direct role for H-NS in regulating cellular motility was recently observed whereby H-NS was found to directly bind, organize and stabilize the FliG rotor subunits of flagella which help provide torque for the rotating flagellum (Paul *et al.*, 2011). StpA was also shown to have a similar but less effective interaction with FliG (Paul *et al.*, 2011).

For efficient nutrient uptake, *S.* Typhimurium and *E. coli* utilize several outer membrane bound porins (De la Cruz & Calva, 2010, Nikaido, 2003). Importantly, the

major outer membrane porins, OmpF and OmpC, allow passive diffusion of hydrophilic nutrient molecules across the membrane barrier (De la Cruz & Calva, 2010). Owing to differences in their pore size the relative abundance of OmpF and OmpC fluctuates according to nutrient availability and environmental changes (Yoshida *et al.*, 2006). Regulation of *ompF* and *ompC* is tightly controlled such that in *E. coli ompC* is favourably expressed at high temperature, high osmotic pressure and nutrient rich environments while *ompF* is expressed in low temperature, low osmotic pressure and nutrient poor environments (Yoshida *et al.*, 2006, Nikaido, 2003). H-NS has been shown to directly repress transcription of *ompC* and both H-NS and StpA are involved in the regulation of *ompF* via a small regulatory RNA molecule, MicF (Nikaido, 2003, Suzuki *et al.*, 1996, Deighan *et al.*, 2000). MicF is known to bind with *ompF* mRNA, sequestering the translation start signal and thus preventing translation (Suzuki *et al.*, 1996). H-NS and StpA function separately to regulate the MicF-*ompF* interaction with H-NS directly binding to and repressing transcription of the *micF* promoter. StpA, however, binds with and de-stabilises the MicF antisense RNA (Suzuki *et al.*, 1996, Deighan *et al.*, 2000). In addition to regulating OmpF and OmpC levels, H-NS and StpA stimulate the substrate-specific maltose regulon by enhancing the translation of the MalT master regulator (Johansson *et al.*, 1998, Park *et al.*, 2010). De-repression of the regulon results in expression of the LamB porin which facilitates uptake of maltodextrins in the absence of maltose (Johansson *et al.*, 1998).

In the same study that identified H-NS and StpA as regulators of the maltose regulon Johannson *et al.* discovered a reduction in the level of the cyclic-AMP (cAMP) receptor protein (CRP) in both *hns* and *hns/stpA* mutant strains of *E. coli* (Johansson *et al.*, 1998). Subsequent characterisation of the StpA regulon in *S.* Typhimurium demonstrated that, in the absence of StpA, several CRP-cAMP regulated genes were down-regulated and CRP levels decreased 1.6-fold (Lucchini *et al.*, 2009). This StpA dependency, however, was specific for LEP cultures only (Lucchini *et al.*, 2009). CRP is a global transcription factor that, in the presence of its allosteric effector cAMP, binds DNA and primarily activates genes required for catabolism of alternate sources of

carbon (Cameron & Redfield, 2006, Saier *et al.*, 1995). Upon depletion of a preferred carbon source in the environment, its uptake slows or stops and the adenylate cyclase enzyme increases the intracellular cAMP concentration (Cameron & Redfield, 2006, Saier *et al.*, 1995). Binding of cAMP to CRP allows for activation of the CRP regulon which contains several operons whose gene products are responsible for the uptake and/or metabolism of less preferred carbon sources such as maltose and galactose (Lucchini *et al.*, 2009, Gosset *et al.*, 2004). StpA is therefore intimately linked with the response of *S.* Typhimurium to stationary phase growth and nutrient depletion firstly by preventing unnecessary expression of the RpoS regulon in MEP and LEP and secondly by up-regulating the CRP regulon in LEP (Lucchini *et al.*, 2009).

During exponential growth, expression of ribosomal RNA (rRNA) and tRNA operons increases to match the increased protein synthesis requirement of the rapidly dividing bacteria. rRNA and tRNA gene expression is tightly controlled to ensure maximal expression during times of nutrient availability and rapid growth while the energetically expensive expression of these operons declines during nutrient depletion and slow growth. In *S.* Typhimurium and *E. coli* H-NS has been shown to bind to and repress the P1 promoters of rRNA operons by trapping RNA polymerase, particularly during stationary phase growth (Pul *et al.*, 2008, Dame *et al.*, 2002, Navarre *et al.*, 2006, Dillon *et al.*, 2010). De-repression of such operons upon a nutritional up-shift is achieved by binding of the FIS protein to an UP element within the rRNA promoter regions that overlaps with H-NS binding sites (Paul *et al.*, 2004).

To maintain transcriptional control of such a large number of genes and to mediate a response to environmental changes, expression of the *hns* and *stpA* genes in *S.* Typhimurium and *E. coli* is carefully controlled to ensure sufficient amounts of H-NS and StpA protein are present in the cell in any given environment. As outlined in Chapter 3 the specific expression patterns of *hns* and *stpA* supply the bacterium with relatively constant levels of H-NS protein throughout growth while StpA protein levels are maximal in mid-exponential phase before dropping to a steady state level for the remainder of growth. Thus the H-NS and H-NS-like protein population is dynamic

throughout growth which facilitates growth phase-dependent alterations to the transcriptional output of the H-NS and StpA regulons. For example, during exponential phase in *S.* Typhimurium the high levels of StpA help to prevent premature expression of the RpoS regulon while at the onset of stationary phase when nutrient availability is decreased, a drop in StpA levels coincides with efficient expression of the RpoS regulon and StpA also helps activate the CRP regulon to aid in nutrient scavenging (Lucchini *et al.*, 2009).

Despite the species specific differences, the unique expression patterns of *hns* and *stpA* in *S.* Typhimurium and *E. coli* are likely to be optimized for growth of either bacterium at 37^{o}C in a nutrient rich environment. To adapt to changing environmental conditions, however, each bacterium must manage the population of H-NS and H-NS-like proteins in order to elicit an efficient transcriptional response. Indeed during cold-shock there is a greater cellular requirement for H-NS and cells lacking functional *hns* have a cold-sensitive phenotype (La Teana *et al.*, 1991). As such the *hns* gene has evolved to respond to the cold-shock response regulator CspA such that binding of CspA within the *hns* regulatory region increases *hns* transcription (La Teana *et al.*, 1991). Conversely, as recently shown by Troxell *et al.* in *S.* Typhimurium, *hns* is down-regulated in iron rich environments where the presence of high concentrations of Fe^{2+} leads to activation of the Fur transcriptional regulator. Fur was shown to bind within the *hns* promoter region and repress transcription (Troxell *et al.*, 2011). The resultant decrease in H-NS levels gave rise to activation of the H-NS-repressed SPI-1 virulence genes (Troxell *et al.*, 2011). Environmental specific up-regulation of *stpA* is also observed *S.* Typhimurium in response to DNA damage via the RecA-dependent SOS response while in *E. coli stpA* expression increases in response to carbon starvation and temperature up-shifts (Benson *et al.*, 2000).

Studies looking at the extremes of *hns* and *stpA* expression i.e. over-expression or a complete loss of expression, highlight how important it is for the bacterium to maintain tight control of H-NS and StpA levels. Loss of the *hns* gene from *S.* Typhimurium or *E. coli* results in pleiotropic changes in gene expression which are

manifested as a range of phenotypes. Importantly, loss of *hns* alters the general colony morphology to give a mucoid phenotype, renders the bacterium non-motile and *hns* mutants are also cold-sensitive and osmo-sensitive (Hinton *et al.*, 1992, Soutourina *et al.*, 1999, Amit *et al.*, 2003). At 37°C, *hns* mutants exhibit a reduced rate of growth which becomes exaggerated at lower temperatures resulting in a cold-sensitive phenotype (Spurio *et al.*, 1992, Hinton *et al.*, 1992). In *E. coli* the reduced growth rate phenotype may be the result of altered cell cycle parameters in the absence of H-NS. Cells deficient for *hns* were shown to have fewer origins per cell, increased generation times and required a larger replication initiation mass (Atlung & Hansen, 2002). *E. coli* cells are thought to require a relatively constant H-NS:DNA ratio. As such, expression of *hns* has evolved to be coupled with the process of chromosome replication such that *hns* expression is up-regulated upon an increase in the rate of DNA replication to supply sufficient amounts of H-NS to both mother and daughter chromosomes (Free & Dorman, 1995). Interestingly, Atlung and Hansen demonstrated that by artificially varying cellular H-NS concentrations a range of cell sizes and cell cycle parameters were achievable (Atlung & Hansen, 2002). Concomitant with an increase in H-NS concentration was an increase in the number of replication origins per cell and cell size up to a point where the levels of H-NS became toxic to the cell (Atlung & Hansen, 2002). Toxic overproduction of H-NS was also demonstrated previously to result in an immediate shutdown of RNA and protein synthesis, gave rise to many morphological changes and resulted in highly condensed spherical nucleoids (Spurio *et al.*, 1992).

Although StpA appears to have a limited role in *E. coli*, over-expression of *stpA* has been observed to compensate for the loss of *hns* in the regulation of several genes such as *bgl*, *proU* and *adi* (Shi & Bennett, 1994, Williams *et al.*, 1996, Johansson *et al.*, 2001). In *S.* Typhimurium, on the other hand, alterations in StpA levels give rise to several phenotypes, as of yet unclassified in *E. coli*, many of which are growth-phase-specific (Lucchini *et al.*, 2009). During mid-exponential phase growth the complete loss of StpA significantly increased the resistance of *S.* Typhimurium when challenged with

high salt, low pH or oxidative stress conditions and in the presence of the antimicrobial peptide Polymyxin B relative to wild-type cells (Lucchini *et al.*, 2009). Conversely, strains over-expressing *stpA* were significantly less resistant to the same stresses than either that of wild-type or *stpA* knockout strains during mid-exponential phase growth (Lucchini *et al.*, 2009). Loss of StpA during late-exponential phase had little effect on resistance to a high salt challenge, however, when over-expressed resistance was again significantly less than that of wild-type or *stpA* knockout strains (Lucchini *et al.*, 2009).

Modulation of expression of the H-NS and StpA regulons appears to be one method by which *S.* Typhimurium and *E. coli* have evolved to cope with and adapt to the multiple environmental stresses each faces while at the same time remaining nutritionally competent. Expression of genes regulated by one or both proteins is dynamic, adapting appropriately to the prevailing environmental conditions. A key factor to modulating both regulons is the distinct expression patterns of *hns* and *stpA* which are, themselves, environmentally regulated particularly with respect to temperature and nutrient availability. Having unique expression patterns means that the relative levels of H-NS and StpA are dynamic, changing throughout growth and in response to environmental changes. This allows for flexibility in the transcriptional output of their regulons. This study aimed to determine the importance of each unique pattern of expression by altering the specific timing and levels of expression of *hns* and *stpA* in *S.* Typhimurium where StpA has a more prominent role than in *E. coli*. Both beneficial and detrimental effects have been described for the loss of and/or over-expression of *hns* or *stpA*. This study also aimed to investigate the possibility that more subtle expressional changes might be beneficial to the bacterium and confer an adaptive advantage relative to wild-type in certain environments. Furthermore, given the vast number of genes under the control of H-NS and StpA and their pleiotropic nature it was of particular interest to determine to what extent the transcriptional output of *S.* Typhimurium could be altered as a result of changes to the expression of *hns* and *stpA*.

4.2 Results

4.2.1 Re-wiring the *hns* and *stpA* open-reading frames (ORFs)

To alter both the timing and expression levels of *hns* and *stpA*, it was decided to interchange/swap their open-reading frames. Thus, in theory, *hns* would receive all the regulatory inputs of *stpA* and inherit a *stpA*-like pattern of expression and *vice versa*. These changes were carried out on the chromosome in a multistep process using λ-red mediated recombination. Firstly, to facilitate selection of the new transcriptional variants, primers were designed to PCR-amplify the tetracycline resistance genes, *tetRA*, from *Shigella flexneri* strain BS185 which harbours the *tetRA* genes within the chromosomally integrated transposon Tn10 (Kane & Dorman, 2011). Primers were designed such that amplicons contained 40 bp of homology with bps 4 – 44 immediately downstream of the *stpA* stop codon at the 5' end and with homology to base pairs 50 – 90 downstream of the *stpA* stop codon at the 3' end (Fig 4.2). Amplicons were then electroporated into electrocompetent wild-type *S.* Typhimurium SL1344 expressing the λ-red recombination genes and the resistance cassette was chromosomally integrated by homologous recombination immediately downstream of the *stpA* ORF. In a similar manner, the kanamycin cassette, *kan*, was integrated downstream of the *hns* ORF. Next, using chromosomal DNA as a template, each ORF excluding the regulatory region, was PCR amplified with its respective resistance cassette using primers that yielded 40 bp of homology at the 5' and 3' ends with the immediate upstream and downstream regions of their cognate ORF. For example, the *stpA* ORF and *tetRA* genes were amplified to yield amplicons with homology to the upstream and downstream regions of the *hns* ORF (Fig 4.2). Amplicons were again chromosomally-integrated into wild-type SL1344 using λ-red mediated recombination resulting in the wild-type *stpA* ORF being replaced by the *hns* ORF and *kan* cassette and *vice versa* (Fig 4.2). This produced two new strains: (i) SL1344^{2Xhns} containing two copies of the *hns* ORF, one under the control of its native promoter and one under the control of the *stpA* promoter and ii) SL1344^{2XstpA} containing two copies of the *stpA* ORF, one under the control of its native promoter and one under the control of the

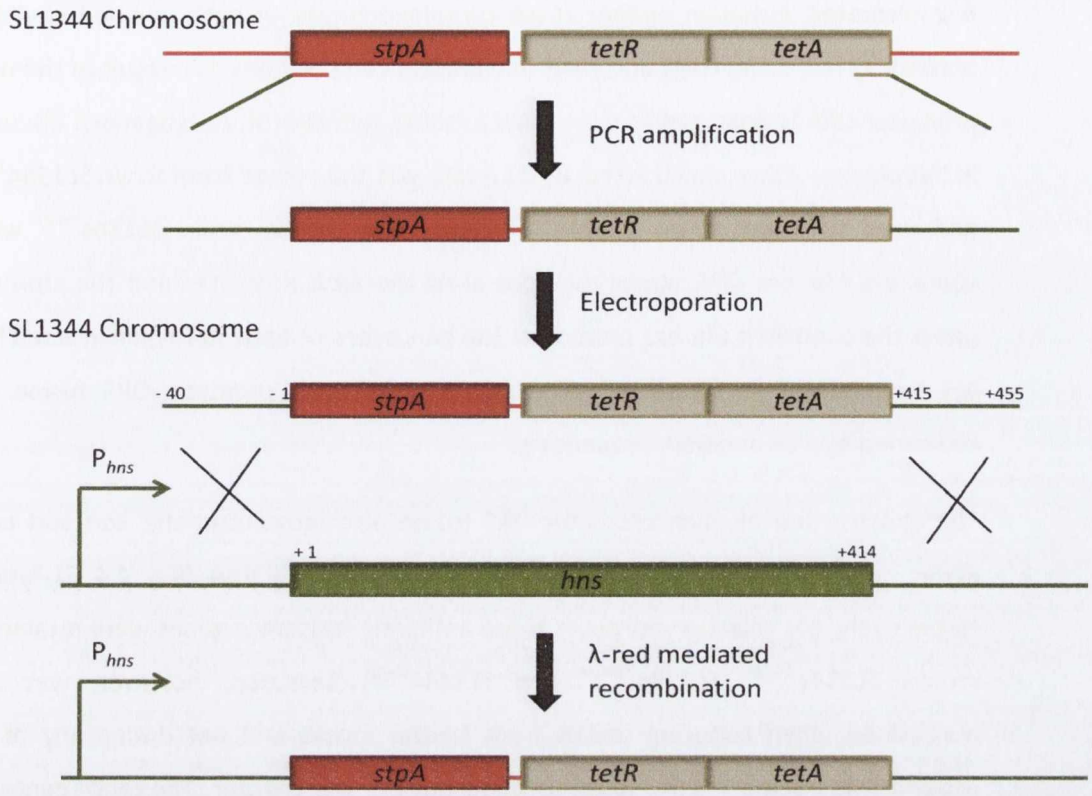


Fig. 4.2 Chromosomal interchanging of the *hns* and *stpA* open reading frames.

Diagrammatic representation of the steps involved in placing the *stpA* ORF under the control of the *hns* promoter. Firstly, the tetracycline resistance genes, *tetRA* (grey box) were PCR amplified from Tn10 and integrated onto the SL1344 chromosome downstream of the *stpA* ORF by λ-red mediated recombination. Primers specific for the first 20 base pairs of *stpA* (red/green line) and the last 20 base pairs of the *tetA* gene (grey/green line) which contained 40 base pairs of homology to the region upstream and downstream of the *hns* ORF (green box) respectively were used to PCR amplify *stpA* and *tetRA* as a single product. The resultant PCR product was electroporated into SL1344 and integrated onto the chromosome to replace the *hns* ORF by λ-red mediated recombination. The *hns* ORF was placed under the control of the *stpA* promoter in a similar manner using the kanamycin resistance gene (*kan*) from plasmid pSUB11 as a genetic marker.

hns promoter. Although neither strain contained copies of both *hns* and *stpA* they potentially had novel H-NS and H-NS-like protein compositions as a result of the novel promoter-ORF fusions and so were used in some subsequent investigations discussed in this chapter. Once constructed, a P22 lysate was then made from strain SL1344$^{2X stpA}$ and used to infect strain SL1344$^{2X hns}$. This gave rise to strain SL1344^{swap} which contained the *hns* ORF under the control of the *stpA* promoter and the *stpA* ORF under the control of the *hns* promoter. The genotypes of each strain constructed here are summarised in Figure 4.3. The structure of each promoter-ORF fusion was confirmed by PCR and DNA sequencing.

The construction of each promoter-ORF fusion also introduced the *kan* and *tetRA* genes downstream of the P_{stpA}-*hns* (Fig. 4.4 A) and P_{hns}-*stpA* (Fig. 4.4 B) fusions, respectively. For selection purposes these antibiotic resistance genes were retained in strains SL1344$^{2X hns}$, SL1344$^{2X stpA}$ and SL1344^{swap}. Selection, however, was only maintained when culturing strains from freezer stocks and not during any of the experiments outlined in the subsequent sections of this chapter. The *tetRA* genes are maintained in a repressed state in the absence of the inducer tetracycline (Muthukrishnan *et al.*, 2012). Thus in the absence of selection *tetRA* genes should not be expressed. Expression of the *kan* gene, however, is constitutive (Dulmage, 1953). Low levels of constitutive 'leaky' expression of *tetRA* and/or constitutive *kan* expression may influence results obtained in some of the following sections and will be taken into consideration.

4.2.2 Alternate expression of *hns* and *stpA* in SL1344^{swap}

By creating strain SL1344^{swap} the intention was to confer an *hns*-like pattern of transcription on the *stpA* ORF and a *stpA*-like pattern of transcription on the *hns* ORF. Thus, in theory, *hns* mRNA levels when expressed via the P_{stpA}-*hns* hybrid gene should now be significantly more abundant than that of *stpA* throughout growth and should be maximal during early- to mid-exponential growth before declining to a steady state level. Conversely, *stpA* mRNA levels should now be greatly reduced when expressed

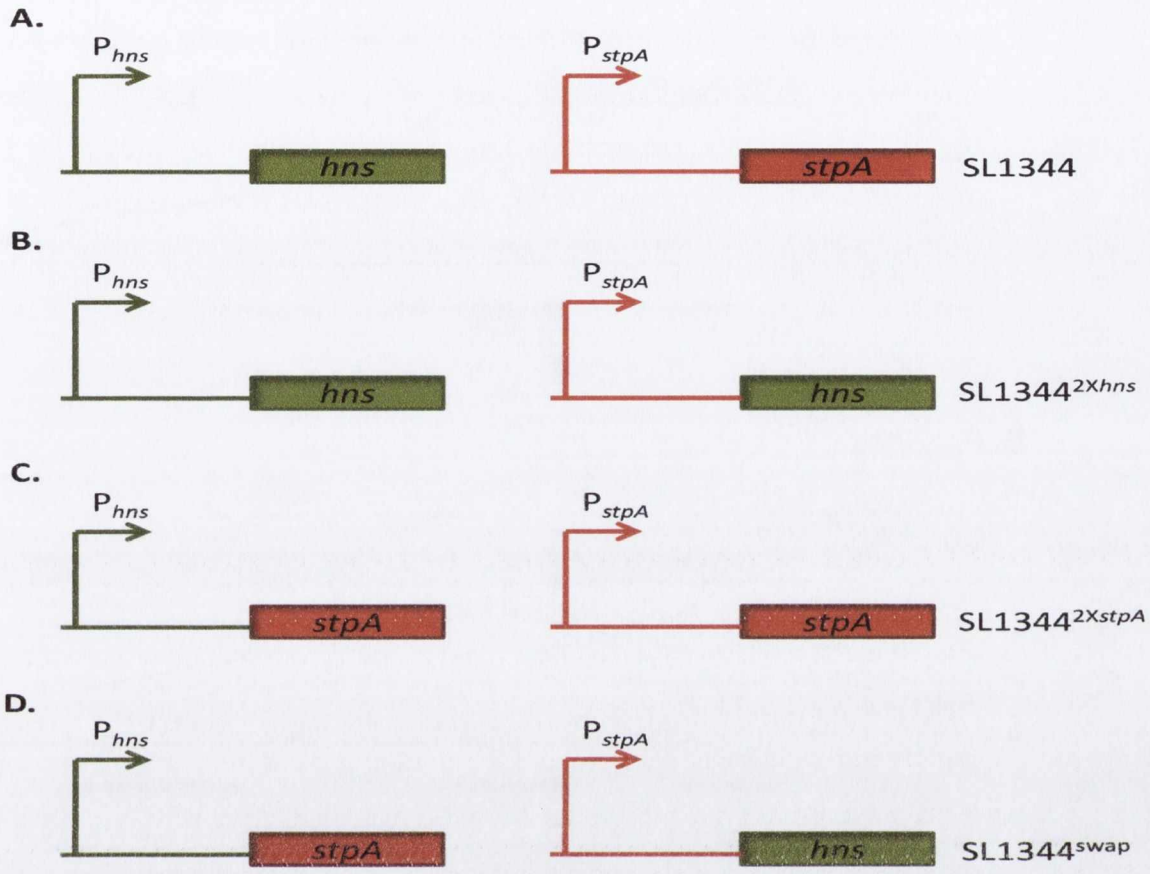
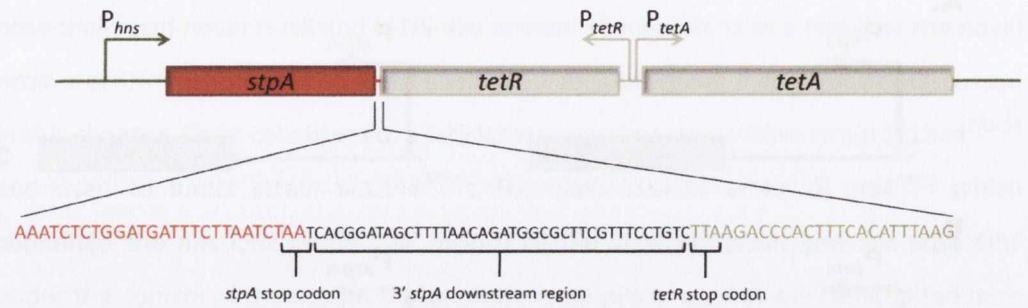


Fig. 4.3 Genotypes of wild-type SL1344, SL1344^{2Xhns}, SL1344^{2XstpA} and SL1344^{swap}.

Diagrams depicting the wild-type SL1344 (A), SL1344^{2Xhns} (B), SL1344^{2XstpA} (C) and SL1344^{swap} (D) genotypes. In wild-type SL1334 the *hns* ORF (green box) is expressed via the *hns* promoter (green lines and arrow) and the *stpA* ORF (red box) is expressed via the *stpA* promoter (red lines and arrow). SL1344^{2Xhns} retains the native *hns* allele and also has a copy of the *hns* ORF expressed via the *stpA* promoter. SL1344^{2XstpA} retains the native *stpA* allele and possesses a copy of the *stpA* ORF expressed via the *hns* promoter. In SL1344^{swap} the *hns* ORF is expressed via the *stpA* promoter and the *stpA* ORF expressed via the *hns* promoter.

A. P_{hns}-*stpA*



B. P_{stpA}-*hns*

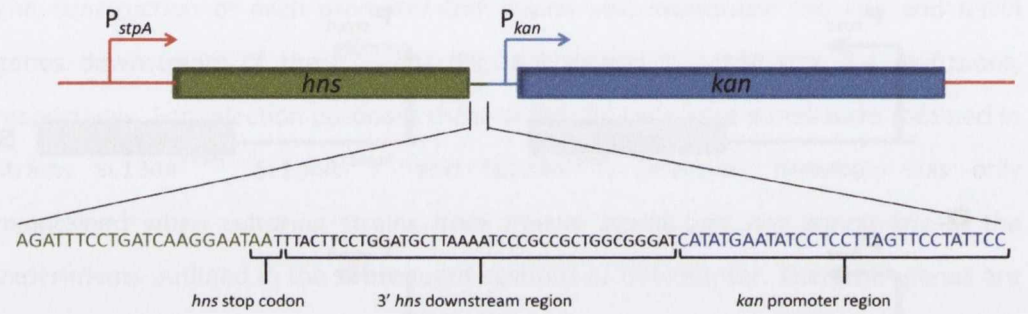


Fig. 4.4 Diagram of the P_{hns}-*stpA* and P_{stpA}-*hns* chromosomal fusion and downstream resistance cassettes.

The *stpA* ORF (red box) is depicted fused with the *hns* promoter (green lines and arrow) (A) and the *hns* ORF (green box) with the *stpA* promoter (red lines and arrow) (B). The *tetRA* (grey boxes) and *kan* (blue box) resistance marker genes and their promoters (grey and blue arrows, respectively) are shown downstream of P_{hns}-*stpA* and P_{stpA}-*hns* fusions. The nucleotide sequences at the 3' ends of the *stpA* and *hns* ORFs are highlighted and show the last 18-20 bps of each ORF (red and green bps, respectively), 40 bp of their flanking 3' sequences and 20 -25 bp of the downstream resistance marker sequences (grey and blue bps, respectively).

via the P_{hns}-*stpA* hybrid gene compared to the levels observed in wild-type SL1344 and should be expressed maximally during early- to mid-exponential growth before sharply declining to a low steady state level of expression for the remainder of growth. To test if interchanging these two ORFs resulted in a direct regulatory and expressional swap, RT-qPCR was performed on samples of total RNA harvested throughout the growth regime of $SL1344^{SWAP}$ at 37 °C in LB broth using primers specific for the *hns-stpA* and *stpA-hns* hybrid mRNA molecules.

Interestingly, despite the *hns* and *stpA* ORFs inheriting the promoter inputs of each other in $SL1344^{swap}$, each ORF now exhibited a new and distinct pattern of expression (Fig. 4.5). When controlled via the *hns* promoter the *stpA* mRNA abundance was significantly reduced relative to wild-type *stpA* mRNA levels (Fig. 4.5 A). Furthermore, instead of inheriting an *hns*-like pattern of expression, *stpA* displayed a novel expression pattern when controlled by the *hns* promoter. Maximal levels of *stpA* were observed in early- to mid-exponential growth akin to wild-type expression of *hns*, the onset of significant *stpA* expression, however, occurred earlier during growth after 1 h of growth (Fig. 4.5 A). This was in contrast to wild-type *hns* expression where significant levels of *hns* mRNA were not detected until 2 h post-inoculation. Furthermore, instead of a sharp decline to a steady state low level of expression immediately after peak expression during early- to mid-exponential growth, *stpA* expression via the *hns* promoter declined in a much more gradual manner.

When controlled by the *stpA* promoter, *hns* was indeed expressed at significantly higher levels than when under the control of its native promoter. However, *hns* did not inherit a pattern of expression equivalent to that of wild-type *stpA* (Fig. 4.5 B). Levels of *hns* mRNA now peaked later in mid-exponential growth phase compared with wild-type *stpA* expression and instead of declining to a steady state level thereafter, *hns* mRNA levels remained somewhat static until stationary phase growth before any decline in levels was observed. Furthermore, *hns* mRNA levels were also greater than those observed for *stpA* mRNA when controlled by the *stpA* promoter. Although a direct swap in the expression patterns of *hns* and *stpA* was not achieved by

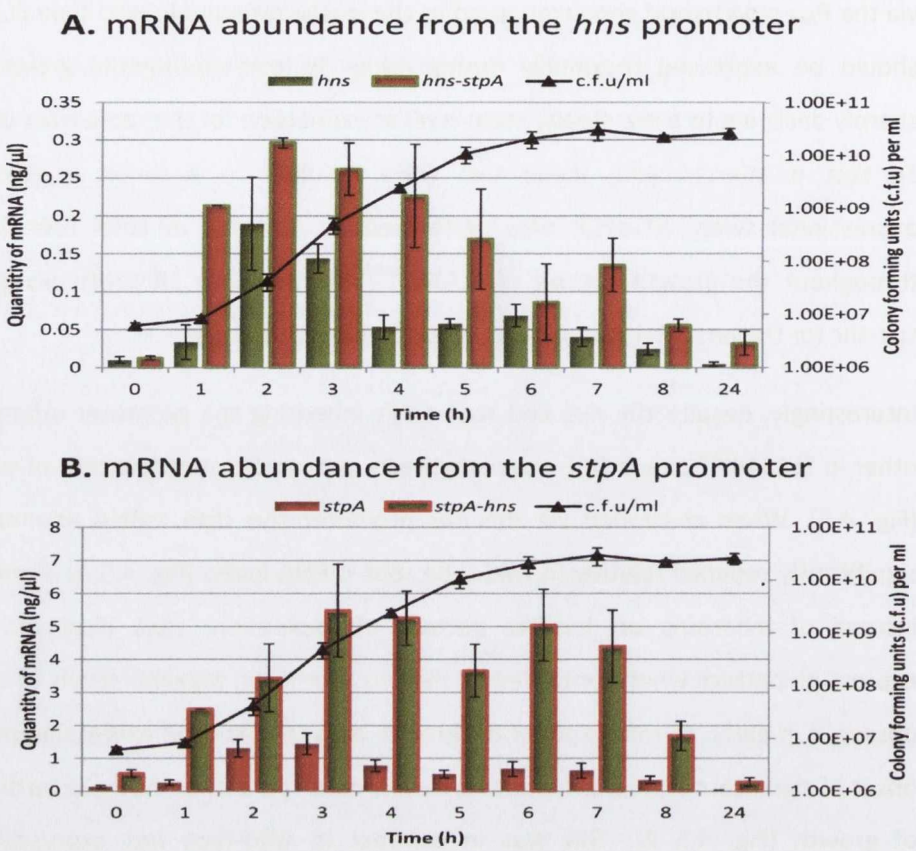


Fig. 4.5 Abundance of *hns-stpA* and *stpA-hns* mRNA in SL1344^{swap} as a function of growth.

The expression patterns of *hns-stpA* (red bars with green border) (A) and *stpA-hns* (green bars with red border) (B) were monitored during growth of SL1344^{swap} by RT-qPCR using gene specific primer pairs. Transcript abundance was quantified (primary y-axis) relative to a standard curve generated from a serially diluted preparation of SL1344^{swap} genomic DNA of known quantity (70 ng/µl). Transcript levels are depicted as a function of the growth regime of SL1344 (black diamond) which was monitored by enumeration of viable colonies (secondary y-axis) at equivalent time points. For comparison the wild-type *hns* (green bars) (A) and *stpA* (red bars) (B) have been included. Data for transcript abundance and viable colony counts were obtained from three independent experimental replicates (n = 3). Error bars represent the standard deviation of transcript abundance between experimental replicates.

simply interchanging their open reading frames, each did attain a new alternate pattern of expression. These new expression patterns nonetheless have the potential to alter the population of H-NS-like proteins throughout growth and give rise to new phenotypes.

As mentioned in section 1.2.1 the antiobiotic resistance gene cassettes were retained downstream of each promoter-ORF fusion. As Fig 4.4 shows, both the *kan* and *tetRA* genes were integrated such that both the *hns* and *stpA* ORFs retained their native stop codons and 40 bps of their immediate downstream regions. It is predicted based on RNAseq data that transcription termination of both genes occurs within these 40 bp regions (Kroger *et al.*, 2012). Thus both ORFs have also retained their native transcription termination signals. The presence of each resistance cassette downstream is therefore unlikely to have influenced the observed patterns of transcription of *hns* and *stpA* in SL1344^{swap}. Transcription of *kan* and/or *tetRA*, however, could potentially influence transcription of the P_{stpA}-*hns* and P_{stpA}-*stpA* fusions, respectively. Transcription of the *kan* gene would generate negative supercoils at the 5' end of the elongating RNAP complex (Rovinskiy *et al.*, 2012). Such supercoils could be inhibitory to the elongating RNAP complex transcribing the P_{stpA}-*hns* fusion which requires the generation of positive supercoils at its 3' end (Rovinskiy *et al.*, 2012). Fig 4.4 depicts the first 30 bps of a 250 bp fragment containing the *kan* gene promoter. Thus the distance between the end of the *hns* ORF and the start of any elongating complexes transcribing *kan* is ≈ 290 bp. Negative supercoiling is therefore unlikely to be having an effect on expression of the *hns* ORF. Furthermore, no inhibitory effect was observed on *hns* ORF expression which was expressed at a higher level than the wild-type *stpA* ORF. As shown in Fig 4.4 the *tetR* and *hns* promoters are transcribed convergently. As such, any readthrough transcription of the *tetR* gene could cause transcriptional interference by elongation complex (EC) collisions between the *tetR* EC and the P_{hns}-*stpA* EC or by the *tetR* EC colliding with the P_{hns}-*stpA* EC poised at the *hns* promoter (Shearwin *et al.*, 2005). Such collisions would be inhibitory to transcription of P_{hns}-*stpA* but as with the P_{stpA}-*hns* fusion transcription

of the *stpA* ORF via the *hns* promoter was increased in SL1344^{swap} relative to the wild-type *hns* allele. The presence of *tetRA* therefore is unlikely to be influencing the observed patterns of expression.

4.2.3 Expression of H-NS and StpA proteins in SL1344^{swap}

Interchanging the open reading frames of *hns* and *stpA* in SL1344^{swap} resulted in each ORF being expressed at different levels relative to that observed in wild-type and each attained an alternative pattern of expression. Importantly, these changes are likely to affect the H-NS-like protein population dynamics throughout growth in terms of protein abundance and the timing of their maximal expression. To determine whether or not the transcriptional alterations made to *hns* and *stpA* resulted in alterative H-NS and StpA protein populations during growth, Western immunoblotting was performed on total protein samples taken throughout the growth of SL1344^{swap} at 37^{o}C in LB broth. For this two new variant SL1344^{swap} strains were created, one in which the *hns* ORF was fused with a 3X FLAG-tag sequence at the 3' end and one in which the *stpA* ORF was fused with a 3X FLAG-tag sequence at the 3' end. The resultant H-NS and StpA proteins would then contain a C-terminal 3X FLAG-tag epitope which was detectable using FLAG-tag antibodies.

Although when controlled by the *stpA* promoter, *hns* exhibited a new mRNA expression pattern, the pattern of H-NS protein expression throughout growth was unaffected by the transcriptional changes (Fig. 4.6 A). As in wild-type SL1344, H-NS protein levels were static at all stages of growth (Fig. 4.6 B). The increased level of *hns* expression in SL1344^{swap}, however, resulted in significantly more H-NS being present in the cell when compared with wild-type H-NS levels, with 1.8-fold more H-NS, on average, present during growth in SL1344^{swap} (Fig. 4.6 B).

In strain SL1344^{swap}, *stpA* mRNA was clearly detectable when expressed via the *hns* promoter and had a distinctive pattern of expression. The *stpA* mRNA was also more abundant than the *hns* transcript when driven from the same promoter. Despite

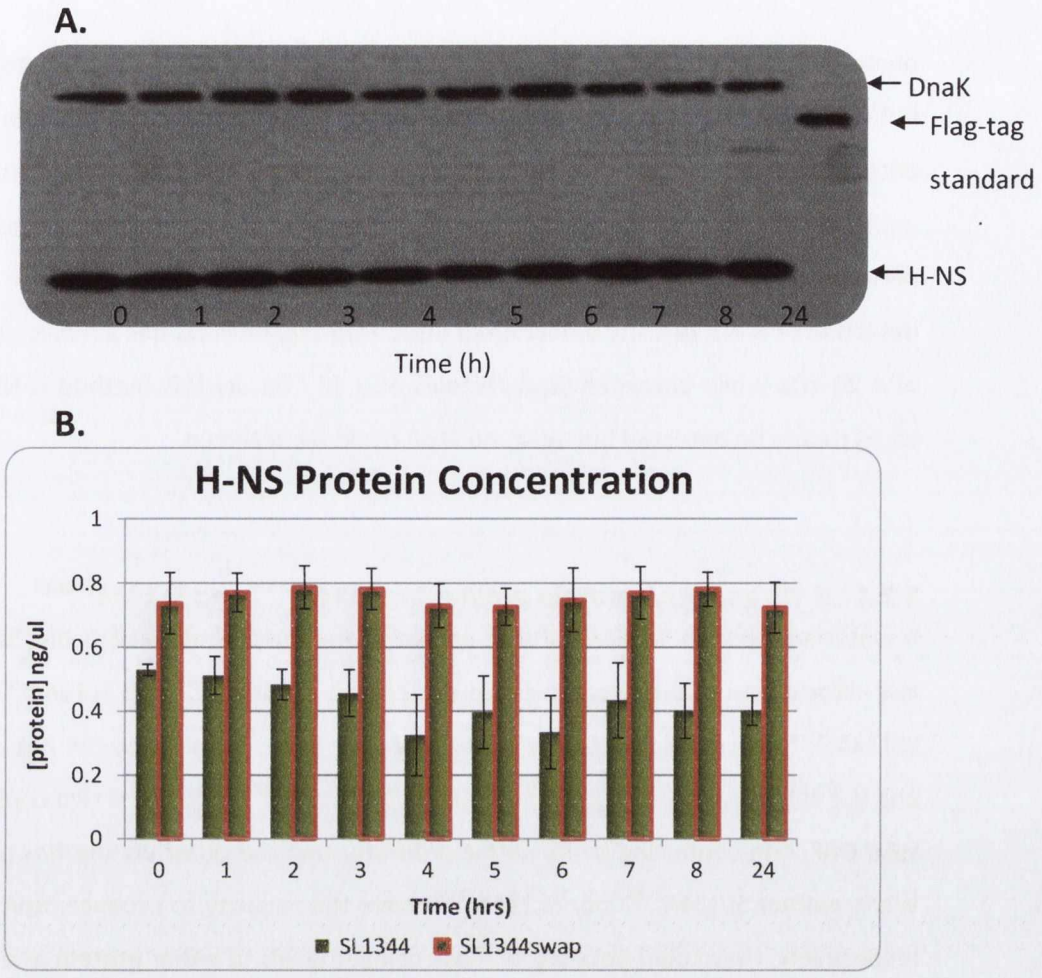


Fig. 4.6 H-NS protein abundance in SL1344^{swap}.

The abundance of H-NS (green bars with red border) was monitored by Western blotting analysis throughout growth of SL1344^{swap} at 37 °C in LB medium (A). FLAG-tagged H-NS protein was detected by Western immunoblotting using a monoclonal FLAG-tag antibody. H-NS abundance in SL1344^{swap} is also depicted alongside the observed abundance in wild-type SL1344 for comparison (B). Data were generated from three individual experimental replicates (n = 3). Error bars represent the standard deviation of densitometric readings of protein bands on Western immunoblots between experimental replicates.

numerous attempts, the StpA protein was undetectable at any point during growth of SL1344^{swap}. To eliminate the possibility that this may be the result of an inability to detect the FLAG epitope, Western immunoblotting was also performed using a StpA antibody. As this antibody is known to cross-react with H-NS, this assay was performed in SL1344^{swap} containing FLAG-tagged H-NS in order to distinguish between detection of H-NS and any detection of StpA. Flag-tagged H-NS has a molecular weight of ≈ 20 kDa while untagged StpA remains at ≈ 15 kDa. By this method H-NS protein could clearly be detected but again no StpA could be detected.

4.2.4 H-NS and StpA protein profiles in SL1344$^{2X\textit{hns}}$ and SL1344$^{2X\textit{stpA}}$

It was mentioned in Section 4.2 that as part of the process of constructing SL1344^{swap} two alternative strains were created designated SL1344$^{2X\textit{stpA}}$ and SL1344$^{2X\textit{hns}}$. Strain SL1344$^{2X\textit{hns}}$ possesses two copies of the *hns* ORF, one controlled by the *hns* promoter and the other by the *stpA* promoter. Similarly, SL1344$^{2X\textit{stpA}}$ contains two copies of the *stpA* ORF, one controlled by its native promoter and the other via the *hns* promoter. While neither SL1344$^{2X\textit{hns}}$ nor SL1344$^{2X\textit{stpA}}$ have the capacity to produce StpA or H-NS, respectively, they could possibly increase cellular levels of either protein as a result of harbouring two identical ORFs. These strains remain uncharacterized transcriptionally. However, the hybrid promoter-ORF fusion present in each strain (i.e. *hns* ORF expressed via the *stpA* promoter in SL1344$^{2X\textit{hns}}$) was chromosomally FLAG-tagged in order to observe protein expression profiles resulting from each fusion.

H-NS protein levels were again observed to be constant at all stages of growth when expressed via the *stpA* promoter in SL1344$^{2X\textit{hns}}$, as had been seen in SL1344^{swap} where *hns* is also under the control of the *stpA* promoter (Fig. 4.7 A). Intriguingly, while no StpA protein could be detected in SL1344^{swap} when the *stpA* ORF was expressed via the *hns* promoter in SL1344^{2stpA}, StpA could be detected at all stages of growth (Fig. 4.7 B). Furthermore, despite inheriting the *hns* regulatory inputs, the StpA protein profile in SL1344$^{2X\textit{stpA}}$ was similar to that of wild-type StpA with maximum levels observed in early exponential growth after which levels declined but StpA was still

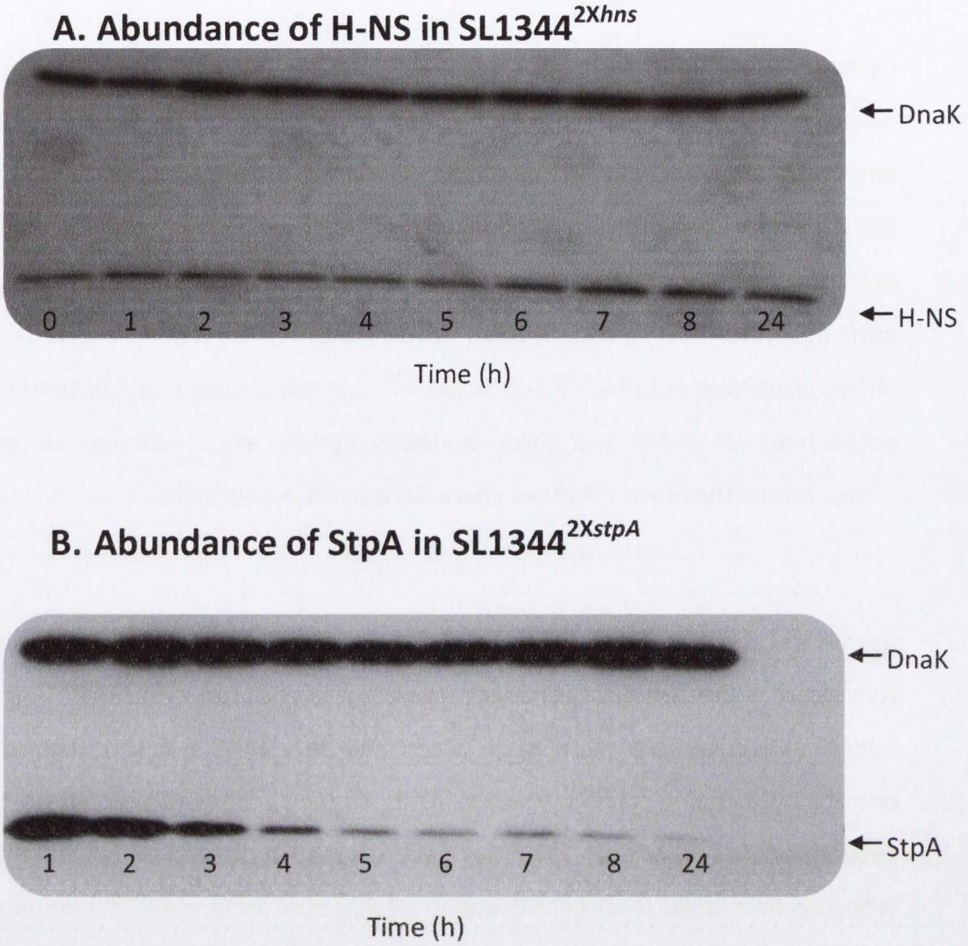


Fig. 4.7 Protein profile of H-NS and StpA when expressed via the *stpA* and *hns* promoters in SL1344^{2xhns} and SL1344^{2xstpA}.

Western immunoblot images of H-NS protein abundance (A) when *hns* was expressd by the *stpA* promoter in SL1344^{2Xhns} and StpA protein abundance (B) when *stpA* was expressed via the *hns* promoter in SL1344^{2XstpA}, respectively, during grwoth. H-NS and StpA were epitope tagged in each strain with a 3X FLAG-tag blots and detected using FLAG-tag monoclonal antibodies. To ensure equal amounts of protein were loaded in each lane blots were also probed for DnaK whose concentration was found not to change as a function of growth. Images are representative of the results from three independent experimental replicates (n = 3).

detected in stationary-phase. This suggests that the presence of H-NS and/or its increased abundance in SL1344^{swap} may be a contributing factor to the lack of detectable StpA protein in this strain via negative regulation of the *hns* promoter. SL1344$^{2X stpA}$ is incapable of producing H-NS and as a result only StpA protein was detected throughout growth. Detection of H-NS and StpA at all stages of growth via each promoter-ORF fusion coupled with expression of the wild-type *hns* and *stpA* alleles present in SL1344$^{2X hns}$ and SL1344$^{2X stpA}$, respectively, could potentially alter the populations of H-NS and StpA in these strains as a function of growth and consequently these were further characterised phenotypically.

4.2.5 Altered stability of hybrid mRNA molecules in SL1344^{swap}

As a result of the regulatory changes made to *hns* and *stpA* in SL1344^{swap}, the resultant mRNA molecules are hybrids in which the *hns* ORF is fused with the *stpA* 5' untranslated region and *vice versa*. While these changes cannot affect the protein sequences of H-NS or StpA they may have resulted in altered mRNA stability. Coupled with the decreased level of expression of P_{hns}-*stpA* in SL1344^{swap} compared to wild-type, a reduced stability of the hybrid *hns-stpA* mRNA molecule may help to account for the lack of detection of StpA protein. Similarly, combined with the increase in *stpA-hns* mRNA levels in SL1344^{swap} an increase in the stability of the hybrid *stpA-hns* mRNA molecule may contribute to the observed increase in H-NS protein abundance. To test this hypothesis, as outlined in Chapter 3 for the wild-type *hns* and *stpA* mRNA molecules, gene expression was inhibited by the addition of rifampicin to exponential phase cultures of SL1344^{swap} (OD_{600} ≈ 0.13). Total RNA was then sampled in a time-course experiment and the stability of each hybrid mRNA molecule was followed by RT-qPCR.

In contrast to the results observed for *hns* and *stpA* mRNA stability in wild-type SL1344, both the *hns-stpA* and *stpA-hns* hybrid mRNA molecules appeared to undergo an equivalent rapid degradation within the first 5 min in the presence of rifampicin with the level of each decreasing to 29% and 27% that of their starting quantities,

respectively (Fig. 4.8 A). Thereafter the hybrid *hns-stpA* mRNA molecule was stable and was detected at levels between 25% and 40% of the quantity present at time t = 0. Whilst significantly lower levels of *hns-stpA* mRNA were observed in SL1344^{swap}, a similar trend was observed to that of the *hns* mRNA molecule whereby after an initial degradation within 5 min post-transcriptional inhibition, *hns-stpA* mRNA levels were relatively constant thereafter (Fig. 4.8 B). As the *hns-stpA* mRNA molecule was more stable than the wild-type *stpA* mRNA this suggests that the apparent lack of StpA protein detection in SL1344^{swap}, is the result of low levels of transcription when expressed via the *hns* promoter coupled with proteolytic degradation of StpA. In contrast, the levels of the *stpA-hns* hybrid mRNA molecule were further depleted to 9% that of the starting level after 15 min and were then maintained at this level. This pattern of degradation observed for *stpA-hns* mimics the degradation of the wild-type *stpA* mRNA molecule (Fig. 4.8 C). Interestingly, when the *hns* ORF was expressed via the *stpA* promoter, almost 1.5-fold more H-NS protein was detected despite the *stpA-hns* mRNA molecule being less stable than wild-type *hns* mRNA (Fig. 4.8 B).

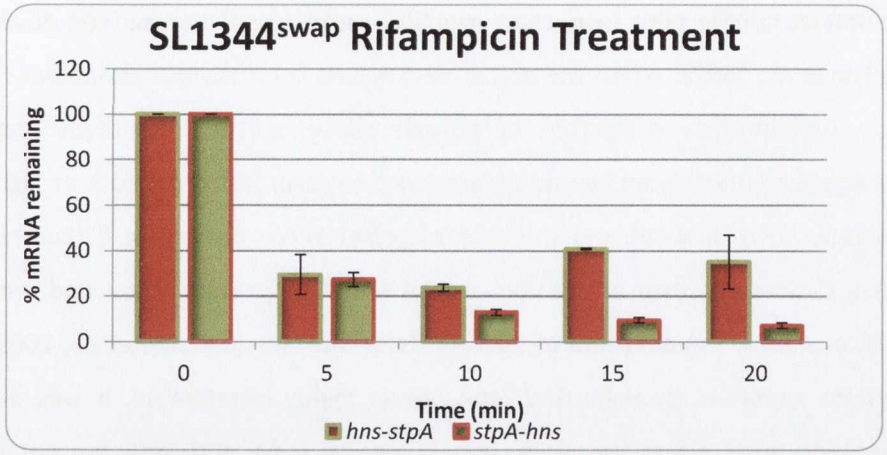
4.2.6 Phenotypic variations resulting from novel H-NS-like protein compositions.

Owing to the pleiotropic nature of gene regulation by H-NS and StpA, alterations in the levels of either protein have been shown to result in several seemingly unrelated phenotypes. Historically, much work has focused on phenotypes arising from strains in which the *hns* gene has been deleted or which possess a truncated copy of *hns* (Hinton *et al.*, 1992, Sonden & Uhlin, 1996). Such studies undertaken in both *S.* Typhimurium and *E. coli* have demonstrated that, depending on the status of the *hns* gene, a wide variety of phenotypic outcomes are achievable. For instance, in *S.* Typhimurium, using a series of truncated *hns* mutants, Hinton *et al.* showed using a reporter plasmid that depending on the nature of the *hns* mutation, various levels of negative DNA supercoiling were achievable (Hinton *et al.*, 1992). Hinton *et al.* also demonstrated using the same series of mutants that each had an idiosyncratic

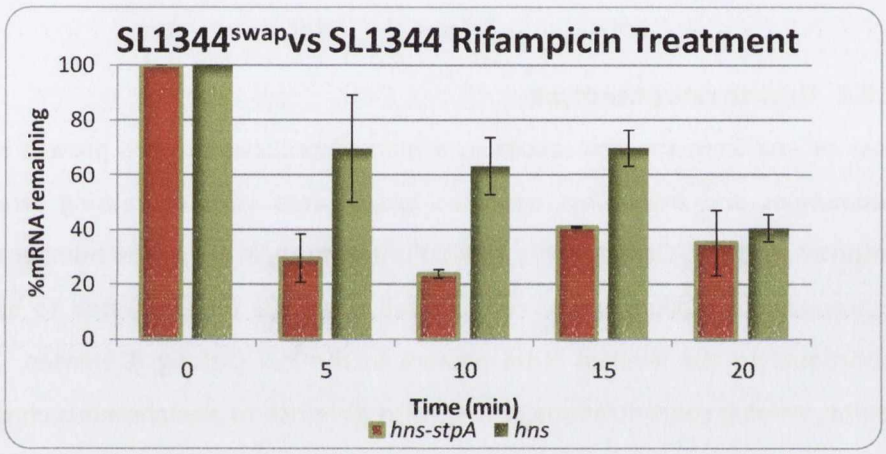
Fig. 4.8 Stability of the hybrid *hns-stpA* and *stpA-hns* mRNA molecules generated in SL1344^{swap}.

The relative stabilities (A) of the *hns-stpA* (red bars with green border) and *stpA-hns* (green bars with red border) mRNA molecules was assessed in SL1344^{swap}. Samples were analysed by RT-qPCR to quantify the abundance of each mRNA molecule as a function of time after the inhibition of transcription. Abundance is expressed as a percentage of the starting quantity of either *hns-stpA* or *stpA-hns* mRNA at time T = 0. For comparison the stabilities of the *hns-stpA* mRNA molecule has been plotted with that of wild-type *hns* mRNA (green bars) (B) and the *stpA-hns* mRNA molecule with that of wild-type *stpA* mRNA (red bars) (C). Data represent three independent experimental replicates (n = 3). Error bars represent the standard deviation of percentage transcript remaining between experimental replicates.

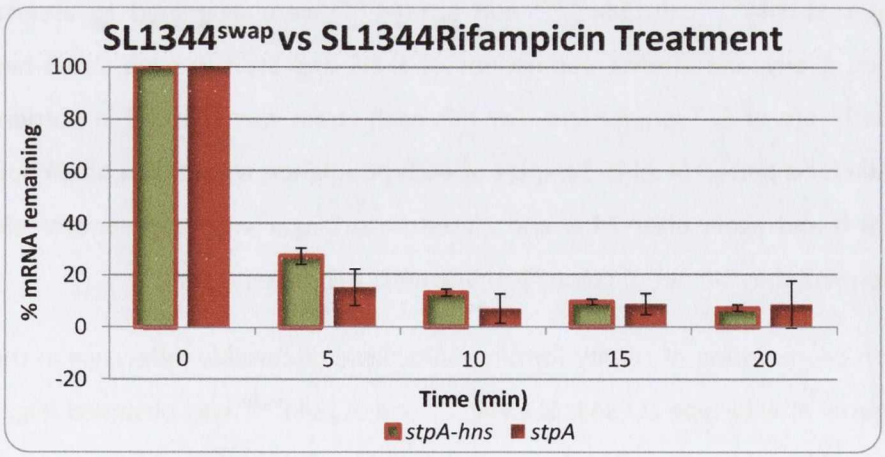
A.


SL1344swap Rifampicin Treatment
% mRNA remaining
120
100
80
60
40
20
0
0
5
10
15
20
Time (min)
hns-stpA
stpA-hns

B.


SL1344swapvs SL1344 Rifampicin Treatment
%mRNA remaining
100
80
60
40
20
0
0
5
10
15
20
Time (min)
hns-stpA
hns

C.


SL1344swap vs SL1344Rifampicin Treatment
% mRNA remaining
100
80
60
40
20
0
-20
0
5
10
15
20
Time (min)
stpA-hns
stpA

phenotypic profile with respect to motility, osmo-sensitivity and cell doubling-time (Hinton *et al.*, 1992). Whilst the loss of *stpA* results in no obvious phenotypes in *E. coli*, in *S.* Typhimurium a number of growth phase specific phenotypes have been characterized; namely an increased resistance to several stresses such as high osmotic pressure, H_2O_2, low pH and the anti-microbial agent polymyxin B (Lucchini *et al.*, 2009). Conversely, over-expression of *stpA* had the opposite effect and reduced the resistance of *S.* Typhimurium to each of these stresses (Lucchini *et al.*, 2009). As the regulons governed by H-NS and StpA are so highly interrelated, it was decided to determine what effect the novel populations of H-NS and StpA present in strains SL1344$^{2X hns}$, SL1344^{2XstpA} and SL1344^{swap} on several *hns* and *stpA* related phenotypes.

4.2.6.1 Growth rate phenotype

A loss of *hns* from the cell results in a marked reduction in the growth rate of *S.* Typhimurium and this effect becomes exaggerated with decreasing temperature (Hinton *et al.*, 1992, Dersch *et al.*, 1994). Furthermore, in *E. coli* the number of origins of chromosome replication per cell and cell size were demonstrated to be directly proportional to the level of H-NS present in the cell (Atlung & Hansen, 2002). In addition, varying concentrations of H-NS also gave rise to asynchronous chromosome replication (Atlung & Hansen, 2002, Nagarajavel *et al.*, 2007). As the levels of H-NS appeared to be intimately connected with cell cycle parameters, the growth rates of strains SL1344$^{2X hns}$, SL1344^{2XstpA} and SL1344^{swap} were examined to ascertain what effect, if any, the altered populations of H-NS and StpA in each strain had on the growth rate of *S.* Typhimurium. For this each strain was cultured in nutrient rich LB media for a period of 24 h. Samples of each population were taken on an hourly basis for 8 h and again after 24 h and plated onto L-agar with appropriate selection to determine the number of colony forming units per ml (c.f.u./ml).

Upon enumeration of colony forming units, little discernible difference in the growth patterns of wild-type SL1344, SL1344$^{2X hns}$ and SL1344^{swap} was observed (Fig. 4.9 A). A cell doubling time (t^d) of 23 min was estimated for SL1344$^{2X hns}$ which was equivalent to

that of the wild-type. This indicated that the loss of StpA coupled with an increased level of H-NS did not significantly affect the growth of *S.* Typhimurium. The increased H-NS levels alongside undetectable levels of StpA protein in SL1344^{swap} served to decrease the generation time of *S.* Typhimurium as a doubling time of t^d = 20 min was estimated. Consistent with a loss of H-NS from the cell, however, the growth rate of SL1344^{2XstpA} was significantly reduced (Fig. 4.9 B). The increased level of StpA, particularly during exponential growth phase, present in SL1344^{2XstpA} did not compensate for the loss H-NS as a doubling time of t^d = 32 min was estimated. Furthermore, SL1344^{2XstpA} appeared to have a more prolonged period of exponential growth and did not reach stationary phase until after 8 h of growth.

4.2.6.2 Expression of the *proU* operon.

The *proU* locus is a three-gene operon (*proVWX*) that encodes a high affinity uptake system for osmoprotectants such as glycine-betaine and proline (Gowrishankar & Manna, 1996, Kane & Dorman, 2011). In conditions of high osmotic pressure or osmotic up-shifts, expression of the *proU* operon is induced and its levels of expression are directly proportional to the osmolarity of the given environment (Gowrishankar & Manna, 1996). At low osmotic pressures, however, *proU* is maintained in a repressed state in order to prevent both wasteful expression and the uptake of unnecessary compatible solutes (Gowrishankar & Manna, 1996). Repression of the operon is achieved via H-NS binding within two regions of the locus designated the upstream regulatory element (URE) and the downstream regulatory element (DRE) (Jordi & Higgins, 2000, Rajkumari *et al.*, 1996). When bound to these regions protein-protein interactions between H-NS bound at the URE and DRE serves to form a repressive loop-complex that either traps or occludes RNA polymerase at the promoter thus preventing open complex formation and transcription (Kane & Dorman, 2011, Nagarajavel *et al.*, 2007). With increasing osmotic pressure alterations in DNA topology and the physical properties of H-NS have been proposed to prevent formation of the repressive loop-complex by H-NS, allowing for expression of *proU* (Ní

Bhriain *et al.*, 1989, Higgins et al., 1988). In strains lacking H-NS, repression cannot be maintained and the *proU* operon is expressed irrespective of osmotic pressure. De-repression of *proU* at low osmotic pressures has been used in both *S.* Typhimurium and *E. coli* as a phenotypic indication of the loss of functional H-NS. It was therefore decided to examine expression of *proU* under various osmotic conditions in strains SL1344$^{2X\textit{hns}}$, SL1344$^{2X\textit{stpA}}$ and SL1344^{swap}.

In order to assay expression of *proU* in each strain, a chromosomal transcriptional fusion was first created. For this the *lacZ* gene, encoding the β-galactosidase enzyme, from *E. coli* was PCR amplified and integrated onto the chromosome of wild-type SL1344 downstream from the DRE within the *proU* locus using λ-red mediated recombination. Once integrated, the *proU-lacZ* transcriptional fusion was then transduced into into SL1344$^{2X\textit{hns}}$, SL1344^{2XstpA} and SL1344^{swap} using bacteriophage P22. Each strain and wild-type SL1344, also harbouring the *proU-lacZ* fusion, were then grown for 16 h in Nutrient Broth (NB) of varying osmolarity. The activity of the β-galactosidase enzyme was then assayed as a measure of *proU* expression. In the absence of NaCl (0 mM) minimal β-galactosidase was observed for all strains and thus complete repression of *proU* was maintained (Fig. 4.10 A). Upon increasing osmotic pressure, repression was relieved to a similar extent in wild-type SL1344, SL1344$^{2x\textit{hns}}$ and SL1344^{swap} as marked by equivalent levels of enzyme activity for each strain in the presence of 100 mM and 200 mM NaCl. The lack of functional H-NS was apparent for strain SL1344$^{2X\textit{stpA}}$ as *proU* was de-repressed in the absence of NaCl and was 2-fold greater than the baseline level observed for the other strains tested. Expression of *proU* then steadily increased in SL1344$^{2X\textit{stpA}}$ with increasing osmolarity before reaching a plateau at osmotic pressures equivalent to 200 mM NaCl or higher. Consistently, under each osmotic condition tested, enzyme activity and thus *proU* expression was 11-fold higher than that achieved by wild-type SL1344. Interestingly, while the pattern of *proU* expression in SL1344$^{2x\textit{hns}}$ mimicked that of wild-type SL1344 and continued to increase with osmotic pressure, *proU* expression in SL1344^{swap} appeared to decrease at NaCl concentrations above 200 mM. As significant levels of H-NS were maintained

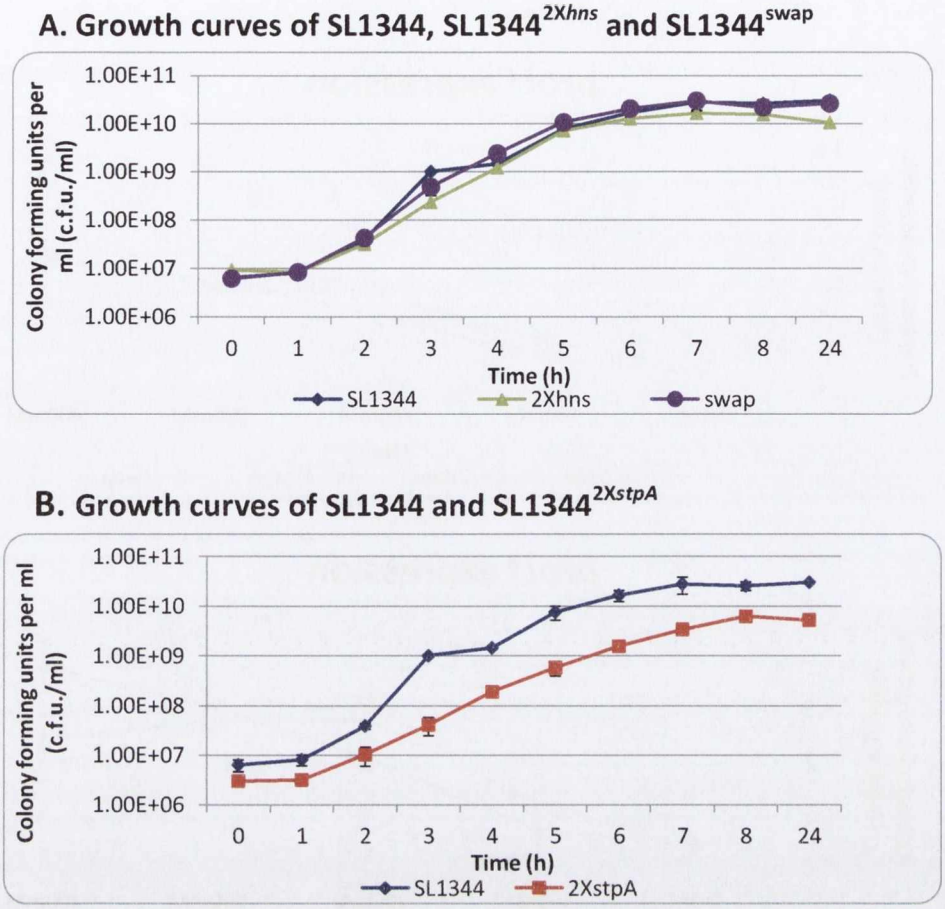
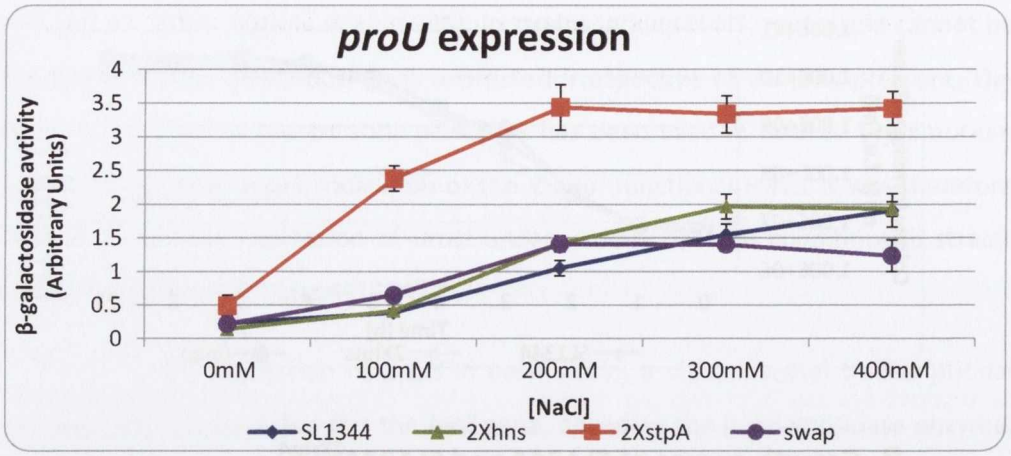


Fig. 4.9 Growth rate phenotype of SL1344^{2Xhns}, SL1344^{2XstpA} and SL1344^{swap}.

Growth of SL1344^{2Xhns} (green triangles), SL1344^{2XstpA} (red squares) and SL1344^{swap} (purple circles) was monitored over time by enumeration of viable colonies for a period of 24 h. Colony forming units per ml (c.f.u/ml) were estimated at each sample time point and used to plot the growth curves for each strain. The growth curves of SL1344^{2Xhns} and SL1344^{swap} are plotted alongside that of wild-type SL1344 (blue diamond) (A). As SL1344^{2XstpA} (red squares) was impaired in its ability to grow relative to wild-type SL1344 it was plotted separately for comparison (B). Results represent the average total viable colony counts for each strain from two experimental replicates (n = 2). Error bars show the standard deviation between total colony counts between experimental replicates.

A.



B.

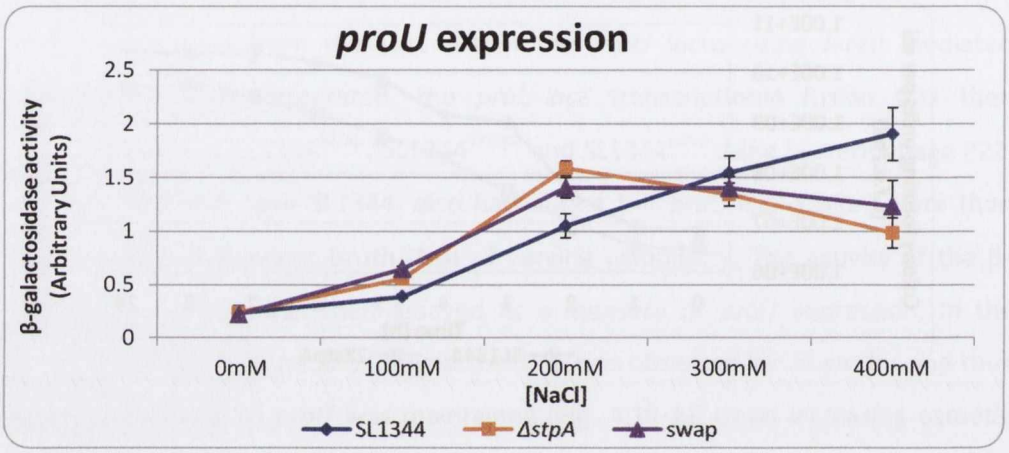


Fig. 4.10 Osmotic induction of the *proU* locus in SL1344^{2Xhns}, SL1344^{2XstpA} and SL1344^{swap}.

Expression of the osmotically inducible *proU* locus was monitored by means of a transcriptional *lacZ* fusion to the first gene within the *proU* operon, *proV*. Osmotic induction of *proU* was measured in SL1344 (Blue diamond), SL1344^{2Xhns} (green triangles), SL1344^{2XstpA} (red squares) and SL1344^{swap} (purple circle) each strain was in the absence of NaCl (0 mM)and in the presence of 100 mM, 200 mM, 300 mM or 400 mM NaCl (A). The level of β-galactosidase activity was measured kinetically for each strain after 16 h of growth for each concentration of NaCl tested. Enzyme activity was also measured in SL1344 *ΔstpA* (orange squares) to explain results observed for SL1344^{swap} at higher NaCl concentrations (B). Results represent the average of three independent experimental replicates. Error bars show the standard deviation between replicates.

in SL1344^{swap} this effect may be the result of the lack of StpA in this strain. As such, *proU* expression was monitored in a *stpA* mutant derivative strain at the same concentrations of NaCl. Expression of *proU* in the absence of StpA again followed a similar pattern to that observed for wild-type SL1344 up to an osmolarity of 200 mM NaCl (Fig. 4.10 B). At both 300 mM and 400 mM NaCl, however, *proU* expression was analogous to that observed with strain SL1344^{swap}. This suggested that StpA plays a previously unidentified role in controlling the expression of the *proU* operon at high osmotic pressures.

4.2.6.3 Motility phenotype.

Loss of motility phenotype was first identified as an *hns*-associated phenotype in *S.* Typhimurium by Hinton *et al.* (Hinton *et al.*, 1992). A series of truncated *hns* mutant derivatives was used to demonstrate that intermediate levels of motility are achievable depending on the nature of the *hns* mutation (Hinton *et al.*, 1992). Furthermore, *hns* knock-out mutants lack flagella and are non-motile (Hinton *et al.*, 1992). Subsequent studies have revealed that the effect of H-NS on motility is bi-modal. Firstly, H-NS represses the *hdfR* gene encoding a repressor of the flagellar operons master regulator *flhDC.* When H-NS is present, repression of *hdfR* is maintained allowing for expression of *flhDC* and in turn the flagellar gene operons (Soutourina *et al.*, 1999, Soutourina & Bertin, 2003). Conversely, in the absence of H-NS, repression of *hdfR* is relieved and the flagellar gene operons are repressed to render the cell non-motile (Soutourina & Bertin, 2003). In a recent *E. coli* study, Paul *et al.* showed that H-NS also had a more direct role in the regulation of motility via protein-protein interactions with the FliG sub-units of the flagellar motor which are responsible for generating torque (Paul *et al.*, 2011). Binding of H-NS to FliG was shown to be required for the correct organisation of these sub-units at the base of the flagellar motor (Paul *et al.*, 2011). Furthermore, over-expression of *hns* enhanced both FliG-FliG interactions and motility in conditions of high ionic strength (Paul *et al.*, 2011). Paul *et al.* also deduced a similar but less significant role for StpA in mediating

FliG organisation and showed that over-expression of *stpA* could restore motility to half that of wild-type in an *hns* deletion strain (Paul *et al.*, 2011).

To determine if the regulatory changes made to *hns* and *stpA* in SL1344^{2Xhns}, SL1344^{2XstpA} and SL1344^{swap} had any impact on the motility of *S.* Typhimurium, the motility of each strain was examined on swarm agar plates and compared to wild-type SL1344. From overnight cultures, 2 µl of each strain (OD_{600} = 1.0) was spotted onto tryptone swarm agar plates and incubated at 37°C for 6 h before measuring the swarm radius. In good agreement with StpA playing a negligible role in the regulation of motility, upon comparison with wild-type SL1344 no difference in motility was observed for SL1344^{2Xhns} with each consistently reaching a swarm radius of ≈ 2.0 cm (Fig. 4.11). Conversely, strain SL1344^{2XstpA} was rendered completely non-motile in lacking a copy of the *hns* gene and no compensation was observed resulting from the increased levels of StpA. Interestingly, despite possessing an *hns* ORF and increased levels of H-NS protein, SL1344^{swap} displayed a 50% reduction in motility with a swarm radius of 1 cm. This is in contrast to SL1344^{2Xhns}, which also lacks StpA but has increased levels of H-NS protein, where wild-type levels of motility were observed.

4.2.6.4 Alternate timing of expression of the RpoS σ-factor.

The alternative σ-factor RpoS plays a key role in the adaptation and survival of *S.* Typhimurium and *E. coli* to environmental stresses and starvation (Ibanez-Ruiz *et al.*, 2000, Klauck *et al.*, 2007). In rapidly dividing cells RpoS is not present at significant levels (Klauck *et al.*, 2007, Bougdour *et al.*, 2008). Upon entry into stationary phase and/or exposure to environmental stresses such as acid, heat or osmotic stress, the RpoS protein rapidly accumulates and multiple genes required for stress adaptation that are under the control of RpoS are up-regulated (Klauck *et al.*, 2007, Lucchini *et al.*, 2009, Ibanez-Ruiz *et al.*, 2000). In *E. coli* it is estimated that up to 10% of its genes are regulated by RpoS (Klauck *et al.*, 2007). To prevent wasteful and inappropriate expression of such a large number of genes the transcription, translation and proteolysis of RpoS is tightly regulated (Ibanez-Ruiz *et al.*, 2000).

In *S.* Typhimurium and *E. coli*, both H-NS and StpA play key roles in the growth-phase-specific regulation of RpoS levels via control of expression of anti-adaptor proteins (Lucchini *et al.*, 2009, Bougdour *et al.*, 2008, Tu *et al.*, 2006, Battesti *et al.*, 2012). During exponential growth RpoS is targeted by the RssB adaptor protein for degradation by the ClpXP protease but becomes stabilised and accumulates in the absence of H-NS or StpA (Bougdour *et al.*, 2008, Klauck *et al.*, 2007). Stabilisation of RpoS in the absence of H-NS in *E. coli* is the result of de-repression of two anti-adaptor proteins, IraM and IraD, which when present bind to and sequester RssB thus preventing targeted proteolysis of RpoS (Battesti *et al.*, 2012). Similarly in *S.* Typhimurium the loss of StpA results in de-repression of the *rssC* gene whose protein product, RssC, also serves as an anti-adaptor molecule which functions, akin to that of IraM, to prevent degradation of RpoS and results in detection of RpoS during exponential growth (Lucchini *et al.*, 2009

As outlined in Section 4.1, both H-NS and StpA are involved in controlling the growth-phase-specific RpoS protein profile. As such, accumulation of RpoS protein as a function of growth was investigated in strains SL1344, SL1344^{2Xhns}, SL1344^{2XstpA} and SL1344^{swap}. Each strain was grown at 37 ^{o}C and total protein samples were extracted at the start of the growth (t = 0) and on an hourly basis thereafter for a total of 8 h. Samples were analysed by Western immunoblotting using a monoclonal anti-RpoS antibody for detection of RpoS in each strain. In wild-type SL1344 low levels of RpoS were detected at t = 0 representing residual RpoS protein present in the overnight stationary phase culture used to inoculate the sample culture (Fig. 4.12 A). Consistent with its role as a stationary phase σ-factor, RpoS was not detected again until after 5 h of growth at the onset of stationary phase. Peak abundance was observed after 6 h of growth and levels remained relatively constant thereafter. In strain SL1344^{2Xhns}, which lacks *stpA* but produces greater amounts of H-NS, a markedly different RpoS protein expression profile was observed. Again residual RpoS was detected at time t = 0 but unlike wild-type, cultures of SL1344^{2Xhns} did not reach stationary phase before RpoS was again detected (Fig. 4.12 B). This time RpoS began to accumulate after 3 h during

exponential growth phase and reached maximal abundance after 4 h. In a similar manner observed in wild-type, RpoS was then maintained at this level for the remainder of the growth. In SL1344^{2XstpA}, which lacks *hns* but generates greater amounts of StpA, accumulation of RpoS was not detected until after 4 h growth at 37°C (Fig. 4.12 C). The level of RpoS at this time point appeared to be the maximum as no further accumulation was apparent and levels were static for the remainder of the experiment. Yet another pattern of RpoS protein expression was observed in SL1344^{swap}. In this strain the H-NS and StpA protein population present in the cell resulted in detection of RpoS after 2 h during early exponential growth phase (Fig. 4.12 D). Levels of RpoS further increased after 3 h of growth and were maintained at this level for the next two hours before a further increase was apparent to reach maximal observed levels after 6 h. RpoS protein levels then appeared to decrease steadily. These results demonstrated that any deviation from a wild-type pattern of H-NS and StpA expression seemed to abolish the growth phase-specific protein expression pattern of RpoS which could be detected during exponential growth in all strains except wild-type SL1344. Furthermore, depending on the relative abundance of H-NS and StpA during growth, the exact timing at which RpoS was detected as a function of growth could be altered.

4.2.6.5 Carbon source utilisation

Both H-NS and StpA are involved in the regulation of genes required for the uptake and utilisation of alternative sources of carbon such as β-glucoside sugars (salicin and arbutin) and maltose (Johansson *et al.*, 1998, Free *et al.*, 2001). The ability to acquire and hydrolyse β-glucosides in *E. coli* is conferred by the gene products of the *bgl* locus (Schaefler & Malamy, 1969). This locus has been termed 'cryptic' as it remains unexpressed under all laboratory conditions that have been tested (Sankar *et al.*, 2009). Repression of the locus requires H-NS and involves a mechanism that is akin to the *proU* upstream and downstream promoter DNA sequence elements (Sankar *et al.*, 2009, Nagarajavel *et al.*, 2007, Free *et al.*, 2001). In strains lacking *hns,* the *bgl* genes

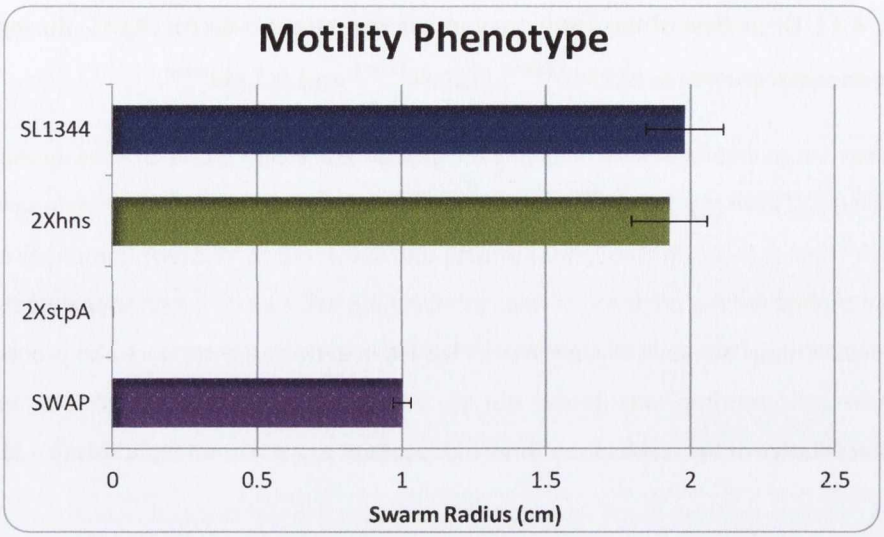
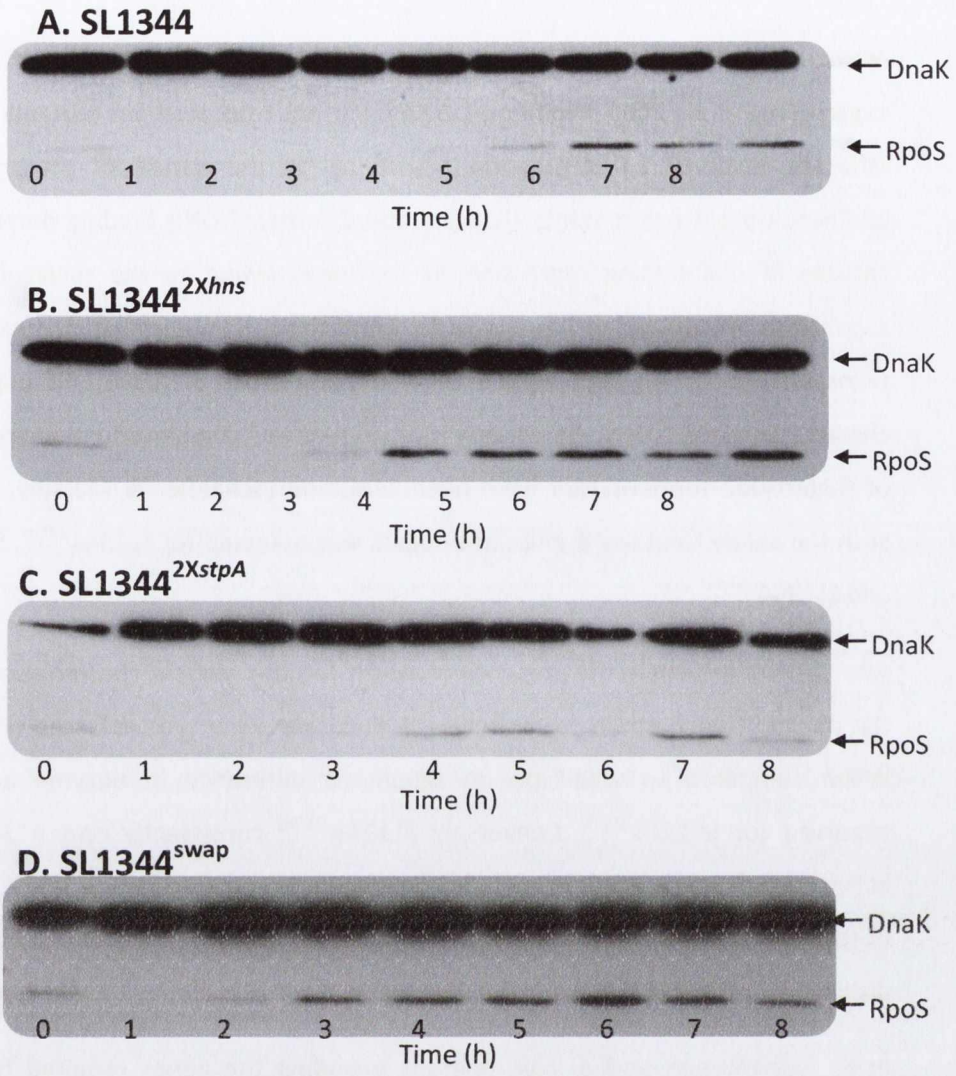


Fig. 4.11 Alterations to the cellular motility of SL1344^{2Xhns}, SL1344^{2XstpA} and SL1344^{swap}.

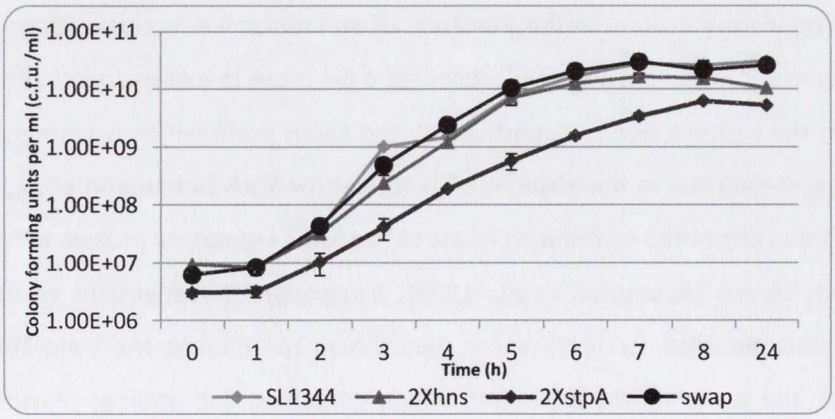
The motility of SL1344 (blue bar), SL1344^{2Xhns} (green bar), SL1344^{2XstpA} (non-motile) and SL1344^{swap} (purple bar) was assessed on tryptone swarm plates. 2 µl volumes of each strain (OD_{600} = 1.0) were spotted onto tryptone swarm plates and incubated for 6 h at 37°C. Results represent the average motility from ten biological replicates taken from three experimental replicates. Error bars are a measure of the standard deviation observed between experimental replicates.

Fig. 4.12 Detection of the stationary phase and stress σ-factor, RpoS, during exponential growth in SL1344$^{2X hns}$, SL1344$^{2X stpA}$ and SL1344^{swap}.

Western immunoblot images depicting the protein expression profile of RpoS during growth in wild-type SL1344 (A), SL1344$^{2X hns}$ (B), SL1344$^{2X stpA}$ (C) and SL1344^{swap} (D). Protein samples were taken from each strain throughout growth and subjected to Western Immunoblotting. Blots were probed for the presence of RpoS at each time point using a monoclonal RpoS antibody. To ensure equal amounts of protein were loaded in each lane blots were also probed for DnaK whose concentration was found not to change as a function of growth. Images are representative of the results from three independent experimental replicates (n = 3).



E. Growth curves

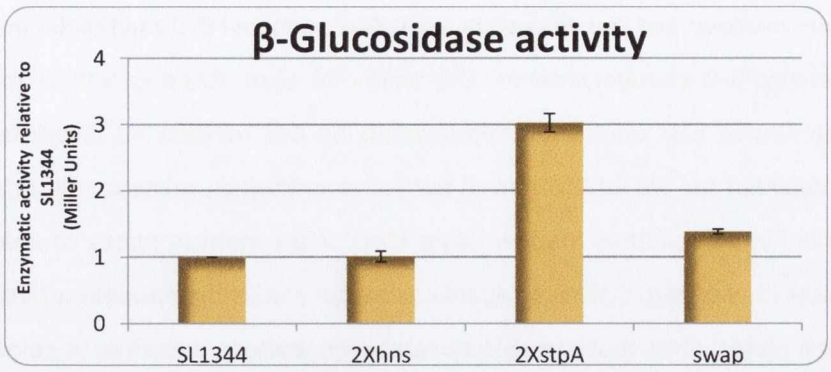


are up-regulated and *E. coli* is capable of proliferating in the presence of β-glucoside sugars (Free *et al.*, 2001, Wolf *et al.*, 2006). Various truncated *hns* mutants give rise to different levels of *bgl* expression depending on the nature of the mutation. In addition, certain *hns* mutants that lack the C-terminal DNA binding domain are still capable of maintaining repression of the locus owing to the molecular adaptor capabilities of StpA which was shown to target the H-NS N-terminal domain to the *bgl* promoter (Wolf *et al.*, 2006, Free *et al.*, 2001, Ueguchi *et al.*, 1996). Although less well characterised, the cryptic *bgl* locus is also present in *S.* Typhimurium where low levels of β-glucoside fermentation have been observed (Schaefler & Malamy, 1969)#. As such the ability to utilise β-glucoside sugars was assessed for SL1344^{2Xhns}, SL1344^{2XstpA} and SL1344^{swap}.

After 12 h of growth in the presence of salicin and the artificial chromogenic substrate the ability of each strain to hydrolyse β-glucoside sugar was assessed (Fig. 4.13 A). When compared to wild-type no significant difference in enzyme activity was observed for SL1344^{2Xhns}. Conversely SL1344^{2XstpA} consistently had ≈ 3-fold more activity which was a result of no H-NS present in this strain allowing for de-repression of the *bgl* locus. An intermediate level of activity was observed for SL1344^{swap} which displayed a 1.38-fold (P-value = 0.03) increase in activity relative to wild-type.

In *S.* Typhimurium and *E. coli*, operons encoding the genes required for efficient uptake and catabolism of maltose and maltodextrin sugars are up-regulated by the MalT regulatory protein in the presence of an inducer i.e. maltose (Boos & Shuman, 1998). In *E. coli*, Johannson *et al.* observed a decrease in expression of genes encoded within the maltose operons, particularly the LamB porin which is responsible for the uptake of maltose, in the absence of H-NS and/or StpA (Johansson *et al.*, 1998). This effect was attributed to reduced levels of the MalT regulatory protein in *hns* and *stpA* mutant strains (Johansson *et al.*, 1998). Subsequent investigations revealed a new regulatory function for H-NS which was shown to enhance the translation of *malT* mRNA and other mRNA molecules which contained sub-optimal ribosome binding sites (Park *et al.*, 2010). Using a similar assay, this time varying the culture broth to

A.



B.

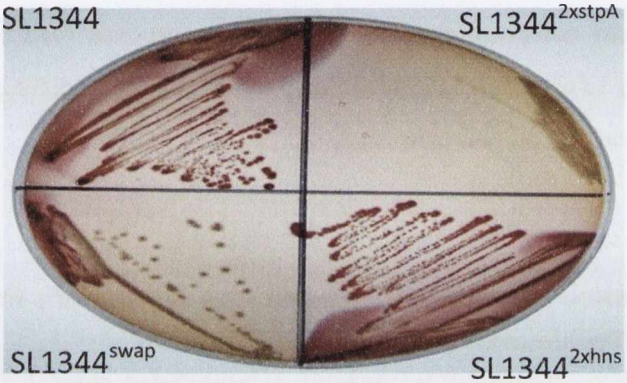


Fig. 4.13Utilisation of the alternative carbon sources β-glucoside and Maltose by SL1344^{2Xhns}, SL1344^{2XstpA} and SL1344^{swap}.

The ability to utilise β-glucoside (A) and maltose (B) was assessed for SL1344^{2Xhns}, SL1344^{2XstpA} and SL1344^{swap}. β-glucoside catabolism was measured quantitatively by assaying the utilisation of chromogenic substrate p-nitrophenyl-β-D-glucopyranoside for SL1344^{2Xhns}, SL1344^{2XstpA} and SL1344^{swap} which was compared to wild-type SL1344. Data represent the average fold increase from two experimental replicates (n = 2). Error bars show the standard deviation between each replicate. The ability to utilise maltose as a carbon source was assessed qualitatively by growing wild-type SL1344, SL1344^{2Xhns}, SL1344^{2XstpA} and SL1344^{swap} on MacConkey agar plates containing 1% (v/v) Maltose. Pink/red colony colouration is indicative of metabolism of maltose with intensity proportional to sugar uptake and utilisation. White colony colouration demonstrates inability to utilise maltose.

include maltose instead of salicin and 4-nitrophenyl-β-D-maltoside instead of p-nitrophenyl-β-D-glucopyranoside, the ability of each strain to utilise maltose as a carbon source was assessed. Unfortunately by this method no colorimetric change was observed for any of the strains tested. Nonetheless, maltose utilisation could be observed in a qualitative manner using MacConkey maltose plates containing the pH indicator phenol red. Colonies capable of maltose utilisation appear a pink/red colour on such plates while those unable to catabolise maltose are white in colour (Fig. 4.13 B). SL1344$^{2X\textit{hns}}$ once more displayed a phenotype equivalent to that of SL1344 as both strains appeared as intense pink colonies when grown on MacConkey maltose agar. SL1344$^{2X\textit{stpA}}$ was compromised in its ability to catabolise maltose and appeared as white colonies while again SL1344^{swap} displayed an intermediate phenotype as a mild pink colony colouration was observed.

4.2.7 Expression alterations to *hns* and *stpA* can confer a fitness advantage for *S.* Typhimurium.

Owing to their short generation times, large population sizes and the ability to freeze and maintain viable strain stocks, bacteria have been the organism of choice for the study of adaptive evolution (Conrad *et al.*, 2011, Crozat *et al.*, 2005, Philippe *et al.*, 2007). In such studies bacteria are continually cultured under a selective pressure, such as increased temperature or carbon source limitation, forcing the accumulation of mutations within the population (Crozat *et al.*, 2005, Riehle *et al.*, 2003). Mutations that are beneficial for the given environment and enhance the ability of the bacterium to survive and proliferate become fixed in the population over-time while non-beneficial mutations are lost through natural selection (Conrad *et al.*, 2011, Orr, 2005a). The net effect of accumulation of beneficial mutations is manifested phenotypically as an increase in fitness of the evolved bacterial population relative to its pre-evolved ancestor (Lenski, 1991). With the potential to alter the transcriptional output of multiple genes, mutations affecting global regulators of gene expression are frequently identified from adaptive evolution experiments as the cause of the

observed fitness increase of evolved populations (Pelosi *et al.*, 2006, Conrad *et al.*, 2011, Ferenci, 2005). In this study, alterations with respect to the timing of expression of two global regulators, *hns* and *stpA,* and their relative protein abundance was achieved, although this did not occur via the process of adaptive evolution. The observed changes to the expression and abundance of H-NS and StpA in SL1344$^{2X hns}$, SL1344$^{2X stpA}$ and SL1344^{swap} have thus far resulted in several phenotypic alterations when compared with wild-type SL1344 (Section 4.2.5). These changes could also potentially incur a fitness cost or benefit to *S.* Typhimurium in certain environmental conditions as a result of the downstream changes in expression of the H-NS and StpA regulons.

To test if the regulatory changes made to *hns* and *stpA* in strains SL1344$^{2X hns}$, SL1344$^{2X stpA}$ and SL1344^{swap} affected the fitness of *S.* Typhimurium, competition experiments were performed. For this, each strain was co-cultured with wild-type SL1344 for a period of 24 h in a given set of environmental conditions. After 24 h of competitive growth the fitness of SL1344$^{2X hns}$, SL1344$^{2X stpA}$ and SL1344^{swap} relative to wild-type SL1344 was calculated as the ratio of the net growth rates obtained for the two competing strains (Lenski, 1991). By this method if the net growth rate of a strain is equal to wild-type then a fitness index (f.i.) value equal to 1 is obtained. An increase in fitness is thus > 1 and a fitness deficit < 1. As expression of *hns* and *stpA* are up-regulated upon temperature downshifts and up-shifts, respectively and *hns* mutant strains exhibit a cold sensitive phenotype it was decided to compete each strain against wild-type at a range of temperatures: 25°C, 37°C and 42°C.

When competed against wild-type SL1344 at 25°C, the regulatory changes made to *hns* and *stpA* in SL1344$^{2X hns}$, SL1344$^{2X stpA}$ and SL1344^{swap} conferred a fitness deficit to each strain which were out-competed by wild-type SL1344 (Fig. 4.14 A). Strain SL1344$^{2X hns}$ was least affected in terms of fitness and had a fitness index closest to that of wild-type (f.i. = 0.97) (Table 4.1). This indicated that the increased concentration of H-NS in this strain resulting from expression of *hns* via the *stpA* promoter can somewhat compensate for the loss of functional StpA at 25°C. This was supported by

data from SL1344^{swap} in which the fitness index dropped further to an f.i. = 0.88 due to the replacement of the native *hns* ORF with the *stpA* ORF. While this copy of the *stpA* ORF produces mRNA, no StpA protein could be detected. Thus, the further reduction in fitness relative to SL1344$^{2X hns}$ could be attributed to a reduction in the pool of H-NS available to the cell in the absence of the wild-type allele. Strain SL1344$^{2X stpA}$ exhibited the greatest fitness deficit (f.i. = 0.77) due to a complete lack of functional H-NS. The genotype of SL1344$^{2X stpA}$ resulted in a growth handicap at 37°C (Section 4.2.5.1) which would become exaggerated at 25°C, as has been observed previously for strains lacking *hns*, allowing SL1344 to out-compete SL1344$^{2X stpA}$. Unlike SL1344$^{2X hns}$ where an increase in the abundance of H-NS could almost completely compensate for the loss of StpA, an increase in the levels of StpA in SL1344$^{2X stpA}$ did not compensate for the lack of H-NS.

Upon increasing the temperature of competition to 37°C, the genotypes of SL1344$^{2X hns}$, SL1344$^{2X stpA}$ and SL1344^{swap} produced a fitness benefit as each now had a fitness index ≥ 1 (Fig. 4.14 B and Table 4.1). Strain SL1344$^{2X hns}$ again showed little deviation from a wild-type fitness level (f.i. = 1.02) (Table 4.1) showing a functional compensation for the loss of StpA by increased H-NS levels. The presence of the native *hns* allele in SL1344$^{2X hns}$ conferred a fitness benefit to *Salmonella* at 25° when compared with SL1344^{swap} in which the native allele is absent. At 37°C, however, the presence of the allele was detrimental to fitness as without it SL1344^{swap} now had a significant fitness advantage (f.i. = 1.72, t-test, P-value = 0.026) relative to wild-type and SL1344$^{2X hns}$. Thus, although the H-NS protein profile was not altered during growth in SL1344^{swap}, the increased levels of H-NS resulting from increased expression of *hns* via the *stpA* promoter, coupled with a lack of detectable StpA, appeared to be quite advantageous for *S.* Typhimurium at 37°C. Intriguingly, despite having a growth defect compared to wild-type at 37°C (Section 4.2.5.1) strain SL1344$^{2X stpA}$ showed a marked increase in competitive fitness (f.i. = 1.18) at this temperature compared with that observed at 25°C. The observed fitness index value of 1.18, although greater than that of wild-type, was not statistically different (t-test, P-value = 0.43) from that of

wild-type SL1344 (f.i. = 1). Nonetheless, a net increase in fitness index value of 0.42 was achieved by SL1344$^{2X stpA}$ relative to that seen at 25 ^{o}C indicating that the genotype of SL1344$^{2X stpA}$ and the resulatant alterations in stpA expression is more beneficial for S. Typhimurium at higher temperatures and that a total loss of H-NS from the bacterium is better tolerated at 37 ^{o}C.

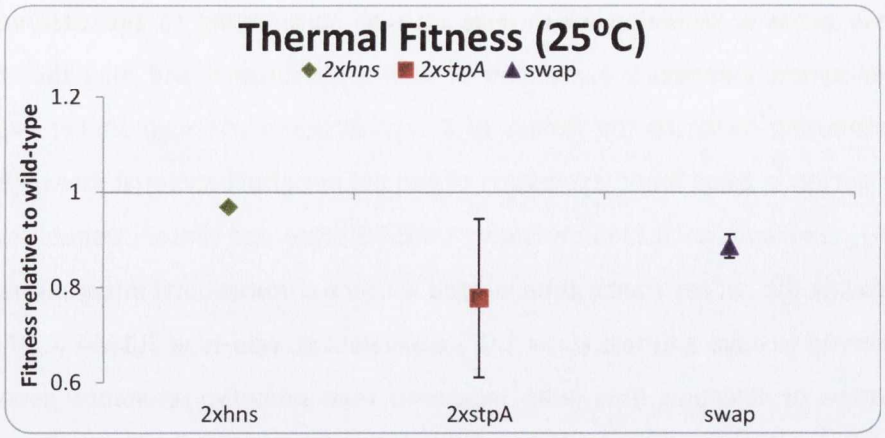
Conducting the experiment at 42 ^{o}C yielded similar results to those observed at 25 ^{o}C for both SL1344$^{2X hns}$ and SL1344^{swap} (Fig. 4.14 C). Both strains had a fitness index equivalent to that observed at 25 ^{o}C (Table 4.1) indicating that each genotype was only beneficial to *S.* Typhimurium at 37 ^{o}C. Upon deviations from 37 ^{o}C the SL1344$^{2X hns}$ genotype incurred a fitness deficit while SL1344^{swap} remained as competitive as wild-type. Despite competing equally with wild-type at both 25 ^{o}C and 37 ^{o}C, SL1344$^{2X hns}$ was unable to remain competitive at 42 ^{o}C (f.I. = 0.79) (Table 4.1). This suggested that StpA plays a key role in the survival and proliferation of *S.* Typhimurium at elevated temperatures, a function which cannot be compensated for by increased cellular levels of H-NS. Taken together these results demonstrate that alterations to the expression patterns and abundance of H-NS and StpA can significantly alter the competitive fitness of *S.* Typhimurium with respect to temperature. A broad range of fitness index values were achievable which depended on both the populations of H-NS and StpA in each strain and the environmental temperature.

These results have demonstrated that the genetic changes made in SL1344$^{2X hns}$, SL1344$^{2X stpA}$ and SL1344^{swap} have incurred a strain specific fitness phenotype with respect to temperature. This has been largely attributed to the significant alterations in the H-NS and StpA populations in each strain. However, each strain has also attained an antibiotic resistance gene(s) cassette by the process of their construction. Furthermore, each strain has a different complement of resistance cassettes with SL1344$^{2X hns}$ encoding the *kan* gene, SL1344$^{2X stpA}$ encoding the *tetRA* genes and SL1344^{swap} encoding both the *kan* and *tetRA* genes. For each competition experiment no antibiotic selection was maintained therefore it is unlikely that the tetRA genes were expressed. It cannot be ruled out, however, that some 'leaky' expression of

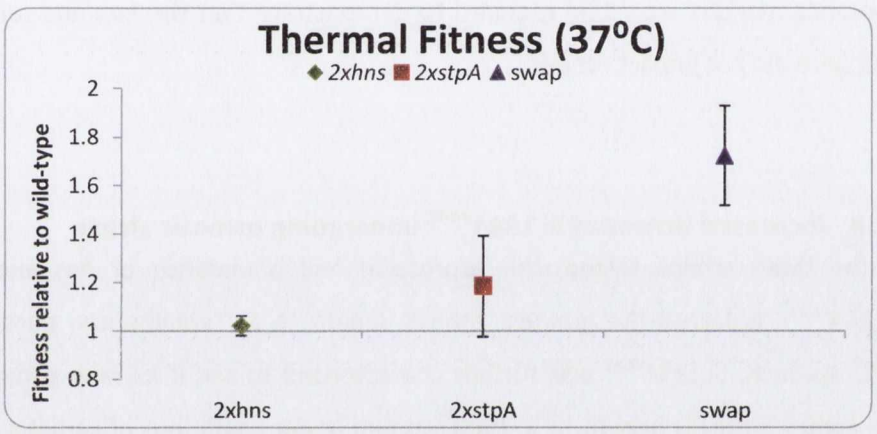
Fig. 4.14 Competitive fitness of strains SL1344$^{2X hns}$, SL1344^{2XstpA} and SL1344^{swap} at 25°C, 37°C and 42°C.

The fitness of SL1344$^{2X hns}$ (green diamonds), SL1344^{2XstpA} (red squares) and SL1344^{swap} (purple triangles) relative to wild-type SL1344 was assessed at 25°C (A), 37°C (B) and 42°C (C). A fitness index f.i. = 1 indicates an equivalent fitness to wild-type. Fitness indices > 1 or < 1 signify a fitness advantage or disadvantage, respectively. The fitness of each strain is depicted relative to the wild-type reference fitness index f.i. = 1. Results presented are the average fitness values obtained from three experimental replicates (n = 3). Error bars denote the standard deviation in fitness values between experimental replicates.

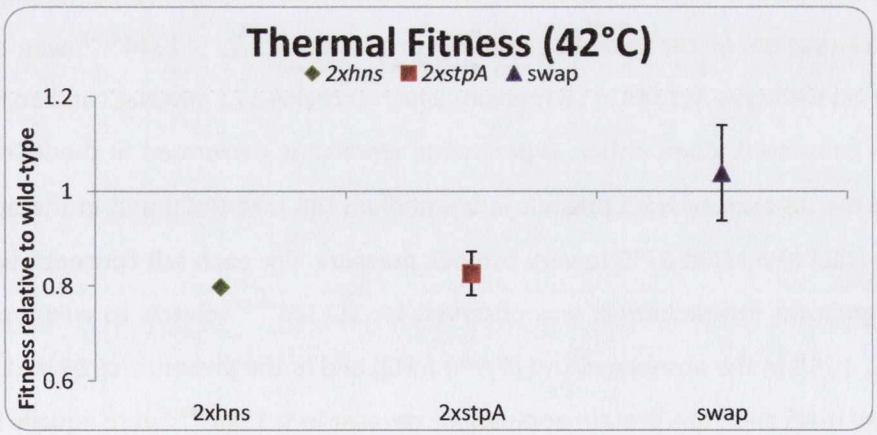
A.


Thermal Fitness (25°C)
2xhns
2xstpA
swap
Fitness relative to wild-type
1.2
1
0.8
0.6
2xhns
2xstpA
swap

B.


Thermal Fitness (37°C)
2xhns
2xstpA
swap
Fitness relative to wild-type
2
1.8
1.6
1.4
1.2
1
0.8
2xhns
2xstpA
swap

C.


Thermal Fitness (42°C)
2xhns
2xstpA
swap
Fitness relative to wild-type
1.2
1
0.8
0.6
2xhns
2xstpA
swap

these genes is occurring which may also be contributing to the observed fitness phenotypes. Conversely expression of *kan* is constitutive and may therefore be a contributing factor to the fitness of S. Typhimurium although similar experiments conducted in *E.coli* found expression of *kan* did not effect bacterial fitness (Blot *et al.*, 1991). Similarly, an SL1344 derivative (JH3570) (Rice and Hinton, unpublished strain) encoding the *cat* resistance gene located within a chromosomal intergenic region was observed to have a fitness (f.i. = 1.07) equivalent to wild-type SL1344 at 37 ^{o}C in the absence of selection. This is an indication that antibiotic resistance genes do not impact bacterial fitness in the absence of selection. Further competition experiments using strains SL1344$^{2X\textit{hns}}$, SL1344$^{2X\textit{stpA}}$ and SL1344^{swap} which lacked their respective resistance markers would be required to demonstrate that the *kan* and *tetRA* genes used here did not impact fitness.

4.2.8 Increased fitness of SL1344^{swap} undergoing osmotic stress

Of the three strains tested, the expression and abundance of *hns* and *stpA* in SL1344^{swap} conferred the greatest fitness benefit to *S.* Typhimurium particularly at 37^{o}C. As such, SL1344^{swap} was further characterised to see if its new genotype may also confer a fitness benefit to *S.* Typhimurium under conditions of osmotic stress. As before SL1344^{swap} was co-cultured and competed with SL1344 for 24 h, this time under different osmotic stress conditions which were achieved by varying the salt concentration of the growth medium. In section 4.2.7, SL1344^{swap} was competed against wild-type SL1344 in LB medium which contains 172 mM NaCl and an f.i. of 1.72 was calculated. Competition experiments were thus performed in media containing half the amount of NaCl present in LB-medium (86 mM NaCl) and in the absence of any NaCl (0 mM) at 37^{o}C to vary osmotic pressure. For each salt concentration tested a significant fitness benefit was observed for SL1344^{swap} relative to wild-type SL1344 (Fig. 4.15). In the absence of salt (0 mM NaCl) and in the presence of 86 mM NaCl, the novel H-NS and StpA protein populations present in SL1344^{swap} were equally beneficial for *S.* Typhimurium as fitness index values of 1.29 and 1.22 were observed,

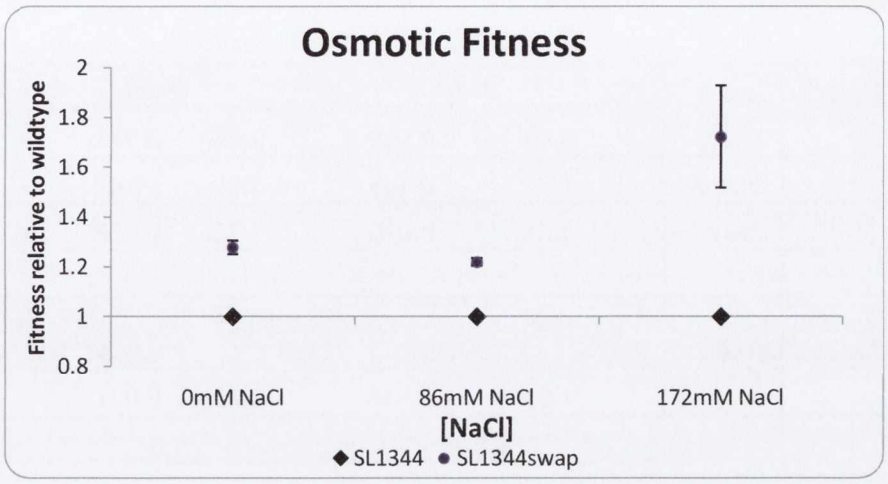


Fig. 4.15 Increased osmotic fitness conferred upon SL1344^{swap}.

The competitive fitness of SL1344^{swap} relative to wild-type SL1344 was assessed at a range of osmotic pressures by co-culturing methods. Osmotic pressure was varied by the addition of NaCl to the culture medium. SL1344^{swap} (purple circles) was competed against wild-type SL1344 (black diamond) at osmotic pressures imposed by the absence (0 mM) and presence (86 mM) of NaCl in the culture medium. The fitness of SL1344^{swap} observed at 37 ^{o}C (Fig. 4.14) was assessed in LB broth containing 172 mM NaCl, is also included.. A fitness index f.i. = 1 indicates an equivalent fitness to wild-type. Fitness indices > 1 or < 1 indicate a fitness advantage or disadvantage, respectively. Results are presented relative to the wild-type SL1344 reference f.i. = 1 and represent the average fitness values obtained from three experimental replicates (n = 3). Error bars denote the standard deviation in fitness values between experimental replicates.

Table 4.1 Fitness index (f.i.) values achieved for SL1344^{2Xhns}, SL1344^{2XstpA} and SL1344^{swap}.

Thermal Fitness	Strain	25°C	P-value	37°C	P-value	42°C	p-value
	2x*hns*	0.97	0.019	1.02	0.614	0.79	0.01
	2x*stpA*	0.77	0.145	1.18	0.43	0.83	0.118
	swap	0.88	0.015	1.72	0.026	1.03	0.578
Osmotic Fitness	**Strain**	**0 mM NaCl**	**P-value**	**86 mM NaCl**	**P-value**	**172 mM NaCl**	**P-value**
	swap	1.28	0.044	1.22	0.031	1.72	0.026

respectively. The regulatory changes made to *hns* and *stpA* in SL1344^{swap}, however, were most beneficial to *S.* Typhimurium at the higher osmotic pressures as SL1344^{swap} failed to attain a similar fitness index (f.I. = 1.72) when competed against wild-type at concentrations of NaCl below the 172 mM present in LB-medium. In conjunction with the results from the temperature competition experiments these results suggest that the novel H-NS and StpA protein composition present in SL1344^{swap} and resultant phenotypic gene expression alterations to the H-NS and StpA regulons has pre-adapted *S.* Typhimurium to cope better with a range of osmotic pressures and conferred a significant fitness advantage over wild-type SL1344 at 37^{o}C.

4.2.9 Characterisation of global transcriptional changes arising from altered *hns* and *stpA* expression in SL1344^{swap}

As H-NS and StpA, directly or indirectly, regulate 13% and 5% of the *S.* Typhimurium genome, respectively, the changes made to their patterns of expression and relative protein abundance in SL1344^{swap} could significantly alter the global transcriptional output (Lucchini *et al.*, 2009, Navarre *et al.*, 2006). Phenotypically these alterations have given rise to new patterns of expression to both *proU* and RpoS with likely downstream activation of RpoS-regulated genes. In addition, the motility of SL1344^{swap} and its ability to utilize alternative carbon sources were compromised relative to wild-type SL1344. Nonetheless, these phenotypic alterations along with unidentified transcriptional alterations arising from the regulatory changes made to *hns* and *stpA* in SL1344^{swap} have imposed a significant fitness advantage upon *S.* Typhimurium particularly at 37^{o}C and at a range of osmotic pressures. To characterise fully the new phenotypic transcriptional output resulting in the observed fitness increases of SL1344^{swap} relative to wild-type, transcriptional microarray experiments were performed as outlined in Section 2.13. For this total RNA was extracted from wild-type SL1344 and SL1344^{swap} during exponential growth (OD_{600} = 0.3) and stationary phase of growth (OD_{600} = 3.0) and used to generate pools of double-stranded cDNA. Each double-stranded cDNA pool sample was then mixed in 1:1 ratio with reference

genomic DNA of a known quantity (200 ng/µl) and mixed samples were hybridised to transcriptional microarray slides containing 44,000 probes representing the total number of genes found in the *S.* Typhimurium SL1344 genome. Gene transcript abundance in SL1344 and SL1344^{swap} for each sample was first quantified relative to the reference genomic DNA. As the same reference genomic DNA was used in every sample, direct comparisons of transcript levels between SL1344 and SL1344^{swap} for each growth phase could then be made and transcript abundance in SL1344^{swap} was expressed relative to wild-type SL1344.

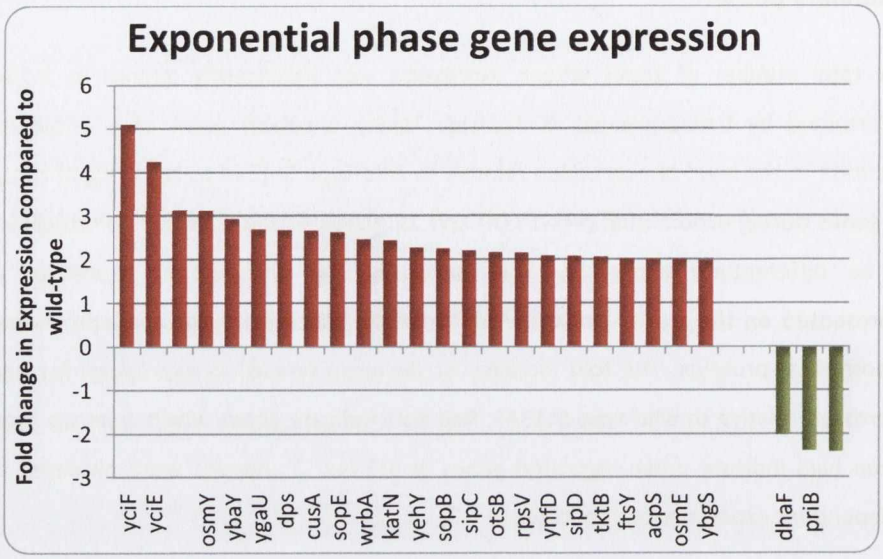
4.2.9.1 Minor transcriptional changes result in increased fitness of SL1344^{swap}

Despite having the potential to alter the expression of many genes in *S.* Typhimurium, the regulatory changes made to *hns* and *stpA* in SL1344^{swap} resulted in only a small set of genes whose transcription was significantly altered during either exponential or stationary phase growth. Taking a cut-off point = 2-fold increase or decrease in gene expression level relative to wild-type SL1344, the expression of only 26 genes (Fig. 4.16 A) during exponential phase and 29 genes (Fig. 4.16 B) during stationary phase was significantly altered in SL1344^{swap}. Functional grouping of differentially-expressed genes in SL1344^{swap} revealed the up-regulation of four virulence genes encoded in the SPI-1 pathogenicity island of *S.* Typhimurium during exponential growth (discussed in Section 4.2.9.2) (Srikanth *et al.*, 2011). The majority of the genes up-regulated during exponential growth, however, were found to be controlled by the stationary phase and stress sigma factor RpoS (Lucchini *et al.*, 2009, Ibanez-Ruiz *et al.*, 2000, Lacour & Landini, 2004). These genes will be discussed further in the following section (Section 4.2.9.3). From the remaining set of genes whose expression was altered in SL1344^{swap} during exponential growth, two (*ftsY* and *priB*) encode proteins involved in cell division and primosomal replication, respectively, *acpS* encodes a holo-[acyl-carrier-protein] synthase, two were identified as pseudogenes (*cusA* and *dhaF*) and the remainder encode uncharacterised hypothetical proteins.

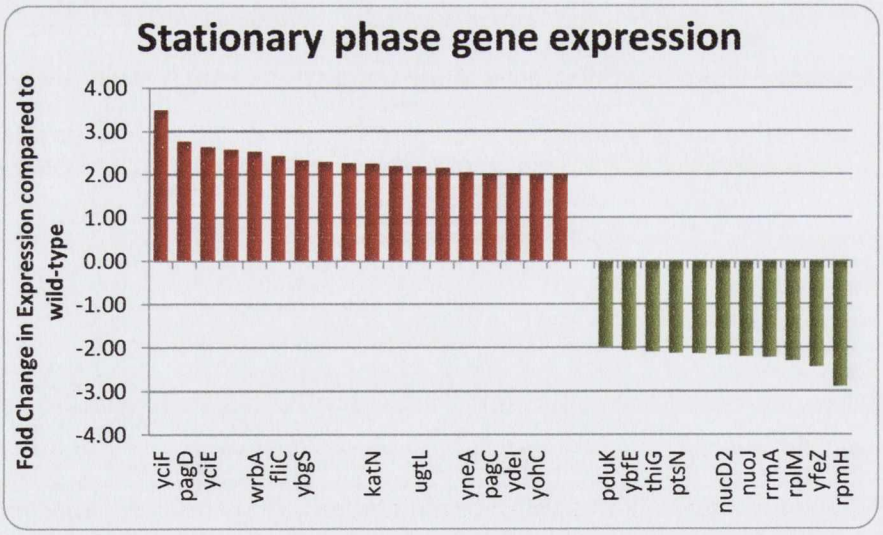
Fig. 4.16 Differentially expressed genes in SL1344^{swap} during exponential and stationary phase.

The total number of genes whose expression was significantly altered in SL1344^{swap} was determined by transcriptional microarray. Taking a cut-off point of a 2-fold increase or decrease in the level of expression relative to wild-type SL1344 as being significant, a total of 26 genes during exponential growth (A) and 29 genes during stationary phase(B) were found to be differentially expressed. The names of the differentially expressed genes are represented on the x-axis. Unnamed differentially expressed genes encode uncharacterised hypothetical proteins. The fold increase or decrease (y-axis) in expression for each gene is expressed relative to wild-type SL1344. Red bars indicate genes which were up-regulated and green bars indicate down-regulated genes in SL1344^{swap}. Results were obtained from three independent experimental replicates (n = 3).

A.


Exponential phase gene expression
Fold Change in Expression compared to wild-type
6
5
4
3
2
1
0
-1
-2
-3
yciF
yciE
osmY
ybaY
ygaU
dps
cusA
sopE
wrbA
katN
yehY
sopB
sipC
otsB
rpsV
ynfD
sipD
tktB
ftsY
acpS
osmE
ybgS
dhaF
priB

B.


Stationary phase gene expression
Fold Change in Expression compared to wild-type
4.00
3.00
2.00
1.00
0.00
-1.00
-2.00
-3.00
-4.00
yciF
pagD
yciE
wrbA
fliC
ybgS
katN
ugtL
yneA
pagC
ydeI
yohC
pduK
ybfE
thiG
ptsN
nucD2
nuoJ
rrmA
rplM
yfeZ
rpmH

During stationary phase growth a number of RpoS-regulated genes were found to be up-regulated and also a number of genes under the control of the PhoP/Q two-component regulatory system (Discussed below in Section 4.2.9.3) (Charles *et al.*, 2009, Zwir *et al.*, 2005). With the exception of *fliC*, which encodes the alternative flagellin sub-unit, FliC, the remaining up-regulated genes during stationary phase were members of the RpoS regulon (Table 4.4) (Discussed below in Section 4.2.9.3). The remaining genes were down-regulated during stationary phase growth in SL1344^{swap} members of the RpoS regulon (Table 4.4) (Discussed below in Section 4.2.9.3). The remaining genes were down-regulated during stationary phase growth in SL1344^{swap} and were involved in cellular metabolism and protein biosynthesis (Table 4.2) (Soutourina *et al.*, 1999, Cheng *et al.*, 2011, Leonardi & Roach, 2004, Choi *et al.*, 2010, Archer & Elliott, 1995).

4.2.9.2 Expression of SPI-1 virulence genes during exponential growth.

During exponential growth, four of the up-regulated genes (*sipC*, *sipD*, *sopE* and *sopB*) (Fig 4.16 A) arising from the alternate expression patterns of *hns* and *stpA* in SL1344^{swap} were identified as *S.* Typhimurium virulence genes encoded within the horizontally acquired *Salmonella* Pathogenicity Island 1 (SPI-1) (Srikanth *et al.*, 2011, Ellermeier & Slauch, 2007). The SPI-1 region encodes a type three secretion system (TTSS) that enables *S.* Typhimurium to invade the epithelial cell lining of the mammalian small intestine (Ellermeier & Slauch, 2007). The TTSS is comprised of a motor, a needle-like structure and a translocon which together mediate contact between the bacterium and epithelial cells and facilitate the transfer of effector proteins into the host cell to mediate the uptake of the bacterium (Srikanth *et al.*, 2011). The *sipD* gene product, SipD, is a component of the TTSS translocon apparatus (Srikanth *et al.*, 2011). Both *sopE* and *sopB* encode two translocated effector proteins, SopE and SopB respectively, which upon translocation into the host cell activate host signal transduction pathways via interaction with Rho GTPases to prime the host cell for entry of the bacterium (Srikanth *et al.*, 2011). Although also a part of the TTSS

4.2 Down-regulated genes involved in metabolism in SL1344^{swap} during stationary phase.

Gene	fold change in expression	P-value	Function
pduK	-1.99	0.001	propanediol utilization protein
thiG	-2.10	0.014	thiamine biosynthesis protein
ptsN	-2.13	0.051	nitrogen regulatory IIA protein
nuoJ	-2.21	0.014	NADH dehydrogenase I chain J
rrmA	-2.23	0.001	rRNA guanine-N1-methyltransferase
rplM	-2.31	0.120	50S ribosomal subunit protein L13
rpmH	-2.90	0.054	50s ribosomal protein l34

translocon, SipC (encoded by *sipC*) acts similarly to an effector protein and along with SopB further aids the entry of *S.* Typhimurium by stimulating host cell actin cytoskeletal rearrangements to induce membrane ruffling and engulfment of the bacterium (Srikanth *et al.*, 2011). The regulation of SPI-1 genes is complex and expression is co-ordinated by several environmental cues (low O_2, high osmolarity and acidic pH) and multiple transcriptional regulator proteins (HilC, HilD, RtsA, Hfq). Each environmental cue and transcriptional regulator ultimately acts via control of the SPI-1 master regulator HilA which directly binds to and activates virulence gene operons located in the SPI-1 region (Srikanth *et al.*, 2011, Troxell *et al.*, 2011, Ellermeier & Slauch, 2007).

In aerobic culture conditions expression of SPI-1 genes is normally observed during late exponential or stationary phase of growth. Thus, the regulatory changes made to *hns* and *stpA* have altered the timing of SPI-1 virulence gene expression (Ibarra *et al.*, 2010). Consistent with its role in binding to and silencing horizontally acquired genes H-NS binds to several regions within SPI-1 and is required for the thermal up-regulation of *hilA*, *hilC* and *hilD* (Ono *et al.*, 2005, Dillon *et al.*, 2010). In a separate study, Troxell *et al.* demonstrated that upon up-regulation of *hns* expression, in a strain of *S.* Typhimurium lacking the Fur transcriptional regulator, expression of *hilA* was significantly decreased (Troxell *et al.*, 2011). H-NS however was still present and its relative abundance was increased in $SL1344^{swap}$ therefore the up-regulation and inappropriate timing of expression of *sipC*, *sipD*, *sopE* and *sopB* was surprising. As part of the PhoP regulon, the *hilA* gene is known to be transcriptionally repressed by the PhoP protein (Ellermeier & Slauch, 2007, Charles *et al.*, 2009). Interestingly, in a recent study several genes whose expression is dependent on the PhoP/Q two-component regulatory system were identified as being up-regulated in the absence of *stpA* in exponential phase cultures and CHIP-chip analysis showed that StpA bound within the regulatory regions of these PhoP regulated genes (Lucchini *et al.*, 2009). Furthermore, expression of the *phoP* gene itself and many genes within the PhoP regulon are also bound and repressed by H-NS in high Mg^{2+} environments (Kong *et al.*, 2008). Perhaps

the lack of detectable StpA protein in conjunction with increased cellular H-NS levels in SL1344^{swap} mediates the observed up-regulation of *sipC*, *sipD*, *sopE* and *sopB* via regulation of *phoP* expression and activity.

4.2.9.3 Up-regulation of the RpoS and PhoP/Q dependent genes.

In a recent transcriptomic study, aimed at defining the StpA regulon in *S.* Typhimurium, Lucchini *et al.* observed that over one third of the genes de-repressed in mid-exponential phase cultures of SL1344 in the absence of StpA were also activated by the stationary phase and general stress sigma factor RpoS (Lucchini *et al.*, 2009). Subsequent experiments showed that expression of this set of RpoS regulated genes during exponential growth resulted from stabilisation of the RpoS protein during exponential phase in the absence of StpA which facilitated expression of the RpoS regulon (Lucchini *et al.*, 2009). Consistent with these observations, the StpA protein could not be detected in SL1344^{swap} and the RpoS sigma factor was detected at significant levels during exponential growth. Not surprisingly these alterations also resulted in de-repression of several RpoS-dependent genes during exponential phase in SL1344^{swap} (Table 4.3). In agreement with Lucchini *et al.*, three of the most highly up-regulated genes identified in this study during exponential phase were *yciE*, *yciF* and *katN* which are part of the *yciGFEkatN* operon in *S.* Typhimurium (Beraud *et al.*, 2010, Lucchini *et al.*, 2009). No physiological role has been assigned to this operon, however, *katN* is known to encode a manganese catalase, the overproduction of which increases the resistance of *S.* Typhimurium to oxidative stress (Beraud *et al.*, 2010, Robbe-Saule *et al.*, 2001). The *yciGFE* operon is conserved in *S.* Typhimurium and *E. coli* and is repressed by H-NS in both species. Intriguingly, *katN* is found in *Salmonella* but not *E. coli* and due to divergence of the *yciGFEkatN* regulatory region this operon is only regulated by RpoS in *S.* Typhimurium (Beraud *et al.*, 2010). The majority of the RpoS dependent up-regulated genes identified as part of this study (*osmY*, *osmE*, *yehY*, *ygaU*, *ybaY*, *otsB*, *wrbA*) encode proteins were either demonstrated or thought to be involved in osmotic or oxidative stress resistance in *S.*

Table 4.3 Differentially expressed RpoS regulated genes in SL1344^{swap} during exponential growth.

Gene	fold change in expression	P-value	Function
yciF	5.10	2.85^{E-07}	Conserved hypothetical protein
yciE	4.24	1.26^{E-07}	Conserved hypothetical protein
osmY	3.13	2.09^{E-05}	Hyperosmotically inducible periplasmic protein
ygaU	2.69	2.29^{E-03}	Conserved hypothetical protein
Dps	2.68	2.87^{E-03}	DNA protection during starvation protein
wrbA	2.46	1.23^{E-02}	trp repressor binding protein
katN	2.44	1.58^{E-03}	Catalase
yehY	2.28	4.29^{E-03}	Hypothetical permease transmembrane component
otsB	2.17	8.08^{E-04}	Trehalose phosphatise
rpsV	2.16	1.73^{E-03}	30S ribosomal protein S22
tktB	2.06	2.62^{E-03}	Transketolase 2
osmE	2.01	2.48^{E-03}	Osmotically inducible lipoprotein E precursor
ybaY	2.93	7.42^{E-04}	Conserved hypothetical lipoprotein

Typhimurium and *E. coli* (Klauck *et al.*, 2007, Lacour & Landini, 2004, Ibanez-Ruiz *et al.*, 2000, Lucchini *et al.*, 2009). The remaining genes identified encoded a transketolase (*tktB*) of redundant function in *E. coli*, a 30 S ribosomal protein (*rpsV*) and importantly the DNA binding protein Dps (*dps*) (Harinarayanan *et al.*, 2008, Grainger & Busby, 2008, Grainger *et al.*, 2008). Dps is normally undetected in exponentially growing cells but can be detected in significant amounts in starved cells where it binds DNA and drives biocrystallisation of the nucleoid to protect DNA from stress-induced damage (Grainger *et al.*, 2008). The observed de-repression of multiple RpoS-dependent genes involved in stress adaptation in SL1344^{swap} during exponential growth could serve to prime and pre-adapt *S.* Typhimurium to cope with environmental stresses and is likely the major contributing factor behind accounting for the observed increase in relative fitness of SL1344^{swap}. Interestingly, during stationary phase of SL1344^{swap} several of the RpoS dependent genes that were up-regulated in exponential phase remained up-regulated (Table 4.4) and yet another RpoS dependent gene (*ydeI*) which is involved in the resistance to anti-microbial peptides such as polymyxin B was identified as up-regulated (Erickson & Detweiler, 2006).

An increase in resistance of *S.* Typhimurium was also observed by Lucchini *et al.* in the absence of StpA which was not RpoS dependent (Lucchini *et al.*, 2009). Lucchini *et al.* observed an up-regulation of several genes including *ugtL*, *pagC* and *pagP* involved in the modification of membrane bound lipopolysaccharide (LPS) which increased resistance of the bacterium to polymyxin B in the absence of StpA (Lucchini *et al.*, 2009). Further characterisation revealed that many of these genes were also regulated by PhoP (Lucchini *et al.*, 2009). Consistent with SL1344^{swap} having undetectable levels of StpA, several PhoP regulated genes were also identified as up-regulated as part of this study. In agreement with Lucchini *et al.*, *ugtL* and *pagC* were also up-regulated in SL1344^{swap} alongside a third PhoP regulated gene *pagD* (Lucchini *et al.*, 2009). Both UgtL and PagC are outer membrane proteins and have been shown to alter outer membrane permeability and increase resistance to cationic peptides and deoxycholate (Navarre *et al.*, 2005, Shi *et al.*, 2004). PagD is a putative virulence protein that is

secreted into the host macrophage cytosol via the SPI-2 TTSS, although its exact role in virulence is yet to be elucidated (Yoon *et al.*, 2011).

Table 4.4 Differentially expressed RpoS regulated genes in SL1344^{swap} during stationary phase.

Gene	fold change in expression	P-value	Function
yciF	3.47	2.47^{E-05}	Conserved hypothetical protein- *yciGFE-(katN)* operon
yciE	2.62	3.52^{E-05}	Conserved hypothetical protein- *yciGFE-(katN)* operon
wrbA	2.52	1.07^{E-02}	trp repressor binding protein
ybgS	2.31	1.51^{E-03}	Hypothetical exported protein
katN	2.23	3.07^{E-03}	Manganese catalase
ydeI	2.00	4.26^{E-03}	Hypothetical periplasmic protein

4.3 Discussion

The individual expression patterns of *hns* and *stpA* have been well characterised in a number of Gram-negative bacteria (Deighan *et al.*, 2003, Hinton *et al.*, 1992, Free & Dorman, 1995, Free & Dorman, 1997, Lucchini *et al.*, 2009). In general the pattern of *hns* expression is conserved with maximal transcription occurring during exponential growth which results in constant cellular H-NS protein levels (Free & Dorman, 1995). Until recently, expression of *stpA* mRNA and StpA protein was observed to occur as a transient burst at the early stages of exponential growth after which both mRNA and protein declined to undetectable levels (Free & Dorman, 1997, Deighan *et al.*, 2003). This pattern of expression holds true for both *E. coli* and the closely related bacterium *Shigella flexneri* (Beloin *et al.*, 2003, Free & Dorman, 1997). In *S.* Typhimurium, however, expression of *stpA* is sustained at a significant level after the early exponential peak and results in detectable StpA protein at all stages of growth (Lucchini *et al.*, 2009). Furthermore, StpA appears to have a more physiologically defined role in *S.* Typhimurium, particularly in the modulation of resistance to stress as a result of its altered pattern of expression and increased protein abundance (Lucchini *et al.*, 2009). Nonetheless, regardless of species, both *hns* and *stpA* exhibit unique patterns of expression which result in dynamic populations of these two proteins as a function of growth. While many of the regulatory mechanisms controlling the expression of *hns* and *stpA* have been elucidated, the biological significance of their individual expression patterns and the resultant dynamic protein populations of H-NS and StpA remain unknown. As such, this study was aimed at understanding how essential these expression patterns are to the normal biological functioning of *Salmonella* Typhimurium.

To investigate the importance of the individual expression patterns of *hns* and *stpA* in *S.* Typhimurium, this study set out to alter both the timing and abundance of their expression and protein levels, respectively. For this the open reading frames (ORFs) of *hns* and *stpA* were successfully interchanged on the chromosome of *S.* Typhimurium SL1344 to produce strain SL1344^{swap}. In this new strain, the *hns* ORF was fused with the *stpA* regulatory region such that *hns* would now inherit all the regulatory inputs

normally imposed upon *stpA* and *vice versa*. The purpose of these promoter-ORF fusions was to impose an *hns*-like pattern of expression upon *stpA* and a *stpA*-like pattern of expression upon *hns*, thus altering both the timing and abundance of expression of each gene. A direct interchange of expression patterns, however, was not observed. Wild-type expression of the native *stpA* ORF resulted in peak expression after 2 – 3 h of growth in early exponential phase, after which expression decreased to ≈ 50% maximal expression until stationary phase. In SL1344^{swap}, however, upon reaching peak expression after 3 h of growth, the *hns* transcript abundance when expressed via the *stpA* promoter did not decline but remained static until stationary phase before declining after 7 h of growth (Fig 4.5 B). Consistent with high levels of transcription arising from the *stpA* promoter relative to the *hns* promoter (see Chapter 3), the levels of *hns* mRNA detected throughout growth in SL1344^{swap} were significantly increased relative to those observed in wild-type SL1344. Similarly, expression of the *stpA* ORF via the *hns* promoter in SL1344^{swap} resulted in a decrease in the cellular levels of *stpA* mRNA. A new pattern of expression was also imposed upon *stpA* that differed from that of *hns* when under the control of the same promoter. In SL1344^{swap} expression of *stpA* via the *hns* promoter reached peak levels after 2 h of growth during early exponential phase akin to the wild-type pattern of *hns* expression. Thereafter, however, *stpA* underwent a gradual decline in the level of expression. This was in contrast to expression of *hns* via the same promoter in SL1344 which, after peak expression in early exponential phase, the level of *hns* transcription declined rapidly to ≈ 33% that of the maximal level.

Despite not achieving a direct swap in the expression patterns of *hns* and *stpA* in SL1344^{swap}, the novel patterns of expression still had the capacity to alter significantly the relative abundance of H-NS and StpA as a function of growth. Surprisingly, the new pattern of expression conferred upon the *hns* ORF still resulted in a wild-type protein expression profile, as once again H-NS levels were constant at all stages of growth. Thus, SL1344^{swap} was still capable of maintaining a constant H-NS:DNA ratio despite *hns* being controlled by a new set of regulatory inputs (Free & Dorman, 1995). The

ability to maintain a constant H-NS:DNA ratio in *E. coli* has been attributed to the negative auto-regulatory capacity of H-NS whereby the peak in *hns* expression during exponential growth both provides sufficient H-NS to match *de novo* DNA synthesis and simultaneously serves to reduce *hns* expression as cells progress towards stationary phase and DNA replication is ceased (Free & Dorman, 1995). Although removed from the native *hns* promoter region in strain SL1344^{swap}, auto-regulation is still likely to occur as H-NS is known to bind within the *stpA* promoter region and elicit a negative effect on expression (Wolf *et al.*, 2006, Free & Dorman, 1997, Sonden & Uhlin, 1996). Interestingly, quantification of H-NS protein levels in SL1344^{swap} revealed that in concurrence with the observed increase in *hns* mRNA levels, H-NS protein levels were 1.8-fold more abundant throughout growth when compared with wild-type levels. Other than by artificial over-expression of *hns*, there are only two natural scenarios known to increase the abundance of H-NS. Under cold-shock conditions, as part of this study, H-NS protein levels were observed to increase 2-fold. A similar increase in *E. coli* has been suggested to aid in shutting down global transcription to facilitate the process of cold adaptation (Free & Dorman, 1997, La Teana *et al.*, 1991). Conversely, H-NS levels were shown to decrease in anaerobic conditions and in the presence of high Fe^{2+} levels leading to de-repression of SPI-1 genes (Troxell *et al.*, 2011). When artificially over-expressed in *E. coli*, increased H-NS levels were observed to be toxic to the bacterium and altered the cell morphology and certain cell-cycle parameters such as the number of origins per cell (Atlung & Hansen, 2002, Spurio *et al.*, 1992). In SL1344^{swap} the level of H-NS protein achieved by the altered expression of *hns*, however, was well tolerated by *S.* Typhimurium and SL1344^{swap} had a comparable growth rate with wild-type SL1344.

Conversely, despite inheriting a new pattern of expression when expressed via the *hns* promoter in SL1344^{swap}, no StpA protein could be detected at any stage of growth despite the hybrid *hns-stpA* mRNA molecule being more stable than wild-type *stpA* mRNA. This was likely due to a combination of the significantly decreased levels of *stpA* transcript produced in SL1344^{swap} relative to wild-type coupled with proteolytic

degradation of StpA. In contrast to the observed increase in stability of *stpA* when fused with the *hns* 5' untranslated region, analysis of the stability of the *stpA-hns* mRNA molecule revealed a significant reduction in stability relative to the native *hns* mRNA molecule. Thus, the 5' untranslated regions of *hns* and *stpA* appear to play key roles in the regulation of mRNA abundance as inheriting the 5' untranslated region of *stpA* destabilised the *hns* mRNA. A number of possible contributing features in the *stpA* 5' untranslated region were identified in chapter 3 (See section 3.2.3) which could be involved in the destabilisation of the *stpA-hns* hybrid mRNA including extensive base pairing within the secondary structure to occlude the translation start codon and an upstream A/U rich region as a possible target for RNase E or Hfq. Inheriting the *hns* 5' untranslated region, however, served to increase the stability of *stpA* mRNA although not to an equivalent stability as that observed for wild-type *hns* mRNA.

Interchanging the open-reading frames of *hns* and *stpA* to create SL1344^{swap} has thus conferred novel patterns of expression upon both genes which fundamentally altered the H-NS-like protein populations during growth by increasing cellular levels of H-NS and effectively eliminating StpA. Furthermore, two additional *S.* Typhimurium strains, SL1344^{2xhns} and SL1344^{2XstpA}, were generated as part of the process of creating SL1344^{swap}. Neither strain was capable of producing both H-NS and StpA. SL1344^{2xhns} possessed two copies of the *hns* ORF, one controlled by the *hns* promoter and the other via the *stpA* promoter. Similarly SL1344^{2XstpA} possessed two copies of the *stpA* ORF, one controlled by the *stpA* promoter and the other by the *hns* promoter. These strains remain uncharacterised transcriptionally. Protein expression data obtained, however, showed that when fused to the *stpA* promoter in SL1344^{2xhns} expression of *hns* resulted in constant levels of H-NS protein throughout the growth cycle. A similar observation was made in SL1344^{swap} when H-NS levels were examined from the same transcriptional fusion. Interestingly, when expressed via the *hns* promoter in SL1344^{2XstpA}, expression of *stpA* resulted in detectable levels of StpA protein throughout growth and, furthermore, a wild-type pattern of protein expression was

observed. The absence of H-NS in this strain suggested that the presence of H-NS in SL1344^{swap} had a significant repressive effect on its own promoter, as no StpA protein was detected. Coupled with expression of the wild-type *hns* and *stpA* alleles also present in SL1344$^{2X hns}$ and SL1344$^{2X stpA}$ novel H-NS and StpA populations, respectively, could potentially arise and so these strains were further characterised phenotypically.

Having altered the unique expression patterns of *hns* and *stpA* to generate three novel strains with aberrant H-NS and StpA protein populations, the next question to be answered was what effect, if any, these changes had on the biological functioning of *S.* Typhimurium? If indeed the unique expression patterns of *hns* and *stpA* are biologically significant and optimized for *S.* Typhimurium, then the alterations made in SL1344^{swap}, SL1344$^{2X hns}$ and SL1344$^{2X stpA}$ could theoretically have downstream effects on the expression of genes regulated by H-NS and StpA and be manifested as phenotypic alterations. Investigation of several phenotypes associated with the loss of *hns* and/or *stpA* revealed that for each phenotype tested a variety of outcomes was achievable which was dependent on the H-NS and H-NS-like protein composition in each strain. Various *hns* mutations and indeed a complete loss of *hns* have been demonstrated to give rise to variable cell doubling times/growth rates in both *S.* Typhimurium and *E. coli* (Hinton *et al.*, 1992, Atlung & Hansen, 2002). The growth curves of both SL1344$^{2X hns}$ and SL1344^{swap} overlapped with that of wild-type SL1344 (Fig 4.9 A). The growth of SL1344$^{2X stpA}$, however, was severely compromised in relation to wild-type which had an extended log phase of growth and at no time point reached a population density comparable with that of SL1344. From subsequent calculation of cell doubling times for each strain, a comparable doubling time for SL1344 and SL1344$^{2X hns}$ (23 min) was observed. Despite displaying an overlapping growth curve with wild-type SL1344 the new H-NS and StpA protein composition present in SL1344^{swap} served to decrease the doubling time by almost 3 min as a doubling time of 20.6 min was calculated. Conversely, SL1344$^{2X stpA}$ which displayed a slow-growth phenotype had a doubling time of 32 min. This reflects the absence of functional H-NS from the cell, shown previously to result in a slow growth phenotype, and

demonstrates that having two copies of the *stpA* ORF could not functionally compensate for the loss of *hns* (Hinton *et al.*, 1992).

Strain SL1344^{2XstpA} also behaved in a similar manner to an *hns* mutant with respect to several other phenotypes. The genotype of this strain resulted in a de-repression of *proU*, rendered the bacterium non-motile and altered the ability of *S.* Typhimurium to utilize maltose and β-glucoside as carbon sources (Soutourina & Bertin, 2003, Ueguchi *et al.*, 1996, Johansson *et al.*, 1998). Although lacking *stpA* and having increased cellular levels of H-NS, SL1344^{2Xhns} consistently resembled wild-type SL1344 with respect to each of the above phenotypes. Interestingly, while SL1344^{swap} had no detectable StpA and also displayed increased cellular levels of H-NS, an intermediate phenotype between that of SL1344^{2XstpA} and wild-type was consistently observed with the exception of *proU* expression (Fig 4.10 A). SL1344^{swap} had 50% motility compared to wild-type SL1344, low levels of maltose uptake and utilisation and a 1.5-fold increase in β-glucoside utilisation. Expression of the *proU* locus in SL1344^{swap} followed the wild-type pattern of de-repression upon increasing osmotic pressures up to an osmotic pressure equivalent to that of 200 mM NaCl. At osmotic pressures greater than 200 mM NaCl, however, *proU* expression was decreased compared to the wild-type (Fig. 4.10 A). This was attributed to the lack of StpA in SL1344^{swap} as a similar pattern of expression was observed in a *stpA* deletion mutant strain suggesting a possible role for StpA in the regulation of *proU* expression in conditions of high osmotic pressure. Both the timing of expression and the abundance of the RpoS sigma factor was observed to be greatly affected by the transcriptional alterations in each strain. As was expected, RpoS was not detected in wild type SL1344 until after 5 h of growth, at the onset of stationary phase (Fig. 4.12 A). Consistent with H-NS and StpA playing a role in the regulation of RpoS abundance during exponential growth, in SL1344^{swap}, SL1344^{2Xhns} and SL1344^{2XstpA} RpoS was detected during exponential growth, yet depending on the H-NS-like protein population, the timing of its detection was variable. In SL1344^{2Xhns} and SL1344^{2XstpA} RpoS was detected after 3 h and 4 h of

growth, respectively, during early- to mid-exponential growth, whereas in SL1344^{swap} RpoS was detected even earlier at 2 h (Fig 4.12 D).

Taken together, the array of phenotypic outcomes observed for SL1344^{swap}, SL1344$^{2X\textit{hns}}$ and SL1344$^{2X\textit{stpA}}$, highlight the importance of the individual patterns of expression of *hns* and *stpA* for the normal cellular functioning of *S.* Typhimurium. Alterations to their expression resulted in inappropriate expression of both *proU* and, more significantly, the stationary phase sigma factor RpoS. In turn RpoS is capable of up-regulating multiple genes required for general stress and starvation adaptation. In general, RpoS levels are tightly regulated to ensure appropriate expression during stationary phase or under certain stress conditions (Lacour & Landini, 2004). Inappropriate expression of RpoS during exponential growth is thought to be detrimental to the bacterium as a result of wasteful expression of RpoS-regulated genes (Battesti *et al.*, 2011). In addition, as RpoS is a member of the same sigma factor family as the vegetative sigma factor RpoD, RpoS competes with RpoD for binding of core polymerase thus channelling some of the pool of core polymerase away from transcribing genes required during exponential growth (Battesti *et al.*, 2011). Aside from altering the expression of *proU* and RpoS, the regulatory changes made to *hns* and *stpA* in SL1344^{swap}, SL1344$^{2X\textit{hns}}$ and SL1344$^{2X\textit{stpA}}$ significantly altered the motility of *S.* Typhimurium and its ability to utilise alternative carbon sources such as maltose and β-glucosides (Fig 4.13).

Intriguingly, for each phenotype tested the outcome was completely dependent on the H-NS and H-NS-like protein complement in each strain. SL1344$^{2X\textit{stpA}}$ consistently displayed phenotypes associated with the loss of *hns* and the presence of a second *stpA* ORF could not compensate for this loss. On the other hand, a complete loss of *stpA* was well tolerated and compensated for by H-NS in SL1344$^{2X\textit{hns}}$ where each phenotypic outcome was comparable with wild-type with the exception of RpoS expression (Fig. 4.12). This could be attributed to the presence of the native *hns* allele because, when replaced by the *stpA* ORF in SL1344^{swap}, a different set of phenotypic outcomes was observed.

As H-NS and StpA have, respectively, the capacity to regulate 13% and 5% of the *S.* Typhimurium genome, this raised the possibility that, in addition to the phenotypic alterations already discussed, SL1344^{swap}, SL1344$^{2X hns}$ and SL1344$^{2X stpA}$ each could give rise to a more global set of strain specific transcriptional and phenotypic outcomes, some of which may be beneficial to the bacterium in terms of fitness (Navarre *et al.*, 2006, Lucchini *et al.*, 2009). Previous adaptive laboratory evolution experiments have identified mutations that alter the expression of genes such as *rpoD*, *fis*, *hfq*, *crp* and *topA* which have the capacity to influence global patterns of transcription and/or DNA superhelicity. Such mutations confer fitness advantages in the evolved populations relative to the ancestral ones (Crozat *et al.*, 2005, Philippe *et al.*, 2007, Conrad *et al.*, 2009). As H-NS and StpA have been extensively shown to influence both global gene expression and super coiling, a series of competition experiments was performed at various temperatures to examine whether the expressional alterations made to *hns* and *stpA* in SL1344^{swap}, SL1344$^{2X hns}$ and SL1344$^{2X stpA}$ could produce a fitness benefit in *S.* Typhimurium.

In general, a fitness deficit was imposed upon *S.* Typhimurium as a result of the changes made to *hns* and *stpA,* particularly at 25 ^{o}C where each strain was less fit than wild-type SL1344 (Fig. 4.14). Strains SL1344$^{2X hns}$ and SL1344$^{2X stpA}$ again were at a fitness disadvantage relative to wild-type when competed against the wild-type at 42 ^{o}C. However, the genotype/phenotype combination of SL1344^{swap} was on a par with that of the wild-type in terms of fitness at both 25 ^{o}C and 42 ^{o}C. Most interestingly, at 37^{o}C, SL1344^{swap} gained a significant competitive fitness advantage relative to wild-type SL1344 with an observed fitness index (f.i.) of 1.7. Taking experimental error into account the fitness of both SL1344$^{2X hns}$ and SL1344$^{2X stpA}$ was equal to that of wild-type at 37^{o}C. This was an unexpected outcome for SL1344$^{2X stpA}$ because it had a reduced growth rate compared to wild-type (Fig. 4.9). As the genotypes of SL1344$^{2X hns}$ and SL1344$^{2X stpA}$ did not confer any significant fitness benefit upon *S.* Typhimurium, these strains were not used in subsequent competition experiments. As the genotype of SL1344^{swap} conferred a substantial fitness benefit upon *S.* Typhimurium at 37^{o}C its

competitive fitness was further tested at a range of osmotic pressures. Under the osmotic pressures imposed by the absence of NaCl (0 mM) and presence of NaCl (86 mM or 172 mM) in the culture media, the expression changes to *hns* and *stpA* in SL1344^{swap} again conferred a fitness advantage this time in each environmental condition tested. A fitness index (f.i.) of 1.29 and 1.23 was observed for SL1344^{swap} in the absence of NaCl and presence of 86 mM NaCl, respectively. In the presence of 172 mM NaCl fitness was further increased with SL1344^{swap} having a fitness index (f.i.) of 1.72. Thus, it appears that as a result of the altered expression of *hns* and *stpA* and the resulting downstream phenotypic changes, SL1344^{swap} has become primed and somewhat pre-adapted to cope with environmental stress. The presence of the resistance genes located downstream of each promoter-ORF fusion may also be a contributing factor to the observed fitness phenotypes of each strain with respect to temperature and osmotic pressure. To conclusively demonstrate that each resistance marker does not impact fitness, further competition experiments are planned to compete SL1344^{swap}, SL1344$^{2X hns}$ and SL1344$^{2X stpA}$ lacking their respective resistance cassettes against wild-type SL1344 under the same environmental conditions as before. Furthermore, RT-PCR is planned to monitor any transcription of the *kan* or *tetRA* genes and determine if indeed any 'leaky' transcription is occurring.

To determine the extent of the global changes in expression arising in SL1344^{swap} that account for the observed increase in competitive fitness, transcriptional microarrays were performed and the total transcriptional activity for SL1344^{swap} during exponential and stationary phase growth was compared with wild-type SL1344. Surprisingly, despite the vast number of genes controlled by H-NS and StpA, relatively few genes exhibited significant changes in expression during either growth phase. A total of 26 genes during exponential phase and 29 during stationary phase showed statistically significant alterations in their level of expression (Fig. 4.16). Consistent with undetectable levels of StpA in SL1344^{swap}, many of the genes up-regulated during each growth phase were members of the RpoS regulon, several of which were involved in resistance to osmotic stress. Lucchini *et al.* previously showed that in the absence of

stpA, RpoS stability increases and the protein can be detected during exponential growth resulting in the up-regulation of multiple genes controlled by RpoS (Lucchini *et al.*, 2009). In good agreement with these data, RpoS was detected in SL1344^{swap} during early exponential growth and many of the genes identified as up-regulated during exponential growth in SL1344^{swap} were also identified by Lucchini *et al.* in the absence of StpA (Lucchini *et al.*, 2009). In addition several PhoP-regulated genes involved in altering outer membrane bound LPS and increased stress resistance were also identified as being up-regulated during stationary phase in SL1344^{swap}.

The up-regulation of several RpoS-regulated genes involved in resistance to osmotic stress during exponential growth in conjunction with alterations to components of the outer membrane could be a major contributing factor to the enhanced fitness of SL1344^{swap} to environmental stresses such as temperature and osmotic pressure. Increased resistance to stress, however, has been proposed to come at a cost to the bacterium (Battesti *et al.*, 2011, Ferenci, 2005). Aside from competition between RpoS and RpoD for core RNA polymerase and utilising cellular resources for the production of energetically expensive stress protection, the cost of remaining highly resistant to stress comes as a reduction in the ability of the bacterium to compete for and utilise carbon sources in nutrient poor environments (Ferenci, 2005, Battesti *et al.*, 2011). This principle, termed SPANC (self-preservation and nutritional competency), was observed for various *rpoS* mutants in *E. coli*, whereby a direct anti-correlation occurred between the cellular levels of RpoS and the range of substrates the bacterium could metabolize (Ferenci, 2005). Indeed, SL1344^{swap} was somewhat impaired in its ability to utilize maltose as a carbon source but was better equipped to utilize β-glucosides (Fig. 4.13). It would be of interest to undertake a series of competition experiments in which the available carbon source was altered in order to see if the apparent increased stress resistance imposed upon SL1344^{swap} had detrimental effects on its nutritional competency.

This study has demonstrated the importance of maintaining the unique expression patterns of *hns* and *stpA* in *S.* Typhimurium. While alterations to their expression were

well tolerated and gave rise to a variety of phenotypic outcomes, in general, such alterations were found to be detrimental to *S.* Typhimurium in terms of competitive fitness. The expression patterns of these two genes have likely been optimized by the process of evolution such that the right amounts of H-NS and StpA are present at the right time during growth in order to maintain homeostasis (Babu & Aravind, 2006). Nonetheless, environmental adaptation requires alterations in gene expression patterns and optimization of their level of expression (Babu & Aravind, 2006, Orr, 2005b). This study has highlighted an apparent genetic flexibility inherent in the expression of *hns* and *stpA* such that every alteration made to their patterns of expression and relative protein abundance resulted in strain-specific phenotypic alterations. In the case of SL1344^{swap} these changes pre-adapted *S.* Typhimurium through relatively few global transcriptional modifications to cope better with stressful environments. While the alterations made in SL1344$^{2X hns}$ and SL1344$^{2X stpA}$ did not produce a fitness advantage with respect to temperature, perhaps these alterations might confer a fitness advantage in other environments. In light of the SPANC principle, these strains may be more nutritionally competent than wild-type SL1344. An intricate relationship between the expression and function of H-NS and H-NS-like proteins is apparent as, despite significant structural and functional overlap, each has distinct yet overlapping roles in *S.* Typhimurium which is in part facilitated by their individual patterns of expression.

In recent years, studying the significance of H-NS and H-NS-like protein expression patterns in has become more complex due to the ability of *S.* Typhimurium and other closely related species to acquire a third plasmid borne H-NS protein, Sfh (Beloin *et al.*, 2003, Doyle *et al.*, 2007). The gene encoding Sfh is located on the large self-transmissible IncHI plasmid pSf-R27 (Doyle *et al.*, 2007). Upon acquisition of this plasmid, Sfh was demonstrated to have a stealth-like role in silencing plasmid-encoded genes whose expression was detrimental to the bacterium (Doyle *et al.*, 2007). In addition, Sfh is known to interact with both H-NS and StpA to form heterodimers, it binds at many of the same locations as H-NS throughout the

chromosome and can, in certain cases, functionally compensate for the loss of *hns* (Deighan *et al.*, 2003). Like *hns* and *stpA*, *sfh* also has a unique pattern of expression. In keeping with *hns* and *stpA*, peak expression of the *sfh* gene occurs during early exponential growth but Sfh protein remains undetected until stationary phase (Doyle & Dorman, 2006). Integration of Sfh into the *S.* Typhimurium gene network has the capacity to alter significantly the H-NS and H-NS-like protein populations in the cell particularly during stationary phase. Interactions with the native H-NS and StpA protein populations would likely influence global transcriptional patterns, thus enhancing the evolvability of *S.* Typhimurium and potentiating pre-adaptation to new environmental niches.

Chapter 5 General discussion

5.1 Discussion

The bacterial chromosome is a malleable structure which is constantly being re-shaped and manipulated in response to growth transitions and environmental signals . Changes in DNA supercoiling and the action of nucleoid-associated proteins (NAPs) mediate such chromosomal alterations in order to elicit an appropriate transcriptional response to the prevailing environmental conditions and maintain homeostasis (Hatfield & Benham, 2002, Dorman, 2006). Plectonemic DNA supercoiling is thought to account for approximately half of all negative supercoils present in the chromosome and serves to compact the nucleoid and provide free energy to help drive transcription (Travers & Muskhelishvili, 2005, Tupper et al., 1994). This energy is introduced into the DNA polymer by the activity of the DNA gyrase enzyme in an ATP dependent manner (Rovinskiy *et al.*, 2012, Dorman, 2006). Nucleoid-associated proteins are responsible for introducing the remaining negative supercoils and play an essential role in regulating global gene transcription (Dorman, 2006, Dame, 2005)

Key to understanding the roles played by NAPs during growth transitions and fluctuations in environmental conditions is an understanding of their patterns of expression. Each of the twelve species of NAPs characterised in *E. coli* and *S.* Typhimurium displays its own unique pattern of expression during growth and is also subject to differential environmental regulation (Ali Azam *et al.*, 1999). This gives rise to dynamic populations of NAPs, of which the abundance and timing of expression of each is variable as a function of growth. Having multiple binding loci throughout the chromosome allows NAPs to simultaneously influence the transcription of hundreds of genes (Dillon & Dorman, 2010). For example, in *E. coli*, FIS binds at approximately 1500 chromosomal loci effecting the transcription of ≈400 genes (Kahramanoglou *et al.*, 2011). The unique expression pattern of *fis*, however, ensures that FIS protein is only present at significant amounts during early- to mid-exponential growth (Ball *et al.*, 1992). FIS is therefore constrained by its pattern of expression as to when it can

exert its effect over the genes which it regulates. Furthermore, while each NAP governs its own specific regulon, the regulons of certain NAPs often overlap, such as those of H-NS and FIS, which sometimes act antagonistically (Falconi *et al.*, 1996). Thus, naturally occurring fluctuations in the expression and abundance of each NAP, allows the bacterium to manipulate patterns of global gene expression. This helps ensure the correct timing of expression of genes required for survival and proliferation as the bacterium progresses through the growth cycle and experiences varying environmental conditions (Sobetzko *et al.*, 2012).

The first part of this work aimed to further understand the unique patterns of expression of the closely related NAPs, H-NS and StpA, specifically in *Salmonella* Typhimurium. While the pattern of *hns* expression is conserved in both *E. coli* and *S.* Typhimurium, information about the regulatory factors governing its expression has been derived solely from studies undertaken in *E. coli* (Free & Dorman, 1995, Hinton *et al.*, 1992). In *S.* Typhimurium, the FUR transcriptional regulatory protein was recently shown to repress the *hns* gene in the presence of iron (Troxell *et al.*, 2011). FUR regulation of *hns* has not been shown in *E. coli* indicating that the *hns* regulatory region has undergone a certain amount of genetic divergence in *S.* Typhimurium. It was therefore of interest to determine which regulatory factors governing *hns* expression in *E. coli* were conserved in *S.* Typhimurium. In *E. coli* expression of *stpA* mRNA and StpA protein is confined to a transient burst during mid-exponential growth after which both decline to undetectable levels (Free & Dorman, 1997). In *S.* Typhimurium, while the same overall pattern of *stpA* expression was conserved, both *stpA* mRNA and *StpA* protein were detectable at significant levels at all stages of growth (Lucchini *et al.*, 2009). StpA was also found to be more biologically active in *S.* Typhimurium and was found to regulate 5% of genes in *S.* Typhimurium (Lucchini *et al.*, 2009). This study thus aimed to unearth some of the possible regulatory factors responsible for this sustained expression of *stpA*.

5.1.1 Anti-correlation between mRNA and protein abundance

The patterns of *hns* and *stpA* expression observed in *S.* Typhimurium as part of this study were found to be in good agreement with those previously published by Hinton *et al* and Lucchini *et al*, respectively (Lucchini *et al.*, 2009). In chapter 3, maximum expression of *hns* occurred during exponential growth and H-NS protein levels were constant (Fig. 3.2 A and 3.5 A). Similarly, both *stpA* mRNA and StpA protein could be detected at all stages of growth with both exhibiting a transient peak of expression during early exponential phase (Fig. 3.2 B and 3.5 B). To characterise the *stpA* pattern of expression, Lucchini *et al.* (2009) relied on activity from a *gfp* reporter gene fusion as a read out of the level of transcription. This, however, is limiting as it allows for the expression of only one gene at a time to be examined. Using RT-qPCR as part of this study, the relative abundance of both the *hns* and *stpA* transcripts was monitored from the same experimental samples. By this method significantly higher levels of *stpA* mRNA were detected at all stages of growth when compared to *hns* mRNA (Fig. 3.3). This was in stark contrast to observations in *E. coli* where *stpA* mRNA is only detectable during early exponential growth or in an *hns* mutant strain (Free & Dorman, 1997, Sonnenfield *et al.*, 2001).

Subsequent quantification of the relative amounts of H-NS and StpA protein revealed an anti-correlation between mRNA and protein abundance as H-NS protein levels were at least 2.5-fold greater than that of StpA at all times during growth (Fig. 3.6) whereas the *stpA* mRNA levels were up to 15-fold higher than *hns* mRNA. This suggested that *stpA* and/or *hns* were the subject of post-transcriptional regulation. Analysis of the stability of the *hns* and *stpA* mRNA molecules revealed that the *stpA* mRNA molecule was highly unstable. Upon inhibition of transcription, levels of *stpA* mRNA dropped to 10% of the starting level within 5 min (Fig. 3.7). The *hns* mRNA molecule, however, was quite stable in comparison, with 60% of the starting level persisting 15 min post rifampicin treatment to inhibit RNA polymerase. The observed instability of the *stpA* mRNA could be attributed largely to the 5' untranslated region as when this region was fused with the *hns* ORF in SL1344^{swap} the resulting hybrid *stpA-hns* mRNA was equally unstable. Conversely, when fused with the *stpA* ORF in

SL1344^{swap} the *hns* 5' untranslated region enhanced the stability of the resulting *hns-stpA* mRNA relative to the wild-type *stpA* transcript indicating that the *hns* 5' untranslated region aids in the stabilisation of *hns* mRNA.

Examination of the *stpA* 5' untranslated region revealed the presence of a shortened RBS consisting of only one AGG triplet which was located 11 bp upstream from the start codon. Secondary structure predictions of the *stpA* mRNA molecule show the RBS to be located within a single-stranded loop readily accessible to ribsomes (Fig. 3.8). The sub-optimal RBS sequence and spacing, however, would be a hindrance to ribosome recruitment and thus translation of the *stpA* mRNA. It is accepted that translation affects mRNA turnover as mutations which prevent or hinder translation have been observed to reduce the half-life of mRNA (Delvillani *et al.*, 2011). Thus, inefficient translation of *stpA* mRNA arising from the presence of a sub-optimal RBS may be contributing to the observed instability of *stpA* mRNA and the anti-correlation between *stpA* mRNA and protein levels. Further experiments aimed at replacing the *stpA* RBS with that of the full length consensus RBS (AGGAGG) at optimal spacing (8 bp) from the translation start codon would determine if, indeed, inefficient translation played a role in the apparent anti-correlation. In this more optimal scenario it would be expected that both the stability of *stpA* mRNA and the levels of StpA protein would increase.

Interestingly, the remaining *stpA* 5' untranslated region upstream of the RBS was 76 % A/U rich and contained one or more poly-U tracts (Fig. 3.8). Such A/U rich regions upstream of RBS sequences act as translational enhancers and play a role in recruiting ribosomes (Vimberg *et al.*, 2007). This would appear contradictory to the above hypothesis whereby the 5' untranslated region is inhibitory to translation. Such A/U rich regions, however, are also a target for binding of the Hfq regulatory protein (Hajnsdorf & Boni, 2012). As Hfq enhances the rate of duplex formation between sRNA molecules and their target mRNA molecules it is therefore a possibility that *stpA* mRNA may be the subject of targeted degradation by an as of yet unidentified sRNA in *S.* Typhmurium (Papenfort & Vogel, 2009). If true, Hfq mediated degradation of *stpA*

mRNA could, in part, explain why the high levels of *stpA* mRNA observed in this study do not correlate with StpA protein levels. Analysis of *stpA* mRNA stability in *hfq* mutant derivatives would help validate this hypothesis as, in the absence of functional Hfq, the *stpA* mRNA stability should be enhanced if indeed sRNA regulation is at play. As sRNA molecules usually bind complementary to their mRNA targets, sequence based searches of the currently known sRNA molecules present in *S.* Typhimurium would further aid in the search for a specific sRNA targeting the *stpA* mRNA.

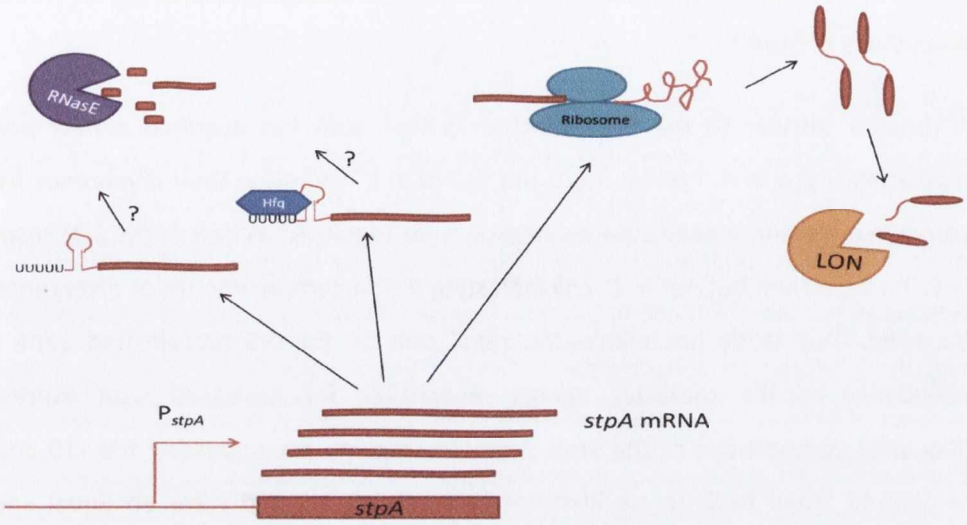
In *E. coli* the StpA protein is known to be subject to proteolysis by the Lon protease in the absence of H-NS (Johansson & Uhlin, 1999). While efforts to show Lon degradation of StpA in *S.* Typhimurium were unfruitful, evidence of StpA proteolysis was observed using strain SL1344$^{2Xstp A}$. In this strain, which lacks H-NS, no StpA could be detected after 2 h of growth suggesting that StpA may indeed be degraded in *S.* Typhimurium (Fig. 3.9). This result, however, was observed at 42 °C and a similar pattern was not observed at 37 °C. One possible explanation for this is that the increased levels of StpA protein observed in *S.* Typhimurium relative to *E. coli* are masking any degradation at 37 °C. Conversely, at 42 °C a number of proteases are more highly expressed and more active and degradation was therefore apparent at this temperature (Goff *et al.*, 1984). Future experiments inhibiting protein synthesis and monitoring any subsequent StpA degradation at 37 °C in SL1344$^{2Xstp A}$ would confirm this hypothesis and demonstrate StpA degradation in a similar manner to that observed in *E. coli*. Furthermore, similar experiments conducted in strains lacking proteases such as Lon or ClpXP would determine which protease is responsible for the proteolysis of StpA in *S.* Typhimurium.

The work presented in this study suggests that *S.* Typhimurium has evolved to have a higher level of *stpA* transcription than *E. coli*. Despite significant instability of the *stpA* mRNA and proteolysis of the StpA protein, this increased level of transcription appears to provide sufficient amounts of mRNA and thus protein to combat these processes of degradation and account for the *Salmonella*-specific pattern of StpA expression (Fig. 5.1). Two pertinent questions, however, remain unanswered: How is this higher level

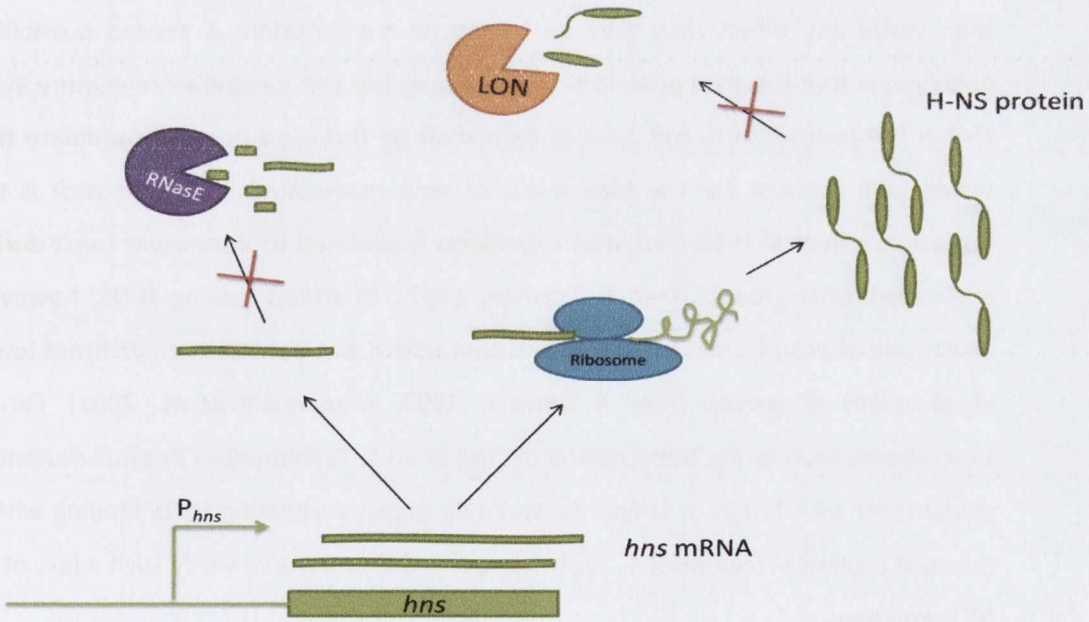
5.1 Model of *hns* and *stpA* expression in *S.* Typhimurium.

In *S.* Typhimurium, *stpA* mRNA (red ribbon) (A) is expressed at significantly higher levels than that of *hns* mRNA (green ribbon) (B). Unlike *hns* mRNA, *stpA* mRNA is rapidly degraded. The presence of secondary mRNA structures and U-rich tracts in the *stpA* mRNA 5' untranslated region may facilitate degradation by acting as target sites for binding of RNasE (purple circle) or Hfq (blue hexagon). Lack of secondary structure in the *hns* mRNA and accessible RBS and translation start codon facilitate translation of the stable H-NS protein. Once translated the StpA protein is also subject to protelytic degradation reducing the levels of cellular StpA

A.


RNaseE
Ribosome
?
?
Hfq
LON
P_{stpA}
stpA mRNA
stpA

B.


LON
H-NS protein
RNaseE
Ribosome
P_{hns}
hns mRNA
hns

of *stpA* expression achieved? And, what is the reason for having an increased abundance of StpA?

A possible answer to the first question is that *stpA* has acquired a new positive regulatory input in *S.* Typhimurium not found in *E. coli* since their divergence from a common ancestor. Indeed *stpA* expression is up-regulated as part of the SOS response in *S.* Typhimurium but not in *E. coli* indicating that a certain amount of divergence has occurred. This study has eliminated regulation by the FIS protein and gene copy abundance as the causative agents responsible for increased *stpA* expression. Sequence comparisons of the *stpA* promoter regions encompassing the -10 and -35 sequences show both to be identical in each species and have an equal spacing between them. Comparisons of a broader region of the *stpA* promoter regions of *S.* Typhimurium and *E. coli*, however, could narrow the search for the proposed transcriptional enhancer by identifying any possible consensus binding sequences for known transcriptional regulators present in *S.* Typhimurium and not in *E. coli*. Subsequent monitoring of *stpA* expression in the absence of possible regulators would then verify any effect they may be having on transcription. A second possibility, however, is that the *stpA* gene in *S.* Typhimurium has lost a negative regulatory input that is still present in *E. coli*. Loss of regulation by H-NS is a possible candidate that would help account for the high levels of *stpA* expression. In *E. coli*, such is the repressive effect of H-NS that *stpA* expression is confined to a transient burst during mid-exponential growth (Free & Dorman, 1997). In strains lacking H-NS, however, expression of *stpA* increased 10-fold and *stpA* mRNA was detected at significant levels at all stages of growth (Free & Dorman, 1997, Sonnenfield *et al.*, 2001). Future experiments monitoring transcription of *stpA* in an *S.* Typhimurium *hns* null mutant in conjunction with EMSA or DNase footprinting assays comparing H-NS binding within the *stpA* promoter regions of *S.* Typhimurium and *E. coli* would verify such a loss of H-NS regulation.

Despite *E. coli* and *S.* Typhimurium occupying similar environmental niches, *S.* Typhimurium naturally expresses significantly more StpA than *E. coli*. Increased

cellular levels of StpA arising from a point mutation in the *stpA* gene were previously shown to compensate for the loss of *hns* and *rpoS* in long term stationary phase cultures of *E. coli* (Johansson & Uhlin, 1999). While this mutation was forced to evolve in the absence of both *hns* and *rpoS* it demonstrates that increasing cellular levels of StpA is one mechanism by which the cell can cope with the loss of *hns*. Despite diverging from a common ancestor over 100 million years ago the genomes of *S.* Typhimurium and *E. coli* still display significant similarity including large regions of synteny (Fookes *et al.*, 2011). A distinguishing feature of the *S.* Typhimurium genome, however, has been the acquisition of several large pathogenicity islands by horizontal gene transfer (Sabbagh *et al.*, 2010). Such islands are characterised by having a high AT content and H-NS has been shown to bind extensively at many of these loci in *S.* Typhimurium (Navarre *et al.*, 2006). An attractive hypothesis, therefore, is that over evolutionary time, in order to facilitate the integration of these islands, in its capacity as a xenogeneic silencer H-NS was progressively sequestered from the core genome. As a compensatory measure, *S.* Typhimurium thus evolved to increase cellular levels of StpA. This situation is analogous to that described for the acquisition of the pSf-R27 plasmid by *S.* Typhimurium. This plasmid encodes an H-NS-like protein, Sfh, which acts as a 'stealth factor' to silence plasmid encoded genes whose expression is detrimental to the host bacterium (Doyle *et al.*, 2007). Doyle *et al.* demonstrated that acquisition of the pSf-R27 plasmid lacking the *sfh* gene incurred a significant cost upon the fitness of *S.* Typhimurium and that this fitness cost was due to sequestration of H-NS from the core genome to silence the incoming plasmid encoded genes. It would be of significant interest to examine the pattern of *stpA* expression in the closely related species *S. bongori*. Of the twenty two so-called pathogenicity islands described in *S. enterica*, *S. bongori* has acquired just seven over evolutionary time. If the above hypothesis of H-NS sequestration is correct, then a *stpA* pattern of expression more closely related to that observed in *E. coli* would be expected in *S. bongori*.

5.1.2 Cold-shock regulation in *S.* Typhimurium

For the most part, the documented regulatory mechanisms involved in the expression of *hns* and *stpA* in *E. coli* were found herein to be conserved in *S.* Typhimurium. Expression of *hns* was observed to be coupled with the process of DNA replication (Fig. 3.11) and as such was maximal during exponential growth. In addition, Fis was a contributing factor to the increase in *hns* expression during exponential phase as the level of expression was reduced 2-fold in a *fis* mutant strain while no effect was observed on *stpA* expression (Fig. 3.12). The *hns* gene was also cold-shock regulated (Fig. 3.13) as reported previously in both *E. coli* and *S.* Typhimurium (La Teana *et al.*, 1991, Hinton *et al.*, 1992). Interestingly in *E. coli* a 2- to 4-fold induction of *hns* expression was reported upon a temperature downshift from 37 ^{o}C to 10 ^{o}C. In contrast, *hns* expression increased 5-fold under similar conditions as part of this study. Furthermore, expression continued to rise as a function time spent at 10 ^{o}C until *hns* mRNA levels reached a plateau level 12-fold higher than observed at the onset of cold-shock. The accumulation of a more stable *hns* mRNA was also found to be a contributing factor to its increased abundance at 10 ^{o}C (Fig. 3.14). Despite such high levels of transcript, however, preliminary data suggested that only a 2-fold increase in the level of H-NS protein occurred. At 10 ^{o}C cold-shock mRNAs are preferentially translated by modified translational machinery (Giuliodori et al., 2004, Gualerzi et al., 2003) . Importantly, in *E.coli* the levels of Initiation factors (IF 1, IF 2 and IF 3) increase substantially during cold-shock of which IF 3 was shown to stimulate translation of *hns* mRNA up to 10-fold more than non-cold-shock mRNAs (Gualerzi *et al.*, 2003). Reduced levels of IF 3 in *S.* Typhimurium relative to those in *E. coli* may be a plausible explanation for the apparent inefficient translation of *hns* mRNA. Experiments to monitor both transcript and protein abundance of IF 3 and monitoring H-NS levels in cells over-expressing IF 3 during cold-shock would help validate this hypothesis.

Cold-shock induction of *hns* expression in *E. coli* is dependent on the cold-shock regulator, CspA, binding within the *hns* regulatory region (La Teana *et al.*, 1991). In *S.* Typhimurium, however, cold-shock induction of *hns* was still observed in a *cspA*

mutant derivative although *hns* mRNA failed to reach peak levels until after 150 min (Fig. 3.16). CspA also seemed to regulate *hns* post-transcriptionally as the observed 2-fold induction of H-NS upon cold-shock in the wild-type background was abolished in a *cspA* mutant. CspA and CspA homologs have been proposed to act as RNA chaperones that facilitate 'melting' of RNA secondary structures to allow for translation to occur (Phadtare & Severinov, 2010). One hypothesis, therefore, is that in *S.* Typhimurium, CspA may be acting to destabilise secondary structures within the *hns* mRNA during cold-shock to increase translation of *hns* mRNA. This would help explain why, in the absence of CspA *hns* expression still increased during cold-shock with no concomitant increase in H-NS protein levels.

5.1.3 Novel H-NS and StpA populations

While the first part of this study focused on regulatory factors governing the expression patterns of *hns* and *stpA* in *S.* Typhimurium, the second part aimed to investigate the biological significance of these highly specific expression patterns. Irrespective of species differences, *hns* and *stpA* in both *S.* Typhimurium and *E. coli* exhibit highly individual patterns of expression which lead to dynamic populations of these two proteins in either bacterium as a function of growth and environmental conditions. Both H-NS and StpA can form homodimers, heterodimers and higher order homo- and heteroligomers(Leonard *et al.*, 2009). Importantly, the formation higher-order multimeric complexes occur in a concentration dependent manner which is thus dictated by the expression pattern of either gene (Leonard *et al.*, 2009, Arold *et al.*, 2010, Lim *et al.*, 2011). Furthermore, H-NS and StpA exhibit distinct DNA binding properties and form unique nucleoprotein complexes (Arold *et al.*, 2010) (Lim *et al.*, 2011, Sonnenfield *et al.*, 2001). Owing to these differential properties of H-NS and StpA alterations in their relative levels have the potential to alter global levels of supercoiling and gene expression patterns.

To understand the importance of the dynamic populations of H-NS and StpA, three strains were constructed in which the *hns* and *stpA* ORFs were interchanged. In each new strain the changes made resulted in altered populations of H-NS and StpA as a function of growth. The best characterised of these three strains was SL1344^{swap} in which the *hns* and *stpA* ORFs were chromosomally interchanged. By swapping the location of their open reading frames in this manner it was intended to directly interchange the expression patterns of *hns* and *stpA*. This, however, did not occur and each ORF inherited a new pattern of expression in SL1344^{swap}. When fused with the *stpA* promoter, the pattern of *hns* expression was equivalent to that of the wild-type *stpA* transcript (Fig. 4.5 B). The hybrid *stpA-hns* mRNA, however, was now expressed at significantly higher levels than those observed for wild-type *stpA* mRNA. This higher level of transcription was also manifested in protein abundance as H-NS levels were 1.8-fold higher in SL1344^{swap}. Despite inheriting a *stpA*-like pattern of transcription, a wild-type H-NS protein profile was maintained with equivalent levels of H-NS present at all times (Fig. 4.6). When expressed via the *hns* promoter *stpA* displayed a new pattern of transcription as after reaching peak abundance the levels of *hns-stpA* mRNA were then maintained at this level until late in stationary phase (Fig. 4.5 A). Transcript levels were also higher throughout growth for the hybrid *hns-stpA* mRNA relative to the wild-type *hns* mRNA. No StpA protein, however, could be detected in SL1344^{swap}.

A question arising from these results is why was a direct interchange in expression patterns not observed after swapping their open reading frames? This can, in part, be explained by taking post-transcriptional regulation into account. It was suggested in chapter 3 that *S.* Typhimurium achieves its unique StpA pattern of expression by increasing transcription of the *stpA* gene to combat processes of mRNA and protein degradation. Significantly reduced levels of *hns-stpA* mRNA were observed in SL1344^{swap} which, although *hns-stpA* mRNA is more stable than *stpA* mRNA, may be insufficient to produce enough protein to avoid proteolytic degradation. Conversely, although the *stpA-hns* mRNA was significantly less stable than that of the wild-type *hns* transcript, it was expressed at significantly higher levels than *hns*. The H-NS

protein is also quite stable over time and when coupled with increased mRNA abundance would account for the observed increase in H-NS levels in SL1344^{swap}. In addition, the translational efficiency may have been affected by the creation of hybrid mRNA molecules resulting in enhanced translation of the *stpA-hns* mRNA and diminished translation efficiency of the *hns-stpA* mRNA. *In vitro* translation assays performed on both wild-type and hybrid mRNAs would help resolve if, indeed, translational efficiency has been affected and plays a role in increasing H-NS levels and reducing the levels of StpA in SL1344^{swap}.

Post-transcriptional regulation, however, does not account for the increased abundance of *stpA-hns* mRNA relative to that of the wild-type *stpA* transcript and *vice versa* despite being expressed via the same promoter. A study undertaken in *E. coli* looking at expression of similar promoter-ORF fusions between various transcription factors demonstrated that certain ORFs seemed to dictate their level of expression regardless of promoter strength (Isalan *et al.*, 2008). In SL1344^{swap}, the *hns* and *stpA* ORFs may thus be dictating their own level of expression when fused with their cognate promoters. Alternatively the increase in expression may be a reflection of the auto- and cross-regulatory properties of H-NS and StpA. The lack of StpA protein in SL1344^{swap} would remove any repression by StpA at both the H-NS and StpA promoters and contribute towards the increased levels of both hybrid mRNAs. In SL1344^{swap} H-NS is present at significant levels and can therefore maintain any repressive effect it may be having at the *hns* and *stpA* promoters. In SL1344$^{2X stpA}$, StpA protein was clearly detected when expressed via the *hns-stpA* promoter-ORF fusion also present in SL1344^{swap} where no StpA could be detected. A key difference between these strains is that SL1344$^{2X stpA}$ lacks H-NS. Detection of StpA may therefore be a result of removal of auto-repression at the *hns* promoter to increase *hns-stpA* mRNA levels and produce StpA. Experiments monitoring the mRNA and protein abundance of *hns* and *stpA* in strains lacking these genes would be required to confirm any such auto- and cross-regulatory properties of H-NS and StpA which, although previously

observed in *E. coli*, have not been shown in *S.* Typhimurium (Sonden & Uhlin, 1996, Free & Dorman, 1997).

5.1.4 Phenotypic variations

If the unique expression patterns of *hns* and *stpA* are biologically significant then altering the timing and level of expression of these genes, as was achieved in SL1344$^{2X stpA}$, SL1344$^{2X hns}$ and SL1344^{swap} should result in phenotypic changes. This was the case as each strain had its own unique set of phenotypic outcomes with respect to several *hns* and/or *stpA* related phenotypes (Fig. 4.9 – Fig. 4.13). Furthermore, the results of these phenotypic tests revealed a functional hierarchy between H-NS and StpA. For each phenotype tested, strain SL1344$^{2X hns}$ consistently mimicked wild-type SL1344 indicating that a total loss of StpA was well tolerated and expression of *hns* via the *stpA* promoter could functionally compensate for its loss. Conversely, expression of *stpA* from the *hns* promoter could not compensate for the loss of H-NS from the cell in SL1344$^{2X stpA}$ which gave rise to a set of phenotypes equivalent to an *hns* deletion strain. Strain SL1344^{swap}, however, demonstrated that a fine balance also exists between the expression and abundance of H-NS and StpA as it displayed intermediate phenotypes for each test.

The changes made to *hns* and *stpA* in each strain were quite well tolerated as, with the exception of SL1344$^{2X stpA}$, no growth defect was imposed upon *S.* Typhimurium relative to wild-type. This prompted competitive fitness experiments at a range of temperatures and osmotic conditions to examine if the subtle changes in expression and resulting phenotypes made in SL1344$^{2X stpA}$, SL1344$^{2X hns}$ and SL1344^{swap} could be advantageous to *S.* Typhimurium (Fig. 4.14 and Fig. 4.15). Despite having equivalent generation times to SL1344 at 37 ^{o}C, when competed against SL1344 at 25 ^{o}C the regulatory changes made in SL1344$^{2X hns}$ and SL1344^{swap} imposed a fitness deficit upon *S.* Typhimurium. Similarly, SL1344$^{2X stpA}$ was also severely comprised in terms of fitness at 25 ^{o}C. At 42 ^{o}C the fitness of SL1344$^{2X stpA}$ and SL1344$^{2X hns}$ was again compromised

while SL1344^{swap} was as competitive as SL1344. The alterations in expression of *hns* and *stpA* were best tolerated at 37 ^{o}C as both SL1344$^{2X stpA}$ and SL1344$^{2X hns}$ were as fit as wild-type SL1344. The genotype/phenotype combination of SL1344^{swap}, however, gave rise to a significant fitness benefit relative to SL1344 at 37 ^{o}C. SL1344^{swap} also successfully out-competed wild-type SL1344 in the absence of NaCl and in the presence of 86 mM NaCl.

Together, the results from these experiments highlight an inherent genetic flexibility in *S*. Typhimurium as subtle changes in the expression patterns of *hns* and *stpA* can give rise to a variety of phenotypic outcomes and significantly impact the fitness of the bacterium in an environmentally dependent manner. The full extent of achievable outcomes, however, is unknown. As intermediate levels of β-glucoside production, maltose utilization and motility were observed for SL1344^{swap} it is a possibility that altering the levels of H-NS and StpA is, in turn, altering the level of expression of genes under their control. It would therefore be of interest to further examine the scope of phenotypic outcomes using an artificial inducible plasmid based system to subtly tweak the cellular levels of H-NS and StpA. Information from such experiments could be useful toward the creation of expression vectors in which the level of expression of multiple genes could be simultaneously controlled by manipulating the supply H-NS and StpA.

In a more natural context variations in the cellular level of H-NS and StpA occur during growth and in response to changes in environmental conditions such as temperature. Using SL1344^{swap} this study has demonstrated that more permanent changes to the expression patterns of H-NS and StpA can significantly increase the fitness of *S*. Typhimurium in a range of physiologically relevant environmental conditions. This raises the question as to why such patterns of H-NS and StpA expression have not naturally evolved in *S*. Typhimurium? This may be explained by the principle of SPANC (self-preservation and nutritional competency) which proposes that strains more resistant to environmental stress are compromised in their ability to compete for and utilise carbon sources in nutrient poor environments (Ferenci, 2005). The genotype of

SL1344^{swap} greatly increased the fitness *S.* Typhimurium in several environmental stresses. By the principle of SPANC, however, the same genotype may be a hindrance to *S.* Typhimurium in environments depleted of nutrients. Conversely, SL1344$^{2X stpA}$ and SL1344$^{2X hns}$, which did not display increased fitness under stress, may be better equipped for nutrient scavenging and utilisation. It is therefore likely that the *hns* and *stpA* patterns of expression in *S.* Typhimurium have become optimised over evolutionary time to minimise the impact of this trade-off within each of the environments it regularly encounters. Fitness assays competing SL1344$^{2X stpA}$, SL1344$^{2X hns}$ and SL1344^{swap} with wild-type SL1344 under a plethora of environmental stresses and in media depleted in various nutrients would help validate this hypothesis.

5.1.5 Altered expression of RpoS and RpoS regulated genes

As H-NS and StpA have the potential to regulate 13 % and 5 % of genes in *S.* Typhimurium, respectively, it was possible that global changes in gene transcription due to altered *hns* and *stpA* expression was accounting for the fitness increases observed for SL1344^{swap}. Surprisingly, despite controlling such a vast number of genes, transcriptional microarray analysis revealed only 26 genes during exponential growth and 29 genes during stationary phase to be differentially expressed in SL1344^{swap} compared to wild-type (Fig. 4.16). Functional grouping of these genes showed that over half of those differentially expressed during exponential growth and several in stationary phase belonged to the RpoS regulon (Table. 4.3). Subsequent monitoring of the occurrence of RpoS in SL1344^{swap}, SL1344$^{2X stpA}$ and SL1344$^{2X hns}$ showed that, in contrast to wild-type SL1344, RpoS could be detected during exponential growth in each strain thus explaining the results observed by transcriptional microarray. Furthermore, the exact timing of expression of RpoS was variable between each strain with detection occurring after 2 h, 3 h and 4 h of growth in SL1344^{swap}, SL1344$^{2X hns}$ and SL1344$^{2X stpA}$, respectively (Fig. 4.12). This again highlights the importance of the wild-type patterns of expression of *hns* and *stpA* as

any variations results in aberrant expression of RpoS and thus RpoS regulated genes. Such inappropriate expression of RpoS regulated genes is thought to be costly to the bacterium in terms of fitness in the absence of stress conditions (Battesti *et al.*, 2011). These results, however, also demonstrate that varying the cellular levels of H-NS and StpA is a possible mechanism by which *S.* Typhimurium controls the appropriate expression of the RpoS regulon in order to mediate a stress response. Subtle variations in the levels of H-NS and StpA were shown here to toggle the timing of RpoS expression during growth. In the wild-type context the *hns* and *stpA* genes may act as environmental sensors whereby stresses which alter their expression, such temperature shifts, in turn subtly alter the relative abundance of H-NS and StpA within the cell to mediate expression of RpoS. Indeed, StpA has been proposed to link RpoS expression with sugar availability as a StpA dependent decrease in RpoS levels was observed in the presence of glucose (Lucchini *et al.*, 2009). It is not known, however, if this regulation is mediated by an alteration in the level of StpA. Future experiments aiming to monitor environmentally dependent changes in the abundance of H-NS and/or StpA in conjunction with RpoS abundance during growth would help provide evidence that *hns* and *stpA* possibly act as environmental sensors to control expression of the RpoS regulon.

5.1.6 Future prospects

With the advent of multiple drug resistant strains of bacteria and an ever diminishing supply of effective antibiotics, the search for novel therapeutics has intensified. The work presented in this study has furthered our knowledge regarding the control of expression of *hns* and *stpA* in *S.* Typhimurium. Importantly, H-NS and StpA are known regulators of virulence gene expression in multiple bacterial species where they generally exert a repressive effect. Troxell *et al.*, (2011) have shown that the FUR regulatory protein mediates virulence gene expression in *S.* Typhimurium via controlling the cellular levels of H-NS and the absence of FUR results in an avirulent phenotype. This potentiates the control of *hns* expression as a novel target in the

search for and design of new antibacterial drugs. Indeed, a recent study screening natural compounds identified capsaicin, a compound found in chillies, as a potent inhibitor of Cholera Toxin (CT) production in the newly emerged *V. cholerae* O1 El Tor strain (Chatterjee *et al.*, 2010). The presence of capsaicin resulted in repression of the *ctxAB* and *tcpA* genes, encoding the CT sub-units and toxin co-regulated pilli, respectively and the virulence gene master regulator *toxT* (Chatterjee *et al.*, 2010). A significant increase in *hns* expression was also observed in the presence of capsaicin leading the authors to hypothesis that the mode of action of capsaicin was to increase cellular levels of H-NS leading to repression of virulence genes (Chatterjee *et al.*, 2010). Furthermore, a similar inhibitory effect was also observed in strains belonging to different serogroups. Given the widespread occurrence of H-NS and H-NS-like proteins in the α, β and γ sub-divisions of proteobacteria and in certain Gram-positive species (Tendeng & Bertin, 2003, Gordon *et al.*, 2008), understanding the factors which govern their expression and cellular levels can greatly aid in the identification and design of potentially broad-spectrum antibacterial drugs.

A potential caveat with this approach is that H-NS is very often accompanied by one or more H-NS-like proteins which, like StpA, could each have an individual pattern of expression and influence the activity and expression of H-NS in different ways (Tendeng & Bertin, 2003). This study has highlighted just how intricate the relationship between just H-NS and StpA can be with subtle variations in their relative cellular abundances giving rise to multiple phenotypes and fitness outcomes. Any design, therefore, of potential antibacterial drugs to target *hns* expression would require further knowledge of such relationships between H-NS and H-NS-like proteins in any given species. Nonetheless, certain changes in the populations of H-NS and StpA in *S.* Typhimurium were herein observed to significantly increase the fitness of the bacterium in a number of physiological conditions relative to the human host. Such a highly competitive strain, if also avirulent, has the potential for use as a vaccine or indeed could be introduced as a therapeutic to out-compete its virulent counterpart within the host. This study has thus started the process of understanding the complex

relationship between H-NS and H-NS-like proteins and their roles in global gene regulation and also provides a basis for the potential genetic engineering of a highly competitive avirulent vaccine strain of *S.* Typhimurium based on controlling the expression of *hns* and *stpA*.

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