

**Consequences of Exchanging the NAP-
Encoding Genes, *fis* and *dps* in *Salmonella*
enterica serovar Typhimurium**



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By Marina Mary Bogue

Declarations

I declare that this thesis has not been submitted as an exercise for a degree at this or any other university and it is entirely my own work, with the following exceptions:

Library preparation for RNA-seq and ChIP-seq were performed by Vertis Biotechnologie AG.

Bioinformatic analyses of whole genome sequencing, RNA-seq and ChIP-seq experiments were performed by Dr. Aalap Mogre.

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Summary

In γ -Proteobacteria, there is an observable synteny of genes along the two replichores of the bacterial chromosome, especially with genes encoding transcription factors, sigma factors and nucleoid associated proteins. Previous studies have elucidated a number of factors influencing gene expression at a given location on the chromosome, including the gene dosage effect, gene orientation, neighbouring genes, and local DNA topology. In a near-future scenario, biological systems may be designed to fulfil specific purposes, yet, many of the governing factors of chromosome biology remain unclear.

This study sought to add to the knowledge of chromosome biology by exchanging the gene position of two NAP-encoding genes, *fis* and *dps*, which are natively located in the NS-Left and Right macrodomains, respectively. One exchange produced a strain which included the *cis*-acting regulatory sequences of the genes (GX), and the other included the open reading frames (OX). Strains which harboured two copies of either *fis* or *dps* (2x) were created in the process. The genomes of all constructed strains were sequenced, which confirmed the exchanges, and identified single nucleotide polymorphisms.

Next, this study identified changes in gene dosages of *fis* and *dps*, and consequential changes in these genes expression in the exchange strains. The gene dosage gradient from Ori to Ter was examined, and a change in gene dosage gradient was observed in OX, which was related to a change in growth rate in this strain. GX exhibited lower gene dosages of *fis* and *dps*, which resulted in changes in gene expression, where *fis* expression was reduced, and *dps* expression was increased. The same effect was observed in Fis and Dps production in the GX strain. In contrast, the change in gene dosage gradient in OX, resulted in less changes in *fis* and *dps* gene dosages. In this strain, the *cis*-acting regulatory sequences was the governing factor in changes in *fis* and *dps* expression; *dps* was expressed at the point in growth that *fis* is normally expressed, and *vice versa*.

Next, this study sought to examine how the change in *fis* and *dps* position affected global gene expression. RNA-seq was performed in exponentially growing cells, and in early stationary phase. Few genes were differentially expressed in GX,

with 11 and 17 differentially expressed genes for exponential and early stationary phases, respectively. Conversely, there was a large number of differentially expressed genes in OX, with 1,207 and 1,543 differentially expressed genes in exponential and early stationary phases, respectively. Many well defined pathways of the Fis regulon were differentially expressed including genes involved in motility, metabolism, and virulence. Further, a large number of genes encoding transcription factors were differentially expressed, highlighting Fis' indirect role on gene expression.

Additionally, this study investigated changes in global Fis binding patterns were affected by the change in *fis* position. Here, ChIP-seq was performed on the strains in exponential phase. Overall, there was a lower binding intensity in GX compared to WT, which was even lower in OX. Additionally there was an observable bias in Fis binding intensity, with the greatest decrease in binding intensity around *oriC*.

Finally, this study examined how the exchanges of *fis* and *dps* affected bacterial physiology. Here, the “2x” intermediate strains, and *fis* and *dps* knockout strains were also examined to help elucidate the exact cause of any observed phenotypes. The phenotypes chosen to be investigated were directly related to either Fis or Dps. Defects in growth, cell morphology, motility and SPI expression were observed in strains lacking the *fis* gene. Defects in oxidative stress resistance and stationary phase viability were observed in strains lacking the *dps* gene. A reduced resistance to oxidative stress was also observed in OX, and this was related to the changes in the *cis*-acting regulatory sequences for *dps* in this strain. OX exhibited a growth defect, and delayed SPI expression, while GX had a reduction in SPI expression. Finally, the competitive fitness between WT, GX and OX was assessed. GX was competitively fitter than WT, while OX was competitively weaker than WT and GX.

Overall, this study examined the effect of changing *fis* and *dps* gene position, and *fis* and *dps* *cis*-acting regulatory sequences on bacterial physiology. Changing the position of the *fis* and *dps* genes, affects the production of these gene products in a gene dosage-dependent fashion, which has a minimal impact on bacterial physiology. Conversely, exchanging the open reading frames of *fis* and

dps resulted in a reversal of expression patterns of these genes, which had a large impact on bacterial physiology.

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Chapter 1:

General Introduction

1.1 General Introduction

1.1.1 Bacterial DNA replication

The bacterial growth cycle is characterised by three distinct phases of growth; a lag phase in which bacteria are adapting to the environment, followed by an exponential phase, in which bacteria are rapidly dividing, followed by stationary phase, in which nutrients have been depleted, and bacterial growth rate is equal to the death rate (Fig 1.1) (Zwietering *et al.*, 1990).

DNA replication begins at the origin of replication, *oriC*, and terminates at the *ter* sites, in a tightly regulated process that ensures that replication begins in the correct location and at the correct time (Reyes-Lamothe *et al.*, 2012, Reyes-Lamothe and Sherratt, 2019). In bacteria, replication initiation begins with DnaA, a protein which binds to specific sequences flanking *oriC*, promoting unwinding of the DNA helix (Fuller *et al.*, 1984, Katayama, 2017). In *Escherichia coli*, there are five copies of the 9-mer DnaA-binding sequences, known as DnaA boxes, and all are required to be bound by DnaA for replication initiation (Fuller *et al.*, 1984, Weigel *et al.*, 1997). Therefore, replication initiation is dependent on the intracellular concentration of DnaA, whose concentration varies throughout growth. The active DNA-binding form of DnaA is ATP dependent, providing a direct link between DNA replication and nutrient availability (Kawakami *et al.*, 2005). The conversion of DnaA-ADP to DnaA-ATP occurs at the DnaA assembly regions (DARS) (Fujimitsu *et al.*, 2009).

Upon binding to DnaA boxes, DnaA can self-oligomerise through its N-terminal and ATP-binding domains, forming a nucleoprotein complex, a process that is assisted by DiaA (Katayama, 2017, Ishida *et al.*, 2004). This nucleoprotein complex promotes unwinding of the DNA helix at the A/T-rich DNA unwinding element (DUE), located adjacent to *oriC* (Fig 1.2) (Kowalski and Eddy, 1989). The ATP-binding helicase loading protein, DnaC, then loads the hexameric helicase, DnaB, forming the prepriming complex (Kobori and Kornberg, 1982). DnaC undergoes ATP hydrolysis, and the primase, DnaG interacts with DnaB, causing dissociation of DnaC from the complex (Fang *et al.*, 1999, Davey *et al.*, 2002). The β -clamp of the DNA polymerase III, DnaN, is subsequently loaded forming the replisome (Fang *et al.*, 1999).

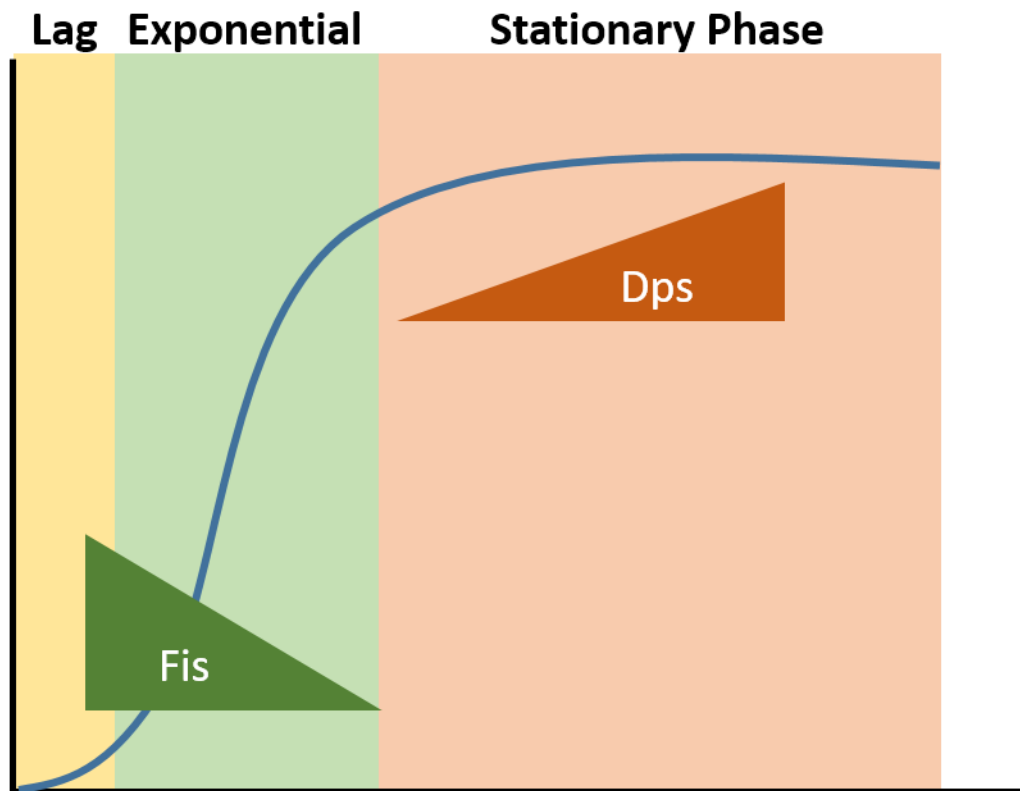


Fig 1.1. The bacterial growth cycle. The bacterial growth cycle consists of three growth phases, lag phase (yellow), exponential phase (green) and stationary phase (orange). Changes in Fis and Dps production throughout growth are indicated by triangles.

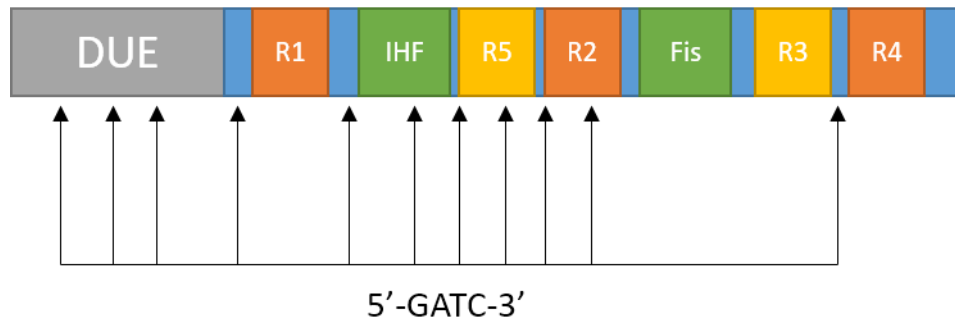


Fig 1.2. Structure of *oriC*. DNA replication begins with binding of DnaA to five sites in the vicinity of *oriC* (R1-R5). Orange indicates high-affinity sites and yellow indicates low affinity sites. Binding sites for the NAPs Fis and IHF are indicated in green. Hemimethylated sites which SeqA binds to are indicated by black arrows.

Several factors regulate the initiation of chromosome replication. In *E. coli*, there are eleven copies of the 5'-GATC-3' sequence which are subject to methylation by the DNA adenine methylase (Dam) (Fig 1.2) (Campbell and Kleckner, 1990, Bach and Skarstad, 2005). Immediately following replication initiation, these motifs are hemimethylated, and bound by the protein SeqA (Lu *et al.*, 1994). This inhibits methylation by Dam, binding of DnaA to DnaA boxes, and untimely replication reinitiation (Lu *et al.*, 1994, Bach and Skarstad, 2005, Nievera *et al.*, 2006). The methylation of this motif by Dam causes SeqA to dissociate from the motif, and allows for DnaA binding. The *dnaA* gene is located in the proximity of *oriC* and the binding of SeqA to hemimethylated DNA also prevents transcription of *dnaA* (Riber and Lobner-Olesen, 2005). By this method, DnaA also negatively autoregulates itself, by binding to a DnaA box within the *dnaA* promoter region (Hansen *et al.*, 2007).

Another method by which DnaA binding to DnaA boxes is regulated is through the DnaA homologue protein (Hda), by a process called regulatory inactivation of DnaA (RIDA) (Kato and Katayama, 2001, Su'etsugu *et al.*, 2008). A complex consisting of the DNA loaded clamp, Hda and ADP interacts with DnaA-ATP, hydrolysing it to ADP, and therefore repressing replication initiation (Su'etsugu *et al.*, 2008).

DnaA is also titrated away from binding at *oriC* by the *datA* locus, which contains five DnaA boxes and a single binding site for the nucleoid-associated protein (NAP), IHF (Kitagawa *et al.*, 1998, Kasho *et al.*, 2017, Nozaki *et al.*, 2009). Binding of IHF to the *datA* locus stimulates binding of DnaA to this region by bending the DNA through an angle of up to 180°. IHF production occurs in late exponential growth, and the titration of DnaA away from *oriC* by IHF, directly relates to a period in the growth cycle where growth rate is slowing down (Dillon and Dorman, 2010, Ditto *et al.*, 1994).

Other NAPs are involved in regulating replication initiation through interactions with the *dnaA* gene. The Factor for Inversion Stimulation (Fis) binds directly to the promoters of *dnaA* and *dnaN*, and is a repressor of the two genes (Froelich *et al.*, 1996). There are multiple other binding sites for Fis at *oriC*, and *in vitro* studies have shown an inhibitory effect of Fis on replication initiation, while *in vivo*

studies have shown a contrary, stimulating effect (Fig 1.2) (Flatten and Skarstad, 2013, Wold *et al.*, 1996, Gille *et al.*, 1991). Binding of the two NAPs, Fis and IHF, coordinate the exchange of ADP to ATP at the DARS2 region (Kasho *et al.*, 2014, Riber *et al.*, 2016).

Once DNA replication has been initiated, the replisomes travel along the two replichores to the point of termination in the Ter region, which is directly opposite *oriC* (Hill *et al.*, 1987). In *E. coli* there are 10 *ter* sites (A-J), which can be bound by the Terminus utilization substance (Tus) (Neylon *et al.*, 2005, Dewar and Walter, 2017). These sites allow the passage of the replisome in one direction only, and the point of termination is in the centre of these sites. In the absence of Tus, aberrant replication reinitiation occurs at a greater frequency, indicating that its primary role may not be in determining the site at which termination occurs (Ivanova *et al.*, 2015, Rudolph *et al.*, 2013).

As the two replication forks approach one another, a class of enzymes known as topoisomerases alters the DNA superhelical density around the replisomes, and without their action, replication would stall due the torsional stress placed on DNA by the movement of the replisome along the DNA (Schvartzman and Stasiak, 2004, Schvartzman *et al.*, 2013). Topoisomerase IV is particularly important here, as it has the ability to decatenate DNA (Zechiedrich *et al.*, 1997). While it is unclear whether the two forks clash, it is proposed that there is a gap in unreplicated DNA between the two forks, which must be filled either before, during or after replisome disassembly. Recent studies have indicated that when the forks meet, a 3' flap is formed, the helicases dissociate, the gap is filled and DNA polymerase I processes the final Okazaki fragment (Fig 1.3) (Rudolph *et al.*, 2013, Markovitz, 2005). However, there is still much to determine about the exact sequence of events during the termination of replication.

During DNA replication, homologous recombination events can occur between the two sister chromatids, which could interfere with the segregation of the two chromosomes into two separate cells by creating a dimeric chromosome (Steiner and Kuempel, 1998). To combat this, dimeric chromosomes are resolved at the *dif* sites by site-specific recombination (Leslie and Sherratt, 1995). For this to occur, a membrane binding protein required for chromosome partitioning, FtsK,

must be in place at the mid-cell (Steiner *et al.*, 1999). FtsK translocates DNA until the *dif* sites are co-localized. The tyrosine integrases XerCD, bind either side of the *dif* site, and FtsK's translocation activity halts when XerCD is bound to the *dif* site (Fig 1.4) (Kennedy *et al.*, 2008, Graham *et al.*, 2010).

Bacterial DNA replication is intrinsically linked to the environment in which the bacterium resides. Therefore, alterations to genes or proteins which are involved with certain growth conditions can have consequences for bacterial growth rate. Indeed, two NAPs which are studied in this thesis are involved in regulating bacterial DNA replication, where Fis is a positive regulator of DNA replication and Dps is inhibitive of DNA replication (Flatten and Skarstad, 2013, Chodavarapu *et al.*, 2008).

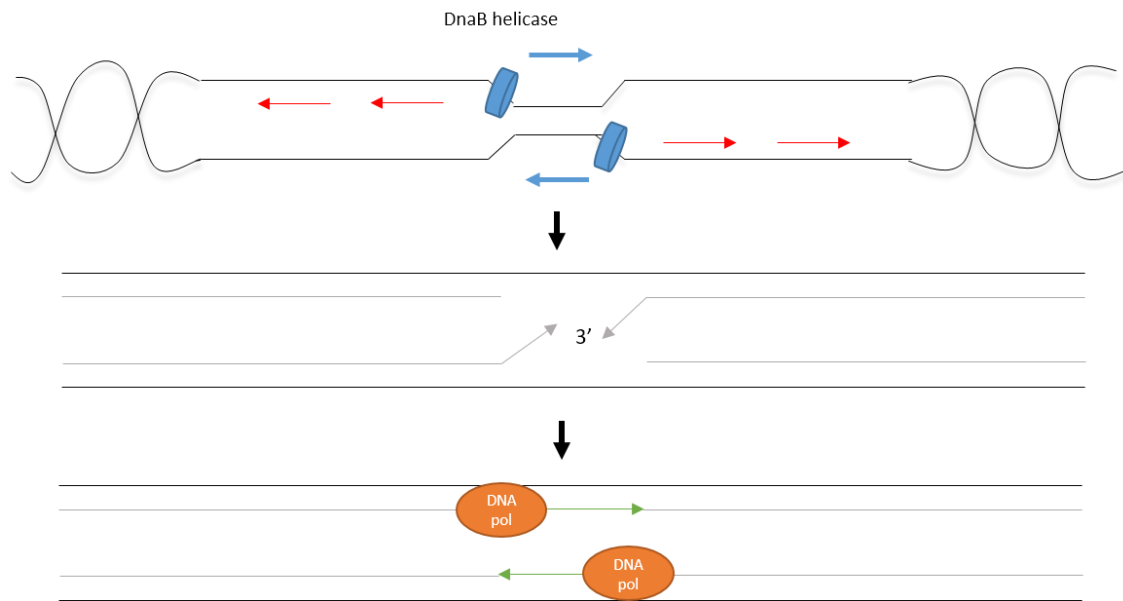


Fig 1.3 Termination of DNA replication. Top: The replication forks converge at some point between *ter* sites, with precatenane formation, and resolution by topoisomerase IV, Okazaki fragments are indicated in red. Middle: Okazaki fragments mature, and collision of DnaB helicases generates 3' overhang, leading to their dissociation. Bottom: The 3' overhang is removed, and the gap is filled by DNA polymerase (DNA pol).

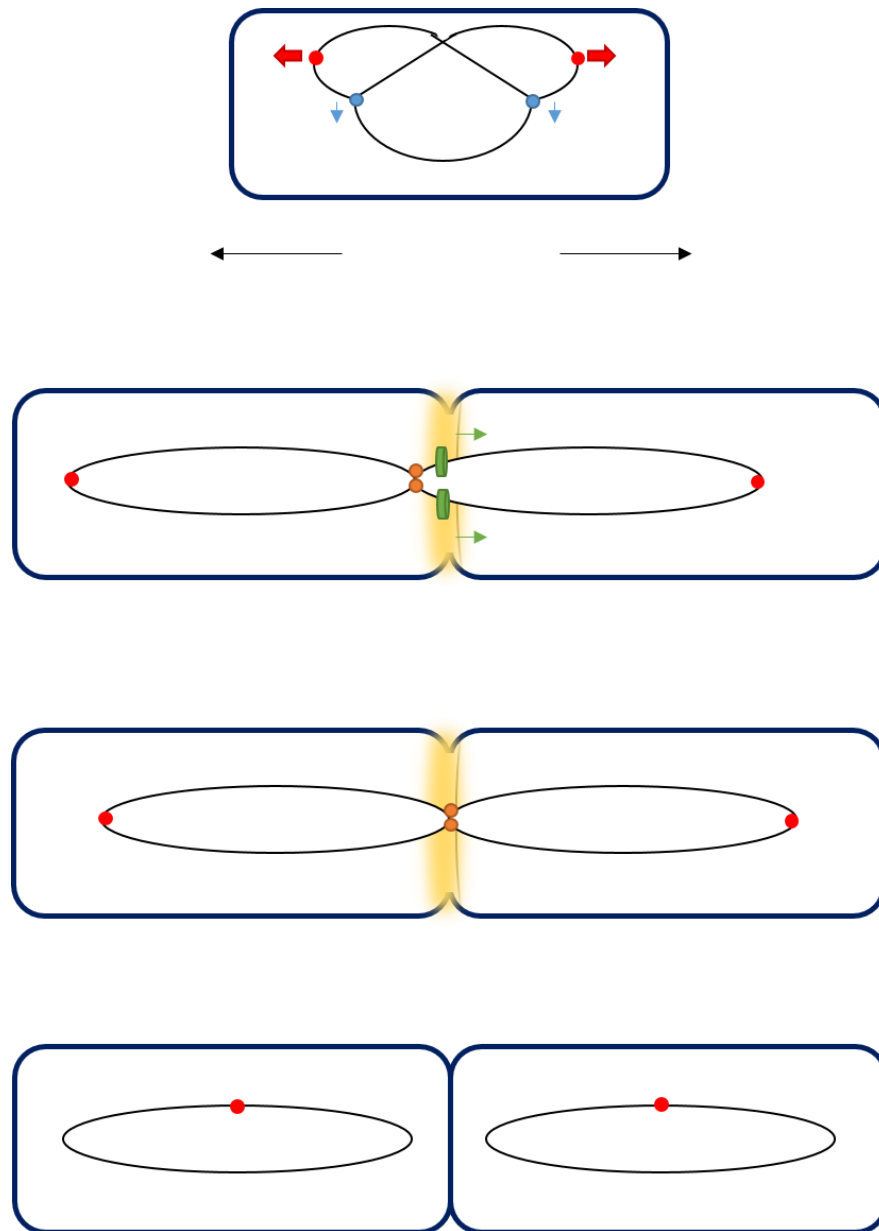


Fig 1.4. Resolution of sister chromatids. As DNA replication progresses, the *oriC* (red circles) of sister chromatids are oriented towards the poles of the cell, newly replicated DNA is hemimethylated, and unreplicated DNA is methylated. The cell wall expands bi-laterally (indicated by black arrows) and the Z-ring (yellow) composed of FtsZ is laid down mid-cell. The formation of the Z-ring leads to the recruitment of FtsK (green), a DNA-binding protein which is attached to the cell wall via a flexible linker. FtsK translocates the DNA (indicated by green arrows) until it reaches the *dif* site bound by XerCD (orange) recombinases. Recombination occurs and the sister chromatids are resolved, and distributed to each cell. . Once sister chromatids have been resolved, essential septation proteins, FtsQ, FtsL and FtsB are recruited, and cell division progresses.

1.1.2 The nucleoid

The 4.8 Mbp chromosome of *Salmonella* has to be compacted into the bacterial cell in such a way that does not impede gene expression or DNA replication (McClelland *et al.*, 2001, Dillon and Dorman, 2010). Based on early observations of intact bacterial chromosomes by electron microscopy, a scaffold model of the chromosome was proposed (Saitoh *et al.*, 1995, Kavenoff and Bowen, 1976). In this model, the chromosome consists of wound loops and domains protruding from a proteinaceous matrix in the centre (Rybenkov *et al.*, 2014). The formation of these loops or domains is important as it allows them to be topologically distinct from one another, while the proteinaceous scaffold prevents the diffusion of energy between domains. In *Salmonella* and *E. coli* these microdomains are estimated to be approximately 10 kb in size (Deng *et al.*, 2004, Postow *et al.*, 2004).

In addition to the above, the chromosome of *Salmonella* and *E. coli* consists of four macrodomains, Ori, Ter, Right and Left, and two lesser structured domains, NS-Right and NS-Left (Fig 1.5) (Valens *et al.*, 2004, Dame *et al.*, 2011). Macrodomains can be defined as distinct regions of DNA which do not interact with one another, and were first defined by Valens *et al.* (2004), by determining the frequency of recombination between λ phage *att* sites which are engineered at locations throughout the *E. coli* chromosome. Since then, chromosome conformation capture (3C), combined with deep sequencing has elucidated a more refined picture of the boundaries within the chromosome structure, and the factors which help maintain nucleoid structure (Lioy *et al.*, 2018). This structure appears to be present in *Salmonella* too (Cameron *et al.*, 2017).

One protein which forms part of the proteinaceous scaffold is the condensin complex, MukBEF. MukBEF is a structural chromosome maintenance (SMC) protein complex, first identified in *E. coli*, when searching for factors which influenced chromosome segregation, but not plasmid segregation (Hiraga *et al.*, 1989, Niki *et al.*, 1992). ATP-dependent MukB consists of a DNA-binding domain, and a long coiled coil domain (Niki *et al.*, 1992). The long coiled coil domain functions in bringing MukB-DNA complexes together, the basis for the scaffold of which DNA loops protrude from. MukE and MukF together form a stable complex

which transiently interacts with MukB and function in facilitating MukB-DNA interactions (Rybenkov *et al.*, 2014). MukBEF binds throughout the genome, although with a bias towards the Ori macrodomain. This may be due to MukBEF's role during the cell cycle, whereby it guides the replicated Ori towards the poles of the cell, promoting chromosome segregation (Danilova *et al.*, 2007).

During the cell cycle, the macrodomains occupy distinct locations within the cell. By fluorescently labelling distinct genetic loci within *E. coli*, Wiggins *et al.* (2010) were able to determine how the various macrodomains localized within the cell throughout the growth cycle. It is now known that Ori occupies the mid-cell in *E. coli*, while the Ter macrodomain is a much less condensed, filament like region (Wu *et al.*, 2019). MukB plays a role in maintaining macrodomain localization, and if removed, Ori localizes at the cell poles (Danilova *et al.*, 2007, Badrinarayanan *et al.*, 2012).

Some proteins involved in structuring the bacterial nucleoid are macrodomain specific (Dame *et al.*, 2011). Most notable of these are three proteins, MatP, SlmA and SeqA. MatP binds specifically to *matS* sites located exclusively in the Ter macrodomain (Mercier *et al.*, 2008). There are 23 *matS* sites in *E. coli*, and binding of MatP to these sites prevents premature chromosome segregation at the Ter region (Mercier *et al.*, 2008). Conversely, the proteins SlmA and SeqA are excluded from the Ter macrodomain (Sanchez-Romero *et al.*, 2010, Tonthat *et al.*, 2011).

As described in Section 1.1.2, SeqA is an important factor in preventing the over-initiation of replication through SeqA's ability to recognise, and to bind to hemimethylated DNA (Lu *et al.*, 1994). However, chromatin immunoprecipitation (ChIP) studies have found several SeqA binding sites distal to *oriC*. It is now believed that SeqA may also play a role in orienting the chromosome during DNA replication through its regulation of genes involved in the cell cycle (Sanchez-Romero *et al.*, 2010).

SlmA was the first nucleoid occlusion factor identified in *E. coli*, and it can bind directly to FtsZ, preventing Z ring formation throughout the nucleoid (Bernhardt and de Boer, 2005, Schumacher and Zeng, 2016). ChIP studies have shown that there are 50 SlmA binding sites within the *E. coli* genome, none of which is in the

Ter macrodomain (Tonthat *et al.*, 2011). Interestingly, structural studies have shown that binding of SlmA to DNA causes a conformational change which then allows SlmA to interact with FtsZ (Schumacher and Zeng, 2016). Together, these studies indicate that SlmA, bound to DNA, prevents Z ring formation, except in the Ter macrodomain, therefore ensuring that cell division occurs at the correct point during the cell cycle.

In stationary phase, the bacterial nucleoid condenses, increasing DNA's resistance to DNA damaging agents. The condensation is achieved through a change in negative supercoiling of the DNA and the NAP content of the cell. Two NAPs in particular are specific to stationary phase in *E. coli*, Dps and CbpA (Almiron *et al.*, 1992, Ali Azam *et al.*, 1999, Chintakayala *et al.*, 2013). Dps functions in protecting DNA from damaging agents, while CbpA has a role in maintaining DNA topology in stationary phase (Nair and Finkel, 2004, Chintakayala *et al.*, 2013).

Not all bacteria position their nucleoids in the manner that *E. coli* does. In *Caulobacter crescentus*, the *oriC* is localised to the cell pole, and the SMC protein ParB is responsible for partitioning the chromosome during segregation (Jensen and Shapiro, 1999). As the chromosome of *C. crescentus* Ori-Ter is positioned from pole to pole in the cell, the two replichores of the chromosome are able to interact with one another (Tran *et al.*, 2017). This is in contrast to *E. coli*, and *B. subtilis* where the two replichores are directed to either pole of the cell, preventing interactions (Fig 1.6). Interestingly, the pathogen *Streptococcus pneumoniae* harbours a non-essential ParB homolog, indicating that it has another method of partitioning chromosomes (Minnen *et al.*, 2011). These observations indicate that regardless of how the nucleoid is organized, bacteria have evolved mechanisms to maintain nucleoid structure.

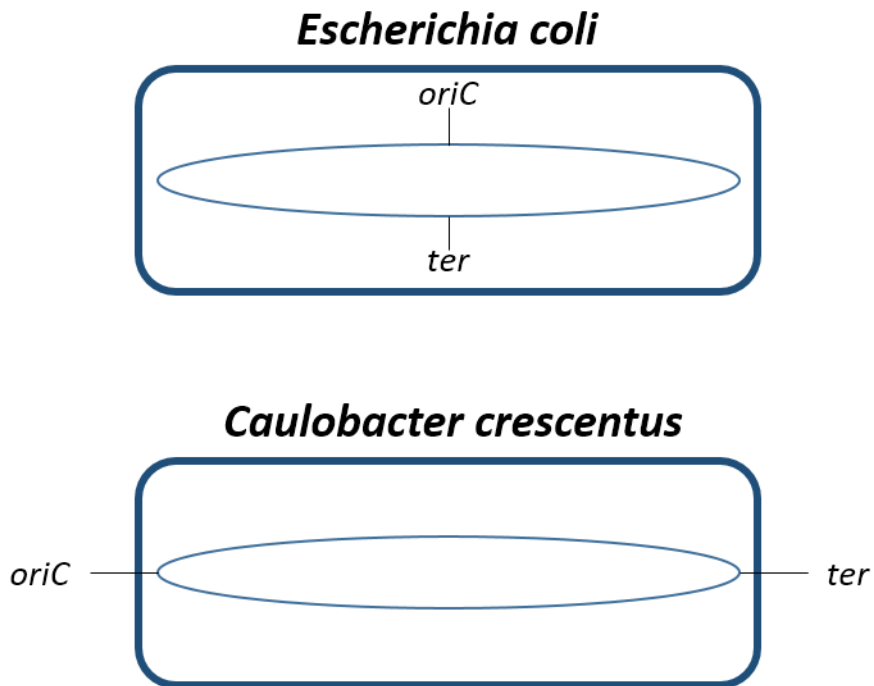


Fig 1.6. Differences in nucleoid organisation between *E. coli* and *C. crescentus*. In *E. coli*, the nucleoid is organised in such a way so that *oriC* and *Ter* sit mid-cell with the two replichores extending to the poles of the cell. In contrast, the nucleoid of *C. crescentus* is organised in such a way that *oriC* and *Ter* extend to the poles.

1.1.3 DNA Supercoiling and Chromosome Dynamics

In prokaryotes, the DNA of the genome exists in a negatively supercoiled state (Drlica, 1992). DNA supercoiling arises from the over- or under-winding of DNA, where under-wound DNA is negatively supercoiled, and over-wound DNA is positively supercoiled. The supercoiled state of DNA is defined by three factors, the twist, writhe and linking numbers (Schvartzman *et al.*, 2013). The linking number is the number of times one strand of DNA passes over another strand in the double helix, while the twist is the number of helical turns along the axis of the double helix, and the writhe is number of superhelical turns (i.e. the number of times the helix passes over itself). The degree of supercoiling is defined by the superhelical density, a measure of the difference between the linking number in the DNA's relaxed and supercoiled states. For DNA free of protein, the superhelical density is -0.05 (Vologodskii *et al.*, 1979).

Negative supercoiling is one of the methods through which DNA is compacted into the bacterial nucleoid, and is controlled by many factors on global and local scales (Dillon and Dorman, 2010, Drlica, 1992). Negatively supercoiled DNA, with its elevated energy content, is also more favourable towards DNA transactions that depend on strand separation, such as DNA replication and transcription (Witz and Stasiak, 2010). Indeed, DNA replication and transcription machinery also alter the supercoiling state as they unwind DNA (Schvartzman *et al.*, 2013).

Topoisomerases are found in all domains of life, and are essential for biological function (Ahmad *et al.*, 2017). In *E. coli* there are four topoisomerases of two types, I and II, summarised in Table 1.1 (Drlica, 1992, Steck *et al.*, 1993, Gellert *et al.*, 1976). Type II enzymes are ATP-dependent, and consist of DNA gyrase and DNA topoisomerase IV. DNA gyrase is responsible for introducing negative supercoils, while topoisomerase IV's primary function is as a decatenase (Gellert *et al.*, 1976, Zechiedrich *et al.*, 1997). DNA gyrase has an A₂B₂ structure, and the subunits are encoded by *gyrA* and *gyrB*. The promoters of *gyrA* and *gyrB* are sensitive to the supercoiling state of the DNA, with their expression being induced by relaxation in DNA, and they are repressed by increased negative supercoils (Menzel and Gellert, 1983). Conversely, topoisomerase I is required for removing negative supercoils from DNA. It is not ATP-dependent, as it can use the stored

Name	Mass (kDa)	Gene	Function
<i>Type I</i>			
Topoisomerase I	97	<i>topA</i>	Relaxes DNA in an ATP-independent manner by making a transient cut to one strand of the DNA duplex.
Topoisomerase III	73.2	<i>topB</i>	Can relax DNA, but main function is in decatenase activity in association with RecQ.
<i>Type II</i>			
DNA gyrase	105	<i>gyrA</i>	Introduces negative supercoils in an ATP-dependent manner by making a transient cut in one strand of the DNA duplex and passing it over the other
	95	<i>gyrB</i>	
Topoisomerase IV	75	<i>parA</i>	ATP-dependent decatenase activity, and can relax positive supercoils
	70	<i>parC</i>	

Table 1.1 The topoisomerases of *E. coli* and *Salmonella*. Type I topoisomerases are ATP-independent and Type II topoisomerases are ATP-dependent. Type II topoisomerases consist of an A₂B₂ structure. Topoisomerase I majorly functions in relieving negative supercoils, while DNA gyrase is responsible for introducing negative supercoils. Topoisomerase IV functions in decatenating sister chromosomes. Topoisomerase III functions in resolving precatenanes formed ahead of the replication fork during DNA replication.

energy of negative supercoils to function (Steck *et al.*, 1993, Drlica, 1992). Topoisomerase I is encoded by *topA*, and its expression is induced by negative supercoils or repressed by a relaxed state (Dekker *et al.*, 2002). Therefore, there is a feedback loop between DNA gyrase and Topoisomerase I activities, creating a homeostatic balance of negatively-supercoiled and relaxed DNA within the nucleoid.

DNA gyrase activity is dependent on the ATP/ADP ratio in the cell, providing another link between the environment, metabolic activity and supercoiling state of the genome (van Workum *et al.*, 1996). As the ATP/ADP ratio is dependent on the growth phase, it is of no surprise that the superhelical density of the genome varies with growth conditions (Conter *et al.*, 1997, Colgan *et al.*, 2018, Dorman *et al.*, 1988). Many genes require an optimal supercoiling state for their expression to be induced. Here, the twist of DNA can affect the spacing between the -35 and -10 hexamers, recognised by RNA polymerase (Travers and Muskhelishvili, 2005). Changes in DNA topology have other implications on protein binding and the activities of RNA polymerase. Alterations in twist influence the groove width in DNA, which in turn affect the DNA binding ability of a protein (Stella *et al.*, 2010). Further, altering the twist of the DNA duplex can result in the formation in single stranded bubbles, which are important in DNA replication and transcription, while alterations in writhe affects the probability of contacts between RNA polymerase bound to promoters and regulatory proteins bound at a distance (Jeon and Sung, 2008).

Changes in ATP/ADP ratios can therefore act as one method by which bacteria can rapidly react to changes in the environment, as a change in superhelical density can activate genes whose products are responsible for responding to a particular stress (Blot *et al.*, 2006). Upon nutrient upshift, the ATP/ADP ratio increases, and the activity of DNA gyrase increases (Conter *et al.*, 1997). Consequently, the superhelical density of DNA in exponentially growing cells is lower than that of stationary phase cells (Cameron *et al.*, 2011). Here, the NAP, Fis has a central regulatory role; its expression is induced by negative supercoils created by ATP-dependent DNA gyrase, while itself regulates genes encoding DNA gyrase and DNA topoisomerase I (Fig 1.7) (Travers *et al.*, 2001, Schneider *et al.*, 2000). Depending on the supercoiling state, Fis binds to the

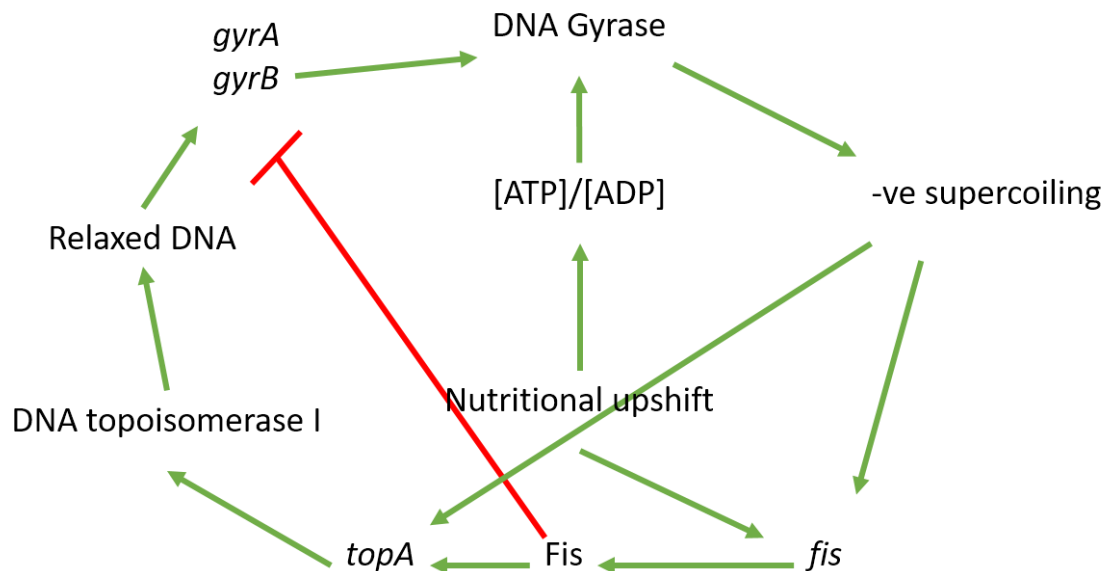


Fig 1.7. The homeostatic control of DNA supercoiling. Upon nutrient upshift the ratio of ATP/ADP increases, and ATP-dependent DNA gyrase introduces negative (-ve) supercoils. The expression of *fis* is stimulated by nutrient upshift and negative supercoils. In turn the Fis protein and negative supercoils activate the expression of *topA*, encoding DNA topoisomerase I. Simultaneously, Fis represses *gyrA* and *gyrB*, encoding the two subunits of DNA gyrase. DNA topoisomerase I relaxes negatively supercoiled DNA, which stimulates *gyrA* and *gyrB* expression. Together, these factors highlight the homeostatic balance of DNA topology in *Salmonella* and *E. coli*.

promoters of *topA*, either activating it or repressing it, while Fis is a repressor of *gyrA* and *gyrB* (Cameron *et al.*, 2011, Weinstein-Fischer and Altuvia, 2007, Keane and Dorman, 2003). In response to oxidative stress, Fis activates the expression of *topA*, leading to a relaxation in DNA (Weinstein-Fischer *et al.*, 2000). This relaxation of DNA simultaneously represses *fis* expression, and induces the expression of genes whose products function in oxidative stress survival. Additionally, upon binding DNA, Fis can act as a topological buffer and can preserve negative supercoils. Such can be seen at stable tRNA promoters such like that of *tyrT*, where binding of three Fis dimers upstream is required for transcriptional activation. Binding of three Fis dimers forms a microloop, constraining supercoils, and allows for the untwisting at the -10 hexamer (Auner *et al.*, 2003). Further, Fis binding upstream of promoters such as that of *leuV*, can translocate the superhelical density from the promoter-distal portion of a supercoiling-induced DNA duplex destabilized (SIDD) region to the promoter-proximal portion of the *leuV* promoter, allowing for unwinding of the helix and transcription by RNA polymerase (Opel *et al.*, 2004). Thus, Fis' role in maintaining the homeostatic balance of the nucleoid makes it different than the conventional transcription factor.

The rapid adaption of the genome to environmental stresses is frequently used in infection, and the superhelical density is affected by temperature, osmolarity, pH, and anaerobiosis (Colgan *et al.*, 2018, Dorman *et al.*, 1988, Goldstein and Drlica, 1984). One example of a virulence factor which is regulated by supercoiling is in Type I fimbriae in *E. coli*. Here, a genetic switch, *fimS*, controls the phase variation of fimbriae expression, where in one orientation of the switch fimbriae are expressed (phase-ON) and in the other fimbriae are not expressed (phase-OFF) (Dorman and Bogue, 2016, Gally *et al.*, 1993). When DNA is relaxed, the switch becomes more biased towards the ON phase (Kelly *et al.*, 2006). Additionally, the expression of virulence genes encoded on *Salmonella* Pathogenicity Islands (SPI) are affected by superhelical density in a manner that is linked to the environment during the *Salmonella* infection cycle. Both SPI-1 and SPI-2 encode Type III secretion systems (Cirillo *et al.*, 1998, Murray and Lee, 2000). SPI-1 genes, important for invasion of the epithelium of the small intestine, are upregulated by negative supercoiling, such are created by the conditions

experienced in the gut lumen. SPI-2 genes are essential for survival within the macrophage, and the expression of these is induced by relaxed DNA, and such relaxation has been observed during the infection cycle (O Cróinín *et al.*, 2006). Together, the link between Fis, ATP/ADP, DNA gyrase and DNA topoisomerase I highlight the dynamic adaptations to the environment, and the constant push for homeostasis within the cell.

DNA gyrase and topoisomerase I also have functions in DNA replication (Witz and Stasiak, 2010, Schwartzman *et al.*, 2013). As mentioned in Section 1.1.1, the activity of the replisome affects local supercoiling states, with DNA being overwound ahead of the replisome, and underwound behind the replisome. Together, these topoisomerases work together to relieve some of the torsional stress placed on DNA during replication (Gellert *et al.*, 1976). Additionally, the superhelical changes caused by the replisome cause it to swivel around DNA, promoting diffusion of supercoils from unreplicated to replicated regions of DNA. These regions of positive supercoils within the replicated DNA generate precatenanes, and as replication continues, the sister duplexes become increasingly intertwined (Schvartzman *et al.*, 2013). Here ATP-dependent topoisomerase IV resolves the precatenanes (Buck and Zechiedrich, 2004).

1.1.4 Nucleoid Associated Proteins of *E. coli* and *Salmonella*

In eukaryotes, histone proteins are utilised to aid the compaction of chromosome into the nucleus (Dillon and Dorman, 2010). In prokaryotes a group of proteins known as nucleoid associated proteins (NAPs) are responsible for the compaction of DNA into the bacterial nucleoid. NAPs are a family of proteins which are grouped together on functional similarity alone. The twelve species of NAPs in *E. coli* are listed in Table 1.2 (Dillon and Dorman, 2010, Ali Azam *et al.*, 1999). The best characterised NAPs in *E. coli* and *Salmonella* are H-NS, Fis, IHF, Dps and HU. The expression patterns of genes encoding NAPs varies: some NAPs are associated with a particular point in growth or they may be constitutively produced (Talukder and Ishihama, 2015, Ali Azam *et al.*, 1999).

IHF, Lrp, and Fis are NAPs that show some sequence specificity in binding to DNA, where IHF and Lrp bind with great specificity and Fis recognises a degenerate 16-bp motif (de los Rios and Perona, 2007, Cho *et al.*, 2008b, Swinger and Rice, 2004). Conversely, the DNA-binding preferences of the NAPs H-NS, HU, StpA and CbpA is determined by A/T tracts, and the intrinsic curvature of DNA (Shimizu *et al.*, 1995, Dame *et al.*, 2006, Zhang *et al.*, 1996, Chenoweth and Wickner, 2008)

Name	DNA bridging	DNA bending	Mass (kDa)	Binding specificity	Native protomer
H-NS	Yes	ND	15	AT rich DNA	Homodimer or heterodimer (H-NS + StpA)
StpA	Yes	ND	15	AT rich DNA	Homodimer or heterodimer (H-NS + StpA)
Fis	Yes	Yes	11	AT rich DNA	Homodimer
Dps	ND	ND	19	Secondary structures in DNA	Monomer or dodecamer
IHF	ND	Yes	11	(A/T)ATCAANNNN TT(A/G)	Heterodimer (IHF α + IHF β)
HU	ND	Yes	9	AT rich and curved DNA	Heterodimer (HU α + HU β)
Lrp	Yes	ND	18	(T/C)AG(A/T/C)A(A/T)ATT(A/T)T(A/T/G)CT(A/G)	Homodimer and higher order
MukB	Yes	ND	175	ND	Homodimer
CbpA	ND	ND	33	Curved DNA	Homodimer or heterodimer
CbpB	ND	ND	33	Curved DNA	Monomer

Table 1.2 Nucleoid associated proteins of *E. coli*. Table adapted from Dillon and Dorman (2010).

1.1.4.1 Fis

Fis was first characterised by its involvement in site specific recombination and genetic switches catalysed by site-specific recombinases such as Hin, Gin, Min and Cin, where it acts as an enhancer protein (Dorman and Bogue, 2016, Bruist *et al.*, 1987). The 11.2 kDa homodimeric Fis protein is associated with exponentially growing cells, and *fis* expression is characterised by a sharp peak in expression upon nutrient upshift (Ali Azam *et al.*, 1999, Yuan *et al.*, 1991). Fis concentration shifts from less than 100 to over 50,000 copies per cell prior to the first round of cell division (Ball *et al.*, 1992). Intracellular Fis concentration is then diluted through multiple rounds of replication until it is no longer detectable in stationary phase (Ball *et al.*, 1992, Ninnemann *et al.*, 1992). The *fis* gene is located in the NS-Left region of the chromosome, and is part of the bicistronic *dusB-fis* operon (Sobetzko *et al.*, 2012, Gerganova *et al.*, 2015). The presence of upstream *dusB* is essential for the production of Fis, as *fis* lacks its own Shine Delgarno sequence (Nafissi *et al.*, 2012). Instead, the AU rich *dusB* mRNA forms a stem-loop structure allowing for docking of ribosomal protein S1 and *fis* translation (Nafissi *et al.*, 2012). The expression of *fis* is induced by negative supercoiling, which are introduced by ATP-dependent DNA gyrase, and Fis negatively autoregulates itself (Fig 1.8) (Schneider *et al.*, 2000, Ball *et al.*, 1992, Ninnemann *et al.*, 1992). One of the Fis binding sites at its own promoter overlaps with that of RNA polymerase, indicating that repression is achieved by exclusion of RNA polymerase. However, there are five additional binding sites for Fis at its promoter, indicating the possibility that a stable nucleoprotein complex contributes to Fis repression (Ball *et al.*, 1992). The *Salmonella fis* promoter contains a 300 bp insertion when compared to other *Enterobacteriaceae*, and while the first 50 bp are conserved between *Salmonella* and *E. coli*, there is significant divergence in sequence beyond that (Cameron *et al.*, 2013). In particular, the *Salmonella* promoter contains an ArcA element which allows the expression of *fis* in stationary phase under anaerobic conditions, and the *fis* promoter of *Salmonella* lacks at least one of the Fis binding sites (Fig 1.8) (Cameron *et al.*, 2013, O Cróinín and Dorman, 2007). This ArcA element is physiologically relevant, as Fis is required for the activation of SPI-1, encoding a

type III secretion system important for invasion of the epithelium of the small intestine, and this environment is micro-aerobic.

The expression of *fis* is also regulated negatively by the stringent response (Fig 1.8) (Ninnemann *et al.*, 1992). Stringent response is a stress response through which bacteria respond to metabolic and nutritional stresses, such as amino acid, carbon, phosphate or nitrogen limitation (Chatterji and Ojha, 2001). The stringent response is signalled by the alarmone (p)ppGpp, and its production is catalysed by the proteins SpoT and RelA. Stringent response rapidly shuts down genes involved in cell division and upregulates genes to promote survival, such as amino acid biosynthesis pathways (Traxler *et al.*, 2008). A GC-rich motif in the *fis* promoter is responsible for repression by the stringent response, and mutation analyses have found uncharacteristic activity at the *fis* promoter in late exponential and stationary phase if the GC motif is mutated (Ninnemann *et al.*, 1992, Walker *et al.*, 1999). Additionally, another protein, DksA, is required to negatively control the *fis* promoter under amino acid starvation, and DksA works synergistically with (p)ppGpp to repress *fis* expression (Mallik *et al.*, 2006).

The NAP IHF is also involved in the regulation of *fis* expression, and the *fis* promoter is stimulated 3-4 fold by IHF binding (Fig 1.8) (Pratt *et al.*, 1997). Further, the catabolite activator protein CRP can either activate or repress *fis* expression, depending on whether Fis is present. In the presence of Fis, CRP is a repressor of *fis* expression, while in the absence of Fis, CRP can stimulate *fis* expression (Nasser *et al.*, 2001).

Fis binds to DNA as a homodimer and the crystal structure has been resolved, revealing that each monomer consists of 4 α -helices named A-D (Yuan *et al.*, 1991). The N-terminal domain consists of 2 β -sheets, which are responsible for interacting with serine-dependent recombinases in recombination (Safo *et al.*, 1997). The C-terminal domain of Fis forms a helix-turn-helix (HTH) DNA binding motif (composed of helices C and D), which recognises a very degenerate 16-bp consensus sequence (Finkel and Johnson, 1992). Fis preferably binds AT-rich DNA of low persistence length, can bend DNA through an angle of 40-90° on binding, and achieves this through an induced fit mechanism by insertion of the helix-turn-helix motif into the major groove of the DNA while simultaneously



Fig 1.8 Factors influencing the regulation of *fis* expression. The *fis* gene is part of the bicistronic *dusB-fis* operon. The expression of *fis* is negatively regulated by Fis and the stringent response alarmone, (p)ppGpp. The expression of *fis* is positively regulated by negative supercoiling (-ve) and IHF. In *Salmonella*, the *fis* promoter contains an ArcA element, required for expression of *fis* under anaerobic conditions. Not to scale.

compressing the intervening minor groove (Hengen *et al.*, 1997, Finkel and Johnson, 1992, Yuan *et al.*, 1991).

In its role as a transcription factor, Fis can have a positive or negative effect on the transcription of its gene targets, depending on where it binds, and its concentration. The binding of Fis to its targets is also dependent on the topological state of the bacterial nucleoid (Cameron and Dorman, 2012). In *Salmonella*, the Fis regulon encompasses approximately 1,500 genes including those involved in metabolism, motility and virulence (Wang *et al.*, 2013). Using electrophoretic mobility shift assays (EMSA), Kelly *et al.* (2004) showed that Fis directly binds to the promoters of *flhD*, *fliA*, and *fliC*, encoding genes of all three classes of the *Salmonella* flagellar biosynthesis pathway (Fitzgerald *et al.*, 2014). Further, Fis plays an important role in virulence in many species of bacteria. In *Salmonella*, Fis is an activator of *Salmonella* Pathogenicity Islands (SPI) 1, and 2. Fis directly activates the SPI-1 gene encoding the regulator HilA, and can bind directly to the promoters of *ssrA* and *ssaG* of SPI-2 (Kelly *et al.*, 2004, Schechter *et al.*, 2003). Further, Fis modulates the level of supercoiling during infection, an important factor for the timely expression of virulence genes (O Cr  in  n *et al.*, 2006). In Enteroinvasive *E. coli* and in *Shigella*, Fis is an activator of *virF*, an important regulatory protein involved in activating plasmid-borne virulence factors (Falconi *et al.*, 2001).

There are several methods by which Fis can regulate genes upon binding to DNA. Fis can directly activate genes by Class I activation, where Fis can directly contact the C terminal domain of one of the α subunits of holo-RNA polymerase, RpoD, recruiting it to a gene's promoter (Browning *et al.*, 2010). Fis can also activate genes by binding upstream of a promoter, altering the DNA conformation in such a way that it is more favourable for RNA polymerase interaction with the promoter (Opel *et al.*, 2004). In a similar manner, Fis can also repress genes by directly binding to the promoter, preventing access by RNA polymerase. Fis can also displace activators bound to promoters of genes, relieving expression, or bind in proximity of an activator, affecting its function by altering DNA conformation. A final method by which Fis represses transcription is through promoter jamming, whereby simultaneous binding of Fis and RNA polymerase prevent access to other RNA polymerase complexes (Browning *et al.*, 2010).

Fis also possesses the ability to constrain supercoils and stabilize DNA loops. Recent Hi-C analyses have indicated that Fis plays an important role in mediating long-range interactions in the bacterial chromosome (Lioy *et al.*, 2018).

1.1.4.2 Dps

Dps is one of three NAPs whose expression is associated with stationary phase in *E. coli* (Almiron *et al.*, 1992). However, *dps* expression can also be induced by oxidative stress, and *dps* expression can be observed in response to NaCl shock, nitric oxide shock, oxygen shock, anaerobic conditions as well as low Mg^{2+} in *S. Typhimurium* (Kroger *et al.*, 2013, Altuvia *et al.*, 1994). The expression of *dps* can therefore be dependent on either the housekeeping sigma factor, RpoD, or the stationary phase sigma factor RpoS (Fig 1.9). The NAPs Fis and H-NS are required for repression of *dps* during exponential growth (Grainger *et al.*, 2008). However, induction of the oxidative stress responsive protein OxyR leads to RpoD-dependent transcription of *dps* (Fig 1.9). In stationary phase, Fis levels are lower and repression of *dps* by Fis is relieved. Here, the NAP IHF, and RpoS are required to induce *dps* expression (Grainger *et al.*, 2008).

Dps is post-translationally regulated by the protease ClpXP during exponential growth, both by targeting of the protease to Dps directly, and by degradation of RpoS, responsible for *dps* induction in stationary phase (Stephani *et al.*, 2003). However, unlike for RpoS, where RssB is the recognition factor, ClpS acts as an adaptor protein for ClpXP-mediated degradation of Dps during exponential growth (Schmidt *et al.*, 2009, Becker *et al.*, 1999). The ClpAP protease stimulates the production of Dps in stationary phase by facilitating the translation of *dps* mRNA (Fig 1.9) (Stephani *et al.*, 2003).

The 19-kDa Dps protein folds into four-helix bundles, which together form a dodecameric shell. *In vitro* experiments have shown that immediately upon addition of DNA, Dps-DNA complexes self-aggregate and form a crystalline complex (Wolf *et al.*, 1999). Three lysine residues in the N-terminal domain are responsible for the formation of Dps-DNA complexes, and while deletion of two of these residues still allows DNA-binding activity, it is prohibitive to the condensation of DNA. DNase I footprinting experiments revealed that Dps has no DNA-binding specificity. However, more recent ChIP-seq and AFM analyses suggest that Dps may have a preference for secondary structures in DNA, such as inverted repeats, and Dps binding sites largely overlap with those of Fis (Antipov *et al.*, 2017, Melekhov *et al.*, 2015). Dps is a ferritin-like protein,

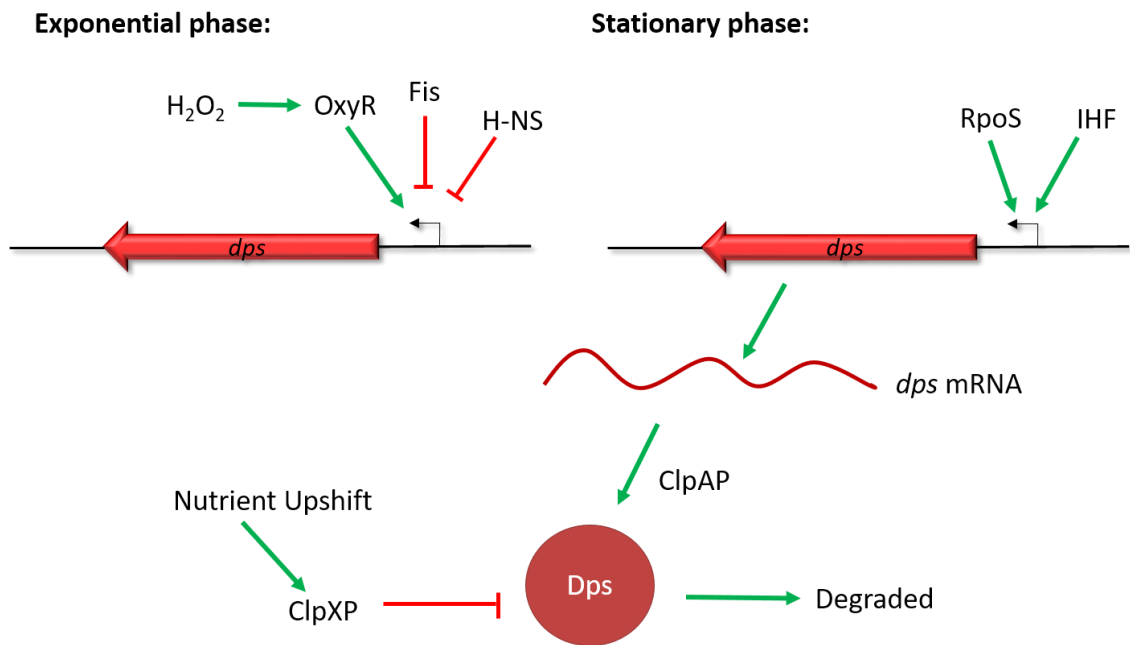


Fig 1.9. The dynamic regulation of *dps* expression and Dps production. During exponential growth Fis and H-NS bind to the *dps* promoter and prevent RpoD-mediated transcription. However, in response to oxidative stress OxyR binds to the *dps* promoter allowing RpoD-mediated transcription. In stationary phase, repression by Fis and H-NS is relieved. Binding of IHF to the *dps* promoter allows for RpoS-mediated expression of *dps*. The protease ClpAP facilitates the translation of *dps* mRNA. Upon nutrient upshift, Dps is rapidly degraded the ClpXP protease. Not to scale.

and beyond its ability to physically shield DNA, it reduces oxidising agents through its ferroxidase activity. Despite Dps' ability to bind and condense DNA, Dps is not known to be a regulator of gene expression. Antipov *et al.* (2017) observed some changes in gene expression. However, RNA-seq studies have shown that *dps* null mutants have few changes in global gene expression, and suggest that Dps has more of an influence on the proteome (Janissen *et al.*, 2018). Further, this study elucidated that Dps-DNA complexes were still accessible to RNAP holoenzyme and transcription factors, indicating that if Dps has a role as a regulator it is at a post-transcriptional level.

The main role of Dps is in shielding DNA from damaging agents, and *dps* null mutants have reduced survival rates in stationary phase, and when challenged with stresses such as pH, temperature and oxidative agents (Almiron *et al.*, 1992, Halsey *et al.*, 2004, Martinez and Kolter, 1997, Nair and Finkel, 2004). However, in *E. coli*, Dps also has a role in regulation of DNA replication, through its interactions with DnaA. Chodavarapu *et al.* (2008) observed that Dps can interact with the N-terminal domain of DnaA, and prevent unwinding of *oriC in vitro*. Further, Dps homologues are found in many bacteria, and largely have a role in oxidative stress resistance, either through their ferroxidase or DNA-binding activities (Haikarainen and Papageorgiou, 2010).

1.1.5 Gene Order

The physical location of a gene is important for gene expression in both prokaryotes and eukaryotes (Rockman *et al.*, 2010, Khvorova *et al.*, 2000). A recent analysis of 131 γ -Proteobacteria found a high conservation of synteny along the two replichores (Sobetzko *et al.*, 2012). Other studies have shown changes in gene expression when genes are moved from their native loci (Bryant *et al.*, 2014, Gerganova *et al.*, 2015). Further, Shaaban *et al.* (2016) observed that horizontally acquired DNA, such as that of prophages are generally integrated at an *oriC*-distal locations, indicative of an advantage of DNA integration at these positions. The conservation of gene order indicates that gene position is important for the timely expression of a gene, and several factors have been found to influence gene expression at a given location.

The first factor which influences gene expression at a given location on the chromosome is the gene dosage effect (Schmid and Roth, 1987, Sousa *et al.*, 1997). During exponential growth *oriC* fires multiple times. Consequently, the genes in the vicinity of *oriC* are of a higher copy number and are transcribed at a higher rate (Fig 1.10) (Khedkar and Seshasayee, 2016). The gene dosage effect is important for genes which require increased expression during exponential phase including those which encode machinery involved in transcription and replication such as the *rrn* clusters in *Salmonella* and *E. coli* (Gyorfy *et al.*, 2015, Couturier and Rocha, 2006). Additionally, the order of NAP-encoding genes along the two replichores approximately reflects their relative abundance throughout the growth cycle; *fis* and *hupA* are expressed early in the growth cycle, and these genes are located at a more *oriC*-proximal position than other NAP-encoding genes which are expressed later in the growth cycle (Sobetzko *et al.*, 2012). This observation is a key indicator that the position of NAP-encoding genes along the two replichores is important for their expression.

Gene orientation is also another important factor which may influence gene expression at a given location. In an actively replicating bacterium with two replisomes, there are up to 2,000 molecules of RNA polymerase, and so bacterial chromosomes have evolved in such a way to minimize collisions between transcription and replication machinery (Stracy *et al.*, 2015). Such collisions are

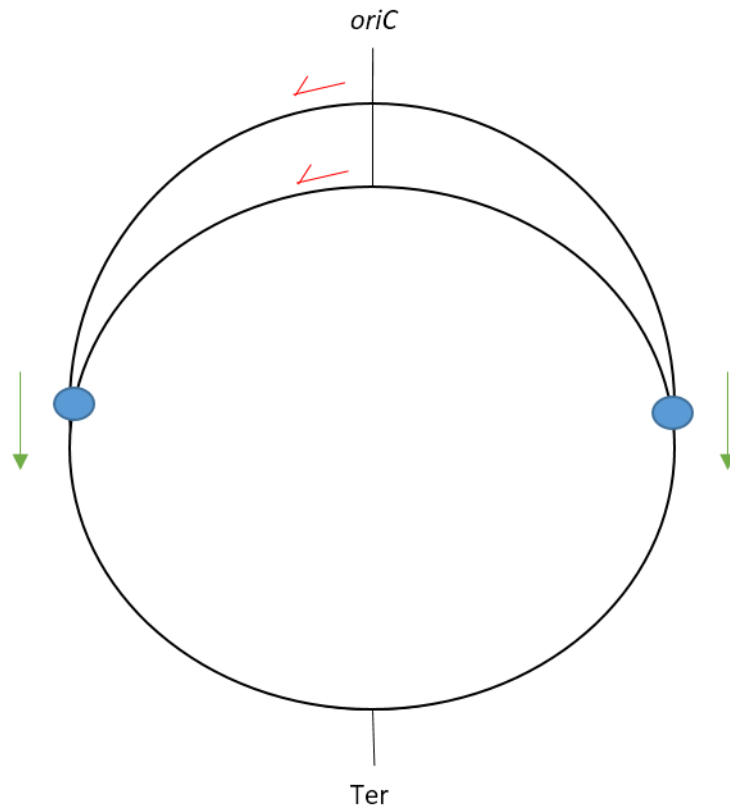


Fig 1.10. The gene dosage effect. During the cell cycle, genes in the proximity of *oriC* (indicated by the red arrows) are present in greater copy number than genes in the proximity of *Ter*. Blue circles, and green arrows indicate the replisome and direction of DNA replication, respectively.

undesirable as DNA polymerase would have a blocking effect on RNA polymerase as they both act on the same stretch of DNA, which results in stalling of the DNA or RNA polymerase complexes (Liu and Alberts, 1995). This can result in backtracking of RNA polymerase, the formation of RNA-DNA hybrids (R-loops), and double-stranded breaks in DNA (Helmrich *et al.*, 2013). If the direction of replication and transcription are the same, then DNA polymerase is far less likely to block transcription. In general, genes which lie in the same direction as the direction of DNA replication tend to be expressed to a higher degree than those which lie in a direction opposite to the direction of replication along a replicore (Zeigler and Dean, 1990, Price *et al.*, 2005). Studies which have reversed the orientation of *rrn* operons have shown detrimental effects on bacterial replication rates (Ivanova *et al.*, 2015, Srivatsan *et al.*, 2010).

The superhelicity of DNA is important for gene expression at a given location. In a negatively supercoiled state, the energy required to form an open complex by RNA polymerase is lowered, and the writhe of DNA can affect transcription factor binding (Dorman and Dorman, 2016). Lal *et al.* (2016) suggests that there is a gradient of negative supercoiling from Ori to Ter in *E. coli* in stationary phase that is not observed during exponential growth. However, recently another study examined the distribution of DNA gyrase cleavage sites across the *E. coli* chromosome (Sutormin *et al.*, 2019). This study found that DNA gyrase cleavage sites were found predominantly in the Ori and NS-Left regions, while Ter and Left macrodomains had fewer cleavage sites, and that DNA gyrase predominantly acted on positively supercoiled DNA, such as those introduced by RNA polymerase during transcription. Therefore, the orientation of neighbouring genes to a gene of interest is also important in gene expression at a given location on the chromosome. In general promoters can be convergent, divergent or in tandem. As RNA polymerase transcribes a gene it generates positive supercoils in front of it, and negative supercoils behind it (Liu and Wang, 1987). These local changes in supercoiling can affect the energy required for open complex formation in neighbouring genes.

Such has been seen at the well-characterised *leu-500* promoter which led to the discovery of *topA*, the gene encoding Topoisomerase I (Margolin *et al.*, 1985, Graf and Burns, 1973). The *leu-500* promoter is the promoter for the *leuABCD*

operon which carries an A to G mutation in the -10 box in *S. Typhimurium*. It was shown that this promoter was still active in a supercoiling-dependent state, such as when the divergent *leuO* was transcribed, generating the negative supercoils required for the activation of the *leu-500* promoter (Chen *et al.*, 1992, Wu *et al.*, 1995).

A final factor to consider with gene location is the proximity of its targets. In *Salmonella* and *E. coli* the genome normally lies with Ori and Ter macrodomains in the centre of the cell and the Right and Left macrodomains located towards either pole (Dame *et al.*, 2011). Gene products are often synthesized close to where the gene is located and the viscous cytoplasm can limit protein diffusion (Schavemaker *et al.*, 2018). As a result proteins which are synthesized at one side of the cell may act on more targets on that side of the cell, than on the opposite side. Such can be seen when taking into account the binding patterns of RpoD and RpoS. The gene encoding RpoD is located closer to *oriC* than that of RpoS, and RpoD binding targets tend to be located closer to *oriC*, while those of RpoS are located in the vicinity of Ter (Fig 1.5) (Sobetzko *et al.*, 2012).

The nucleotide composition of the leading and lagging strand is in such a way that G is more frequently found on the leading strand, and C is more frequently found on the lagging strand (Lobry, 1996). Deamination of cytosine causing C to T mutations is a driving force for the GC skew, and such can happen with ssDNA during DNA replication (Bhagwat *et al.*, 2016)

1.1.6 Nucleoid adaptations during the bacterial growth cycle

Bacteria must adapt to changes in the environment throughout the bacterial growth cycle. Different genes and pathways must be activated or repressed in order to deal with environmental stresses or nutrient limitations. To do this, bacteria have evolved a number of mechanisms including changes in DNA superhelicity, NAP composition, sigma factors, small signalling molecules and gene expression.

As nutrients are depleted, the ratio of ATP/ADP decreases, and consequently DNA gyrase activity is reduced. The result of this is a relaxation of DNA, and expression of another set of supercoiling sensitive genes (Dorman *et al.*, 1988, Schneider *et al.*, 1999, Blot *et al.*, 2006, Cameron *et al.*, 2011).

The composition and ratios of various NAPs change throughout the growth cycle. Fis is undetectable in stationary phase while production of Dps, CbpA, Lrp and IHF are induced (Mangan *et al.*, 2006, Ali Azam *et al.*, 1999). The production of Dps leads to a rapid condensing of the nucleoid in a manner in which gene expression is not affected, while IHF and Lrp are transcriptional regulators (Janissen *et al.*, 2018, Mangan *et al.*, 2006, Cho *et al.*, 2008a)

One of the most notable changes in protein production in stationary phase is the induction of the stress response sigma factor RpoS. RpoS is the stress response sigma factor and it regulates approximately 10% gene in *E. coli* (Weber *et al.*, 2005). Induction of RpoS is regulated at a transcriptional and translational level, and can occur through a multitude of regulatory cascades, depending on the particular stress (Battesti *et al.*, 2011). The RpoS protein is also subject to proteolysis by ClpXP through an adaptor protein, RssB (Schweder *et al.*, 1996, Zhou *et al.*, 2001). During exponential growth, Fis is a repressor of *rpoS* expression and binds at -50 relative to the *rpoS* promoter (Hirsch and Elliott, 2005b). Other proteins that negatively regulate *rpoS* expression include cAMP-CRP, and ArcA (Lange and Hengge-Aronis, 1994, Mika and Hengge, 2005). Induction of the stringent response results in increased levels of *rpoS* mRNA and transcriptional fusion studies indicate that (p)ppGpp acts has a stabilising influence on *rpoS* mRNA (Hirsch and Elliott, 2002). The RNA chaperone Hfq and trans-encoded sRNAs also act on *rpoS* regulation at the mRNA level (Brown and

Elliott, 1997). The 5'-UTR of *rpoS* forms a stable hairpin structure that occludes the ribosome binding site and minimizes translation (Cunning *et al.*, 1998). Hfq simultaneously stabilises *rpoS* mRNA and recruits three sRNAs, DsrA, RprA and ArcZ (Majdalani *et al.*, 1998, Majdalani *et al.*, 2002, Mandin and Gottesman, 2010). These sRNAs have complementary nucleotides to the 5'-UTR of *rpoS* mRNA, and stimulate translation by binding, removing the hairpin, allowing access for the translation machinery. A fourth sRNA, OxyS inhibits *rpoS* translation by titrating Hfq away (Zhang *et al.*, 1998).

Sigma factors recognise the -10 and -35 elements of promoter sequences, and variances in promoter sequence can exclude certain sigma factors from promoters. The housekeeping sigma factor, RpoD and RpoS recognise similar promoters, and many promoters that are dependent on either of these sigma factors can be read by the other *in vitro* (Tanaka *et al.*, 1993). One example of a promoter recognised by both RpoD and RpoS is the *dps* promoter (Fig.1.4) (Grainger *et al.*, 2008). As RpoD is bound preferentially by RNA polymerase, and intracellular RpoD concentrations remain higher than those of RpoS in stationary phase, a number of factors have been identified which either stimulate RpoS-RNA polymerase interactions or limit RpoD-RNA polymerase interactions. The Crl protein is required for maximum expression of the RpoS regulon, and it binds directly to RpoS, contributing to the interface recognised by RNA polymerase (Pratt and Silhavy, 1998, Cavaliere *et al.*, 2015). Conversely the 6S regulatory RNA binds to RpoD holoenzyme specifically in the region which recognises the -35 element downregulating RpoD targets which require a strong -35 element-RpoD interaction (Fig 1.11) (Cavanagh *et al.*, 2008). In a similar manner, the anti-sigma factor Rsd binds RpoD preventing its interaction with the core RNA polymerase (Jishage and Ishihama, 1998). This anti-sigma factor is also upregulated by (p)ppGpp (Durfee *et al.*, 2008). The factors influencing RpoD- and RpoS-dependent transcription, highlight the homeostatic balance involved in responding to environmental stimuli.

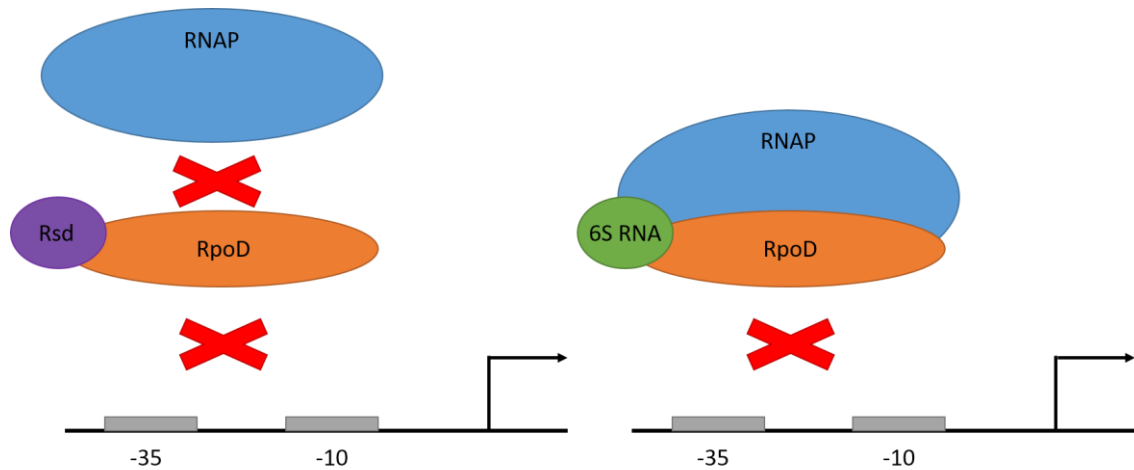


Fig 1.11 Mechanisms by which RpoD dependent transcription is inhibited.

Rsd binds directly to RpoD, preventing it from interacting with RNA polymerase (RNAP). This method frees up the available RNA polymerase core for RpoS. 6S RNA directly interacts with RpoD bound to RNA polymerase, preventing the holoenzyme from recognising promoter elements which require a strong interaction. RNA polymerase core is not made available for RpoS by this method.

1.1.7 Advances in synthetic biology

With our current knowledge on chromosome dynamics, the question arises as to what are the minimum requirements for a functional chromosome, and how should a synthetic chromosome, built for a specific purpose, be genetically organised? Several approaches have been used to aid our understanding of this. The world's first synthetic cells were created in 2010, by synthesizing the 1.08 Mbp genome of *Mycoplasma mycoides* and transferring it into *Mycoplasma capricolum* (Gibson *et al.*, 2010). Since then, Hutchison *et al.* (2016) used this synthetic cell's genome to generate a minimum genome of 531,560 bp containing only 473 genes. Of these 473 genes, 149 are of unknown function. Using a pre-existing genome as a scaffold is a useful tool in determining gene essentiality. The nucleoid structure has not been examined in this microbe, nor has it been assigned a function. However, this advance in synthetic biology paves the way for designing organisms for a specific function.

Other research has been conducted on the logistics of synthetic chromosomes. Schallopp *et al.* (2017) inserted the second chromosome of *Vibrio cholera* into *E. coli* in order to understand replication and folding of secondary chromosomes, so that in future a secondary chromosome can be used to give a bacterium a specific function. Together these studies are aiding our understanding as to what is essential for a synthetic chromosome, and what is required for optimum chromosome replication and gene expression.

As the study outlined in this thesis focuses on exchanging the positions of two NAP-encoding genes, it will contribute also to our basic understanding of chromosome biology, by providing insights into the conservation of gene order in *Salmonella*.

1.1.8 Aims

The gene positions of master transcriptional regulators and NAP-encoding genes have been highly conserved, and leads to the question as to why. Previous studies have shown how a change in gene position in the bacterial chromosome affects that gene's expression (Bryant *et al.*, 2014, Gerganova *et al.*, 2015). Recently, Fitzgerald *et al.* (2015) exchanged the ORFs of *hns* and *stpA*, and observed changes in bacterial competitive fitness, where the *hns-stpA* exchange strain could outcompete wild-type *S. Typhimurium* at 37°C, but was competitively weaker at lower temperatures. This exchange strain did not produce StpA, had upregulated H-NS concentrations and prematurely produced RpoS (Fitzgerald *et al.*, 2015). However, this study looked at an exchange of ORFs, and therefore *hns* was under *stpA* *cis*-regulatory elements, and *vice versa*. Therefore, Fitzgerald *et al.* (2015) examined more than just the effect of gene position, but also the effect of rewiring two orthologous NAP-encoding genes with each other's *cis*-acting regulatory sequences.

To build on this study, two NAP-encoding genes could be exchanged in two ways; one in which the ORFs alone are exchanged, and one in which the genes with their *cis*-acting regulatory genes are exchanged. The NAPs Fis and Dps differ in function. They are expressed at two different stages of growth, Fis is a transcriptional regulator, and Dps is not (Ali Azam *et al.*, 1999, Janissen *et al.*, 2018, Wang *et al.*, 2013). They interact with DnaA for opposite outcomes in the cell cycle (Kasho *et al.*, 2014, Chodavarapu *et al.*, 2008). Additionally, the *fis* and *dps* genes are located in the NS-Left and Right macrodomains, respectively. Within the nucleoid, these gene positions mean that they are physically located distal from each other, where NS-Left and Left are extended towards one pole, and NS-Right and Right are extended towards the other pole (Fig 1.12). It could therefore be hypothesized that the exchange of these two NAP-encoding genes in particular, would maximise the physiological consequences for the bacterium, therefore giving a greater understanding into the conservation of *fis* and *dps* position, expression, and contributions to bacterial physiology.

For this study, *S. Typhimurium* was used as a model organism, as previous work involving exchanging NAP-encoding genes have been performed in this

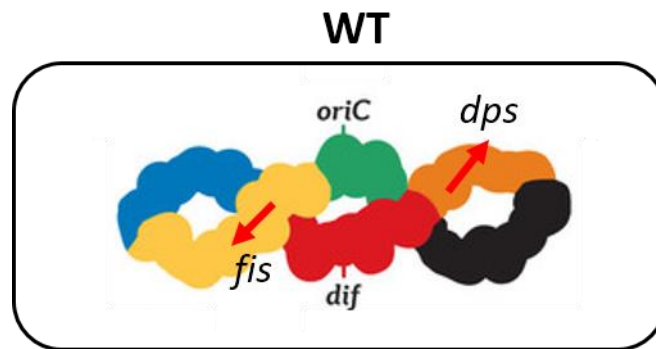


Fig 1.12. Locations of *fis* and *dps* genes within the nucleoid. In WT, the position of the *fis* gene is located in the NS-Left region, and *dps* is located in the Right macrodomain. Green represents Ori, yellow represents NS-Left, blue represents Left, red represents Ter, black represents Right, and orange represents NS-Right. Not to scale. Adapted from Dorman, 2013.

organism (Fitzgerald *et al.*, 2015). Additionally, mechanisms of virulence regulated by Fis are well characterised in *Salmonella*, and is representative of pathways which could be studied in detail in the *fis-dps* exchange strains (Cameron *et al.*, 2013, Wang *et al.*, 2013). Therefore, *S. Typhimurium* is represents a good model organism for this study.

The specific aims of this thesis are:

1. To exchange the positions of the NAP-encoding genes, *fis* and *dps*, producing one bacterial strain in which the genes and their *cis*-acting regulatory sequences are exchanged, and another in which the ORFs are exchanged.
2. To examine the effect of the change in *fis* and *dps* position on gene expression.
3. To examine the impact of the exchanges of *fis* and *dps* on global gene expression.
4. To determine how the exchanges of *fis* and *dps* affect global Fis binding patterns.
5. To elucidate how the exchanges of *fis* and *dps* influence bacterial physiology, by examining phenotypes associated with Fis and Dps functions.

Chapter 2:

Materials and Methods

2.1 Reagents, Strains, Media and Growth Conditions

All chemicals were of analytical grade and unless otherwise stated were purchased from Sigma-Aldrich (Dublin, Ireland). The water used throughout this study was sterile distilled water.

2.1.1 Strains

Bacterial strains used in this study were derivatives of *Salmonella enterica* serovar Typhimurium SL1344. Plasmids were propagated in *Escherichia coli* DH5 α . The strains, their genotype and source are listed in Table 2.1.

2.1.2 Media and Growth Conditions

Bacterial strains were routinely grown in Luria-Bertani media (1% tryptone, 0.5% yeast extract and 1% sodium chloride) supplemented with antibiotics where appropriate. Agar was used at a concentration of 1.5%. Bacterial strains were typically grown as 4 ml overnight cultures prior to subculturing in 25 ml Luria-Bertani broth and grown to desired growth phase. Bacterial strains were grown at 37°C, 200 rpm unless otherwise stated.

2.1.3 Monitoring Growth of Bacterial Cultures

The growth of bacterial cultures was monitored by measuring the optical density at a wavelength of 600 nm. 1 ml of culture was aliquoted into a plastic cuvette and the OD_{600 nm} was measured using the Thermo Scientific BioMate 3S spectrophotometer.

Table 2.1 Strains used in this study.

Strain	Genotype	Description
SL1344	<i>rpsL hisG46</i>	(Siddappa <i>et al.</i> , 2007)
SL1344 <i>fis::kan</i>	<i>fis::kan</i>	Kanamycin resistance cassette inserted 63 bp downstream of <i>fis</i> ORF
SL1344 <i>dps::cat</i>	$P_{dps}::cat$	Chloramphenicol resistance cassette inserted 149 bp upstream of <i>dps</i> ORF
SL1344 2xfG	$\Delta P_{dps} dps::P_{fis} dusBfis$	<i>dps</i> ORF and upstream/downstream regulatory regions replaced with <i>dusBfis</i> operon and regulatory regions
SL1344 2xdG	$\Delta P_{fis} dusBfis::P_{dps} dps$	<i>dusB</i> and <i>fis</i> ORFs and upstream/downstream regulatory regions replaced with <i>dps</i> ORF and regulatory regions
SL1344 2xfO	$\Delta dps::fis$	<i>dps</i> ORF replaced with <i>fis</i> ORF
SL1344 2xdO	$\Delta fis::dps$	<i>fis</i> ORF replaced with <i>dps</i>
SL1344 Δfis	$\Delta fis::cat$	(Kelly <i>et al.</i> , 2004)
SL1344 Δdps	Δdps	<i>dps</i> ORF deletion
SL1344 GX	$\Delta P_{dps} dps::P_{fis} dusBfis$ $\Delta P_{fis} dusBfis::P_{dps} dps$	Exchange of <i>dusBfis</i> and <i>dps</i> and their <i>cis</i> regulatory regions
SL1344 OX	$\Delta dps::fis \Delta fis::dps$	Exchange of <i>fis</i> and <i>dps</i> ORFs
SL1344 <i>fis::8xmyc</i>	<i>fis::8xmyc</i>	Deletion of the <i>fis</i> stop codon and insertion of 8 copies of the Myc epitope tag separated by a 21 bp linker.

SL1344 GX <i>fis::8xmyc</i>	GX <i>fis::8xmyc</i>	Deletion of the <i>fis</i> stop codon and insertion of 8 copies of the Myc epitope tag separated by a 21 bp linker.
SL1344 OX <i>fis::8xmyc</i>	OX <i>fis::8xmyc</i>	Deletion of the <i>fis</i> stop codon and insertion of 8 copies of the Myc epitope tag separated by a 21 bp linker.
SL1344 <i>PprgH</i>	<i>PprgH::gfp[LVA]/R::cat</i>	Destabilised version of GFP fused to SPI-1 promoter, <i>PprgH</i> (Ibarra <i>et al.</i> , 2010)
SL1344 GX <i>PprgH</i>	<i>PprgH::gfp[LVA]/R::cat</i>	Destabilised version of GFP fused to SPI-1 promoter, <i>PprgH</i>
SL1344 OX <i>PprgH</i>	<i>PprgH::gfp[LVA]/R::cat</i>	Destabilised version of GFP fused to SPI-1 promoter, <i>PprgH</i>
SL1344 2xfG <i>PprgH</i>	<i>PprgH::gfp[LVA]/R::cat</i>	Destabilised version of GFP fused to SPI-1 promoter, <i>PprgH</i>
SL1344 2xdG <i>PprgH</i>	<i>PprgH::gfp[LVA]/R::cat</i>	Destabilised version of GFP fused to SPI-1 promoter, <i>PprgH</i>
SL1344 2xfO <i>PprgH</i>	<i>PprgH::gfp[LVA]/R::cat</i>	Destabilised version of GFP fused to SPI-1 promoter, <i>PprgH</i>
SL1344 2xdO <i>PprgH</i>	<i>PprgH::gfp[LVA]/R::cat</i>	Destabilised version of GFP fused to SPI-1 promoter, <i>PprgH</i>
SL1344 Δfis <i>PprgH</i>	<i>PprgH::gfp[LVA]/R::cat</i>	Destabilised version of GFP fused to SPI-1 promoter, <i>PprgH</i>
SL1344 Δdps <i>PprgH</i>	<i>PprgH::gfp[LVA]/R::cat</i>	Destabilised version of GFP fused to SPI-1 promoter, <i>PprgH</i>
SL1344 <i>PssaG</i>	<i>PssaG::gfp[LVA]/R::cat</i>	Destabilised version of GFP fused to SPI-2 promoter, <i>PssaG</i> (Ibarra <i>et al.</i> , 2010)

SL1344 GX PssaG	PssaG::gfp[LVA]/R::cat	Destabilised version of GFP fused to SPI-2 promoter, PssaG
SL1344 OX PssaG	PssaG::gfp[LVA]/R::cat	Destabilised version of GFP fused to SPI-2 promoter, PssaG
SL1344 2xfG PssaG	PssaG::gfp[LVA]/R::cat	Destabilised version of GFP fused to SPI-2 promoter, PssaG
SL1344 2xdG PssaG	PssaG::gfp[LVA]/R::cat	Destabilised version of GFP fused to SPI-2 promoter, PssaG
SL1344 2xfO PssaG	PssaG::gfp[LVA]/R::cat	Destabilised version of GFP fused to SPI-2 promoter, PssaG
SL1344 2xdO PssaG	PssaG::gfp[LVA]/R::cat	Destabilised version of GFP fused to SPI-2 promoter, PssaG
SL1344 Δfis PssaG	PssaG::gfp[LVA]/R::cat	Destabilised version of GFP fused to SPI-2 promoter, PssaG
SL1344 Δdps PssaG	PssaG::gfp[LVA]/R::cat	Destabilised version of GFP fused to SPI-2 promoter, PssaG
SL1344 <i>cmr</i>	SL1483::cat	Insertion of chloramphenicol resistance cassette into the pseudogene SL1483
SL1344 GX <i>cmr</i>	SL1483::cat	Insertion of chloramphenicol resistance cassette into the pseudogene SL1483
SL1344 OX <i>cmr</i>	SL1483::cat	Insertion of chloramphenicol resistance cassette into the pseudogene SL1483
DH5 α	<i>fhuA2 lacΔU169 phoA glnV44 Φ80' lacZΔM15 gyrA96 recA1 relA1 endA1 thi-1 hsdR17</i>	(Taylor <i>et al.</i> , 1993)

2.2 Plasmids, Bacteriophage and Oligonucleotides

2.2.1 Plasmids

All plasmids used throughout this study are listed in Table 2.2. Plasmids were maintained by using either the antibiotic Carbenicillin or Chloramphenicol at concentrations of 100 µg/ml and 25 µg/ml respectively.

2.2.2 Bacteriophage

Bacteriophage P22 HT 105/1 *int-201* was used in this study for all genetic transductions. Stocks of bacteriophage were sterilised using a 0.2 µm PES filter (Millex, Cork, Ireland) prior to storage at 4°C in the dark.

2.2.3 Oligonucleotides

Oligonucleotides used in this study were purchased from IDT technologies (Leuven, Belgium). The sequences of oligonucleotides used in this study are listed in Table 2.3.

2.3 Genetic Techniques

2.3.1 Determination of nucleic acid concentration by spectroscopy

Nucleic acid concentration was determined by measuring the absorbance at 260 nm using a DeNovix DS-11 spectrophotometer.

Table 2.2 Plasmids used in this study.

Name	Selective Agent	Source
pKD3	Amp, Cat	Dorman Research Group
pKD4	Amp, Kan	Dorman Research Group
pKD46	Amp	Dorman Research Group
pCP20	Amp, Cat	Dorman Research Group
pUC18	Amp	Dorman Research Group
pBlueScript II SK (+)	Amp	Dorman Research Group
pBlueScript II SK (+)::P _{fis} <i>dusBdp_{scat}</i>	Amp	This study

Amp = ampicillin (100 µg/ml)

Cat = chloramphenicol (35 µg/ml)

Kan = kanamycin (50 µg/ml)

Table 2.3 Oligonucleotides used in this study.

Name	Sequence
fisVal-F	GCTGTCCGGGTTGTTCTG
fisVal-R	ACCAAATTCCATGTGATGCGT
dpsVal-F	CAGTATGCCGCACCGTTT
dpsVal-R	GCGCTATTACTTCGTCATTTTTTGT
fiskanInsert-F	TAAAAAGGCGCTACTCGGCATGGGGAAGCGCC TTTTTTATGTGCCACCTGCATCGATGGC
fiskanInsert-R	TTCACATTCCGCTTTTCATGACCAAATTCCATGTG ATGCGTCATATGAATATCCTCCTTAG
dpscmInsert-F	GACGAGTTTGCTGTTTGGTGGGTAATAATTCTC ATTTTAAGTGTAGGCTGGAGCTGCTTC
dpscmInsert-R	TACTTCGTCATTTTTTGTTCATTTTTTCTCATTT TTTACCATATGAATATCCTCCTTA
fisReg-F	GTCATTTTTTGTTCATTTTTTCTCATTTTTTACAT TAAAGTGATCTTGTCCCTGAAAC
fisReg-R	CAAATAATTCACCTTTTGTCCGAGGGGAGTACAA GACGTGTCATATGAATATCCTCCTTAG
fisORF-F	TTAATTACCTGGGACACAAACATCAAGAGGATAT GAGATTATGTTCGAACAACGCGTA
dpsReg-F	TTCACATTCCGCTTTTCATGACCAAATTCCATGTG ATGCGTCAAGACGTGTGCACTATT
dpsReg-R	CTTCCGACTGGCAATGGAGAAAAATCACGCGC AGCGGGAACATATGAATATCCTCCTTAG
pBLUE_BB_F	CCAGCTTTTGTTCCTTTAG
pBLUE_BB_R	CCCAATTCGCCCTATAGTG
pBLUE_dusB_F	GTGAATTGTAATACGACTCACTATAGGGCGAATT GGGCGATTCATTGATCTACAACA
pBLUE_dusB_R	GATTAGACGCTTTTGTTCCTTTTACCAGTTTAGCGGT ACTCATAGTTCTGTGTCAGCTCTTTATTTT
pBLUE_dps_F	GAAAATTTTTCGTAAACAGAAATAAAGAGCTGA CAGAACTATGAGTACCGCTAAACTGGT
pBLUE_dps_R	TAGGAACCTTCGGAATAGGAACTAAGGAGGATAT TCATATGCAAGACGTGTGCACTATTTA
pBLUE_cat_F	TGGCACAGGGGTTTTGCACTTAAATAGTGCACA CGTCTTGTCATATGAATATCCTCCTTAG
pBLUE_cat_R	AAGCTCGAAATTAACCCTCACTAAAGGGGAACAA AAGCTGGGTGCCACCTGCATCGATGGC

fis8mycins_F	GGTACGCTGCGTAAAAAATTAAAAAATACGGC ATGAACGTCCGGATCCAGTCTTCGTGAT
fis8mycins_R	GAGTAGCGCCTTTTTAAACAAGCAGTTAGCTAA TCGAAAAATTCCGGGGATCCGTGACC
pBLUE_chq_F	GACGTTGTAAAACGACGGCC
pBLUE_chq_R	CGGCTCGTATGTTGTGTGG
SLiCE_dpsORF_F	ATTTAATGAATTATAAATTTATTTTGCTGTTTTTA AGCACGATTCATTGATCTACAACA
SLiCE_dpsORF_R	TTCACATTCCGCTTTCATGACCAAATTCCATGTG ATGCGTGTGCCACCTGCATCGATGGC

2.3.2 Isolation of nucleic acid

2.3.2.1 Isolation of plasmid DNA

Plasmid DNA was isolated from *Salmonella* and *Escherichia coli* using the QIAprep Spin miniprep kit (QIAGEN, Manchester, UK). For both high (300-700 copies per cell) and low (10-50 copies per cell) copy number plasmids 25 ml of culture was typically processed according to the manufacturers' guidelines, and eluted in 20-50 µl sterile distilled water. This method typically gave yields of 5-10 µg plasmid DNA.

2.3.2.2 Isolation of chromosomal DNA for polymerase chain reaction

Bacterial chromosomal DNA was quickly isolated by harvesting 400 µl overnight culture by centrifugation at 16,000 x g for 1 min. The cell pellet was resuspended in sterile distilled water and boiled at 100°C for 5 min, vortexed and cell debris was pelleted by centrifugation again. The supernatant was mixed with 1 volume of chloroform. The upper aqueous layer was isolated by centrifugation at 16,000 x g for 10 min. The concentration was determined as described in Section 2.3.1 and adjusted to 100 ng/µl prior to storage.

2.3.2.3 Isolation of chromosomal DNA for whole genome sequencing

Chromosomal DNA for whole genome sequencing was prepared by a phenol-chloroform method which caused minimal shearing to DNA as previously described (Sambrook and Russell, 2006b). Briefly, 2 ml of an overnight culture was harvested by centrifugation at 16,000 x g and the cell pellet was resuspended in TE pH 8.0 (100 mM Tris-HCl pH 8.0, 10 mM EDTA pH 8.0). Lysis was performed by the addition of 1% SDS, 2 mg/ml proteinase K and incubating at 37°C for 1 h. DNA was isolated by the addition of 1 volume of phenol pH 8.0: chloroform : isoamyl alcohol (25 : 24 : 1), followed by centrifugation at 16,000 x g to separate the aqueous and organic layers. Following this, 1 volume of chloroform was added to the aqueous layer and centrifugation was repeated. To remove contaminants 0.3 M sodium acetate pH 5.2 and 5 volumes of 100% ethanol were added and precipitated at -20°C for 1 h. The DNA was pelleted by

centrifugation, washed once with 70% ethanol, dried at 65°C for up to 5 min and resuspended in 50 µl sterile distilled water. DNA concentration was determined as described in 2.3.1.

2.3.2.4 Isolation of RNA for RNA-seq

Once at the desired OD_{600 nm}, bacterial cultures were treated with 40% transcription stop solution (5% phenol pH 4.3 in ethanol) at 4°C for 30 min. The bacterial pellets were harvested by centrifugation at 3,220 x g for 10 min. The bacterial pellet was dissolved in TE pH 8.0 (100 mM Tris-HCl pH 8.0, 10 mM EDTA pH 8.0) containing 0.5 mg/ml lysozyme. Lysis was carried out by the addition of 1% SDS and 0.1 mg/ml proteinase K while incubating at 40°C for 20 min. RNA was isolated by the addition of 1 volume of phenol pH 4.3 : chloroform (1 : 1), followed by centrifugation at 16,000 x g to separate the aqueous and organic layers. Following this, 1 volume of chloroform was added to the aqueous layer and centrifugation was repeated. To remove contaminants 0.3 M sodium acetate pH 6.5 and 5 volumes of 100% ethanol were added and precipitated at -20°C for 1 h. The RNA was pelleted by centrifugation, washed once with 70% ethanol, dried at 65°C for up to 5 min and resuspended in 50 µl DEPC treated water. DNA concentration was determined as described in Section 2.3.1.

2.3.2.5 Isolation of RNA for quantitative PCR

Once at the desired OD_{600 nm}, 1 OD unit was harvested by centrifugation, and bacterial cultures were treated with 40% transcription stop solution (5% phenol pH 4.3 in ethanol) at 4°C for 30 min. The bacterial pellets were harvested by centrifugation at 3,220 x g for 10 min. Cell pellets were resuspended in TE pH 8.0 containing 50 mg/ml lysozyme. From this point RNA was isolated using an SV Total RNA Isolation kit (Promega, Wisconsin, USA.) as per manufacturer's instructions.

2.3.3 Template preparation for colony polymerase chain reaction

For routine colony polymerase chain reactions, a single colony was transferred to 200 µl sterile distilled water and lysed by boiling for 10 min at 100°C.

2.3.4 Polymerase chain reaction (PCR)

Polymerase chain reactions (PCR) were first performed *in silico* using the Serial Cloner software (serialbasics.free.fr). Standard PCRs were carried out in a 10 µl reaction volume containing 100 µM dNTPs, 0.2 µM of each oligonucleotide primer (Table 2.3), 1x Taq DNA polymerase buffer, 1 U Taq DNA polymerase (New England Biolabs, Ipswich, MA, USA), 200 ng DNA template or 1 µl of colony template described in Section 2.3.2 and sterile distilled water to 10 µl. High fidelity PCRs were carried out in 50 µl reactions using 1 U high fidelity Phusion DNA polymerase (New England Biolabs) and 1x high fidelity Phusion DNA polymerase buffer. All other components were the same as in the standard PCR reaction. Cycling conditions for PCR reactions were as follows; 95°C for 5 min, followed by 35 cycles of 95°C for 30 s, 50-60°C for 30 s, 72°C for 1-3 min, followed by 1 cycle of 72°C for 7 min.

2.3.5 Purification of polymerase chain reaction DNA products

Purification of PCR products was required for sequencing and transforming products into electro-competent bacteria. PCR products were purified using the QIAquick PCR purification kit (QIAGEN) as per manufacturers' guidelines. Briefly, 5 volumes of buffer PV (5 M Gu-HCl, 30% isopropanol) was added to 1 volume of PCR product and the pH was adjusted using 3 M sodium acetate. The mixture was added to a QIAquick spin column and washed with an ethanol buffer (10 mM Tris-HCl pH 7.5, 80% ethanol), dried and eluted in 30 µl sterile distilled water. DNA concentration was determined as described in Section 2.3.1.

2.3.6 DNA agarose gel electrophoresis

Depending on the sample size gel electrophoresis was performed using 1-2% agarose gels. Agarose gels were made using 1xTAE (40 mM Tris, 20 mM acetic acid, 1 mM EDTA) and immersed in 1xTAE buffer once set. DNA was mixed with 10x loading dye (50% glycerol, 0.1% bromophenol blue) before loading onto the gel. Electrophoresis was carried out at 100 V for 30 min and stained for 20 min in GelRed Nucleic Acid Gel Stain (Biotium, Fremont, CA, USA). Agarose gels were imaged using the Alphamager 2200 UV based imaging system. The molecular size marker used was the HyperLadder 1 kb (Bioline, London, UK).

2.3.7 Sequencing of DNA

2.3.7.1 Sequencing of plasmid DNA and PCR products

DNA sequencing of plasmid DNA and PCR products was carried out by Source BioScience (Waterford, Ireland) with the Sanger method of using fragment specific primers. The resultant sequences were compared to the expected sequence using the computer program SnapGene software (from GSL Biotech; available at snapgene.com).

2.3.7.2 Whole genome sequencing

Whole genome sequencing was performed by the Sanger Institute (Cambridge, UK) and MicrobesNG (Birmingham, UK) using Illumina next generation sequencing technology. The resulting reads were assembled against a reference SL1344 genome using the computer program BreSeq (Deatherage and Barrick, 2014).

2.3.8 Restriction digest of DNA

Unless otherwise stated, all restriction digests were carried out using NEB buffer #2 (NewEngland Bioscience). Restriction digests were carried out in 50µl reactions containing 1x restriction enzyme buffer, 100µg/ml BSA, 10U restriction

enzyme, 1µg template DNA. Digestion was carried out at 37°C for 1h. Digestion product was treated with 2U shrimp alkaline phosphatase (Roche) for 1h at 37°C. Enzymes were heat inactivated at 65°C for 20min. Digested DNA was recovered by ethanol precipitation as previously described. DNA concentration was determined as described in Section 2.3.1.

2.3.9 Quantitative polymerase chain reaction

Quantitative polymerase chain reaction (qPCR) was performed by a 96 well plate method using the StepOnePlus Real Time PCR system and analysed with the accompanying computer software. Each 20 µl reaction contained 1x FastStart Universal SYBR Green Master (Rox) (Merck, Wicklow, Ireland), 0.6 µM each primer pair 8 µl DNA and sterile distilled water to 20 µl. For each primer pair a standard curve of 10-fold serially diluted DNA concentrations was included, to which the concentrations of unknown samples could be determined. A melt curve was included each time a new primer pair was used. Cycle conditions for qPCR reactions were as follows; 95°C for 10 min, followed by 40 cycles of 95°C for 15s, 60°C 1 min. Oligonucleotide primer pairs used in qPCR are listed in Table 2.4.

2.4 *In vivo* Genetic Techniques

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

2.4.1.1 Preparation of high-titre bacteriophage P22 lysates

Overnight cultures of *Salmonella* were diluted 1:1000 into 25 ml Luria-Bertani broth and grown until OD_{600 nm} of 0.1, prior to the addition of 20 µl of high-titre P22 HT 105/1 *int-201* stock which was a multiplicity of infection of ~1. The culture was incubated for a further 4 h at 37°C, 200 rpm after which cell lysis was clearly visible. At this point, 500 µl chloroform was added and the lysed culture was left to stand at room temperature for 10 min to kill any remaining cells. The cell debris was pelleted by centrifugation at 3,220 x g, and the supernatant was passed through a 0.2 µm PES filter prior to storage at 4°C in the dark. Typical phage titres

Table 2.4 Oligonucleotides used for qPCR.

Name	Sequence
RT_fis_F	TGACGTACTGACCGTTTCTACCGT
RT_fis_R	ACGTCCTGACCATTCAGTTGAGCA
RT_dps_F	CACTGACCGATCATCTGGATAC
RT_dps_R	CGGATAGCTTTTCAGTGGAGTT
RT_hemX_F	CGCCTGACGGTATGTTTCTT
RT_hemX_R	CCCAACCAGGACGTCTATTTAC
RT_rpoS_F	TGAATACCCAATCTCACCAAGG
RT_rpoS_R	AGAACGGAGTAGAGGCTTTTG
RT_gyrB_F	CTCGTTCAGCTCGGTAATCAG
RT_gyrB_R	ATGATTGGTCGTATGGAGCG
RT_prpoS_F	TGAGCCCTCAGAATACGTAATTG
RT_prpoS_R	ATCCGGTACGCCAATTACTG
RT_pcarA_F	ATCCGGCTTAACTTCAGAATGC
RT_pcarA_R	CCGTCTTCCAGAACCAATAGC
RT_gidA_F	CAGATTCGGCTGATTCTCCA
RT_gidA_R	TCAGGCAGGTATTCAGTTTAGG
RT_STM1554_F	TTTAGACGCCCGCACTTC
RT_STM1554_R	TAGCTCTCCCGAGTTAGATAATCA

by this method 10^9 - 10^{10} p.f.u. and were determined by spotting 10-fold serial dilutions on an agar plate lawned with wild-type SL1344.

2.4.1.2 Transduction by bacteriophage P22

Transduction by bacteriophage P22 was routinely done during strain construction as for marker rescue and combining mutations or fusions into single strains. All mutant or fusion strains constructed in this study were linked to an antibiotic resistance cassette as a means for selection of the desired mutant. Transductions were performed by first serially diluting the phage to 10^{-2} and combining each dilution with the recipient *Salmonella* overnight culture in a 1 : 1 ratio. Controls containing only bacteriophage and only bacteria were included. Samples were incubated statically at 37°C for 1 h prior to spreading on Luria-Bertani agar plates containing appropriate antibiotic selection and incubated for 12-16 h at 37°C. Colonies from the plate of highest phage dilution were selected for phage clearance.

2.4.1.3 Clearance of bacteriophage P22

To ensure *Salmonella* transduced with bacteriophage contained no pseudolysogens colonies were single colony purified on green agar plates (Luria-Bertani agar with 2 μ M alizarin yellow, 0.84% glucose, 6.6×10^{-3} % aniline blue) with appropriate antibiotic selection, and incubated at 37°C for 12-16 h. A single light green colony was selected and re-streaked again on green agar and incubated in the same manner.

2.4.2 Transformation of *Salmonella* with plasmid DNA

2.4.2.1 Preparation of electrocompetent cells for transformation with plasmid DNA

Overnight cultures of *Salmonella* were diluted 1:100 in 12 ml Luria-Bertani media and grown to an OD_{600 nm} of 0.4. Cells were harvested by centrifugation at 3,220 x g for 10 min, 4°C. The cells were washed once with 10 ml cold sterile distilled

water and 3 times with 1 ml sterile distilled water, after which the cells were finally resuspended in 200 µl sterile distilled water. Cells were used immediately for electroporation.

2.4.2.2 Electroporation of electrocompetent cells

To a pre-chilled electroporation cuvette with a gap width of 2 mm (Cell Projects, Kent, UK), 200-500 ng plasmid DNA and 50 µl electrocompetent cells were added. The cuvette was wiped of any moisture prior to placing in the Gene Pulser chamber (Bio-Rad, Dublin, Ireland). An electrical pulse was administered for 4-5 ms with the Gene Pulser set to deliver 2.5 kV from a 25 µF capacitor with a Pulse Controller resistance of 200 Ω. Immediately after electroporation, 1 ml of pre-warmed (37°C) Luria-Bertani broth was added to the cuvette, the cells were transferred to a test tube and recovered at 37°C for 1 h. After recovery, the cells were harvested by centrifugation, resuspended in 100 µl broth, spread onto Luria-Bertani agar containing appropriate antibiotic selection and incubate at 37°C for 12-16 h. Controls containing no plasmid DNA were included.

2.4.3 Transformation of *Escherichia coli* with plasmid DNA

2.4.3.1 Preparation of chemically competent *Escherichia coli* DH5α

Overnight cultures of *Escherichia coli* were diluted 1 : 100 in 5 ml Luria-Bertani broth and grown to an OD_{600 nm} of 0.5. The cells were harvested by centrifugation at 3,220 x g and resuspended in 200 µl TSS solution (10% PEG 3350, 15 mM MgCl₂.6H₂O, 5% DMSO, LB to 50 ml). Chemically competent cells were stored at -80°C or used immediately.

2.4.3.2 Chemical transformation of chemically competent DH5α

To transform chemically competent DH5α with plasmid DNA 100µl recipient cells (Section 2.4.3.1) were mixed with 50-150 ng plasmid. Samples were incubated on ice for 30 min, heat shocked for 45 s at 42°C and 1 ml of pre-warmed Luria-Bertani broth was added. The cells were recovered at 37°C, 200 rpm for 1-2 h.

After recovery, the cells were harvested by centrifugation, resuspended in 100 μ l broth, spread onto Luria-Bertani agar containing appropriate antibiotic selection and incubate at 37°C for 12-16 h. Controls containing no plasmid DNA were included.

2.4.4 λ -red mediated homologous recombination

2.4.4.1 Induction of γ , β and *exo* genes

λ -red mediated homologous recombination was used to create mutant and fusion strains derived from SL1344 as previously described. The temperature sensitive plasmid pKD46 was first transformed into the strain which was to be modified by the λ -red system as described in Section 2.4.2, with recovery and growth temperatures of 30°C after the transformation. Overnight cultures of the strain harbouring pKD46 were subcultured 1:100 into 50 ml Luria-Bertani broth containing 100 μ g/ml carbenicillin and 100 mM arabinose and grown at 30°C, 200 rpm to an OD_{600 nm} of 0.5.

2.4.4.2 Preparation of linear exogenous DNA

Linear DNA that was to be incorporated into the bacterial chromosome was amplified by PCR as described in Section 2.3.4. Oligonucleotide primers were designed to amplify the DNA to be integrated with 40 bp overhangs homologous to the region of integration.

2.4.4.3 Preparation of electrocompetent cells for λ -red mediated homologous recombination

Overnight cultures of the recipient strain were diluted 1:100 in 50 ml Luria-Bertani broth and grown to an OD_{600 nm} of 0.5. Cells were harvested by centrifugation at 3,220 x g for 20 min at 4°C. The following wash steps were carried out, incubating on ice for 20 min and centrifuging at 3,220 x g for 20 min at 4°C between each wash; 50 ml chilled sterile distilled water, 25 ml sterile distilled water and 5 ml 10% glycerol, before finally resuspending in 500 μ l 10% glycerol. Cells were

electroporated as described in Section 2.4.2.2 using up to 1 µg linear exogenous DNA. Controls including no DNA were included.

2.4.4.4 Determining integration of linear exogenous DNA

Candidate colonies were toothpicked onto agar plates containing appropriate antibiotic selection and grown for 16 h at 37°C. Each colony was screened by PCR to determine whether DNA integration was successful. Oligonucleotide primers were designed against sequences upstream and downstream of the locus of interest and PCR was carried out as described in Sections 2.3.3 and 2.3.4. The PCR products were electrophoresed as described in Section 2.3.6 and the product sizes were compared to those of the ancestral strain. By this method, a single colony could be selected and confirmed by Sanger sequencing (Section 2.3.7.1).

2.4.5 Seamless ligation cloning extract (SLiCE)

2.4.5.1 Preparation of SLiCE extract

SLiCE was performed as previously described (Zhang *et al.*, 2014). An overnight culture of *E. coli* strain TOP10 harbouring pKD46 was subcultured 1:100 in YT media supplemented with appropriate antibiotics and grown at 30°C to an OD_{600 nm} of 2.5. Arabinose was added to a final concentration of 1 mM and the culture was grown for a further 2 h. Cells were harvested by centrifugation at 7,000 x g at 4°C, washed twice with cold sterile distilled water before finally resuspending in 500 µl Cellytic B. The cell suspension was incubated at room temperature for 10 min on the blood tube rotator SB1 (Stuart Scientific, Staffordshire, UK). The cell debris was pelleted by centrifugation at 20,000 x g for 1 min and the supernatant was mixed with 1 volume of 100% glycerol. SLiCE extract was stored at -20°C.

2.4.5.2 SLiCE reaction

Prior to the SLiCE reaction the inserts and the vector backbone were amplified by PCR with oligonucleotide primers with 40 bp regions of homology to the adjacent insert / vector, and subsequently purified (Sections 2.3.4 and 2.3.5). The vector backbone product was digested with DpnI to remove any remaining vector template as described in Section 2.3.8. SLiCE reactions were performed in a 10 µl reaction volume containing an equimolar amount of inserts and vector backbone, as well as 1x ligation buffer (NEB), 1 µl SLiCE extract (Section 2.4.5.1) and sterile distilled water. SLiCE reactions were incubated at 37°C for 1 h prior to transforming into chemically competent DH5α as described in Section 2.4.3.

2.5 Manipulation of RNA *in vitro*

2.5.1 Analysis of RNA integrity by gel electrophoresis

RNA integrity was assessed before and after DNase treatment by observation of large rRNA species using agarose-formaldehyde gel electrophoresis. Briefly, 1 µg RNA was mixed with 1x HT loading buffer (30 mM HEPES, 30 mM Triethanolamine, 400 mM formaldehyde, 0.5 mM EDTA, 0.02% bromophenol blue, 10% glycerol) and denatured at 65°C for 5 min, before cooling to room temperature. The denatured RNA was loaded onto a 2% agarose-HT gel containing 400 mM formaldehyde. The RNA was electrophoresed for 1.5 h at 60 V and stained in GelRed for 20 min prior to imaging using an Alphamager 2200.

2.5.2 DNase treatment of large quantities of RNA

For RNA yields >500 ng, RNA was diluted to 500 ng/µl, denatured at 65°C for 5 min and treated with 10 U RNase-free DNase I (ThermoFisher Scientific, Waltham, USA) in DNase 1 buffer at 37°C for 40 min. DNase treated RNA was isolated using a phenol chloroform method of isolation as described in Section 2.3.2.4.

2.5.3 DNase treatment of small quantities of RNA

For RNA yields under 500 ng, RNA was diluted to 200 ng/μl and DNase treated using a Turbo DNase kit (Invitrogen, California, USA). DNase treatment was performed using a 2-step incubation where half the DNase was added at the start of treatment and the other half after 30 min incubation at 37°C. The samples were incubated for a further 30 min prior to the addition of 0.2 volumes of DNase inactivation reagent. The samples were incubated for a further 5 min at room temperature and the DNase inactivation reagent was removed by centrifugation at 10,000 x g for 1.5 min. Using 200 ng of DNase treated RNA, endpoint PCR was used to ensure there was no DNA contamination. RNA integrity was examined by gel electrophoresis prior to cDNA synthesis.

2.5.4 Reverse transcription of RNA

RNA was reverse transcribed into cDNA using a GoScript Reverse Transcriptase kit (Promega) as per manufacturer's specifications, using 400 ng DNase treated RNA per reaction.

2.5.5 RNA sequencing

RNA-seq experiments were performed by vertis Biotechnologie AG (Freising-Weihenstephan, Germany). Briefly, samples were analysed by capillary electrophoresis, and rRNA was depleted using the Ribo-Zero rRNA Removal Kit for bacteria (Illumina). Ribodepleted RNA samples were fragmented using ultrasound (4x30 s pulses at 4°C). Oligonucleotide adapters were ligated to the 3' end of the RNA molecules. First-strand cDNA synthesis was performed using M-MLV reverse transcriptase and the 3' adapter as primer. The first-strand cDNA was purified and the 5' Illumina TruSeq sequencing adapter was ligated to the 3' end of the antisense cDNA. The resulting cDNA was PCR-amplified to about 10-20 ng/μl using a high fidelity DNA polymerase. The cDNA was purified using the Agencourt AMPure XP kit (Beckman Coulter Genomics) and was analyzed by capillary electrophoresis. For Illumina NextSeq sequencing, the samples were pooled in approximately equimolar amounts. The cDNA pool was size

fractionated in the size range of 200 - 550 bp using a differential clean-up with the Agencourt AMPure kit. An aliquot of the size fractionated pool was analyzed by capillary electrophoresis. Samples were PCR amplified for Truseq according to the instructions of Illumina. The cDNA pool was paired end sequenced on an Illumina NextSeq 500 system using 2 x 75 bp read length.

2.6 Chromatin immunoprecipitation with sequencing

2.6.1 Fixation of cells

Overnight cultures were normalised to a starting OD_{600 nm} of 0.003 in 25 ml Luria Bertani media and grown to an OD_{600 nm} of 0.4. Cells were harvested by centrifugation at 3,220 x g for 10 min at room temperature and resuspended in 50 ml PBS. Formaldehyde was added drop-wise to a final concentration of 1% while continuously stirring the cells. After 10 min of crosslinking cold glycine was added drop-wise to a final concentration of 0.125 M and the cells were stirred for a further 5 min.

2.6.2 Cell lysis and shearing of DNA

Crosslinked cells were harvested by centrifugation at 3,220 x g for 10 min at 4°C. The cell pellet was resuspended in 600 µl lysis buffer (50 mM Tris-HCl pH 8.1, 10 mM EDTA pH 8.0, 1% SDS, Roche protease inhibitor cocktail) and incubated on ice for 10 min, before the addition of 1.2 ml IP dilution buffer (20 mM Tris-HCl pH 8.1, 150 mM NaCl, 2 mM EDTA, 1% Triton X-100, 0.01% SDS, Roche protease inhibitor cocktail). Chromatin was sheared to an average length of 500 bp by sonicating for 30 s with an amplitude of 10 µm, twelve times, with a 1 min incubation on ice between bursts using a Soniprep 150. Sheared chromatin was analysed by agarose gel electrophoresis and stored at -80°C.

2.6.3 Pre-clearing chromatin with normal rabbit IgG

To pre-clear chromatin for input samples, 50 µl normal rabbit IgG (Millipore, Cork, Ireland) was added and samples were incubated for 1 h on a blood tube rotator

SB1 at 4°C. Antibody was removed by the addition of 100 µl homogeneous Protein G-Agarose (Roche) and a further incubation for 3 h. The Protein G-Agarose was removed by centrifugation at 3,220 x g for 2 min and 200 µl was used as input.

2.6.4 Immunoprecipitation of protein-DNA complexes

For all ChIP experiments a mock IP and an experimental IP were set up at the same time using 1,350 µl pre-cleared chromatin and 10 µg of normal mouse IgG (Millipore) and monoclonal mouse anti c-Myc respectively. Samples were incubated at 4°C for 16 h on a blood tube rotator SB1. 100 µl homogeneous Protein G-Agarose was added to each and the samples were incubated for a further 3 h. Protein G-Agarose beads were pelleted by centrifugation at 5,200 x g, washed 4 times with high salt IP wash buffer (50 mM HEPES pH 7.9, 500 mM NaCl, 1 mM EDTA, 0.1% SDS, 1% Triton X-100, 0.1% deoxycholate), and twice with TE pH 8.0. Protein-DNA complexes were eluted from the beads twice with IP elution buffer (100 mM NaHCO₃, 1% SDS).

2.6.5 Reversal of crosslinks

To reverse crosslinks between DNA-protein complexes, NaCl was added to the input, mock and experimental IP samples to a final concentration of 300 mM and samples were incubated for 6 h at 65°C. 90 mg proteinase K was added and the samples were incubated at 45°C for 16 h.

2.6.6 Extraction of DNA

DNA was extracted by the addition of 1 volume of phenol: chloroform: isoamyl alcohol (25: 24: 1), vortexed and the aqueous and organic layers were separated by centrifugation at 16,000 x g for 5 min. The aqueous layer was transferred to a new tube, 1 volume of chloroform was added, vortexed and centrifuged again. The aqueous layer was again transferred to a new tube. To precipitate the DNA, 5 µg glycogen, 300 mM sodium acetate pH 5.2 and 2.5 volumes 100% ethanol

were added and mixed well. DNA was precipitated at -20°C for 1 h. DNA was pelleted by centrifugation at 16,000 x g for 20 min, washed once with 70% ethanol, air-dried and resuspended in sterile distilled water.

2.6.7 ChIP-qPCR

Prior to sequencing, ChIP-qPCR was performed as a control. qPCR was performed as described in Section 2.3.9, diluting input samples 1: 50 and IP samples 1:5. Oligonucleotide primers were designed against promoters that Fis is known to bind to, and promoters which Fis does not bind to (Table 2.4)

2.6.8 ChIP sequencing

ChIP sequencing was performed by vertis Biotechnologie AG using Illumina NextSeq 500 technology. The DNA samples were first fragmented with ultrasound (2 pulses of 30 sec at 4°C). After end-repair, TruSeq sequencing adapters were ligated to the DNA fragments. Finally, the DNA was PCR-amplified to about 10-20 ng/μl using a high fidelity DNA polymerase. Aliquots of the PCR amplified libraries were examined by capillary electrophoresis. For Illumina NextSeq sequencing, the samples were pooled in approximately equimolar amounts. The DNA pool was eluted in the size range of 250 – 550 bp from a preparative agarose gel. An aliquot of the size fractionated library pool was analyzed by capillary electrophoresis. The adapters were designed for TruSeq sequencing according to the instructions of Illumina. The shotgun library pool was sequenced on an Illumina NextSeq 500 system using 75 bp read length.

2.7 Phenotypic assays

2.7.1 Growth curves and viable counts

Overnight cultures were normalised to a starting OD_{600 nm} of 0.0005 in 25 ml Luria Bertani media. OD_{600 nm} were measured hourly for 8 h, and a final measurement at 24 h. At each time point an aliquot of culture was taken and serially diluted so that when spread on LB agar plate, colony numbers were between 30 and 300.

2.7.2 Microscopy

Cultures were grown to mid-exponential phase or stationary phase in LB broth. Cells were harvested by centrifugation at 16,000 x g for 5 min and washed once with PBS, and resuspended in PBS. Cells were spread on a glass slide, allowed to dry and heat fixed. Saffron was used to stain the cells for one minute, and residual saffron was washed away with distilled water. Stained cells were observed at 1,000x using oil immersion microscopy on a Nikon Optiphot microscope and images were taken using the Nikon DS-Fi1 microscope camera.

2.7.3 Motility assays

Bacteria motility was measured by inoculating a 0.3% Luria Bertani agar plate with 1 µl overnight culture and incubating for 5 h at 37°C. For strains with an extended lag phase, the incubation was performed for a further 1 h. After these incubations the diameter of circular swimming was measured.

2.7.4 Resistance to oxidative stress assay

Resistance to oxidative stress assays were performed as described previously. Overnight cultures were diluted to approximately 10⁶ CFU/ml in PBS and exposed to 0, 0.5 or 1 mM hydrogen peroxide over 2 h, after which cells were spread on LB agar plates at an appropriate dilution, and the percentage survival was calculated.

2.7.5 Gene dosage assay

To quantify changes in gene dosage, chromosomal DNA was isolated during exponential growth, early stationary phase and late stationary phase as described in Section 2.3.2.2. Oligonucleotide primers were designed against an *oriC* proximal gene and a Ter proximal gene and qPCR was performed as described in Section 2.3.9. DNA quantities of genes of interest were then compared to those of the *oriC* and Ter proximal genes.

2.7.6 SPI reporter assays

SPI-1 (*PprgH-gfp*[LVA]/R) and SPI-2 (*PssaG-gfp*[LVA]/R) promoter fusions were integrated into the SL1344 by transduction by bacteriophage P22, and selected for on LB agar plates supplemented with 25 µg/ml chloramphenicol (Section 2.4.1) (Ibarra *et al.*, 2010, Sridhar and Steele-Mortimer, 2016). Overnight cultures of SPI-1 and SPI-2 reporter fusion strains were diluted 1:100 in LB. OD_{600 nm} and OD_{510 nm} were recorded every 20 min over a 24 h period using a Synergy H1 microplate reader (Biotek, Vermont, USA).

2.7.7 Competitive fitness assays

To measure bacterial competitive fitness the chloramphenicol resistance cassette was transduced (Section 2.4.1) into the pseudogene *SL1483* of each strain. The conversion of overnight culture OD_{600 nm} measurements to CFU/ml was elucidated for each strain. LB broth was inoculated with 1x10⁸ cells of each a chloramphenicol sensitive and a chloramphenicol resistant strain. Cells were plated onto LB agar with and without chloramphenicol at an appropriate dilution at 0 and 24 h timepoints. From this, the fitness index could be calculated as described previously (Lenski, 1991).

2.8 Protein assays

2.8.1 Preparation of protein lysates for routine use

Overnight cultures were subcultured 1:1,000 and grown to the desired OD_{600 nm}. One OD unit was harvested by centrifugation at 16,000 x g, and washed once with PBS. Cells were resuspended in laemmli buffer (2% SDS, 10% glycerol, 5% 2-mercaptoethanol, 0.002% bromophenol blue, 62.5 mM Tris-HCl pH 6.8), boiled for 10 min at 100°C, and stored at -20°C.

2.8.2 Preparation of protein lysates for quantitative Western immunoblotting

Overnight cultures were subcultured 1:1,000 and grown to the desired OD_{600 nm}. One OD unit was harvested by centrifugation at 16,000 x g, resuspended in 350 µl PBS and transferred to a sonication tube. Cells were lysed by sonicating for 10 s at an amplitude of 10 µm, repeated 10 times, with a 30 s incubation on ice between bursts.

2.8.2 SDS-PAGE

2.8.2.1 SDS-PAGE

SDS-PAGE gels were prepared to have a 12.5% resolving gel (12.5% acrylamide, 375 mM Tris-HCl pH 8.8, 0.001% SDS, 0.001% APS, 0.0001% Temed) and a 5% stacking gel (5% acrylamide, 82.5 mM Tris-HCl pH 6.8, 0.001% SDS, 0.001% APS, 0.0001% Temed). SDS-PAGE gels were immersed in running buffer (25 mM Tris, 190 mM Glycine, 0.1% SDS), and samples were boiled at 100°C for 10 min prior to loading. A broad-range prestained protein ladder (NEB) was used as a molecular marker. Samples were electrophoresed at 60 V through the stacking gel, and 130 V through the resolving gel.

2.8.2.2 Coomassie staining of SDS-PAGE gels

SDS-PAGE gels were washed in distilled water prior to staining. The gel was stained with Coomassie stain (0.1% Coomassie Brilliant Blue R-250, 50% methanol, 10% glacial acetic acid) for 1 h, while rocking on a belly dancer (Stovall Life Science, Greensboro, USA). The stain was removed, and 10% glacial acetic acid was added to destain. Fresh destain solution was added after 1 h and the gel was incubated for a further 16 h. The gel was washed with distilled water prior to imaging with an Alphaimager.

2.8.3 Western Immunoblotting

2.8.3.1 Transfer of protein to PVDF membrane

The wet transfer method was used to transfer total protein from the SDS-PAGE gel to a PVDF membrane of 0.2 μ m pore size. SDS-PAGE gels were first washed in dH₂O to remove excess SDS. The PVDF membrane was activated in 100% methanol for 1 min. The gel, PVDF membrane and 6 pieces of filter paper (all cut to the gel size) were incubated in 1x transfer buffer (20% methanol, 25 mM Tris, 190 mM glycine) for 10 min. The transfer sandwich was assembled in the order of 3 pieces of filter paper, PVDF membrane, PAGE gel, and 3 more pieces of filter paper. The transfer sandwich was electrophoresed for 1 h 45 min at 300 mA.

2.8.3.2 Blocking

Blocking of the membrane was performed for 1 h at room temperature with a 5% blocking solution (5% skimmed milk powder, PBST), while rocking.

2.8.3.3 Probing using protein-specific antibodies

Membranes were probed overnight at 4°C using the following primary concentrations; 1:50,000 Mouse- α -Dnak (cat. no. ab69617), 1:50,000 Mouse- α -C-Myc (cat. no. M4439), 1:2,000 Rabbit- α -Dps (serum kindly provided by Prof. Regine Hengge). All antibodies were diluted to the appropriate concentration in PBST containing 5% BSA. After use, primary antibodies were filter sterilised and stored at 4°C.

2.8.3.4 Probing with HRP-conjugated secondary antibodies

Membranes were washed 4 times with PBST for 5 min each while rocking. HRP-conjugated secondary antibodies were diluted 1:10,000 and 1:2,000 for polyclonal Goat- α -Mouse (cat. no. 170-6516) and Goat- α -Rabbit HRP (cat. no. P0448) respectively. Secondary antibodies were diluted to the appropriate concentration in PBST containing 5% skimmed milk powder. Membranes were

incubated with secondary antibodies for 1 h 30 min at room temperature while rocking. Post-incubation, membranes were washed 3 times with PBST and once with PBS for 10 min each.

2.8.3.5 Imaging Western immunoblots

PVDF membranes were placed between plastic sheets and excess PBS was removed by squeezing onto tissue. ECL components were mixed in a 1:1 ratio and 750 µL was added to each membrane. Membranes were stored in the dark for 5 min at room temperature. Excess ECL was removed, and the plastic sheets were sealed shut. Protein bands were visualised using the ImageQuant LAS 4000 imager. Densitometry was performed on the resulting images using ImageJ.

2.9 Bioinformatic Analyses of Next Generation Sequencing Data

2.9.1 Sequence Alignment

Raw read sequence qualities were assessed using FastQC (<http://www.bioinformatics.babraham.ac.uk/projects/fastqc/>). Burrows Wheeler Alignment tool (Li and Durbin, 2009) was used to align reads to the *S. enterica* subsp. *enterica* serovar Typhimurium str. SL1344 chromosome and plasmids reference sequences (chromosome: NC_016810.1, pCol1B9_SL1344: NC_017718.1, pRSF1010_SL1344: NC_017719.1, pSLT_SL1344: NC_017720.1). Aligned reads were sorted by the reference sequence coordinate and low mapping quality reads (mapping quality < 30) were removed using Samtools (Li *et al.*, 2009). Samtools was also used to summarize mapping statistics (Table 2.5 and Table 2.6). More than 90% of the reads were mapped and of the mapped reads, >98% were unique. Sequence coverages were calculated using Deeptools (Ramírez *et al.*, 2014) and are available as BigWig (.bw) files that can be viewed using IGB (Freese *et al.*, 2016).

Sample	Total Reads	Mapped	% Mapped	Uniquely Mapped Reads	% Uniquely Mapped of Total	% Uniquely Mapped of Mapped Reads
WT OD 0.4(1)	28641006	27964913	97.64	26761115	93.44	95.7
WT OD 0.4(2)	29050026	28418166	97.82	27174148	93.54	95.62
WT OD 3(1)	23153629	22593615	97.58	21928569	94.71	97.06
WT OD 3(2)	24645531	24102962	97.8	23670085	96.04	98.2
GX OD 0.4(1)	33976531	33175667	97.64	31951134	94.04	96.31
GX OD 0.4(2)	23395368	22807304	97.49	21745746	92.95	95.35
GX OD 3(1)	29137038	28619694	98.22	27943665	95.9	97.64
GX OD 3(2)	23545289	23122667	98.21	22695719	96.39	98.15
OX OD 0.4(1)	10247759	9591806	93.6	8350624	81.49	87.06
OX OD 0.4(2)	10573982	9966067	94.25	8692028	82.2	87.22
OX OD 3(1)	15218213	10697427	70.29	4515907	29.67	42.21
OX OD 3(2)	9771302	9266513	94.83	8593718	87.95	92.74

Table 2.5 Mapping statistics from RNA-seq experiment.

Sample	Total Reads	Mapped	% Mapped	Uniquely Mapped Reads	% Uniquely Mapped of Total	% Uniquely Mapped of Mapped Reads
WT IP(1)	8323354	8173918	98.2	8046153	96.67	98.44
WT IP(2)	8136713	8095733	99.5	7976467	98.03	98.53
WT MOCK	7772823	7018580	90.3	6896137	88.72	98.26
GX IP(1)	8853253	8754636	98.89	8625679	97.43	98.53
GX IP(2)	8279321	7936091	95.85	7822533	94.48	98.57
GX MOCK	8907749	8143517	91.42	7998261	89.79	98.22
OX IP(1)	8336838	8220613	98.61	8096583	97.12	98.49
OX IP(2)	7639618	7181785	94.01	7080100	92.68	98.58
OX MOCK	8761787	7984582	91.13	7840045	89.48	98.19

Table 2.6 Mapping statistics from ChIP-seq experiment.

2.9.2 Single Nucleotide Polymorphism Analysis

SNP analysis was performed using Breseq (Deatherage and Barrick, 2014, Barrick *et al.*, 2014, Deatherage *et al.*, 2015). This pipeline performs all the functions including mapping reads using Bowtie2, calling SNPs and other genomic variations.

2.9.3 RNA-seq Analysis

2.9.3.1 Differential Expression Analysis

Counts of number of reads mapping to genomic features (genes or sRNA) were obtained using the R package Rsubread (Liao *et al.*, 2019). The R package EdgeR (Robinson *et al.*, 2010, McCarthy *et al.*, 2012) was used to identify differentially expressed genes. P values were adjusted using the Benjamini Hochberg method (False Discovery Rate, FDR) and a cutoff of 0.05 was used to call differentially expressed genes.

2.9.4 ChIP-seq Analysis

2.9.4.1 Peak Calling

Since differential binding analysis was being performed, duplicate reads were not excluded, as they contain information regarding the binding intensity of the transcription factor being pulled down. MACS2 was used to call peaks against the mock. Each strain (WT, GX and OX) had its own mock sample. A custom R script was used to roughly annotate peaks with the names of neighbouring genes and sRNA. These lists are available as both Excel and .gff files. The sequences of Fis peaks from the WT were used to search for motifs using unbiased MEME (Bailey and Elkan, 1994). This Fis motif was then used to search for all possible motifs in the genome, bound or not in the ChIP, using FIMO (Grant *et al.*, 2011).

2.9.4.2 Differential Binding Analysis

Differential binding analysis was performed in R (R Core Team (2013)) using the DiffBind package (Stark and Brown, 2011). To correct for differences in gene dosage gradient between strains, mocks derived from each strain were used as control.

2.10 Statistical analyses

Statistical analysis was conducted using a pairwise students T-test of biological replicates only. Values below 0.05 were considered statistically significant.

Chapter 3:
Exchanging *fis* and *dps* in *Salmonella*
***enterica* serovar Typhimurium**

3.1 Introduction

The bacterial nucleoid is a highly organised and dynamic structure, which is compacted into the bacterial cell in such a way that transcription and replication are not spatially impeded, while also being able to respond rapidly to environmental factors and stresses (Sobetzko *et al.*, 2012, Talukder and Ishihama, 2015, Bryant *et al.*, 2014). In *Salmonella*, *Escherichia coli* and many other γ -Proteobacteria, it has been observed that the order of genes along the two replichores is largely conserved (Sobetzko *et al.*, 2012). Among the most conserved of these genes are those encoding nucleoid associated proteins (NAPs), subunits of DNA topoisomerases, sigma factors, and other master transcriptional regulators (Sobetzko *et al.*, 2012). Throughout evolution, gene order has largely been conserved, and raises the question as to whether gene order is important for bacterial physiology. Indeed, several factors are known to affect gene expression at a given location on the bacterial chromosome, including gene dosage, DNA negative supercoiling, gene orientation, neighbouring gene orientation and the proximity of genes encoding transcription factors (Lal *et al.*, 2016, Bryant *et al.*, 2014, Sobetzko *et al.*, 2012, Gerganova *et al.*, 2015, Ivanova *et al.*, 2015).

Upon nutrient upshift, bacteria exit the lag phase and enter exponential growth, and the origin of replication, *oriC*, fires multiple times (Fig 1.10) (Khedkar and Seshasayee, 2016). This means that genes located adjacent to *oriC* are present in multiple copies, and therefore more available to transcriptional machinery, and highly expressed genes required during exponential growth tend to be located near *oriC* (Khedkar and Seshasayee, 2016, Sobetzko *et al.*, 2012, Stracy *et al.*, 2019). Here, gene orientation is also an important factor, as collisions between transcription machinery and DNA replication machinery can cause stalling and reduce growth rate, along with R-loop formation, and backtracking of RNA polymerase (Ivanova *et al.*, 2015, Brochu *et al.*, 2018, Nudler, 2012).

Fis and Dps are two growth-phase-specific NAPs, associated with exponential and stationary phase, respectively (Wang *et al.*, 2015, Kelly *et al.*, 2004, Janissen *et al.*, 2018). Fis production is associated with a large peak in *fis* transcription upon the transition from lag into exponential phase (Osuna *et al.*, 1995). *In vitro*

studies have shown that Fis is a target to degradation by the Lon protease in the presence of inorganic polyphosphate. However, the same effect is not observed *in vivo*, due to the stronger bond of Fis to DNA, than to that of inorganic polyphosphate (Kuroda *et al.*, 2006). However, the concentration of the Fis protein becomes diluted through multiple rounds of bacterial cell division until Fis is undetectable in stationary phase (Kelly *et al.*, 2004, Keane and Dorman, 2003). The *fis* gene is part of the bicistronic *dusB-fis* operon, and *dusB* mRNA is essential for the robust translation of *fis* (Fig 1.8) (Nafissi *et al.*, 2012). The expression of *fis* relates directly to the physiological state of the bacterium. The promoter of *fis* is dependent on DNA supercoiling, and expression is induced by negative supercoils, such as those seen upon nutrient upshift (Fig 1.8) (Cameron *et al.*, 2013, Schneider *et al.*, 2000). Fis is also a negative autoregulator of its own gene, and the Fis regulon encompasses approximately 1,500 genes (Wang *et al.*, 2015).

The *dps* gene is a member of the Fis regulon, and along with H-NS, Fis represses *dps* expression during exponential growth (Fig 1.9) (Grainger *et al.*, 2008). However, Dps expression can also be induced by oxidative stress, via OxyR, and *dps* expression can be observed in response to NaCl shock, nitric oxide shock, oxygen shock, anaerobic conditions as well as low Mg²⁺ in *S. enterica* serovar Typhimurium (Kroger *et al.*, 2013, Rothe *et al.*, 2012, Altuvia *et al.*, 1994). While Dps is not known as a transcriptional regulator, its binding throughout the genome is not random, and many of its binding sites overlap those of Fis (Janissen *et al.*, 2018, Antipov *et al.*, 2017). Further, both Fis and Dps have roles in regulating DNA replication, where Fis is an activator of DNA replication, and Dps is a repressor of DNA replication (Flatten and Skarstad, 2013, Chodavarapu *et al.*, 2008).

This study aims to investigate the effects of gene location and regulation of two growth phase specific, NAP-encoding genes, *fis* and *dps* whose gene products differ in function. In order to determine the effect of gene position on bacterial physiology, the *fis* and *dps* genes, including their *cis*-acting regulatory sequences were exchanged. A separate exchange of the *fis* and *dps* ORFs was also made. The impacts of these exchanges were analysed by examining changes in gene dosages, and *fis* and *dps* expression.

3.2 Results

3.2.1 The positions of *fis* and *dps* are conserved among γ -Proteobacteria

In *Salmonella enterica* and *Escherichia coli*, the *fis* and *dps* genes are located in the NS-Left and Right macrodomains, respectively (Sobetzko *et al.*, 2012). Through bioinformatic analyses, it is possible to determine how conserved the location of these genes is, relative to the origin of replication and genome size. The database DoriC contains a comprehensive list of the location of *oriC* and genome size in enterobacteria (Gao *et al.*, 2013). The difference in distance between *oriC* and *fis* or *dps* was calculated and made relative to the genome sizes of a range of *Salmonella* species, subspecies and serovars as well as many other enterobacteria (Fig 3.1). Among *Salmonellae*, the locations of *fis* and *dps* are highly conserved, except in *Salmonella enterica* serovar Typhi, Paratyphi and Gallinarum. The location of *fis* and *dps* are also largely conserved among other enteric bacteria as previously observed (Sobetzko *et al.*, 2012).

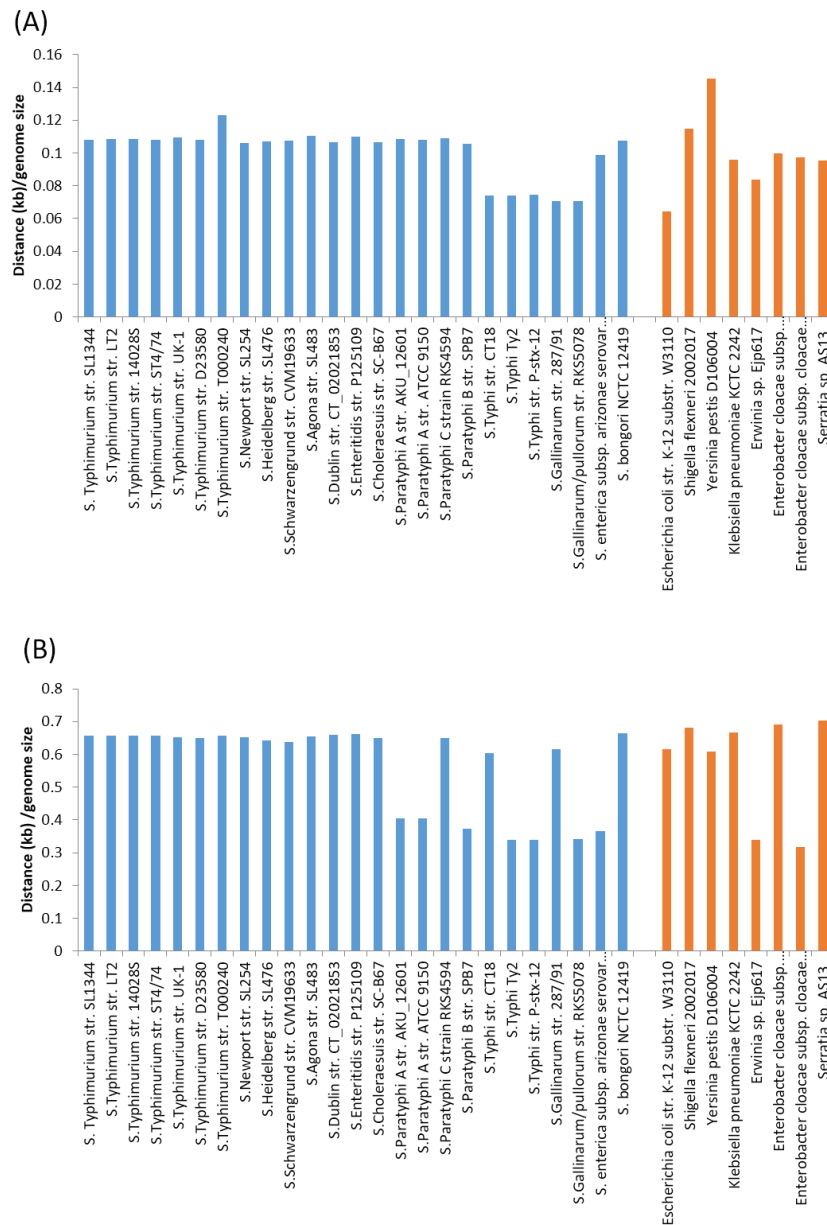


Fig 3.1. The positions of *fis* and *dps* relative to *oriC* in *Salmonella* spp. and other γ -Proteobacteria. The distance between *fis*, *dps* and *oriC* relative to the genome size was calculated from the database, *DoriC*. (A) The position of *fis* relative to *oriC* is largely conserved with the exceptions of *S. enterica* serovars Typhi and Gallinarum. (B) The position of *dps* relative to *oriC* is conserved with the exceptions of *S. enterica* serovars Paratyphi, Typhi and Gallinarum.

3.2.2 Construction of GX and OX strains

Lambda red recombineering (Datsenko and Wanner, 2000) was used to create the *fis* and *dps* exchange strains. Two *fis-dps* exchange strains were proposed; the first would have the *fis* ORF, the *dps* ORF together with their *cis*-acting regulatory sequences, exchanged reciprocally (hereinafter, GX, for "Gene eXchange"). In the second strain, only the *fis* and *dps* ORFs were exchanged reciprocally (hereinafter OX, for "ORF eXchange").

Published information was used to determine how much of the DNA neighbouring *fis* and *dps* to use when making the genetic exchange to produce GX. Because the *dusB* gene, lying immediately upstream of *fis*, is required for robust translation of *fis* mRNA (Nafissi *et al.*, 2012), it was treated as a *cis*-acting regulatory sequence and included in the GX exchange. For the reciprocal exchange of the complete *fis* and *dps* genes, 315 bp upstream of *dusB* and 190 bp upstream of *dps* were included because these regions were known to be important for normal regulation (Cameron *et al.*, 2013, Cameron *et al.*, 2011, Kroger *et al.*, 2013, Grainger *et al.*, 2008, Altuvia *et al.*, 1994).

For both the gene and ORF exchanges, any possible downstream regulatory regions of the moving gene were included in the exchange. This strategy was chosen because RNA-seq data from *Salmonella* indicate the existence of a 3' UTR in *dps* (Kroger *et al.*, 2013). In order to select for the relocated *fis* gene, a kanamycin resistance cassette was inserted 62 bp downstream of the *fis* ORF as a selectable marker. Attempts at inserting an antibiotic resistance cassette downstream of *dps* resulted in no transformants, so for the *dps-fis* gene exchange, the kanamycin resistance cassette was inserted 190 bp upstream of *dps*. As insertion of an antibiotic resistance cassette upstream of *dps* would make it difficult to generate a *dusB-dps* operon, Seamless Ligation Cloning Extract (SLiCE) was used to generate this operon on the plasmid pBluescript SK(+) with a chloramphenicol resistance marker downstream of the operon (Zhang *et al.*, 2014). The *fis::kan*, *kan::dps* and *dusB::dps::cat* constructs were amplified by PCR and inserted in either the *dps* or *fis* locus generating "2x" intermediate strains (Fig 3.2).

In total, four intermediate strains were created, two of which had two copies of *fis* and none of *dps*, and two of which had two copies of *dps*. Of the strains which had two copies of *fis*, "2xfG" (2x *fis* gene) had the *dusB-fis* operon and its *cis*-acting regulatory sequences at both the *fis* and *dps* loci, while "2xfO" (2x *fis* ORF) had the *dusB-fis* operon in its native locus and *fis* under the control of the *dps cis*-acting regulatory sequences at the *dps* locus. Similarly, "2xdG" (2x *dps* gene) had *dps* and its *cis*-acting regulatory sequences in the *dps* and *fis* loci, while "2xdO" (2x *dps* ORF) had *dps* and its *cis*-acting regulatory sequences in its native locus, and the *dusB-dps* operon in the *fis* locus (Fig 3.2). Following the construction of these strains, the kanamycin resistance cassette was removed from the 2x *fis* strain, using the yeast Flp tyrosine integrase site-specific recombinase. This recombinase acts on the copies of the directly-repeated FRT sites that flank the resistance marker to delete it (Datsenko and Wanner, 2000).

The exchange strains, GX and OX were generated by transducing the *dps* construct with bacteriophage P22 from 2xdG to 2xfG and from 2xdO to 2xfO, respectively. These transduction events removed the *dusB-fis* operon from the native locus and replaced it with either the *dps* gene or the *dusB-dps* operon. Finally, the yeast Flp recombinase was again used to remove any remaining antibiotic selection markers. The genetic structures of the constructs were confirmed to be correct at each step in the process by Sanger sequencing.

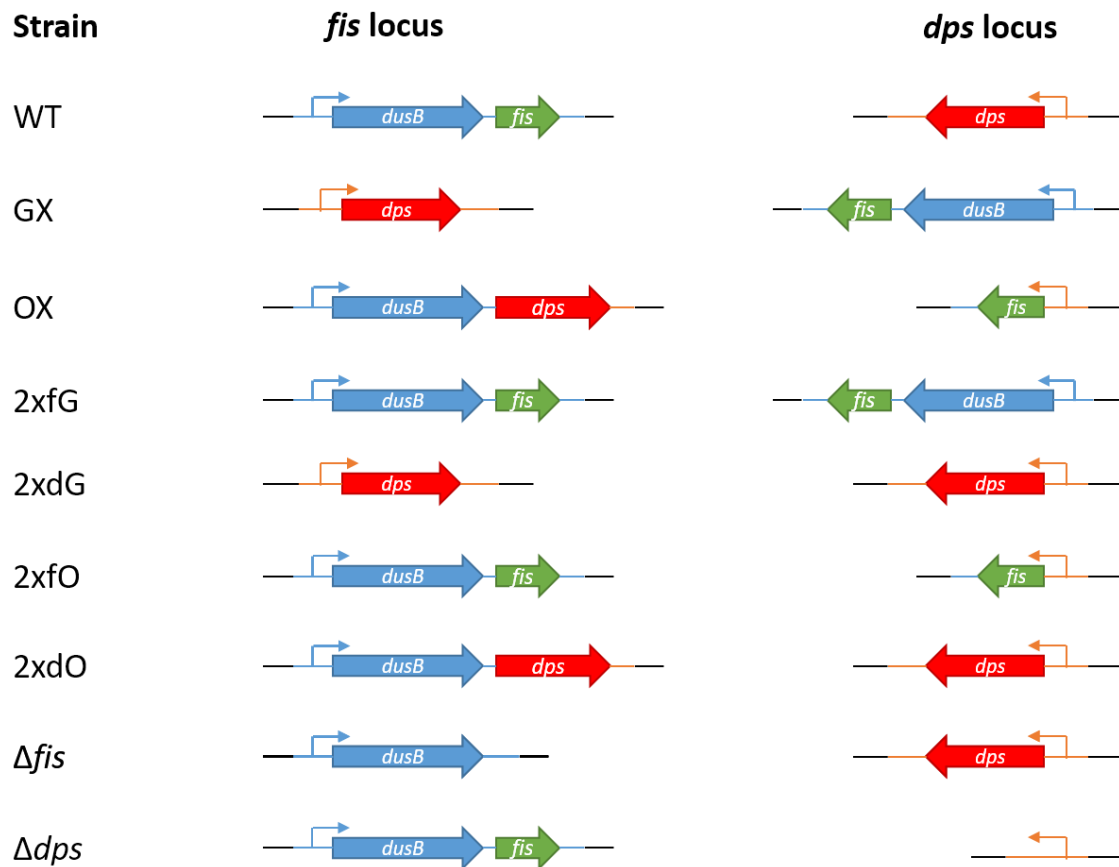


Fig 3.2. Strains constructed in this study. With the exception of Δfis , all strains were constructed by λ -red recombineering. The *fis*-*dps* exchange strains were constructed via “2x” intermediate strains. Orange represents *dps* *cis*-acting regulatory sequences. Blue represents *fis* *cis*-acting regulatory sequences. Not to scale.

3.2.3 Validation of strains by whole genome sequencing

Whole genome sequencing was carried out on all of the strains to check for the presence of genetic changes additional to those that had been introduced deliberately during the strain construction. One concern when large alterations are made in expression of master regulators, such as NAPs, is the appearance of compensatory mutations, or mutations which show a polar effect (Ali *et al.*, 2014). Genomic DNA was prepared, and sequencing was carried out by MicrobesNG and the Sanger Institute. Contigs were assembled against a reference strain (Genbank accession number NC_016810.1) using Velvet, and SNP analyses were carried out using Breseq (Deatherage and Barrick, 2014, Zerbino and Birney, 2008). As expected, new junction formations were identified in all 2x and *fis-dps* exchange strains at the *fis* and *dps* loci, indicative of successful exchange of the genes.

The SL1344 parental strain used in this study has two previously described SNPs, in *manX* and *menC*, compared to the reference genome (Fitzgerald *et al.*, 2015). Other SNPs were found in the strains OX, 2xdG, 2xfO and 2xdO (Table 3.1). Of the four SNPs identified in OX, one (in SL1344_3357) is found in another 2x strain, 2xdO. Given its proximity to the parental *fis* locus, it is probable that this SNP was co-transduced by bacteriophage P22 in the strain construction process (Section 3.2.2). However, this SNP is synonymous in that the protein coding sequence is unaffected by it.

In OX, two SNPs were identified in the proximity of the parental *dps* locus in the hypothetical genes *ybiH* and SL1344_0801. SL1344_0801 is also denoted as *ybiN* in some studies and encodes a methyltransferase in *E. coli*, but remains a hypothetical protein in *Salmonella*, whereas *ybiH* encodes a hypothetical *tetR* transcriptional regulator (Sergiev *et al.*, 2008, Kroger *et al.*, 2013).

In 2xdG SNPs were identified in *symE*, encoding an endoribonuclease, SL1344_1950, encoding a bacteriophage terminase and in SL1344_4453 encoding a hypothetical inner-membrane protein (Kroger *et al.*, 2013). However, the SNP in SL1344_4453 was also synonymous.

On the other hand, 2xfO has SNPs in *ybiR*, *ybiT* and intergenic to *ybiT* and *ybiS* which are all located in the proximity of the parental *dps* locus. The gene *ybiR*

encodes a putative outer membrane protein, while *ybiT* encodes the ATP-binding subunit of an ABC transporter (Kroger *et al.*, 2013). Finally *ybiS* encodes a hypothetical exported protein (Kroger *et al.*, 2013).

In order to determine whether any of the gene products in Table 3.1 had interactions with either Fis or Dps, a Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) was used. This tool collects and consolidates known and predicted protein-protein information (Szklarczyk *et al.*, 2015). It was observed that although these SNPs were found in genes in close proximity to each other, as well as *fis* and *dps*, STRING regulon analyses found no connection between these genes and Fis or Dps in *Salmonella* or *E. coli* (Szklarczyk *et al.*, 2015).

Strain	Position	Mutation	Annotation	Gene	Description
OX	884,095	C→G	V173L (G <u>T</u> G→C <u>T</u> G)	ybiH ←	tetR family transcriptional regulator
	891,073	(TGGGCG) ₃ → ₂	coding (341-346/933 nt)	SL1344_0801 →	hypothetical protein
	3,577,470	C→T	C260C (TGC <u>C</u> →TGT <u>I</u>)	SL1344_3357 →	hypothetical protein
	4,020,999	G→T	intergenic (+200/-3)	SL1344_3765 → / → emrD	hypothetical protein/multidrug resistance protein D
2xdG	2,059,976	T→C	K121R (A <u>A</u> A→A <u>G</u> A)	SL1344_1950 ←	bacteriophage terminase (large subunit)
	4,797,533	G→A	N8N (AAC <u>C</u> →AAT <u>I</u>)	SL1344_4453 ←	putative inner membrane protein
	4,797,697	C→A	S89I (AG <u>C</u> →AT <u>C</u>)	symE ←	endoribonuclease
2xfO	902,861	A→C	D287A (GAT→G <u>C</u> T)	ybiR →	hypothetical protein
	904,295	T→C	intergenic (-217/-2)	ybiS ← / → ybiT	hypothetical protein/ABC transporter ATP-binding protein
	905,822	T→C	M509T (AT <u>G</u> →AC <u>G</u>)	ybiT →	ABC transporter ATP-binding protein
2xdO	3,577,470	C→T	C260C (TGC <u>C</u> →TGT <u>I</u>)	SL1344_3357 →	hypothetical protein

Table 3.1 SNPs identified in strains used in this study. With the exception of the SNPs in *manX* and *menC*, SNPs were only identified in OX, 2xdG, 2xfO and 2xdO.

3.2.4 Changes in the gene dosage gradient in OX

In bacteria, there is a gene dosage gradient from *oriC* to Ter, which is determined by the rate of the cell cycle. During exponential growth, *oriC* fires multiple times, and consequently DNA in the proximity of *oriC* is present at higher copy number than that in the proximity of Ter (Sousa *et al.*, 1997, Schmid and Roth, 1987).

As gene copy number plays an important role in determining the expression of a gene, it was pertinent to determine the gene dosage gradient, and the gene copy numbers of *fis* and *dps* in WT, GX and OX (Bryant *et al.*, 2014, Khedkar and Seshasayee, 2016). To examine the gene dosage gradient in WT, GX and OX, genomic DNA was extracted at various points throughout growth and the copy number of an origin of replication (*oriC*) proximal gene (*gidA*) and a Ter proximal gene (*STM1554*) were quantified by qPCR. There was no change in the gene dosage gradient in GX compared to WT (Fig 3.3 (A)). However, the trend in the gene dosage gradient in OX differs in having a tendency towards a decreased gradient in exponential phase ($OD_{600\text{ nm}} = 0.4$), and an increased gradient in early stationary phase ($OD_{600\text{ nm}} = 3$) (Fig 3.3 (A)). In stationary phase (24 h) the Ori/Ter ratio was 1, indicative of non-replicating bacteria.

One explanation for a change in the gene dosage gradient throughout growth, is a change in growth rate, and so the growth rates per hour were determined from optical density values (Section 3.2.1.1). While the growth rates of GX and WT were similar, a decrease in growth rate was observed in OX in the first hour, and an increased rate in later hours (Fig 3.3 (B)). These data indicate that the exchange of *fis* and *dps* ORFs had a greater effect on the cell cycle than the exchange of the *fis* and *dps* genes.

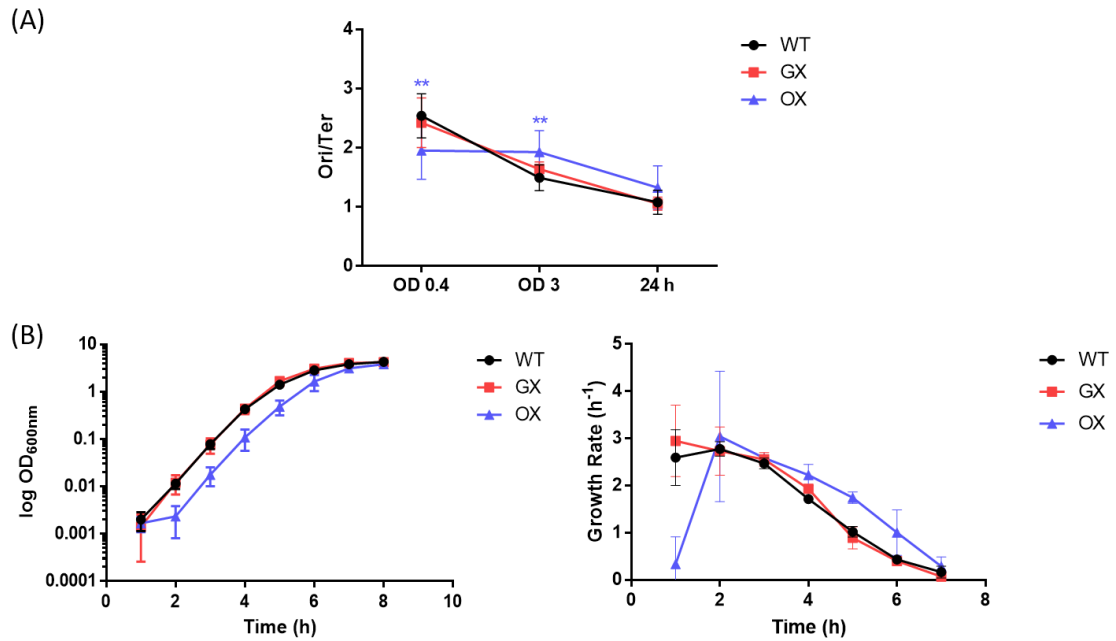


Fig 3.3. The gene dosage gradient and growth rate of *fis-dps* exchange strains. (A) The gene dosage gradient in WT, GX and OX measured by quantifying the copy number of an *oriC*-proximal gene and a *Ter*-proximal gene. In OX, there is a statistically significant change in the gene dosage gradient in exponential and early stationary phases ($p = 0.04$ and 0.03 respectively). (B) Left: Growth curve of WT, GX, and OX over 8 h period showing a change in growth patterns of OX. Right: The growth rate of WT, GX and OX as determined from the growth curve. The shift in growth rate is responsible for the change in gene dosage gradient. Error bars represent standard deviation of at least five biological replicates. Statistical significance was calculated by the student's t-test.

3.2.5 Alterations in *fis* and *dps* gene dosages

Previous studies have highlighted how the proximity of a gene to *oriC* affects the expression of that gene, and many genes, including rRNAs, which are highly expressed during exponential growth are located near *oriC* (Gyorfy *et al.*, 2015, Bryant *et al.*, 2014, Gerganova *et al.*, 2015). In GX and OX, *fis* is located further from *oriC* while *dps* is located closer to *oriC* than in WT, and this could affect the gene dosage, as well as expression throughout growth.

In a similar manner as in Section 3.2.4, genomic DNA was extracted at various time points throughout growth and the copy numbers of *fis*, *dps* and *gidA* were determined by qPCR.

As expected, a decrease in *fis* copy number and an increase in *dps* copy number was observed in GX compared to WT (Fig 3.4). Interestingly, OX did not show the same pattern of *fis* or *dps* copy number throughout growth, and this may be related to the change in gene dosage gradient (Fig 3.3(A)). These data indicate that exchanging *fis* and *dps* affects their copy number in exponential growth and in early stationary phase.

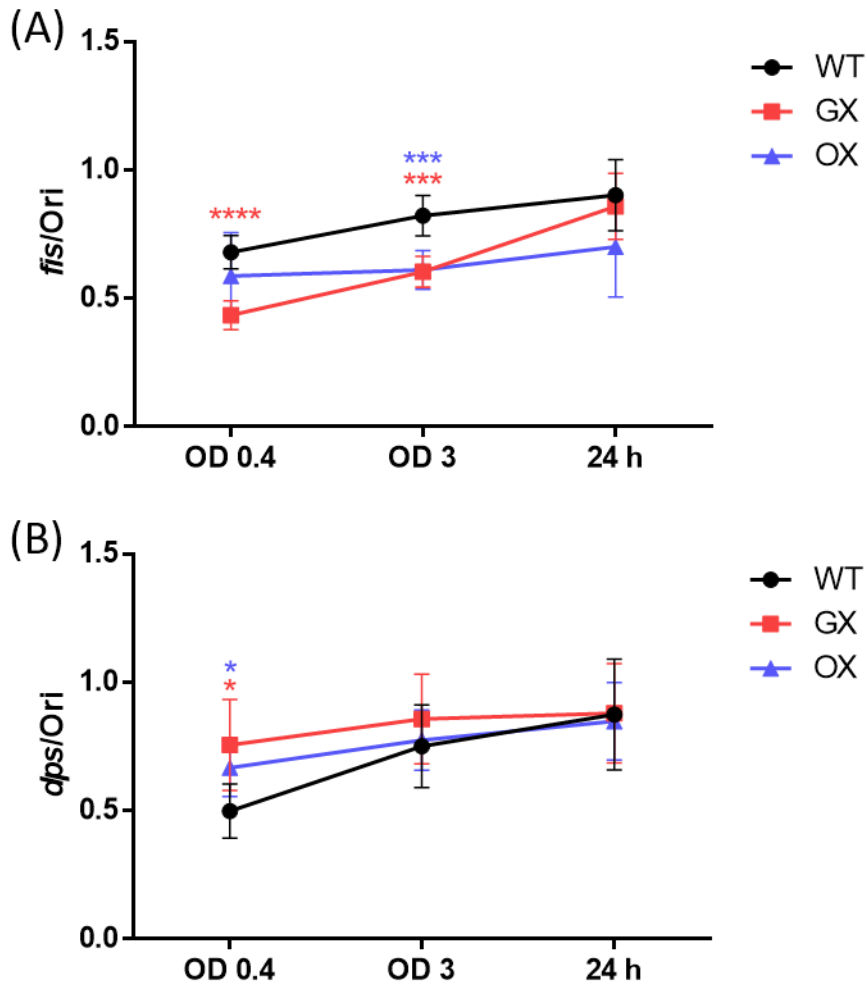


Fig 3.4. *fis* and *dps* copy number in exchange strains. The copy number of *fis*, *dps*, and an *oriC*-proximal gene were quantified by qPCR throughout growth. (A) The copy number of *fis* is reduced in GX compared to WT in exponential and early stationary phase ($p < 0.0001$ and $p = 0.0003$ respectively). The copy number of *fis* is reduced in early stationary phase in OX compared to WT ($p = 0.0008$). (B) The copy number of *dps* is increased in GX and OX during exponential growth compared to WT ($p = 0.01$ and 0.02 respectively). Error bars represent standard deviation of at least five biological replicates. Statistical significance was calculated by the student's t-test.

3.2.6 Expression of exchanged genes

As gene dosage and *cis*-acting regulatory sequences affect gene expression, it was of interest to examine *fis* and *dps* expression patterns in the *fis-dps* exchange strains (Bryant *et al.*, 2014). In order to determine the expression patterns of *fis* and *dps* in WT, GX and OX, total RNA was extracted at various points through growth. The RNA was DNase treated, converted into cDNA and the quantities of *fis* were measured by qPCR. The *hemX* gene was used as an internal control because previous RNA-seq data have shown its expression remains constant under all tested growth conditions and growth phases, except late stationary phase (Kroger *et al.*, 2013). The quantities of *hemX* also remained constant between WT, GX and OX strains. The cDNA quantities of *fis* and *dps* were therefore normalised to *hemX*.

In line with the reduced *fis* copy number, a downward trend in *fis* expression was observed in GX (Fig 3.5 (A)). On the other hand, *fis* expression patterns were reversed in OX, with little expression early in the growth phase, and an increase later in the growth phase.

In GX, there was a statistically significant increase in *dps* expression at all points throughout growth. This is in line with an increase in *dps* copy number in GX (Fig 3.5 (B)). In OX, the expression of *dps* was reversed with *dps* expression peaking in exponential growth, and a reduction in expression in early stationary phase.

These data indicate that in OX, *fis* takes the expression patterns of *dps*, and *dps* takes *fis* expression patterns.

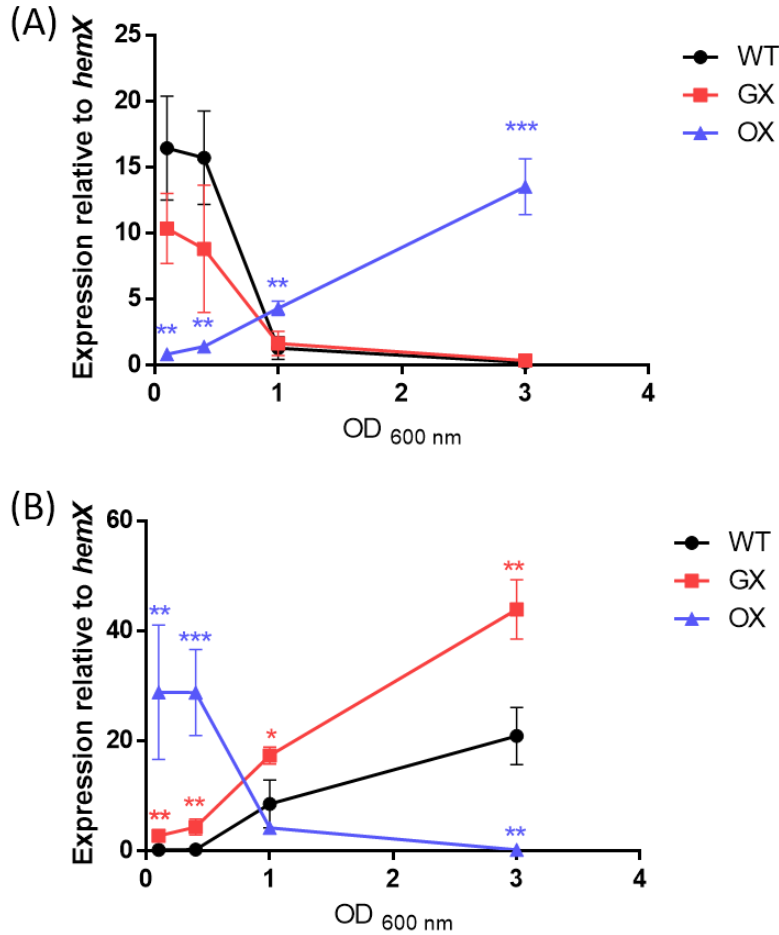


Fig 3.5 Expression of *fis* and *dps* in exchange strains. RNA was isolated at various points throughout growth, DNase treated and converted into cDNA. The abundance of *fis* and *dps* was quantified by qPCR and made relative to the constitutively expressed gene *hemX*. (A) The expression patterns of *fis* in WT, GX and OX. There are statistically significant changes in *fis* expression at all points in growth in OX compared to WT ($p = 0.002$, 0.002 , 0.007 and 0.0004 for OD_{600 nm} values of 0.1, 0.4, 1 and 3 respectively). (B) The expression patterns of *dps* in WT, GX and OX. There are statistically significant increases in *dps* expression at all points in growth in GX compared to WT ($p = 0.002$, 0.001 , 0.02 and 0.006 for OD_{600 nm} values of 0.1, 0.4, 1 and 3 respectively). There are statistically significant increases in *dps* expression at OD_{600 nm} values of 0.1 and 0.4 in OX compared to WT ($p = 0.004$ and 0.0006 respectively), and a statistically significant decrease in *dps* expression at an OD_{600 nm} of 3 ($p = 0.002$). Error bars represent standard deviation of at least three independent biological replicates. Statistical significance was calculated by the student's t-test.

3.2.7 Fis production in *fis-dps* exchange strains

In order to determine the protein levels of Fis throughout growth, the *fis* gene was tagged with the 8xmyc epitope and Fis::8xMyc production was monitored throughout growth. The 8xMyc epitope tag was chosen as it has previously been used, and is not known to alter Fis function (Cameron and Dorman, 2012). First, several timepoints were collected by boiling lysates (Section 2.8.2). By this method an increase in Fis production was observed upon entry into stationary phase in OX (Fig 3.5 (A)). However, due to background it was too difficult to tell whether there were any changes in Fis::8xMyc production in GX compared to WT. Therefore, two timepoints were chosen to be examined further, at OD_{600 nm} of 0.4 and 3. These two timepoints were chosen because at an OD_{600 nm} of 0.4 bacteria are in mid-exponential growth, and intracellular Fis concentration is high, whereas at an OD_{600 nm} of 3, bacteria are transitioning into stationary phase, and intracellular Fis concentration is reduced (Fig 3.6 (A)) (Talukder and Ishihama, 2015).

Samples were prepared with sonication as described in Section 2.8.2. Sonication as a method of cell lysis has the advantage in that DNA is sheared, preventing DNA disrupting in downstream applications (Sambrook and Russell, 2006a). At both timepoints in growth, Fis::8xMyc production was reduced in OX when compared to WT, while Fis::8xMyc production was reduced in exponential phase in GX (Fig 3.6 (B)). These data indicate that the changes in *fis* gene dosage and *fis* expression (Sections 3.2.5 and 3.2.6) have affected Fis production throughout the bacterial growth cycle in GX and OX.

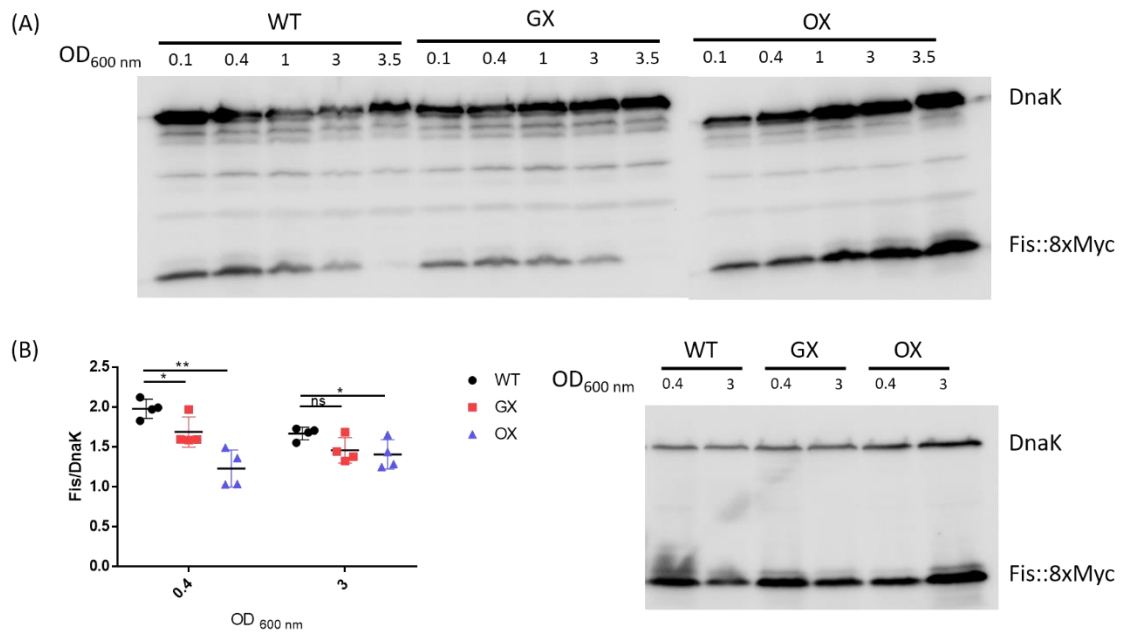


Fig 3.6 Fis::8xMyc production in WT, GX and OX. Western immunoblots of 21.5 kDa Fis::8xMyc and 69.1 kDa DnaK were performed using a 1:50,000 dilution of both mouse α -Myc and mouse α -DnaK (A) Fis::8xMyc production by using the boiled lysate method of sample preparation in WT, GX and OX (Section 2.8.1). Image is representative of three independent biological replicates. (B) Densitometry of Fis relative to DnaK based on Western immunoblots with samples prepared by sonication method (Section 2.8.2). In exponential phase, Fis::8xMyc concentration is reduced in GX and OX compared to WT ($p = 0.04$ and 0.001 respectively). In stationary phase, Fis::8xMyc concentration is reduced in OX compared to WT ($p = 0.04$). Error bars represent standard deviation of four independent biological replicates. Statistical significance was calculated by the student's t-test.

3.2.9 Dps production in *fis-dps* exchange strains

Unlike Fis, epitope tagging of Dps has not been used for *in vivo* studies. As Dps is a 19-kDa protein which forms a dodecamer, it would be difficult to epitope tag without interfering with Dps function (Melekhov *et al.*, 2015, Antipov *et al.*, 2017). Therefore, anti-Dps sera was used to monitor the production of Dps throughout growth. As cross-reacting impurities in serum increase the background signal in Western immunoblotting, it was not possible to calculate the relative concentrations of Dps by densitometry. To further reduce the background signal, only the samples prepared by the sonication method (Section 2.8.2) were used at OD_{600 nm} values of 0.4 and 3. Despite this, there is a clear increase in Dps production in GX in stationary phase (Fig 3.7). Whereas in OX, Dps is produced in mid-exponential phase (Fig 3.7).

These data indicate that the change in *dps* gene dosage and gene expression have affected Dps production throughout the growth cycle in GX and OX.

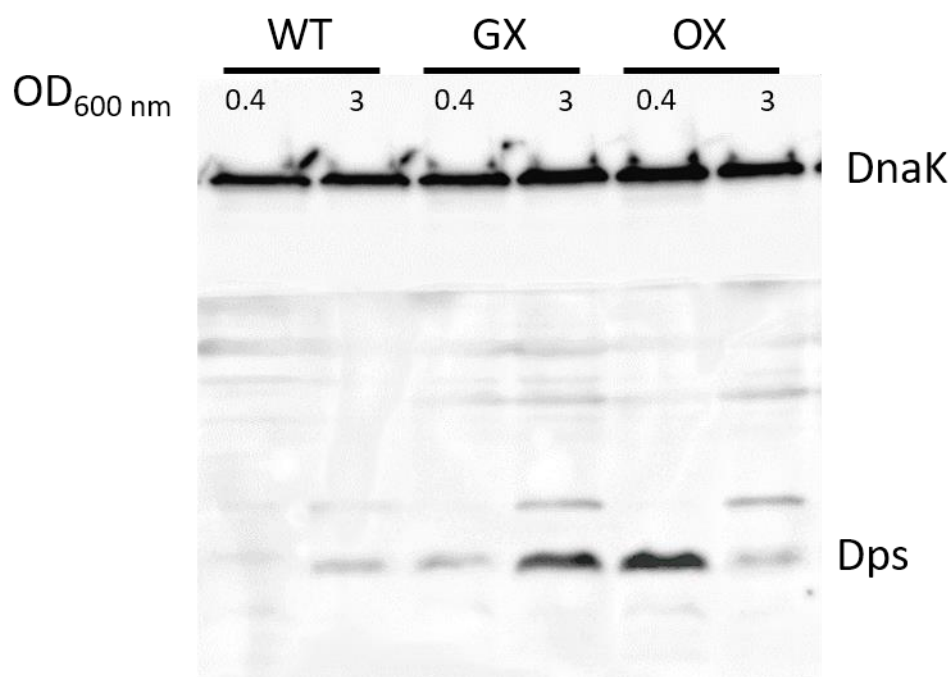


Fig 3.7 Dps production in WT, GX and OX. Western immunoblot of 19 kDa Dps and 69.1 kDa DnaK using a 1:50,000 dilution mouse α -DnaK and 1:2,000 dilution rabbit α -Dps. In exponential phase, there is an increase in Dps production in OX compared to WT. In stationary phase, there is an increase in Dps production in GX compared to WT. Image is representative of four independent biological replicates. Background has been modified using ImageJ.

3.3 Discussion

The positions of genes encoding NAPs, topoisomerases and sigma factors are highly conserved among γ -Proteobacteria, and previous studies have suggested that this conservation is related to the need for highly organised gene expression patterns that can quickly adapt to environmental factors (Sobetzko *et al.*, 2012). This study sought to examine the effects of exchanging the gene position and regulation of two NAP-encoding genes, which products differ in function, Fis and Dps. In γ -Proteobacteria, the genes *fis* and *dps* are located on opposite replicores in the NS-left and right macrodomains respectively, and exchanging their positions would affect their positions relative to *oriC* (Sobetzko *et al.*, 2012, Gerganova *et al.*, 2015).

The positions of *fis* and *dps* are largely conserved among *Salmonella* spp. and further among γ -Proteobacteria (Fig 3.1). Changes in the distance between *fis*, *dps* and *oriC* were observed in the serovars Typhi, Paratyphi and Gallinarum. One possible explanation for these changes is the acquisition of other *Salmonella* pathogenicity islands, which may both affect the distance between *fis* or *dps* and *oriC*, or the overall genome size (Fookes *et al.*, 2011). An example of this is the acquisition of the 133 kb SPI-7 in *S. enterica* serovar Typhi, and the absence of SPI-13 and 14 which is present in serovars Typhimurium, Enteritidis, Gallinarum and Choleraesuis (Fookes *et al.*, 2011, Gerlach and Hensel, 2007).

Two *fis-dps* exchange strains were created in this study, one in which the *dusB-fis* operon and its *cis*-acting regulatory regions were exchanged with those of *dps*, and one which exchanged the *fis* and *dps* ORFs only (Fig 3.2). The construction of these exchange strains was done by a method that has been used previously (Fitzgerald *et al.*, 2015). ORF exchanges of *hns* and *stpA*, and movements in position of the *dusB-fis* operon have been done previously, but the strains constructed in this study differ in that the exchange is between two NAP-encoding genes that are associated with two distinct parts of the growth cycle (Sobetzko *et al.*, 2012, Fitzgerald *et al.*, 2015, Gerganova *et al.*, 2015). In OX, *fis* is under the control of *dps* *cis*-acting regulatory signals, while *dps* is under *fis* *cis*-acting regulatory sequences. In GX, the two genes and their *cis*-acting regulatory sequences have been exchanged. Together, these two strains should provide

insight into exactly why gene order, and timely expression of growth phase-specific NAPs are important in bacterial physiology.

Whole genome sequencing of the *fis-dps* exchange strains and 2x intermediate strains confirmed the successful movements in position of *fis* and *dps*, as well as SNPs in OX, 2xdG, 2xfO and 2xdO strains (Table 3.1). The majority of the SNPs identified were either in genes encoding hypothetical proteins or in close proximity to *fis* or *dps*. As none of these genes are linked to the Fis or Dps regulon, repeating the construction of the strains seemed unnecessary.

In bacteria, a gene dosage gradient exists from Ori to Ter and is growth rate dependent (Khedkar and Seshasayee, 2016). As bacteria exponentially grow, genome replication is occurring at a quicker rate than segregation and bacterial replication. This leads to a scenario where DNA adjacent to the *oriC* is present in multiple copies leading to increased expression of genes which may lie on it (Sobetzko *et al.*, 2012, Schmid and Roth, 1987). The higher the ratio between Ori and Ter DNA, the faster the growth rate is. In line with this, the change in growth rate in OX, led to a change in the gene dosage gradient from Ori to Ter, while in GX there was no change in growth rate, and no change in the gene dosage gradient (Fig 3.3) (Khedkar and Seshasayee, 2016).

The exchange of *fis* and *dps* in this study, affected the distance between *oriC* and these genes. As *dps* is located further from *oriC*, in the left macrodomain, it was hypothesized that *fis* gene dosage would be lowered in the exchange strains (Sobetzko *et al.*, 2012). Indeed, in GX the change in *fis* and *dps* gene dosages are clear, with a decrease in *fis* copy number, and an increase in *dps* copy number in exponential growth (Fig 3.4). These changes in copy number are not observable in stationary phase, when bacteria are no longer replicating (Khedkar and Seshasayee, 2016). The same effect was not as stark in OX, and this is likely due to the change in growth rate, and hence, the overall gene dosage gradient (Fig 3.3)

The movement of any gene in its position along a replicore is known to affect gene expression. Several studies have highlighted how movement of a gene further from *oriC* reduces the expression of the gene in exponential growth. Gerganova *et al.* (2015) observed a reduction in *fis* expression when the *dusB*-

fis operon was moved to the Ter macrodomain. Similarly, Bryant *et al.* (2014) observed a reduction in GFP fluorescence, depending on the location of the gene along the two replichores. Taking into account the reduced *fis* copy number, and increased *dps* copy number, the changes in *fis* and *dps* expression in GX are to be expected (Fig 3.5). In OX, there is a reversal of *fis* and *dps* expression patterns, with *fis* expression increasing in stationary phase, and *dps* expression peaking in exponential growth (Fig 3.5). From this, it is apparent that the *cis*-acting regulatory sequences have a greater role in defining *fis* and *dps* expression patterns.

While there is a downward trend in *fis* expression in GX, it is more noticeable at the protein level, with a 1.17-fold reduction in Fis::8xMyc production in exponential growth (Fig 3.6 (B)). A consequence of the increase in *fis* expression in stationary phase in OX, is that there is some Fis::8xMyc protein upon nutrient upshift, and reduced Fis concentration during exponential growth (Fig 3.6 (B)). In this strain, Fis::8xMyc production increases later in the growth phase. However, in early stationary phase Fis concentration is still less than that of WT.

While it was not possible to determine Dps concentrations accurately relative to the loading control DnaK, there was a clear increase in Dps production in early stationary phase in GX (Fig 3.7). The level of Dps at this point in growth in GX was similar to that in OX during exponential growth. It is unclear, what the consequences of Dps overproduction would have on cellular physiology, as Dps is not known to regulate many genes in stationary phase, when its production is at its peak (Janissen *et al.*, 2018).

While the changes in gene dosage subtly affected the expression of *fis* and *dps* in GX, overall, the effects of exchanging the *fis* and *dps* ORFs, had a more striking impact. As Fis is a major regulator of gene expression, these data indicate that the impact of *fis* and *dps* exchange on bacterial physiology would be much greater in OX (Kelly *et al.*, 2004, Wang *et al.*, 2013). Together the two exchange strains make a novel method of examining the impacts of gene order and regulation on bacterial physiology.

Chapter 4:
The effects of *fis* and *dps* reciprocal exchanges on global gene expression and Fis binding patterns.

4.1 Introduction

Exchanging the regulatory regions (GX) or just the open reading frames (OX) of the *fis* and *dps* genes on the *Salmonella* chromosome led to changes in *fis* and *dps* expression, and to changes in Fis and Dps protein concentrations as functions of growth phase. These effects were particularly striking in OX, where the expression patterns of *fis* and *dps* were reversed, with *dps* expression peaking during exponential growth, and *fis* expression rising at the onset of stationary phase (Fig 3.5). Although studies in which genes have been moved to a new location on the bacterial chromosome have been performed before, none has sought to examine the global effects on gene expression or on protein binding patterns (Fitzgerald *et al.*, 2015, Bryant *et al.*, 2014, Gerganova *et al.*, 2015). Therefore it was of interest to examine both of these factors in GX and OX.

Fis and Dps differ in function, they are produced in different growth phases, and Fis is a regulator of gene expression, while Dps is not (Ali Azam *et al.*, 1999, Kelly *et al.*, 2004, Janissen *et al.*, 2018). The Fis regulon has been well characterised in both *Salmonella* and *E. coli*, and it's now clear that Fis regulates over 1,500 genes, many of which are regulated in an indirect manner (Cho *et al.*, 2008b, Wang *et al.*, 2013, Wang *et al.*, 2015). Indeed, Fis binds to and regulates many genes which are also transcriptional regulators, including *rpoS* and *gyrB* (Keane and Dorman, 2003, Hirsch and Elliott, 2005a). Fis is a regulator of genes involved in motility, metabolism and virulence, and *fis* mutants have reduced macrophage invasion and survival rates in *Salmonella* (Wang *et al.*, 2013). Fis also binds and regulates genes involved in the early, middle and late stages of flagellar biosynthesis (Kelly *et al.*, 2004).

Fis preferentially binds to A/T-rich DNA, and it recognises a degenerate 16-bp binding motif (Cho *et al.*, 2008b, Finkel and Johnson, 1992). Fis can act as either an activator or repressor of gene expression depending on where it binds at a promoter (Browning *et al.*, 2010). Further, Fis can activate genes from promoter-distal sites by binding at stress-induced duplex destabilisation (SIDD) regions, and it acts as a topological buffer, preserving negative DNA supercoils at certain promoters (Opel *et al.*, 2004, Pemberton *et al.*, 2002).

Conversely, Dps is not known to regulate gene expression directly, although it binds to DNA in a non-random manner (Antipov *et al.*, 2017, Janissen *et al.*, 2018). A recent ChIP study has shown that Dps preferentially binds to inverted repeats prone to form secondary structures in DNA, and when comparing the binding sites of Dps to those of other NAPs, the greatest correlation was between Fis and Dps binding sites (Antipov *et al.*, 2017). Instead of transcriptional regulation, the main role of Dps is to shield DNA from damaging agents, such as oxidative stress, thermal stress, and metal toxicity (Almiron *et al.*, 1992, Martinez and Kolter, 1997, Karas *et al.*, 2015). While Dps induction causes rapid compaction of the bacterial nucleoid, Dps is unable to hinder transcription by RNA polymerase (Wolf *et al.*, 1999, Janissen *et al.*, 2018).

Due to the lack of an effect of Dps on the transcriptome, changes in Fis concentration would be the predominant cause of changes in gene expression in GX and OX. However, it could be argued that perhaps the *fis* gene alone should have been moved to a more *oriC*-distal position, while maintaining the native *dps* gene position, for the analyses in this chapter. Previously, Gerganova *et al.*, (2015), relocated the *fis* gene to *oriC*-distal positions in the Ter macrodomain, and observed an increase in *fis* expression which compensated for the reduction in the *fis* copy number. Further, Bryant *et al.*, (2014) observed that gene dosage was not the only factor in determining gene expression at a given location on the chromosome. To account for unknowns from inserting the *fis* gene at any *oriC*-distal position, this study used exchange strains where the expression patterns at each locus had been well characterised.

This study sought to examine the global changes in Fis binding and gene expression patterns. To do this, the *fis* gene was modified so that it expressed an epitope-tagged Fis protein. The presence of the tag did not interfere with the normal functions of Fis. The wild type and the strains with reciprocal exchanges of *fis* and *dps* were compared: the global binding patterns of the Fis protein were compared by ChIP combined with DNA sequencing, and the gene expression patterns were compared by RNA sequencing.

4.2 Results

4.2.1 The 8xMyc epitope tag does not affect Fis function

As generating a monoclonal antibody for ChIP experiments can be quite costly, epitope tagging a protein of interest represents a viable alternative. One drawback of epitope tagging is the risk of interfering with the protein's folding and/or function (Jarvik and Telmer, 1998). The 8xMyc epitope tag was chosen as it has previously been used in this laboratory for Fis studies, and it is not known to disrupt Fis' function (Cho *et al.*, 2008b, Cameron and Dorman, 2012). Regardless, a number of precautionary experiments were performed to compare *fis* gene expression and Fis protein production in the tagged and untagged strains.

To determine whether the 8xMyc tag had an impact on *fis* expression, RNA was isolated from WT, WT *fis::8xmyc*, GX and GX *fis::8xmyc*. It was then DNase treated, converted into cDNA and quantified by qPCR. cDNA amplified from the transcript of the constitutively expressed gene *hemX* was used to normalise the data. The expression of *fis* was unaffected by the presence of the 8xMyc epitope tag (Fig 4.1).

Next, the impact of the 8xMyc epitope tag on Fis function was assessed. Fis is a transcription factor as well as a NAP, and its regulon encompasses over 1,500 genes (Wang *et al.*, 2013). Therefore, the function of Fis could be assessed by examining the expression of genes within the Fis regulon. Three genes of which Fis is a repressor were chosen whose expression were examined for analysis: *dps*, *gyrB* and *rpoS* (Grainger *et al.*, 2008, Keane and Dorman, 2003, Hirsch and Elliott, 2005a). Again, *hemX* was used to normalise the data. The expression of *dps*, *gyrB* and *rpoS* was similar in the tagged and untagged strains (Fig 4.2).

Together these results indicate that the sequence expressing the 8xMyc epitope tag does not affect *fis* expression, nor does the tag affect Fis function.

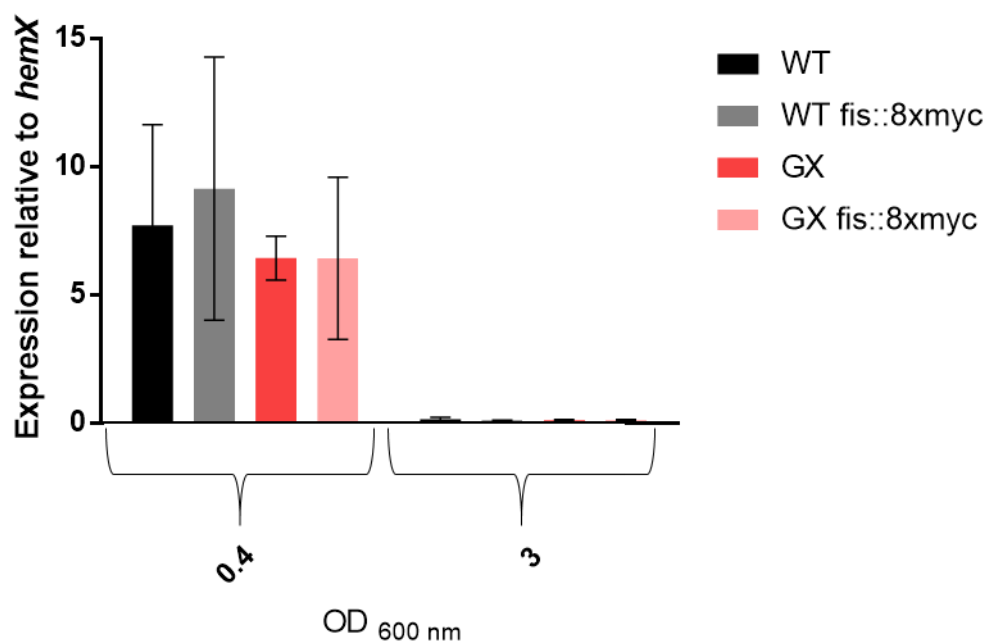


Fig 4.1. The expression of *fis* is unaffected by the 8xMyc epitope tag. RNA was isolated at various points throughout growth, DNase treated and converted into cDNA. The abundance of *fis* was quantified by qPCR normalised to the constitutively expressed gene *hemX*. The expression of *fis* is unaffected by the 8xMyc epitope tag ($p > 0.05$). Error bars represent standard deviation of three independent biological replicates. Statistical significance was calculated by the student's t-test.

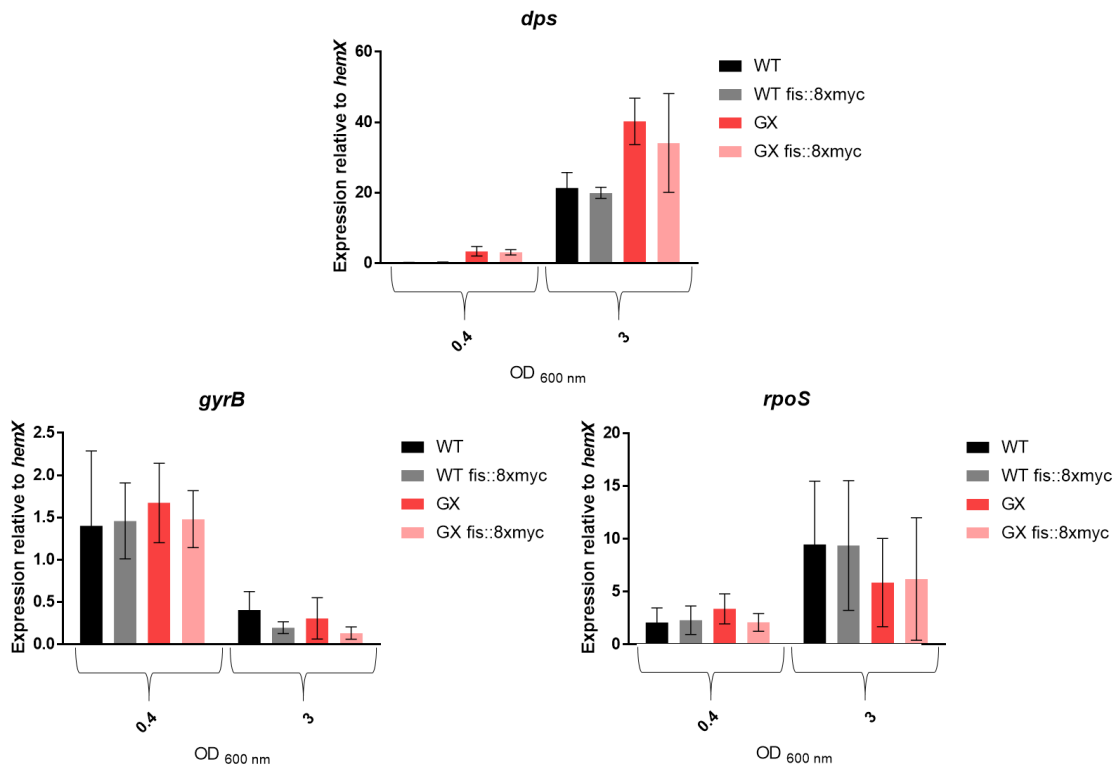


Fig 4.2. The expression of genes regulated by Fis are unaffected by the 8xMyc epitope tag. RNA was isolated at various points throughout growth, DNase treated and converted into cDNA. The abundance of *dps*, *rpoS* and *gyrB* was quantified by qPCR normalised to the constitutively expressed gene *hemX*. The expression of *dps*, *rpoS* and *gyrB* are unaffected by the 8xMyc epitope tag ($p > 0.05$). Error bars represent standard deviation of three independent biological replicates. Statistical significance was calculated by the student's t-test.

4.2.2 Global gene expression in *fis-dps* exchange strains

To assess the impact of the *fis-dps* exchanges on global gene expression, total RNA was isolated at two stages in growth, at OD_{600 nm} values of 0.4 and 3. These OD_{600 nm} values represent points during exponential growth and early stationary phase, respectively. The 8xMyc epitope tagged strains were used for this analysis, so that RNA-seq data would be directly comparable to ChIP-seq data (Section 4.2.3). The RNA was treated with DNase I to remove DNA, and prior to sequencing an aliquot was converted into cDNA and *dps* expression was assessed by qPCR. This ensured that there was little deviation between the two biological replicates which were to be sent for sequencing (Fig 4.3).

Library preparation, rRNA depletion, cDNA synthesis and sequencing were performed by Vertis Biotechnologie AG using single-reads for all samples for two independent biological replicates of each strain at each point in growth (Section 2.5.5). The resulting data were mapped back to the reference genomes, and differential gene expression was calculated using the Rstudio edgeR package. The false discovery rate (FDR) was determined by the Benjamini-Hochberg procedure, whereby p-values are ranked from smallest to largest, and divided by the lower ranked p-values to eliminate false positives generated by type I error (rejection of a true null hypothesis). Differentially expressed genes were determined to be those with an FDR below 0.05. These bioinformatic analyses were kindly performed by Dr. Aalap Mogre. The cDNA reads varied between 10,195,329 and 14,340,160 among samples.

To validate the RNA-seq data, the Log₂ fold changes of *fis* and *dps* in GX and OX were compared to those found by qPCR. The Log₂ fold changes of *fis* and *dps* were similar between these two approaches (Fig 4.4).

During exponential growth, a total of 11 genes was differentially expressed between GX and WT, including four downregulated genes, and seven upregulated genes (Table 4.1). Upregulated genes included *dps*, *fliC*, and *STM2340-2344*. The four downregulated genes were *yhdJ*, *yhdU*, *fljA* and *fljB* (Table 4.1). The genes *yhdJ* and *yhdU* are located directly downstream of the native *fis* locus.

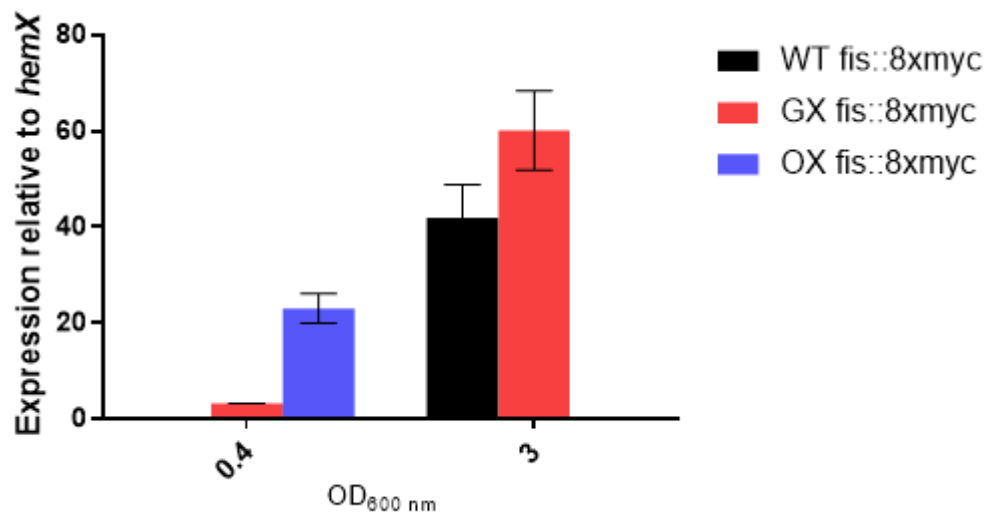


Fig 4.3. The expression of *dps* in samples analysed by RNA-seq. An aliquot of each sample to be analysed by RNA-seq was converted into cDNA. The abundance of *dps* was quantified by qPCR normalised to the constitutively expressed gene *hemX*. There is little deviation between biological replicates. Error bars represent standard deviation of two independent biological replicates.

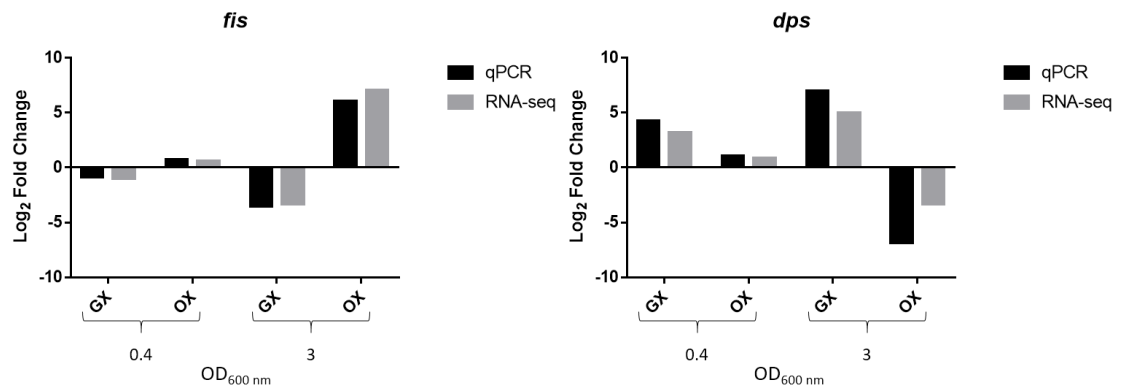


Fig 4.4 Validation of RNA-seq results by comparing to qPCR data. The Log₂ fold changes of *fis* and *dps* from the RNA-seq experiment were compared to those found in the qPCR experiment. For both genes tested the Log₂ fold changes were largely similar.

Gene	Exponential Phase	Early Stationary Phase	Function
	Log ₂ Fold Change	Log ₂ Fold Change	
<i>dps</i>	3.15	0.83	DNA protection during starvation protein
<i>yhdJ</i>	-1.32	3.29	CcrM-like DNA adenine methyltransferase
<i>yhdU</i>	-1.43	1.8	hypothetical exported protein
<i>fljA</i>	-6.72	-6.95	repressor of phase 1 flagellin gene
<i>fljB</i>	-8.78	-8.59	flagellin
<i>fliC</i>	4.08	3.15	flagellin
<i>prpC</i>	N/A	-0.92	2-methylecitate synthase
<i>prpD</i>	N/A	-1.09	propionate catabolic protein
<i>prpE</i>	N/A	-1.04	propionate catabolic protein
<i>STM1967</i>	N/A	1.14	hypothetical exported protein
<i>STM2340</i>	1.63	N/A	hypothetical transketolase C-terminal section
<i>STM2341</i>	2.21	N/A	hypothetical transketolase N-terminal section
<i>STM2342</i>	2.66	N/A	hypothetical membrane protein
<i>STM2343</i>	2.83	N/A	hypothetical sugar phosphotransferase component II B
<i>STM3388</i>	N/A	1.61	hypothetical membrane protein
<i>STM4535</i>	N/A	1.95	PTS transport system, IIA component
<i>STM4537</i>	N/A	1.36	PTS transport system, IIC component
<i>STM4538</i>	N/A	1.45	PTS transport system, IID component
<i>STM4539</i>	N/A	1.39	hypothetical sugar isomerase
<i>SL1344_1928</i>	N/A	-1.32	hypothetical bacteriophage protein

Table 4.1. Differentially expressed genes in GX.

In early stationary phase, 17 genes were differentially regulated between GX, with upregulation of 11 genes, and downregulation of six genes (Table 4.1). Again, *dps* and *fliC* were upregulated, while *fljAB* were downregulated. Interestingly, in contrast to exponential growth, *yhdJ* and *yhdU* were upregulated, indicating that the expression of these genes is dependent on the expression of the upstream gene, which is *fis* in WT (Table 4.1). Three of the downregulated genes are part of the propionate utilisation operon. These genes are involved in the metabolism of propionate, and share a common intermediate with the propanediol utilization operon, a pathway whose expression is affected in *fis* mutants (Kelly *et al.*, 2004, Palacios *et al.*, 2003).

In the case of OX, a total of 1,207 and 1,543 genes were differentially expressed compared to WT in exponential and early stationary phases, respectively (Fig 4.5). A full list of differentially expressed genes in OX is provided in Appendix I, where genes are ranked in order of $\log_2$ fold changes, and includes the $\log_2$ counts per millions, p-values and the FDR. In exponential phase there was a total of 557 upregulated genes, and 650 downregulated genes. In early stationary phase, there was a total of 813 upregulated genes, and 730 downregulated genes (Fig 4.5). Of the differentially expressed genes, 148 and 209 had a $\log_2$ fold change greater than 2 in exponential and early stationary phase respectively. Among the differentially expressed genes there were 50 differentially expressed non-coding RNAs in both growth phases, and these, along with the plasmid replication protein *tapA* were among the genes with the greatest $\log_2$ fold change in expression. Despite the increase in Fis production in early stationary phase to a concentration similar to that of GX (Fig 3.6), there was a far greater number of differentially expressed genes in OX at this growth phase (Fig 4.5). This anomaly highlights the indirect role that Fis has on gene expression through its regulation of genes encoding other transcriptional regulators.

In agreement with previous studies, the increased number of downregulated genes in exponential phase in OX indicates that Fis is predominantly an activator of gene expression, as Fis concentration is lower than WT at this point in growth (Kelly *et al.*, 2004, Wang *et al.*, 2013). Interestingly, in early stationary phase, there was a tendency for genes located around the Ter macrodomain to be

downregulated (Fig 4.5 (C)). These results indicate that the exchange of just the *fis* and *dps* ORFs (OX) has a greater impact on global gene expression than exchanging the ORFs together with their native *cis*-acting regulatory sequences (GX).

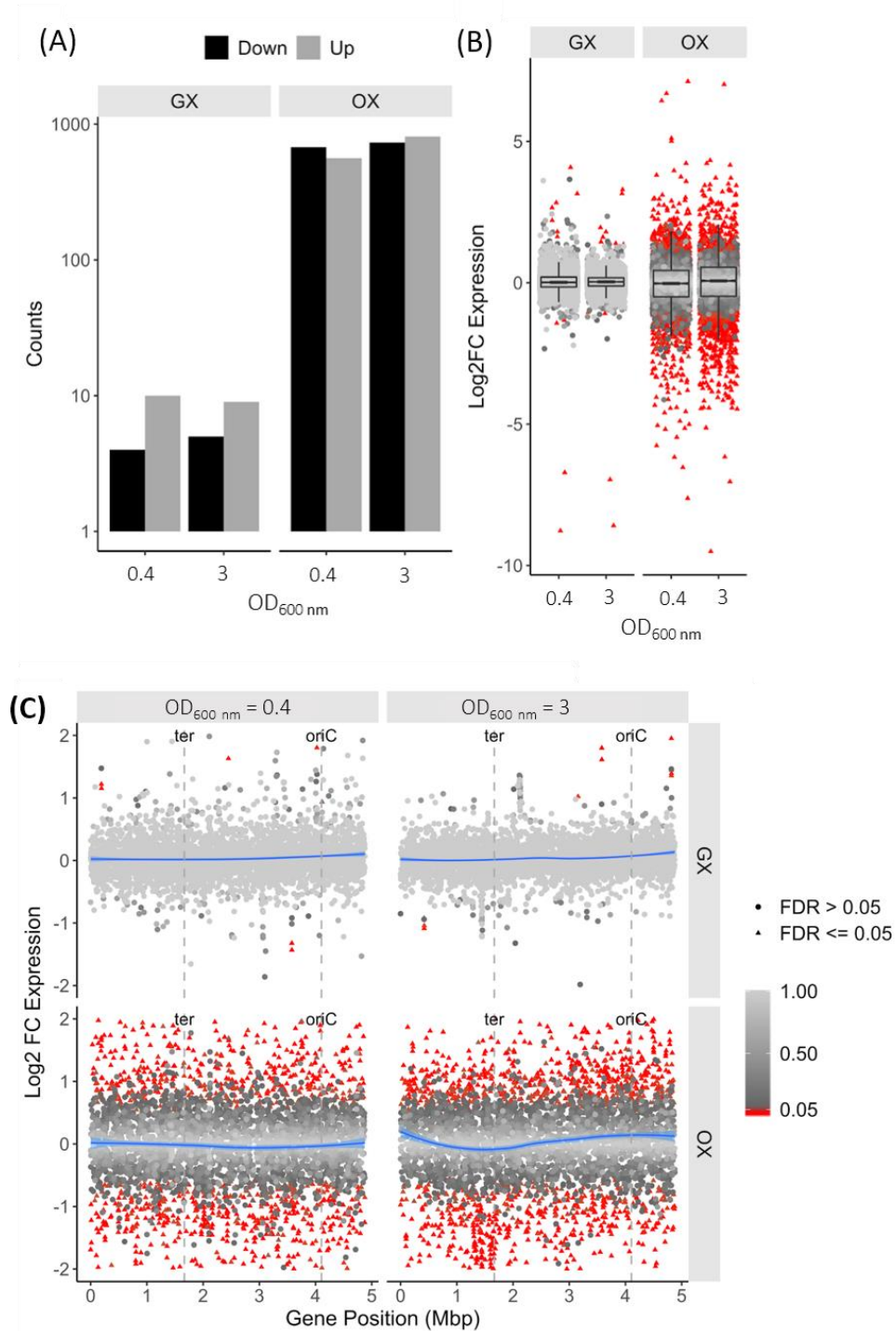


Fig 4.5. Gene expression changes assayed by RNA-seq. (A) Counts of differentially expressed genes. (B) Log₂ fold changes (Log₂FC) in expression of genes in the GX and OX strains. Significant peak intensity changes (FDR < 0.05) are shown in red. (C) Log₂ fold changes in expression scattered along the chromosome. The blue curve shows the local regression peak intensity change. The greater number of genes with an FDR below 0.05 indicates that OX shows greater mis-regulation in gene expression. Figure generated by Dr. Aalap Mogre.

4.2.3 The influence of Fis on motility genes in OX

The Fis regulons of *Salmonella* and *E. coli* have been well characterised, and as there was a large number of differentially expressed genes in OX, genes involved in various Fis-associated pathways were analysed in greater depth. The role of Fis in regulating genes involved with motility and chemotaxis has been well defined, Fis binds directly to genes involved in the flagellar biosynthesis pathway, and reduced motility has been observed in *fis* mutants (Kelly *et al.*, 2004).

In *Salmonella* and *E. coli*, there are over 50 genes involved in flagellar biosynthesis and function, divided into at least 17 operons (Chilcott and Hughes, 2000). These genes are divided into three classes, early, middle and late, depending on the order in which they are expressed. The early genes consist of the transcriptional regulators *flhD* and *flhC* which activate the expression of the middle genes. The middle genes encode proteins which are necessary for the assembly of the flagellar hook-basal body, as well as the transcriptional regulators of the late genes. Late genes encode hook-associated proteins, and proteins required for the late assembly stage, including flagellin. The flagellin of *Salmonella* is subject to phase variation, with two flagellin proteins, FliC, and FljB, which are alternatively expressed. Expression of *fljBA* is dependent on the orientation of the *hin* genetic switch. In the phase on direction, *fljBA* is expressed, where *fljA* encodes a posttranscriptional repressor of *fliC* (Bonifield and Hughes, 2003).

In OX, motility genes from all three classes were differentially expressed in exponential phase, while middle and late genes were differentially expressed in early stationary phase (Fig 4.6). Similar to GX, the genes which had the greatest change in expression were *fliC* and *fljBA* (Table 4.1). Strikingly, motility genes were predominantly downregulated during exponential phase, and upregulated in early stationary phase, in a manner that reflects the alterations in *fis* expression in OX (Fig 3.5).

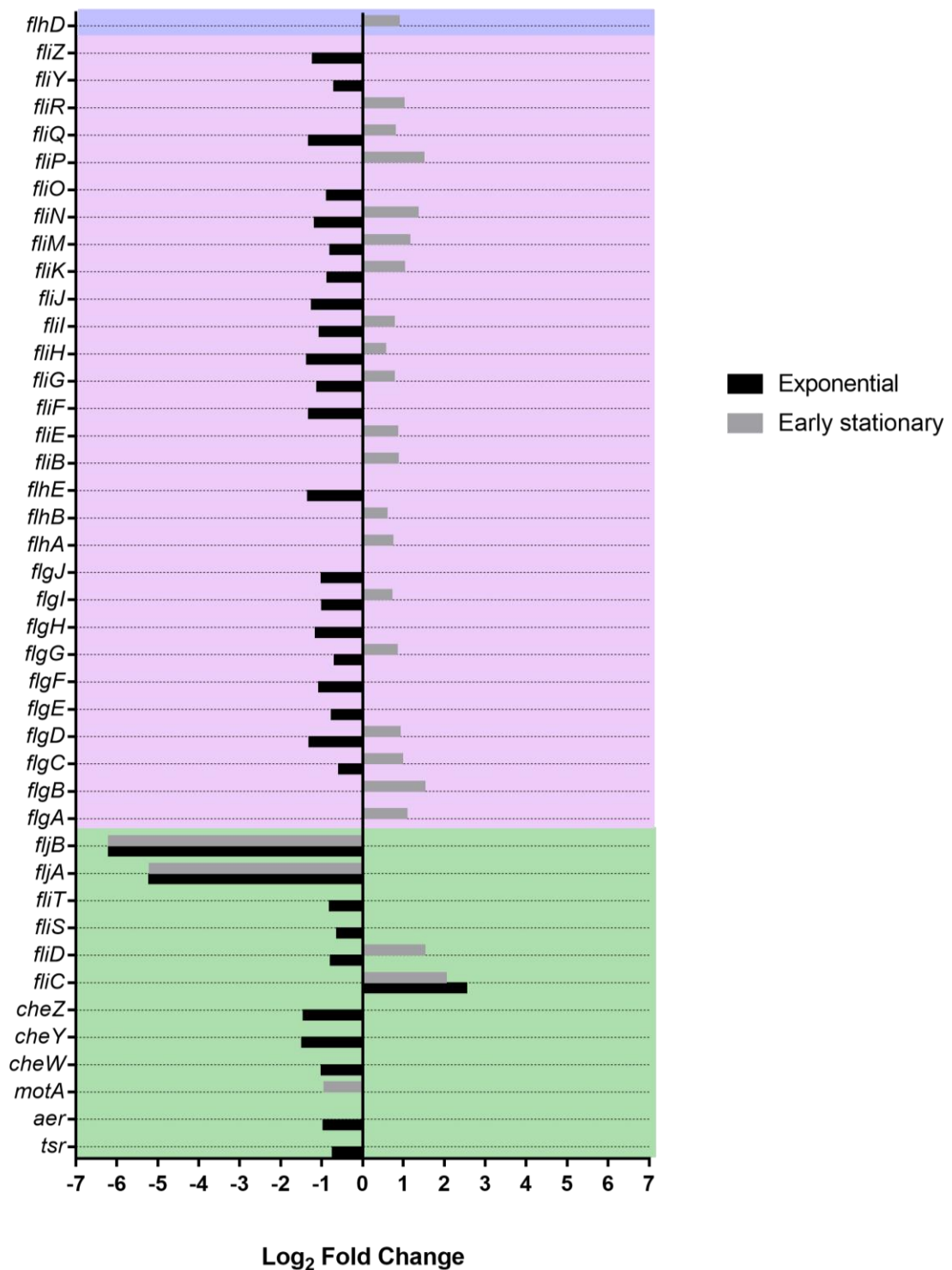


Fig 4.6. Differential expression of motility genes in OX. The log₂ fold change in expression of early (blue), middle (pink) and late (green) genes in OX. Genes which belong to both middle and late classes were assigned as late genes. Genes which were not differentially expressed are not shown. For the most part, motility genes were downregulated in exponential phase and upregulated in early stationary phase.

4.2.4 Differential expression of virulence genes in OX

Among the five major pathogenicity islands in *S. enterica* serovar Typhimurium, SPI-1 and SPI-2 are the best characterised (Marcus *et al.*, 2000). SPI-1 and SPI-2 encode the Type III secretion systems responsible for cell invasion, and survival within the macrophage respectively (Galan, 2001). The role of Fis has been well defined in *Salmonella* virulence, *fis* mutants have reduced ability to invade epithelial cells and survive within the macrophage, and *fis* is expressed into stationary phase under anaerobic conditions, such as those encountered in the gut lumen (Cameron *et al.*, 2013, Wang *et al.*, 2013, O Cróinín and Dorman, 2007).

During exponential growth, SPI-1 genes were predominantly downregulated, including the SPI-1 transcriptional regulators *hilD* and *hilC*, and the early motility genes *flhDC* (Fig 4.7) (Singer *et al.*, 2014, Petrone *et al.*, 2014). These findings are in agreement with studies in *fis* mutants (Kelly *et al.*, 2004, Wang *et al.*, 2013). In early stationary phase, 12 SPI-1 genes were upregulated, while the *sitABCD* operon was downregulated (Fig 4.7).

Only two SPI-2 genes were upregulated during exponential growth, and none was downregulated. However, in early stationary phase the majority of SPI-2 genes were downregulated (Fig 4.7). At this point in growth, Fis concentration is statistically significantly lower than that of WT, and consistent with previous findings indicates that Fis is a positive regulator of SPI-2 genes (Fig 3.6) (Kelly *et al.*, 2004, Wang *et al.*, 2013). The changes in SPI-1 and SPI-2 expression as mapped reads are shown in Fig 4.8. As SPI-1 and SPI-2 expression is low during exponential growth, the differential expression of these genes in OX is difficult to determine by examining mapped reads visually. However, in early stationary phase SPI-1 expression increases, and there are more noticeable differences between WT and OX.

SPI-3 and SPI-4 genes encode proteins which have a role in survival within the macrophage (Blanc-Potard *et al.*, 1999). While the genes of SPI-4 were upregulated in early stationary phase, many SPI-3 and SPI-5 genes were downregulated (Fig 4.9). The *mgtB* and *mgtA* genes, encoding a magnesium transporter, were upregulated.

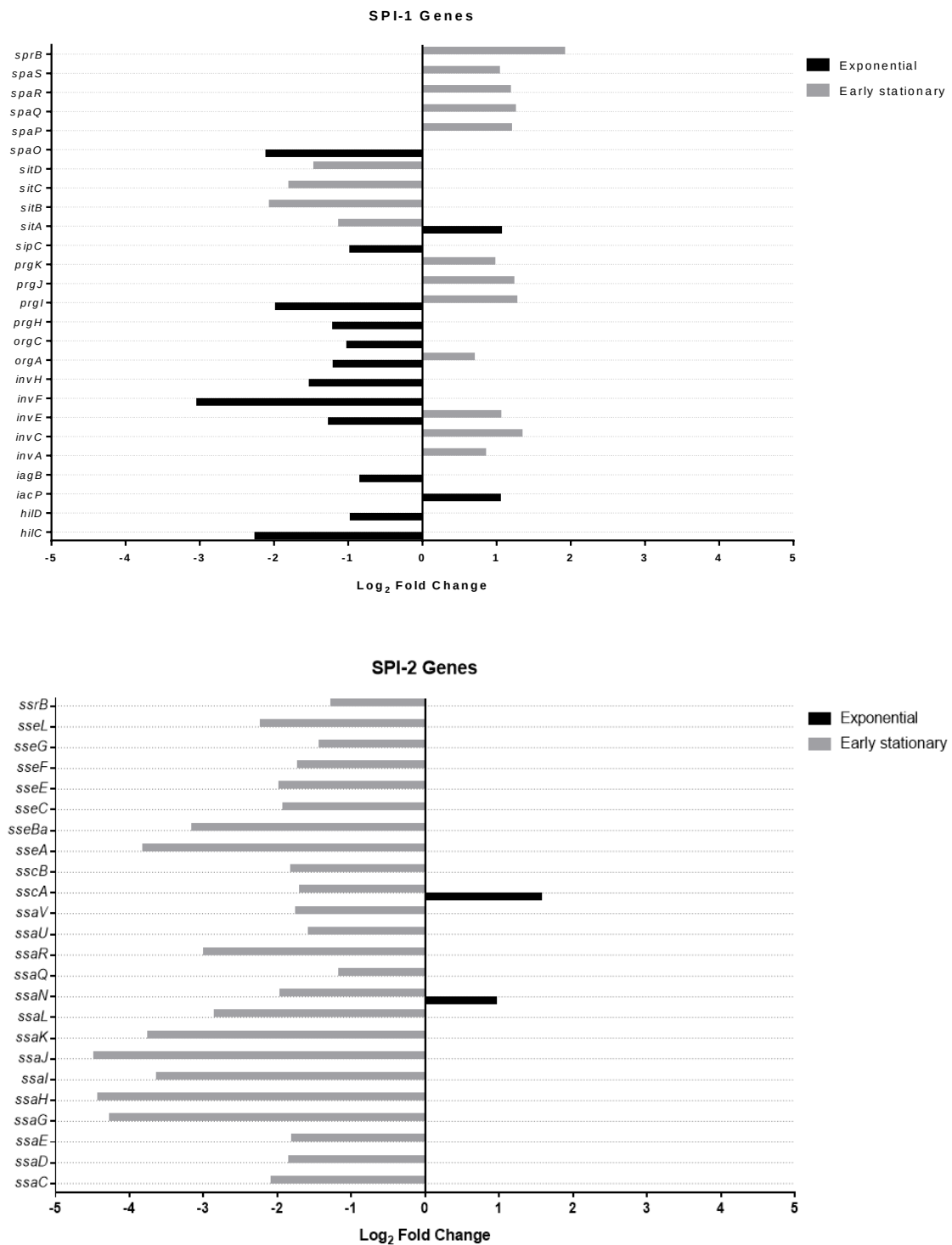


Fig 4.7. Differential expression of SPI-1 and 2 genes in OX. The log₂ fold changes in SPI-1 and SPI-2 genes in OX in exponential and early stationary phases. Genes which were not differentially expressed are not shown. SPI-1 genes were predominantly downregulated during exponential growth. SPI-2 genes were predominantly downregulated in early stationary phase.

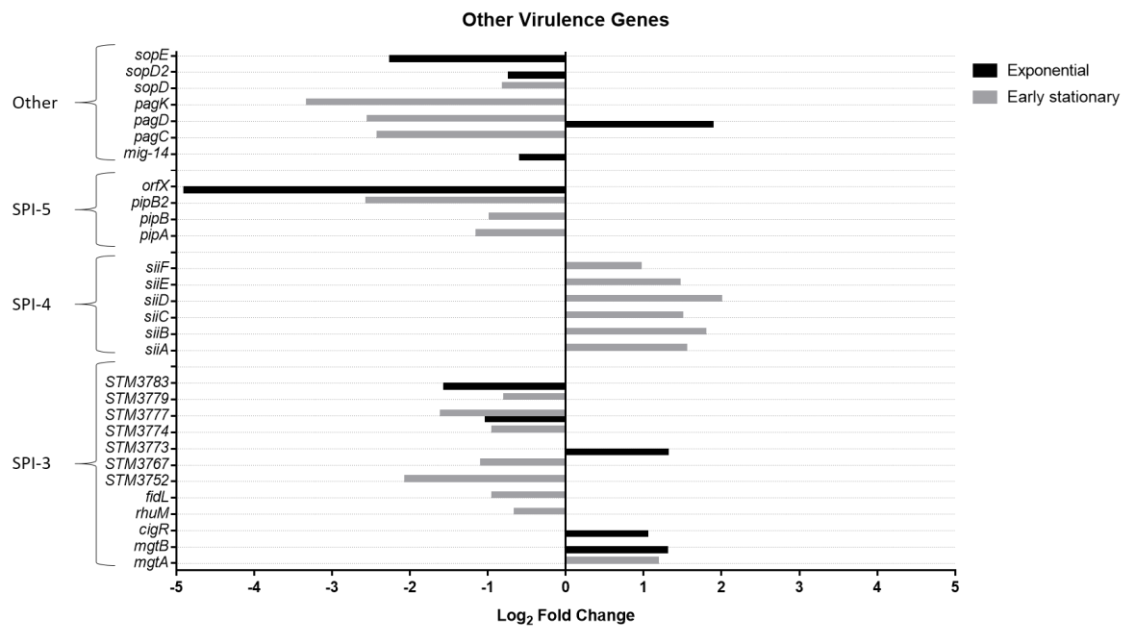


Fig 4.9. Differential expression of other virulence genes in OX. The log₂ fold changes in SPI-3, SPI-5 and other virulence genes in OX. Virulence genes were predominantly downregulated. Genes which were not differentially expressed are not shown.

Other genes involved in virulence, but not located on pathogenicity islands, were also differentially expressed in OX (Fig 4.9). These included the *pag* genes which are regulated by the PhoPQ two-component system (Behlau and Miller, 1993). In agreement with previous results, these data indicate that Fis positively regulates virulence genes (Kelly *et al.*, 2004, Wang *et al.*, 2013).

4.2.5 Differential expression of metabolism and transport genes in OX

Fis regulates many genes involved in metabolism and transport (Cho *et al.*, 2008b, Kelly *et al.*, 2004, Wang *et al.*, 2013). Kelly *et al.* (2004) compiled a list of 54 differentially expressed metabolism and transport genes from microarray studies in a *fis* mutant. This list of genes was used to identify a sample of differentially expressed genes involved in metabolism and transport in OX. From the 54 differentially expressed genes of the Kelly *et al.* (2004) study, 39 and 34 genes in OX from exponential and early stationary phase, respectively, overlapped with those of the previous study. 11 of these genes were shared among the two growth phases (Table 4.2). The other 28 and 23 genes are listed in Tables 4.3 and 4.4.

During exponential growth, metabolism and transport genes were predominantly downregulated (Table 4.3). In early stationary phase, metabolism and transport genes were predominantly upregulated (Table 4.4). Notably, the downregulation of these genes in exponential phase and upregulation in early stationary phase reflected changes in Fis production in OX (Fig 3.6), and therefore indicate that Fis is an activator of metabolism and transport genes. Examples of these genes are those involved in glutamine and glutamate metabolism (*gln* and *glt* genes, respectively). These were downregulated in exponential phase and upregulated in early stationary phase. Notably, these genes are members of the RpoN regulon, and Fis and RpoN synthesis are enhanced by polyamines (Hartman *et al.*, 2016, Terui *et al.*, 2007).

The propanediol utilization pathway was one of the pathways that was upregulated in early stationary phase in OX (Fig 4.10). There are 21 genes in the *pdu* operon (Bobik *et al.*, 1999). Interestingly, differential expression of the *pdu* genes was not observed during exponential growth, which is in contrast to previous findings in a *fis* knockout mutant (Kelly *et al.*, 2004). This pathway requires vitamin B₁₂ as a co-factor, and three *btu* genes, encoding proteins which are involved in vitamin B₁₂ import were upregulated (Table 4.4). Additionally, the *pdu* operon is regulated by the regulatory protein, PocR (Bobik *et al.*, 1999). However, *pocR* was not among the differentially expressed genes in OX.

	Exponential Phase	Early Stationary Phase	
Gene	Log ₂ Fold Change	Log ₂ Fold Change	Description
<i>aldB</i>	1.13	-3.49	aldehyde dehydrogenase B
<i>fabB</i>	-2.11	-0.69	3-oxoacyl-[acyl-carrier-protein] synthase I
<i>fhuB</i>	-1.06	1.02	ferrichrome transport protein FhuB precursor
<i>fhuD</i>	-1.3	1.24	ferrichrome-binding periplasmic protein precursor
<i>fumA</i>	-1.56	0.8	Fumarate hydratase class I, aerobic
<i>fumB</i>	-0.76	0.8	fumarate hydratase class I
<i>glnP</i>	-1.05	0.79	glutamine transport system permease protein GlnP
<i>glnQ</i>	-1.9	0.59	glutamine transport ATP-binding protein GlnQ
<i>gltA</i>	-1.32	0.67	citrate synthase
<i>gltB</i>	-0.74	0.73	glutamate synthase [NADPH] large chain precursor

Table 4.2. Differentially expressed metabolism and transport genes shared among both growth phases in OX. Descriptions were obtained from Kroger *et al.* (2012)

Gene	Log ₂ Fold Change	Description
<i>aceK</i>	1.31	isocitrate dehydrogenase kinase/phosphatase
<i>cadA</i>	1.4	lysine decarboxylase
<i>csgG</i>	1.3	assembly/transport component in curli production
<i>cysD</i>	-3.14	ATP sulfurylase (ATP:sulfate adenylyltransferase)
<i>cysH</i>	-2.27	Phosphoadenosine phosphosulfate reductase
<i>cysJ</i>	-2.71	sulfite reductase (NADPH) flavoprotein beta subunit
<i>cysK</i>	-0.85	cysteine synthase A
<i>cysP</i>	-4.41	thiosulphate-binding protein precursor
<i>cysU</i>	-3.46	sulphate transport system permease protein CysT
<i>cysZ</i>	0.88	hypothetical sulfate transport protein CysZ
<i>fabA</i>	-1.03	D-3-hydroxydecanoyl-(acyl carrier-protein)
<i>fabD</i>	-0.52	malonyl CoA-acyl carrier protein transacylase
<i>fabF</i>	-0.72	3-oxoacyl-[acyl-carrier-protein] synthase II
<i>fabG</i>	-0.45	3-oxoacyl-[acyl-carrier protein] reductase
<i>fabI</i>	-0.66	enoyl-[acyl-carrier-protein] reductase (NADH)
<i>fhuA</i>	-1.08	ferrichrome-iron receptor
<i>fhuC</i>	-1.25	ferrichrome transport ATP-binding protein FhuC
<i>fumC</i>	-0.75	Fumarate hydratase class II
<i>glnH</i>	-1.74	glutamine-binding periplasmic protein precursor
<i>gltD</i>	-1.8	glutamate synthase (NADPH) small chain
<i>gltK</i>	-1.12	glutamate/aspartate transport system permease protein GltK
<i>gltL</i>	-2.12	glutamate/aspartate transport ATP-binding protein GltL
<i>nupG</i>	-1.19	nucleoside permease nupg (nucleoside-transport system protein)
<i>sdhA</i>	-1.33	succinate dehydrogenase flavoprotein subunit
<i>sdhB</i>	-1.63	succinate dehydrogenase iron-sulfur protein
<i>speA</i>	0.76	Biosynthetic arginine decarboxylase
<i>speC</i>	0.51	ornithine decarboxylase, constitutive (ec 4.1.1.17)
<i>speD</i>	-0.68	S-adenosylmethionine decarboxylase, proenzyme

Table 4.3. Differentially expressed metabolism and transport genes during exponential growth in OX. Descriptions were obtained from Kroger *et al.* (2012)

Gene	Log ₂ Fold Change	Description
<i>btuB</i>	1.31	isocitrate dehydrogenase kinase/phosphatase
<i>btuD</i>	1.4	lysine decarboxylase
<i>btuF</i>	1.3	assembly/transport component in curli production
<i>cysA</i>	-3.14	ATP sulfurylase (ATP:sulfate adenylyltransferase)
<i>cysC</i>	-2.27	Phosphoadenosine phosphosulfate reductase
<i>cysI</i>	-2.71	sulfite reductase (NADPH) flavoprotein beta subunit
<i>cysN</i>	-0.85	cysteine synthase A
<i>cysW</i>	-4.41	thiosulphate-binding protein precursor
<i>eutB</i>	-3.46	sulphate transport system permease protein CysT
<i>eutC</i>	0.88	hypothetical sulfate transport protein CysZ
<i>eutH</i>	-1.03	D-3-hydroxydecanoyl-(acyl carrier-protein)
<i>eutK</i>	-0.52	malonyl CoA-acyl carrier protein transacylase
<i>glnA</i>	-0.72	3-oxoacyl-[acyl-carrier-protein] synthase II
<i>glnD</i>	-0.45	3-oxoacyl-[acyl-carrier protein] reductase
<i>ndk</i>	-0.66	enoyl-[acyl-carrier-protein] reductase (NADH)
<i>potC</i>	-1.08	ferrichrome-iron receptor
<i>potF</i>	-1.25	ferrichrome transport ATP-binding protein FhuC
<i>potG</i>	-0.75	Fumarate hydratase class II
<i>psd</i>	0.73	phosphatidylserine decarboxylase proenzyme
<i>rbsC</i>	1.09	high affinity ribose transport protein
<i>sdhC</i>	1.08	succinate dehydrogenase cytochrome b-556 subunit
<i>speB</i>	-0.61	Agmatinase
<i>speG</i>	0.56	spermidine N1-acetyltransferase

Table 4.4. Differentially expressed metabolism and transport genes in early stationary phase in OX. Descriptions were obtained from Kroger *et al.* (2012)

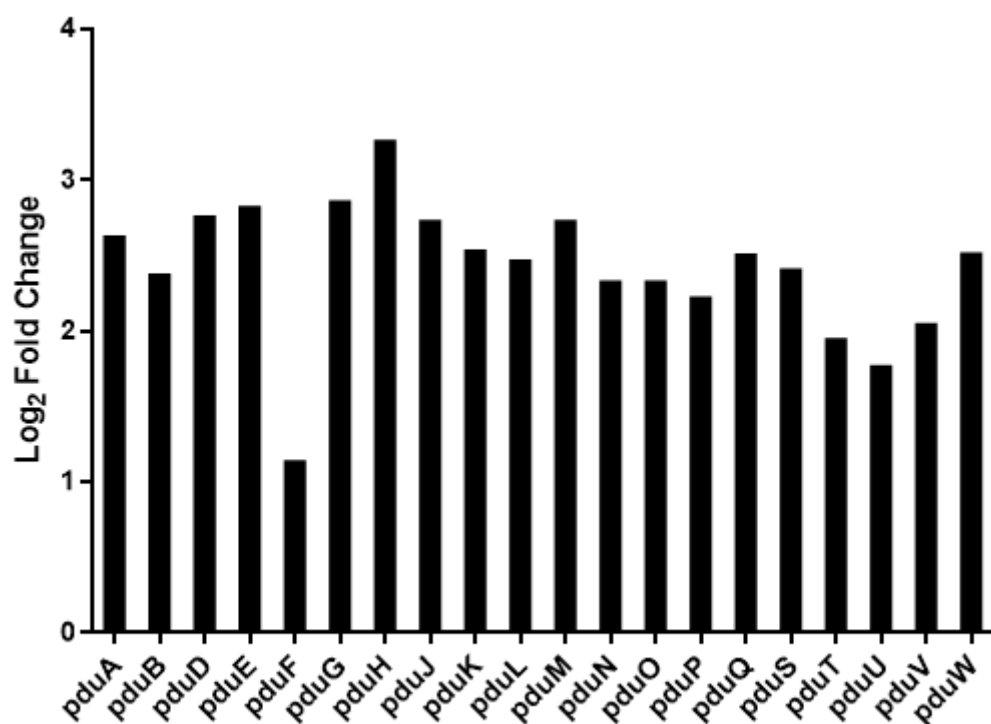


Fig 4.10. Upregulation of propanediol utilization genes in early stationary phase in OX. The log₂ fold change of the *pdu* genes in OX. 20 out of the 21 genes in the propanediol utilization operon were upregulated in OX in early stationary phase.

4.2.6 Differential expression of global regulators and stress genes in OX

Genes involved in stress responses and transcriptional regulators were next identified. Notably genes encoding the β , β' and ω subunits of RNA polymerase were differentially expressed. In exponential phase *rpoB* and *rpoC* were downregulated by a log₂ fold change of 1.08 and 0.8, respectively, while *rpoZ* was upregulated by a log₂ fold change of 1.09 and 1.34 for exponential and early stationary phases respectively. Genes encoding sigma factors were also differentially regulated, especially in early stationary phase (Table 4.5). Sigma factors are the subunits of RNA polymerase holoenzyme which recognise promoter elements, and variance in their expression has consequences for the expression of genes within their regulon (Schellhorn *et al.*, 1998). Fis is a repressor of *rpoS*, and binds directly to its promoter (Hirsch and Elliott, 2005a). Consistent with the changes in Fis production in OX, *rpoS* was upregulated during exponential growth, and downregulated in early stationary phase.

Many NAPs have been characterised as regulators of gene expression, and genes encoding NAPs were differentially expressed in OX (Table 4.6). There was no change in expression in *hupB*, *ihfA*, *ihfB* or *cbpB* in either growth phase in OX. As expected, *dps* and *fis* had the greatest changes in expression, *fis* was downregulated in exponential phase, and upregulated in stationary phase. The expression patterns of *dps* were also opposite to those in WT, and this effect is due to the exchange of the *fis* and *dps* ORFs in this strain. The orthologous genes *hns* and *stpA* were downregulated in both growth phases. These two NAPs are global repressors of gene expression, and the *hns* gene is directly regulated by Fis (Oshima *et al.*, 2006, Falconi *et al.*, 1996). Two NAP-encoding genes, *hupA* and *lrp* were downregulated during exponential growth and upregulated in early stationary phase. HU is a regulator of genes involved with metabolism and virulence, while Lrp regulates genes involved in metabolism (Mangan *et al.*, 2011, Newman *et al.*, 1992).

Other transcription regulators which were differentially expressed in OX included *phoP* and *crp*. PhoP is part of the two-component PhoPQ regulatory system, responsible for adapting to low Mg²⁺ conditions, is a regulator of approximately 5% of genes in *S. Typhimurium*, including acid stress resistance genes, and the

pag virulence genes (Garcia Vescovi *et al.*, 1994, Harari *et al.*, 2010). Expression of *phoP* was upregulated by a log₂ fold change of 0.52 and 0.88 in exponential and stationary phases respectively. CRP plays a major role in catabolite repression, directly binding to approximately 200 genes, and indirectly regulating approximately half of the genes in *E. coli* (Grainger *et al.*, 2005). Expression of *crp* was upregulated 0.53 fold in early stationary phase.

The expression of *rtsA* and *rtsB* encoding a SPI-1 regulator and *flhDC* respectively, were also differentially expressed in OX (Ellermeier and Slauch, 2003). The expression of *rtsA* and *rtsB* were downregulated during exponential growth, and *rtsB* was upregulated in early stationary phase. These changes in *rtsA* and *rtsB* expression are consistent with previous findings (Kelly *et al.*, 2004).

Besides stress-specific sigma factors, other stress response genes were differentially expressed. The *htrA* gene induced by heat shock was upregulated 0.62 fold in early stationary phase (Wick and Egli, 2004). During exponential growth, the *cspA*, *cspB* and *cspC* genes encoding cold shock proteins were downregulated 3.31, 2.47 and 1.06 fold respectively (Czapski and Trun, 2014). If these cold shock proteins are efficiently translated, then they could have implications for the proteome, as cold shock induction causes a transient block in protein synthesis (Etchegaray and Inouye, 1999). Consistent with previous findings, *sodB* encoding Fe-containing superoxide dismutase was differentially expressed in a Fis dependent manner (Kelly, 2004).

Genes	Exponential Phase	Early Stationary Phase	Function
	Log ₂ fold change	Log ₂ fold change	
<i>rpoD</i>	-0.73	-0.75	Housekeeping
<i>rpoS</i>	1.64	-0.99	Starvation/stationary phase
<i>rpoH</i>	-0.65	N/A	Heat shock
<i>rpoE</i>	N/A	-0.9	Extreme heat stress and extracellular proteins
<i>fliA</i>	N/A	-0.77	Flagellar biosynthesis and chemotaxis
<i>rpoN</i>	N/A	0.78	Nitrogen limitation
<i>fecI</i>	N/A	N/A	Ferric citrate transport and metabolism

Table 4.5. Differential expression of genes encoding sigma factors in OX. Descriptions were obtained from Kroger *et al.* (2012)

	Exponential Phase	Early Stationary Phase	
Genes	Log ₂ fold change	Log ₂ fold change	Description
<i>dps</i>	5.01	-3.3	DNA protection during starvation protein
<i>fis</i>	-3.34	7.01	Fis DNA-binding protein
<i>hns</i>	-1.87	-0.73	DNA-binding protein (histone-like protein Hlp-II)
<i>stpA</i>	-1.22	-1.13	DNA-binding protein StpA
<i>hupA</i>	-1.6	1.13	histone like DNA-binding protein HU-alpha
<i>lrp</i>	-0.96	0.7	leucine-responsive regulatory protein
<i>mukB</i>	N/A	0.54	cell division protein
<i>cbpA</i>	0.84	N/A	curved DNA-binding protein

Table 4.6. Differential expression of NAP-encoding genes in OX. Descriptions were obtained from Kroger *et al.* (2012)

4.2.7 The effect of *fis-dps* exchanges on Fis binding patterns

Chromatin immunoprecipitation, combined with DNA sequencing, was used to determine the global binding patterns of Fis::8xMyc in WT, GX and OX. For this experiment, mock IPs and experimental IPs were collected during exponential growth ($OD_{600\text{ nm}} = 0.4$), as this is a time when Fis concentration would be high in WT. Although there was a delay between the growth condition used and addition of the fixative (Section 2.6.1), which may have influenced the results of the experiment, this delay remained constant among all samples. Therefore, the results remained comparable between strains. Despite this, and prior to sequencing, qPCR was used to check the fold-enrichment at a known Fis binding site (the *rpoS* promoter / *PrpoS*), and a site that Fis does not bind to (the *carA* promoter / *PcarA*) (Hirsch and Elliott, 2005a, Wang *et al.*, 2013). There was approximately a 10-fold enrichment between these two promoters, indicating that the ChIP experiment was a success (Fig 4.11).

For the ChIP-seq samples, library preparation and sequencing were performed by Vertis Biotechnologie AG (Section 2.6.8), and peak calling was kindly performed by Dr. Aalap Mogre. This resulted in between 1,209 and 1,497 peaks among all samples. The Benjamini-Hochberg method of determining the false discovery rate (FDR) was used to identify false positives. These peaks were then assessed visually using the Integrated Genome Browser to determine a $\log_{10}$ FDR cut-off. From this, a $\log_{10}$ FDR cut-off of 20 was selected, as peaks below this number appeared to have poor enrichment. This reduced the number of common peaks between replicates to between 762 and 790 peaks, with a median peak length of 534-756 bp among samples. Again, the Fis binding site in the *rpoS* promoter was used as a positive control, and a visualisation of the mapped reads to this peak is showed in Fig 4.12. This observation is in agreement with the findings of the ChIP-qPCR, and are indicative that the ChIP-seq experiment has worked. A full list of all peaks is listed in appendix II.

The Fis binding sites and changes in global transcription patterns were visualised using the online tool CiVi, by inputting lists of peaks, or differentially expressed genes with their corresponding $\log_2$ fold changes in binding intensity, or expression (Fig 4.13) (Overmars *et al.*, 2015).

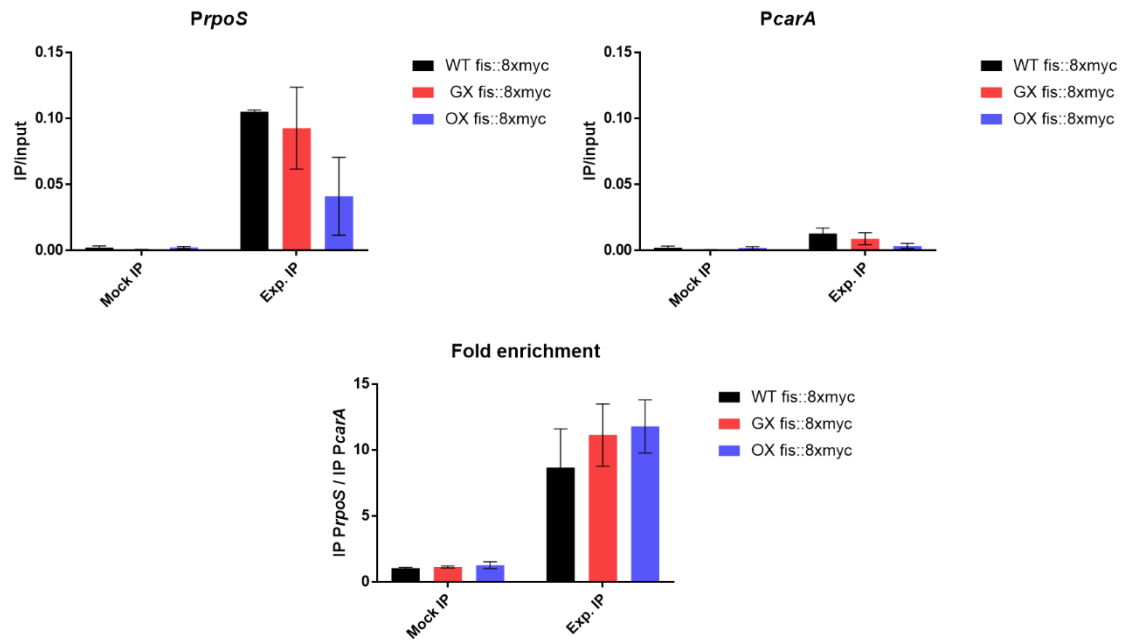


Fig 4.11 ChIP-qPCR of known sites to which Fis does or does not bind. Prior to sequencing, the enrichment at a known Fis binding site (*PrpoS*), and at a site which Fis is not known not to bind (*PcarA*) was quantified by qPCR. For all three strains, there was enrichment at *PrpoS*, but not at *PcarA*. The change in enrichment equates to an approximate 10-fold change. Error bars represent standard deviation of two independent biological replicates.

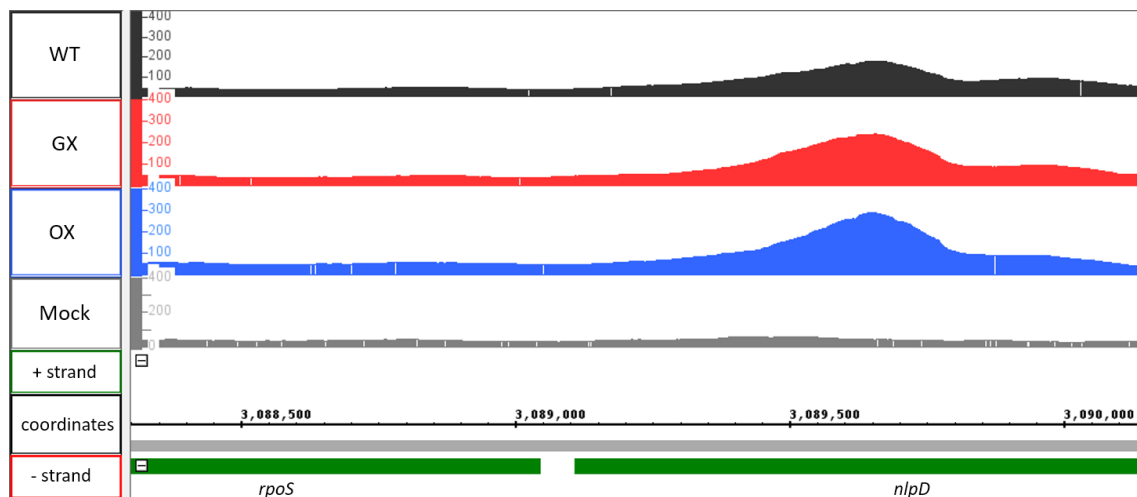


Fig 4.12 Mapped reads to the Fis binding site in the *rpoS* promoter. The mapped reads to the *rpoS* promoter were visualised using Integrated Genome Browser. In WT, GX and OX there was an observable peak in this region compared to the mock IP (grey) indicating Fis binding. These results are in agreement with the ChIP-qPCR data.

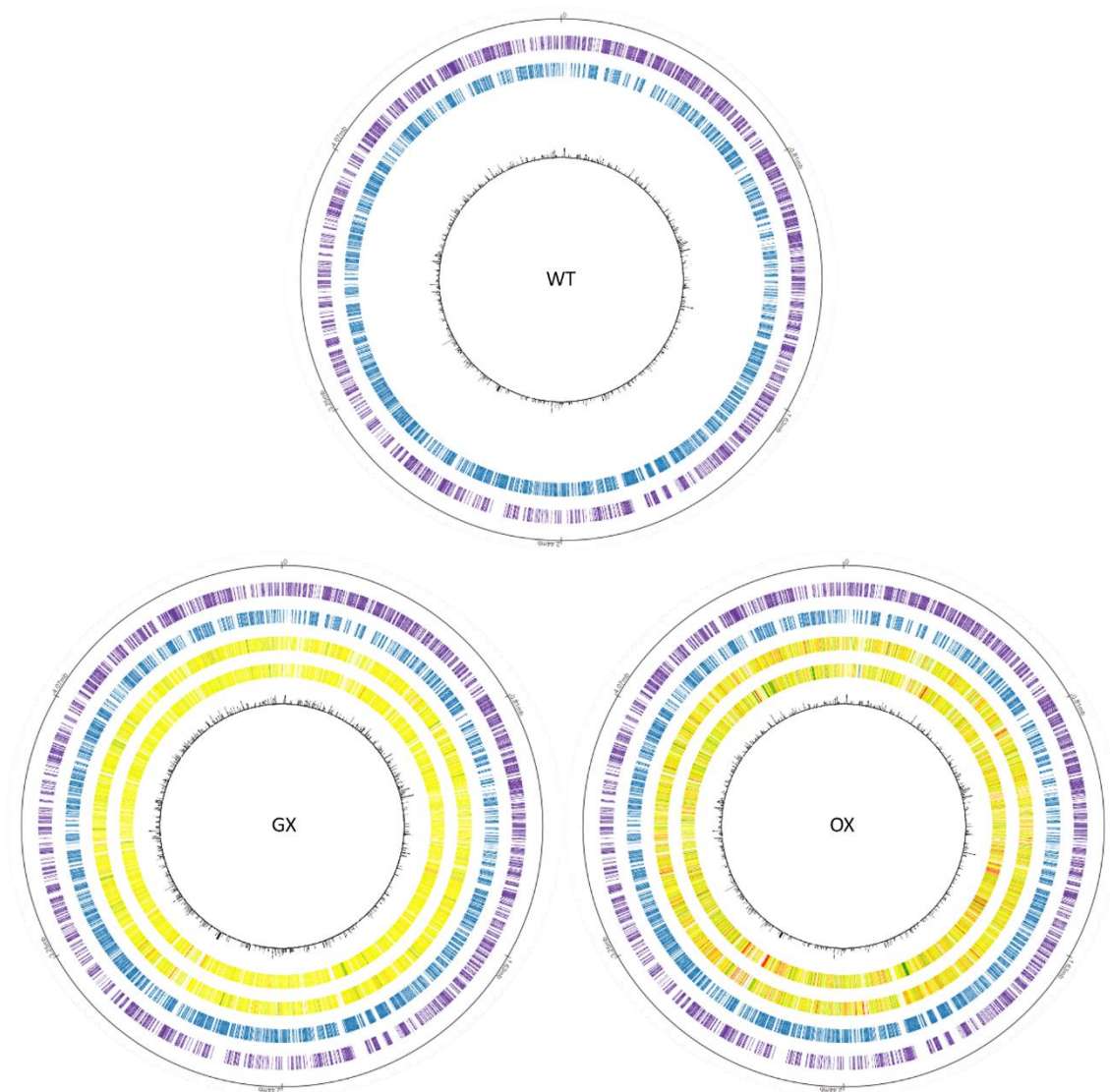


Fig 4.13. The circular chromosomes of WT, GX and OX, with expression data and Fis binding sites. The CiVi online tool was used to visualise the changes in gene expression and Fis binding sites around the chromosome for WT, GX and OX. From the outermost ring to the innermost where ring 1 is the outermost, ring 1 shows the location relative to position zero measured in millions of base pairs (mbp) of the *S. Typhimurium* str. SL1344 genome. Ring 2 shows the genes on the positive strand. Ring 3 shows the genes on the negative strand. Ring 4 (GX and OX only) shows the changes in gene expression in exponential phase compared to WT, with green representing upregulated genes, red represents downregulated genes and yellow represents genes whose expression is unaffected. Ring 5 (GX and OX only) shows the changes in gene expression in early stationary phase using the same colour scheme as ring 4. Ring 6 shows the Fis binding sites across the genome as determined by ChIP-seq.

Next, the overall Fis binding intensity between WT and GX or OX were examined. Overall, there was a decrease in Fis binding intensity with an average Log₂ fold-change of -0.44 and -0.91 for both GX and OX respectively compared to WT (Fig 4.14 (A)). An FDR of 0.05 was determined to be the cut-off for differential binding between WT and GX or OX. The binding intensity of Fis in GX and OX compared to WT was mapped along the chromosome (Fig 4.14 (B)). In GX there were 162 differentially bound peaks compared to WT, while in OX there were 306 differentially bound peaks. The differentially bound peaks are listed in Appendix III. The log₂ fold change of differentially bound peaks with an FDR below 0.05 was greater between OX and WT than between GX and WT, with 111 peaks having a Log₂ fold change less than -2.0 between OX and WT compared to 26 between GX and WT (Fig 4.14 (B)). Interestingly, there was a shift in binding intensity along the chromosome, with a reduction in binding intensity in Ori (Fig 4.14 (B)). This effect was more pronounced in OX than in GX.

In OX, out of the 1,207 differentially expressed genes, 246 of them were found to be bound by Fis (20.4%), and 106 of these genes had an increase in expression, while 140 had decreased expression. Of the differential Fis binding sites with an FDR below 0.05, 41 out of 246 genes were bound by Fis. Eleven of these genes were upregulated, and 30 were downregulated. Consistent with previous findings, these data indicate that Fis predominantly acts as an activator of gene expression (Kelly *et al.*, 2004, Wang *et al.*, 2013). The large number of genes displaying changes in transcription is greater than the number that are bound by Fis (20.4%). This indicates that Fis' predominant effect on gene expression is indirect.

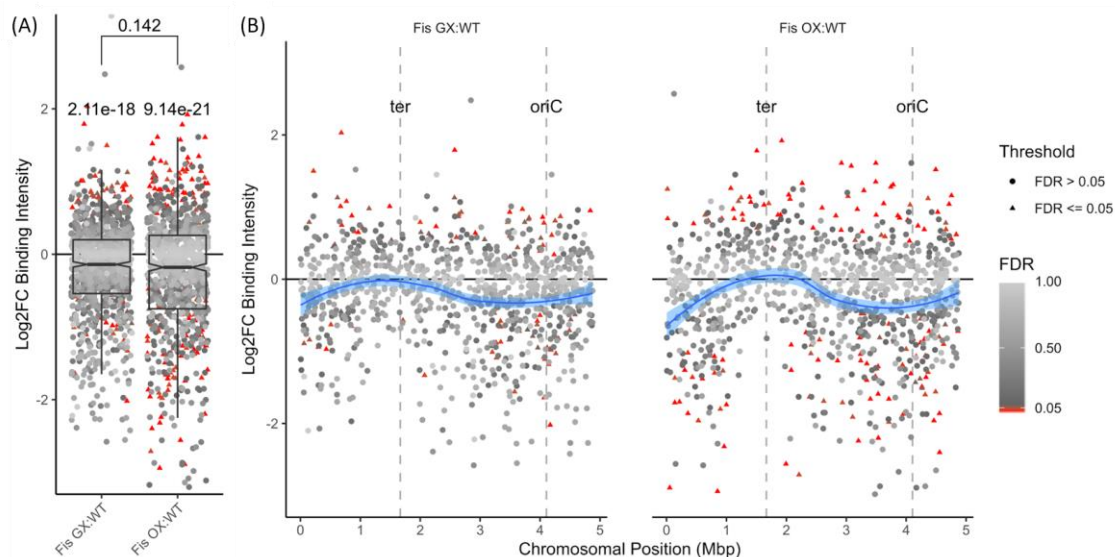


Fig 4.14. Changes in Fis binding assayed by ChIP-seq. (A) ChIP-seq was used to identify Fis peaks in WT, GX and OX. The Log₂ fold change (Log₂FC) in Fis binding intensity was calculated, and significant peak intensity changes (FDR < 0.05) are shown in red. (B) Peak intensity changes plotted as a function of chromosomal position. The blue curve shows the local regression peak intensity change. Both strains show a pattern of increased Fis binding around Ter and decreased binding around *oriC*, with the extent of change being greater in OX. In calculating these intensity changes, the appropriate mocks (WT mock for WT peaks, GX mock for GX peaks, OX mock for OX peaks) were used as controls for ori-ter gradient changes. Figure generated by Dr. Aalap Mogre.

4.3 Discussion

Fis is a global regulator of gene expression, and previous RNA-seq studies have found that Fis regulates ~1,500 genes in *Salmonella*, either in a direct or an indirect manner (Wang *et al.*, 2013, Kelly *et al.*, 2004). The changes in *fis* and *dps* gene position affected both Fis and Dps production in GX and OX (Fig 3.6 and Fig 3.7). Therefore, to elucidate how these changes caused global effects, global transcription patterns and Fis binding patterns were investigated. Epitope tagging of Fis was performed to allow the Fis protein to be detected in global DNA binding experiments with ChIP. The 8xMyc epitope tag did not affect the expression of *fis* or the functions of the Fis protein, as assessed by monitoring the expression patterns of genes known to be regulated by Fis (Fig 4.1 and Fig 4.2).

The exchange of *fis* and *dps* ORFs together with their *cis*-acting regulatory sequences (GX) had only a small effect on global transcription patterns, with just 11 and 17 genes being differentially expressed in exponential and early stationary phases, respectively (Fig 4.5). Conversely, exchanging the *fis* and *dps* ORFs alone (OX) had a greater impact on global transcription patterns, with 1,207 and 1,543 genes being differentially expressed in exponential and early stationary phases, respectively (Fig 4.5). As Dps is not known to affect the expression of any genes, it is probable that changes in Fis concentration are responsible for all of the changes in gene expression that were detected (Janissen *et al.*, 2018). RNA-seq has not been performed previously in a bacterium which has had its *fis* *cis*-acting regulatory sequences, exchanged for those of a different gene, but studies in a Δ *fis* mutant have identified between 1,495 and 1,646 genes within the Fis regulon in *S. Typhimurium* (Wang *et al.*, 2013, Wang *et al.*, 2015, Kelly *et al.*, 2004). These studies were performed in early exponential and mid-exponential phases of the growth cycle. When comparing the gene list generated from this study to that generated from another RNA-seq study in a *fis* mutant conducted by Wang *et al.* (2013), only 333 genes were shared between them. In my investigation, the expression of *fis* increases in OX in stationary phase (Fig 3.5), and no previous study has examined global expression patterns in a strain which expresses *fis* in stationary phase. The dissimilarity between the lists of

differentially expressed genes may be caused by a reduced, but not zero, concentration of Fis in this strain during exponential growth.

Consistent with previous findings, Fis had a large regulatory role in the expression of genes involved in motility and virulence, and many of these genes were differentially expressed in OX (Kelly *et al.*, 2004, Wang *et al.*, 2013). Expression of early, middle and late genes contributing to the flagellar biosynthesis pathway was affected by the exchange of the *fis* and *dps* ORFs (Fig 4.6). Notably, the expression of the genes encoding the master regulator, *flhD* and of the sigma factor associated with flagellar biosynthesis, *fliA* was downregulated. The downregulation of these genes is a probable cause of changes in the expression of other genes involved in motility. Flagellar biosynthesis genes were predominantly downregulated in exponential phase and upregulated in early stationary phase, which reflects the changes in *fis* expression in OX (Fig 4.6). An exception to this was the downregulation of *fljBA* in both GX and OX. The expression of these genes is dependent on the orientation of the *hin* invertible genetic switch, and one oversight when epitope tagging Fis was in determining if the tagged protein could still function as an architectural element at Fis-dependent genetic switches (Bonifield and Hughes, 2003, Dorman and Bogue, 2016). If Fis::8xMyc is unable to function in facilitating *hin* inversion, and if the switch was in a locked off position, then this would explain the downregulation of *fljBA*.

This same effect was observed with genes located in SPI-1, where SPI-1 genes were predominantly downregulated during exponential growth, and upregulated in early stationary phase (Fig 4.7). The exception here, was with the *sit* genes which were downregulated during early stationary phase. The *sitABCD* operon encodes a manganese transporter, and is regulated by the ferric uptake regulator, Fur, and MntR (Janakiraman and Slauch, 2000, Ikeda *et al.*, 2005). Neither *fur* nor *mntR* were differentially expressed in OX, suggesting another means of regulating the *sitABCD* operon. The regulator of SPI-1, RtsA, was downregulated during exponential growth. RtsA induces *hilA* expression, and downregulation of *rtsA* could contribute to reduced SPI-1 expression at this growth phase (Ellermeier and Slauch, 2003).

In contrast, only two SPI-2 genes were upregulated in OX, and these were during exponential growth. These genes were *sscA* and *ssaN*, encoding a chaperone and an ATPase, respectively (Yoshida *et al.*, 2014, Cooper *et al.*, 2013). However, SPI-2 genes were downregulated in early stationary phase (Fig 4.7). Crosstalk between SPI-1 and SPI-2 is mediated by HilD, which displaces H-NS from the promoters of *ssrAB*, and SsrAB is a two-component transcriptional regulator of SPI-2 genes (Martinez *et al.*, 2014). During exponential growth *hilD* was downregulated in OX, and expression returned to normal levels in early stationary phase (Fig 4.7). However, this reduced expression in exponential phase may have knock-on effects for the expression of SPI-2 genes in early stationary phase, and *ssrB* was also downregulated at this point in growth (Fig 4.7).

Previous studies in a *fis* knockout mutant have indicated that Fis is a repressor of the propanediol utilization pathway encoded by the *pdu* genes (Kelly *et al.*, 2004). During exponential growth, there was no observable change in *pdu* expression in OX, while in early stationary phase 20 out of 21 *pdu* genes were upregulated (Fig 4.10). Crp is required for the induction of *pdu* genes, *crp* was upregulated in early stationary phase, and may be responsible for increased *pdu* expression at this growth phase (Ailion *et al.*, 1993).

Genes encoding sigma factors, NAPs and transcription factors were among those that were differentially expressed in OX (Table 4.5 and Table 4.6). The increase in *rpoS* expression in *fis* mutants has been reported before (Kelly *et al.*, 2004, Hirsch and Elliott, 2005a). Surprisingly, there was a decrease in expression of *rpoD* at both points in growth and *rpoD* has never been linked to changes in *fis* expression (Table 4.5). Whether the concentration of RpoD protein has changed in this strain is unknown. Changes in the expression of stress response sigma factors were also observed, the heat shock sigma factor gene *rpoH* was downregulated during exponential growth while *rpoE* and *fliA* were downregulated in early stationary phase (Table 4.5). The large number of differentially expressed genes encoding transcriptional regulators highlight the role Fis has in regulatory hierarchies. Changing the expression patterns of *fis* influences gene expression on a global level.

Fis is a major regulator of tRNA and rRNA synthesis, binding of Fis to *rrn* promoters enhances transcription 5 – 10 fold *in vivo*. Therefore, it would be interesting to examine how changes in *fis* position and *cis*-acting regulatory sequences would affect rRNA and tRNA levels. However, rRNA depletion is one of the primary steps in RNA-seq experiments, as these RNAs are over-represented.

In a similar manner to the RNA-seq experiment, the Fis binding to its DNA targets was more greatly affected in OX. There was a notable change in binding intensity along the chromosome, with a reduction in Fis binding in the vicinity of Ori (Fig 4.14). Again, this effect was more pronounced in OX than in GX. Consequences of this shift in binding intensity across the chromosome may be linked to the increased number of downregulated genes in the vicinity of Ter (Fig 4.5).

One explanation for the change binding intensity along the chromosome, is the movement of the *fis* gene to a more Ter-proximal position at the *dps* locus. However, if this was the only factor influencing this shift in binding intensity, then the effect in GX and OX would be more similar. During exponential growth, intracellular Fis concentration is reduced in GX, and further reduced in OX (Fig 3.6). When Fis concentration is reduced to lower-than-normal levels in exponential phase, perhaps Fis binds preferentially to its binding sites near Ter rather than to those in the vicinity of Ori. However, previous ChIP-seq studies examining Fis binding have focused on exponential phase, when Fis is near its maximum levels (Wang *et al.*, 2015, Kahramanoglou *et al.*, 2011).

In OX, Fis bound to only 20.4% of genes that were differentially expressed. This is in agreement with previous studies, and indicates that Fis predominantly regulates genes in an indirect manner (Cho *et al.*, 2008b, Kahramanoglou *et al.*, 2011, Wang *et al.*, 2013).

Taken together, these results indicate that exchanging the *fis* and *dps* ORFs had greater consequences on the transcriptome and for Fis binding patterns than exchanging the *fis* and *dps* genes with their *cis*-acting regulatory sequences. Strikingly, expression of Fis-dependent genes followed the re-wired expression patterns of *fis* in OX: many genes that are normally activated by Fis were

downregulated in exponential phase in OX, when Fis concentration was low, and were upregulated in early stationary phase, when Fis levels were rising.

Chapter 5:
**Phenotypic Effects of *fis* and *dps* gene and
Open Reading Frame Exchanges**

5.1 Introduction

Exchanging the regulatory regions (GX) or just the open reading frames (OX) of the *fis* and *dps* genes led to changes in *fis* and *dps* expression, global changes in *Salmonella* gene expression and changes in Fis protein binding patterns. These changes were particularly striking in OX, the effects on GX being much more subtle.

Salmonella and *E. coli* strains in which NAP-encoding genes have been relocated have been studied before. Fitzgerald *et al.* (2014) exchanged reciprocally the NAP-encoding ORFs of the *hns* and *stpA* genes and found temperature-dependent changes in bacterial competitive fitness. Gerganova *et al.* (2015) reported that moving the *dusB-fis* operon to the Ter macrodomain in *E. coli* decreased both competitive fitness and oxidative stress resistance. In the present study, the reciprocally-exchanged strains exhibited novel *fis* and *dps* expression patterns, and so it was of interest to elucidate how these changes affected bacterial physiology.

The expression of the *fis* gene is characterised by a sharp peak in the production of the Fis protein upon entry into exponential growth, and subsequently the intracellular concentration of Fis becomes diluted following multiple rounds of bacterial cell division until the protein is no longer detectable in stationary phase (Kelly *et al.*, 2004, Talukder and Ishihama, 2015).

Several studies have examined the Fis regulon in *Salmonella* and *E. coli*. In *Salmonella*, it has been reported that the expression of 1,646 genes are affected by Fis, either directly or indirectly (Wang *et al.*, 2013). Fis can either activate or repress genes, and gene ontology studies have shown that the majority of Fis-regulated genes are involved in metabolism, motility or virulence (Wang *et al.*, 2013, Kelly *et al.*, 2004). Both Fis and Dps have roles in regulating DNA replication, with Fis stimulating replication by binding at *oriC*, and Dps inhibiting replication by interacting with DnaA (Chodavarapu *et al.*, 2008, Flatten and Skarstad, 2013). Further, Fis plays an indirect role in maintaining the homeostatic balance of DNA supercoiling within the bacterial cell, through its regulation of genes encoding topoisomerases (Travers *et al.*, 2001).

In contrast to Fis, Dps does not function as a regulator of gene expression; its role is associated with protecting DNA from damaging agents, such as those encountered in stationary phase or during oxidative stress (Janissen *et al.*, 2018, Almiron *et al.*, 1992, Martinez and Kolter, 1997). Dps production increases during stationary phase, and can be rapidly induced by the hydrogen peroxide-response protein, OxyR (Michan *et al.*, 1999). Upon contact with hydrogen peroxide, OxyR is oxidized, and in this form can activate gene expression of members of its regulon (Kullik *et al.*, 1995). Despite the rapid induction of Dps production and the condensing of the nucleoid, Dps is unable to hinder the activity of RNA polymerase (Janissen *et al.*, 2018).

Given the different roles of Fis and Dps in bacterial physiology, specific phenotypes relating to each protein were chosen for investigation. Phenotypes associated with Fis included growth, cell morphology, motility and virulence gene expression. Phenotypes related to Dps included oxidative stress resistance and viability in stationary phase. With the stark changes in *fis* and *dps* expression in OX and the more subtle ones in GX, it was hypothesised that the phenotypic changes would be greater in OX. In order to elucidate the gene manipulation responsible for any change in phenotypes, the intermediate “2x” strains which harbour either two copies of *fis* or *dps* and deletion strains were also examined phenotypically.

5.2 Results

5.2.1 Altering *fis* expression extends lag phase

5.2.1.1 Bacterial growth monitored by optical density at OD_{600 nm}

The growth patterns of the strains, in which *fis* and *dps* had been exchanged, and of the “2x” strains, were monitored by optical density readings taken hourly over an 8-h period. As *fis* expression peaks during the transition from lag phase to exponential growth, and to investigate this transition, cultures were first normalised to a low starting OD_{600 nm} of 0.0005 (Kelly *et al.*, 2004). Of the two strains in which *fis* and *dps* were exchanged, only OX showed a change in its growth patterns, with an extension in lag phase of 1 h (Fig 5.1(A)). However, by 8 h OX had reached the same OD_{600 nm} as WT and GX.

As 2xfG and 2xfO each have two copies of *fis*, one of which replaces *dps* at the *dps* native locus, their growth patterns were compared to Δdps , while the growth patterns of 2xdG and 2xdO were compared to those of Δfis (Fig 5.1 (B, C)). While the growth patterns of 2xfG and Δdps remained similar to that of WT, the 2xfO displayed an extended lag phase similar to that of OX.

On the other hand, 2xdG, 2xdO and Δfis displayed a decreased OD_{600 nm} throughout the exponential growth phase. Strikingly, the growth patterns of OX and 2xfO are very similar, and this extended lag phase of these strains are suggestive of misregulation of *fis*. Together, these results confirm the importance of Fis in the bacterial growth cycle.

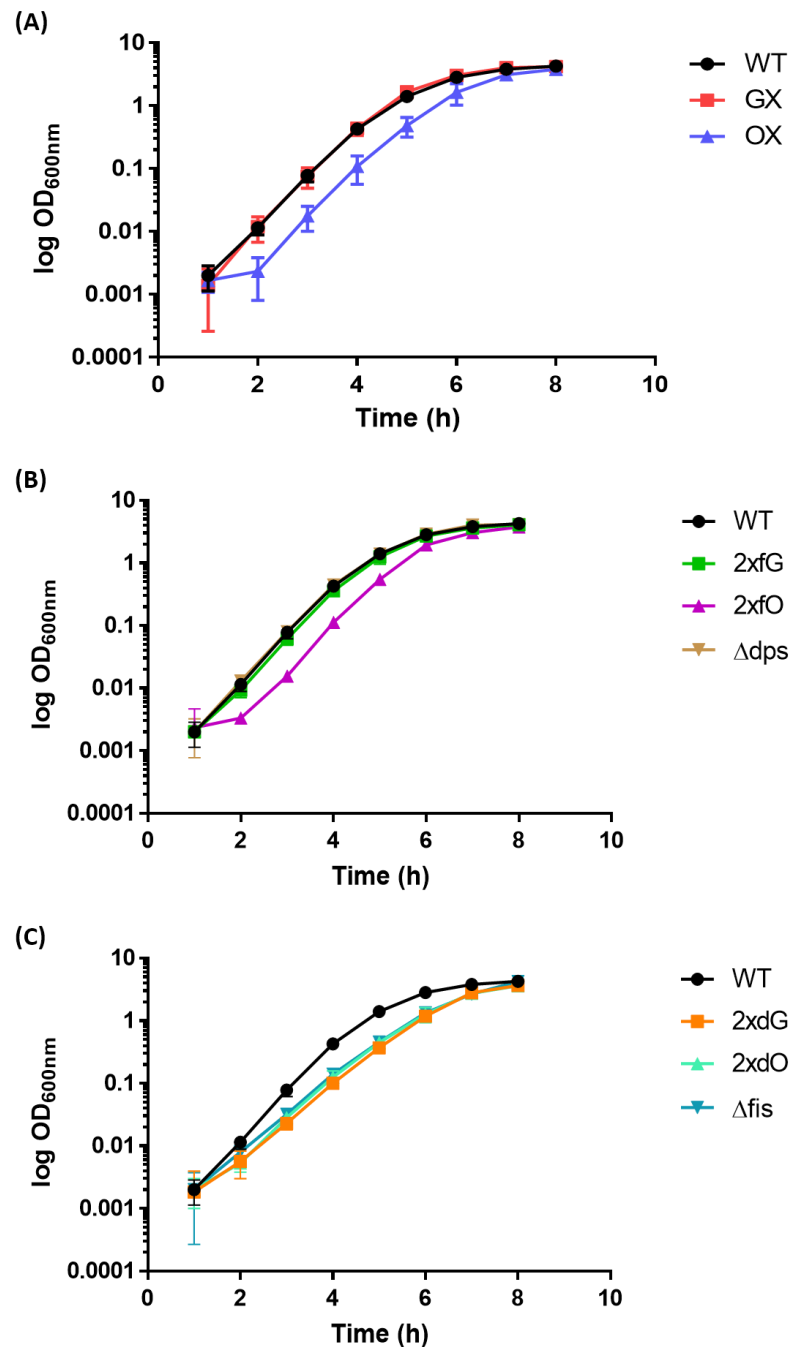


Fig 5.1 Growth of bacterial cultures monitored by OD_{600 nm} readings. OD_{600 nm} readings were measured hourly over an 8-h period and plotted against time. (A) Growth patterns of WT and the *fis-dps* exchanged strains, GX and OX. (B) Growth patterns of strains containing 2 copies of *fis* (2xfG and 2xfO) compared to Δ*dps*. (C) Growth patterns of strains containing 2 copies of *dps* (2xdG and 2xdO) compared to Δ*fis*. Error bars represent standard deviation of at least 3 biological replicates.

5.2.1.2 Growth as determined by viable counts on agar plates

To supplement the data in Section 5.2.1.2, the growth patterns of the strains were determined by counting colony-forming units on agar plates as a function of time. An advantage of plate viable counts over spectrophotometry is that the former detects only live cells, and is less susceptible to inaccuracies due to changes in cell size and/or morphology.

OX exhibited a 1-h extended lag phase, while GX showed no change in growth when compared to the WT (Fig 5.2(A)).

Similarly, the 2x *dps* strains, 2xfO and the Δfis strains had decreased viability throughout exponential growth when compared to the WT (Fig 5.2(B, C)). These results are in agreement with the growth curve observations measured by spectrophotometry (Fig 5.1).

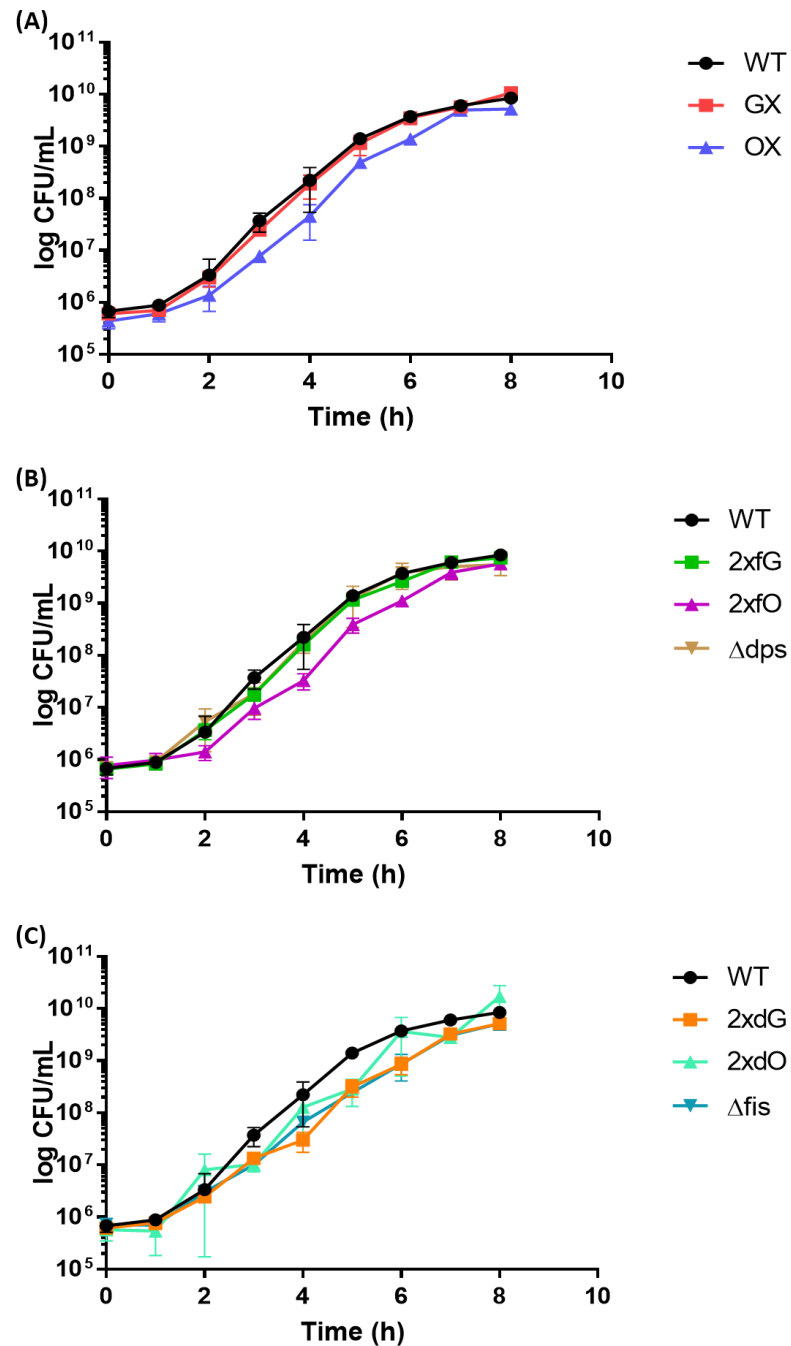


Fig 5.2 Growth determined by viable plate counts. Agar-plate viable counts were measured hourly over an 8-h period and plotted against time. (A) Growth patterns of WT and *fis-dps* exchanged strains, GX and OX. (B) Growth patterns of strains containing 2 copies of *fis* (2xfG and 2xfO) compared to Δ *dps*. (C) Growth patterns of strains containing 2 copies of *dps* (2xdG and 2xdO) compared to Δ *fis*. Error bars represent standard deviation of at least 3 biological replicates.

5.2.2 Viability of *Salmonella* in stationary phase

Dps was first characterised from stationary phase cultures of *E. coli* and its role in DNA protection in stationary phase is well documented (Almiron *et al.*, 1992). Therefore, viable counts at 24 h post-inoculation were determined to examine a phenotype which may be related to alterations in Dps production.

Decreases in viability of 1.7 fold, 2 fold and 1.5 fold were observed in the 2xfG, 2xfO and Δdps strains, respectively, compared to the WT (Fig 5.3). As these strains lack Dps, a reduction of viability would be expected in stationary phase, and previously a study has shown reduced viability of *dps* mutants in stationary phase (Nair and Finkel, 2004).

Interestingly, a 5-fold increase in survival was observed in 2xdO compared to the WT. The 2xdO strain contains 2 copies of *dps*, one of which is under the control of the *dusBfis* promoter. This increase in viability could be related to an increase in Dps production.

No statistically significant changes in viability were observed in GX or OX compared to the WT, indicating a sufficient level of Dps in stationary phase in these strains.

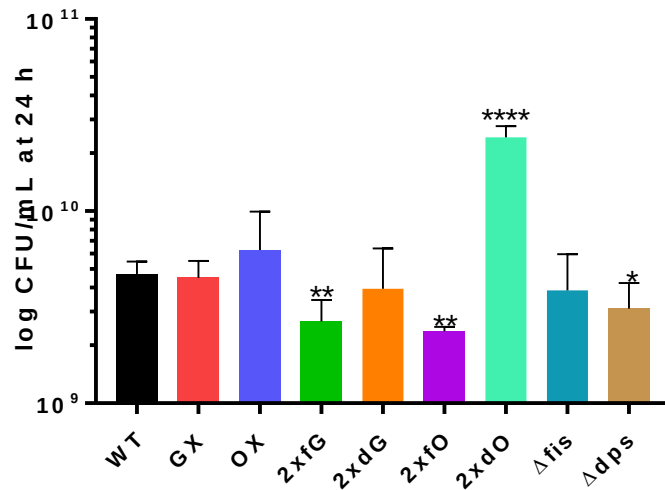


Fig 5.3 Reduced viability of *dps* deletion strains in stationary phase. Viability was measured at 24 h and compared to WT. No statistically significant changes in viability were observed in GX, OX, 2xdG or Δfis . A reduction in viability was observed in 2xfG, 2xfO and Δdps ($p = 0.006$, 0.0001 and 0.02 respectively). An increase in viability at 24 h was observed in 2xdO ($p < 0.0001$). Error bars represent standard deviation of at least 3 biological replicates. Statistical significance was determined by the student t-test.

5.2.3 Resistance to oxidative stress

The OxyR regulon encompasses 30 proteins which are important for oxidative stress resistance, and includes proteins which function in peroxide metabolism, redox balance and peroxide protection (Storz *et al.*, 1990). One such peroxide protection protein is the ferritin-like Dps protein (Martinez and Kolter, 1997). Dps has the ability to sequester iron and prevent oxyradical formation (Halsey *et al.*, 2004). Therefore, oxidative stress was a phenotype chosen to examine the effect of the altered positioning of the *dps* locus in strains constructed in this study.

Unsurprisingly, strains lacking *dps* (Δdps , 2xfG and 2xfO) showed a statistically significant decrease in survival on exposure to 0.5 mM and 1 mM H₂O₂ compared to WT (Fig 5.4). However, survival was most reduced in OX and 2xfO, which could indicate a detrimental effect to the misexpression of *fis* under *dps* regulatory signals.

Strains which contained *dps* and its *cis*-regulatory regions, at both the native *fis* and *dps* loci, had no observable change in oxidative stress resistance compared to WT.

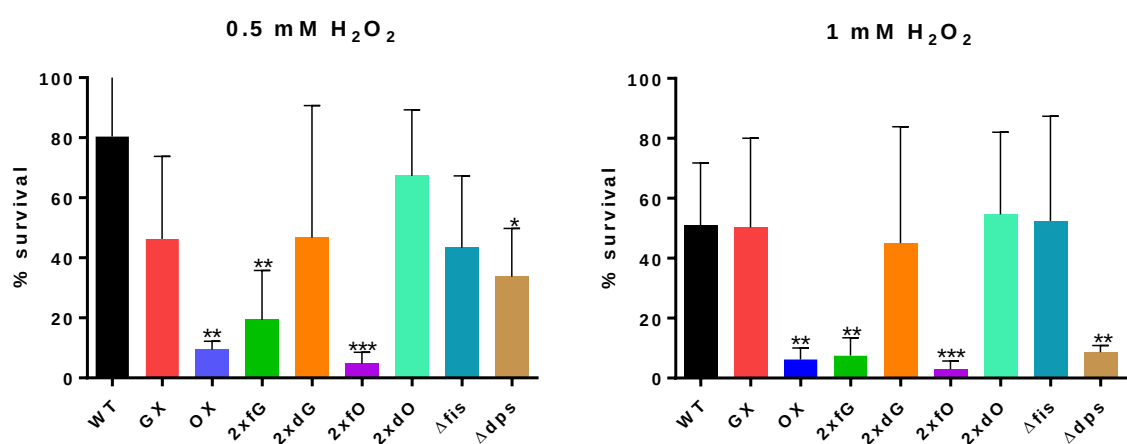


Fig 5.4 Reduced oxidative stress resistance in *dps* deletion strains and OX.

Strains were challenged with 0, 0.5 or 1 mM H₂O₂ over a 2-h period, plate counts were determined and percentage survival was calculated. No increases in oxidative stress resistance were observed. Decreases in oxidative stress resistance were observed in OX, 2xfG, 2xfO and Δ *dps* ($p = 0.006$, 0.003 , 0.0003 and 0.01 respectively). Error bars represent standard deviation of at least 3 biological replicates. Statistical significance was calculated using the student t-test.

5.2.4 Fis is required to maintain cell morphology

In all bacteria, the cell cycle must coordinate DNA replication and inheritance of the chromosome with cell growth, division, cell volume and shape (Reyes-Lamothe and Sherratt, 2019). Therefore, changes in cell morphology are indicative of defects in the cell cycle. As Fis plays roles in chromosome replication in *Salmonella* and *E. coli*, it was important to examine whether cellular morphology is altered in rewired strains for two main reasons (Filutowicz *et al.*, 1992, Flatten and Skarstad, 2013). First, it is important to observe and understand the molecular mechanism with any altered phenotypes. Second, an altered cellular morphology could affect the scattering of light needed to determine the optical density of the culture. In the latter case another method to determine growth rate would be necessary. Therefore, cellular morphology was observed using phase-contrast microscopy.

No changes in cellular morphology were observed in GX, OX, 2xfG, 2xfO, or Δdps in either the exponential or the early stationary phases of growth (Fig 5.5 and 5.6). In contrast, a filamentous cell phenotype was observed in the cases of numerous cells of those strains lacking the *fis* gene in both growth phases (2xdG, 2xdO and Δfis). The filamentous phenotype is consistent with previous observations (Filutowicz *et al.*, 1992, Flatten and Skarstad, 2013).

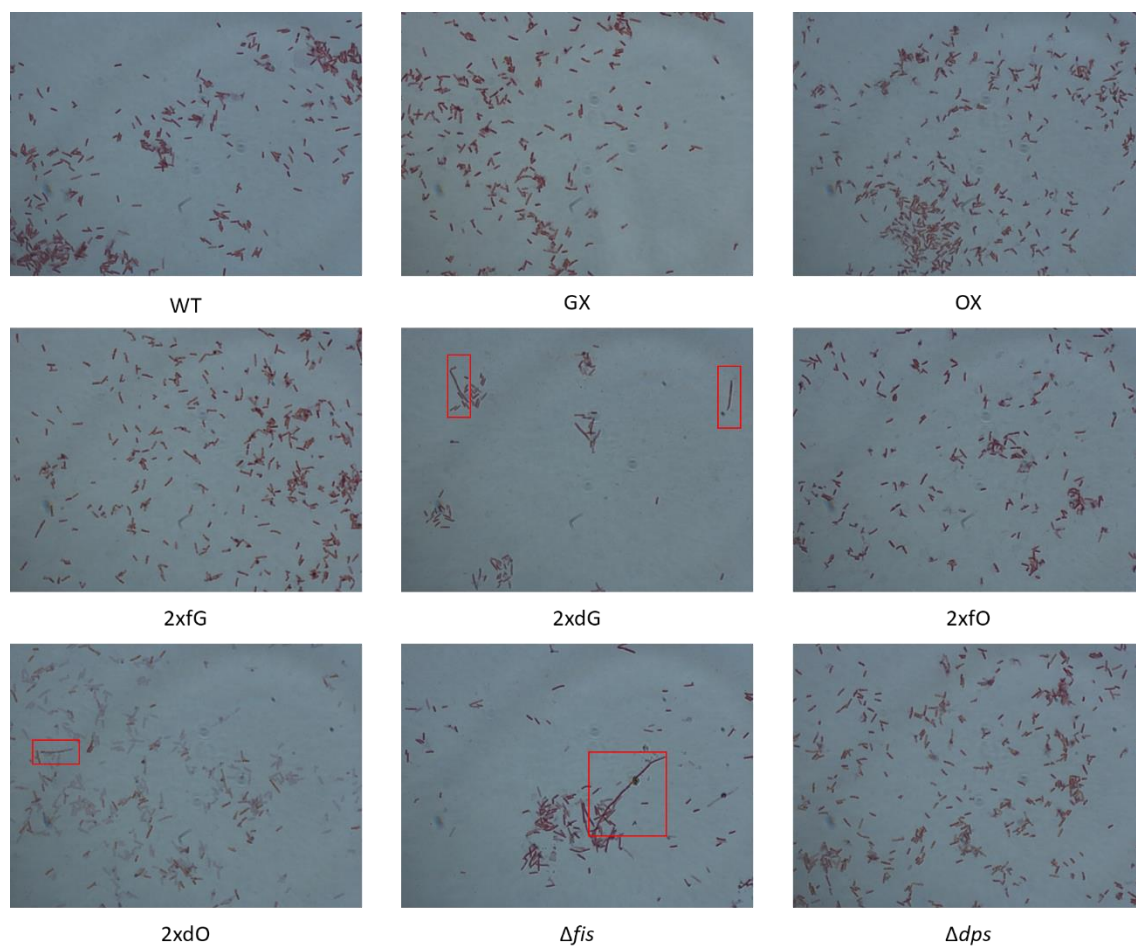


Fig 5.5. Cell morphology during exponential growth. Bacteria were grown to exponential phase, harvested, washed with PBS and stained with safranin. Filamentous cells were observed in 2xdG, 2xdO and Δfis (indicated with red boxes). No morphological changes were observed in any other strains compared to WT. Images are representative of 3 biological replicates.

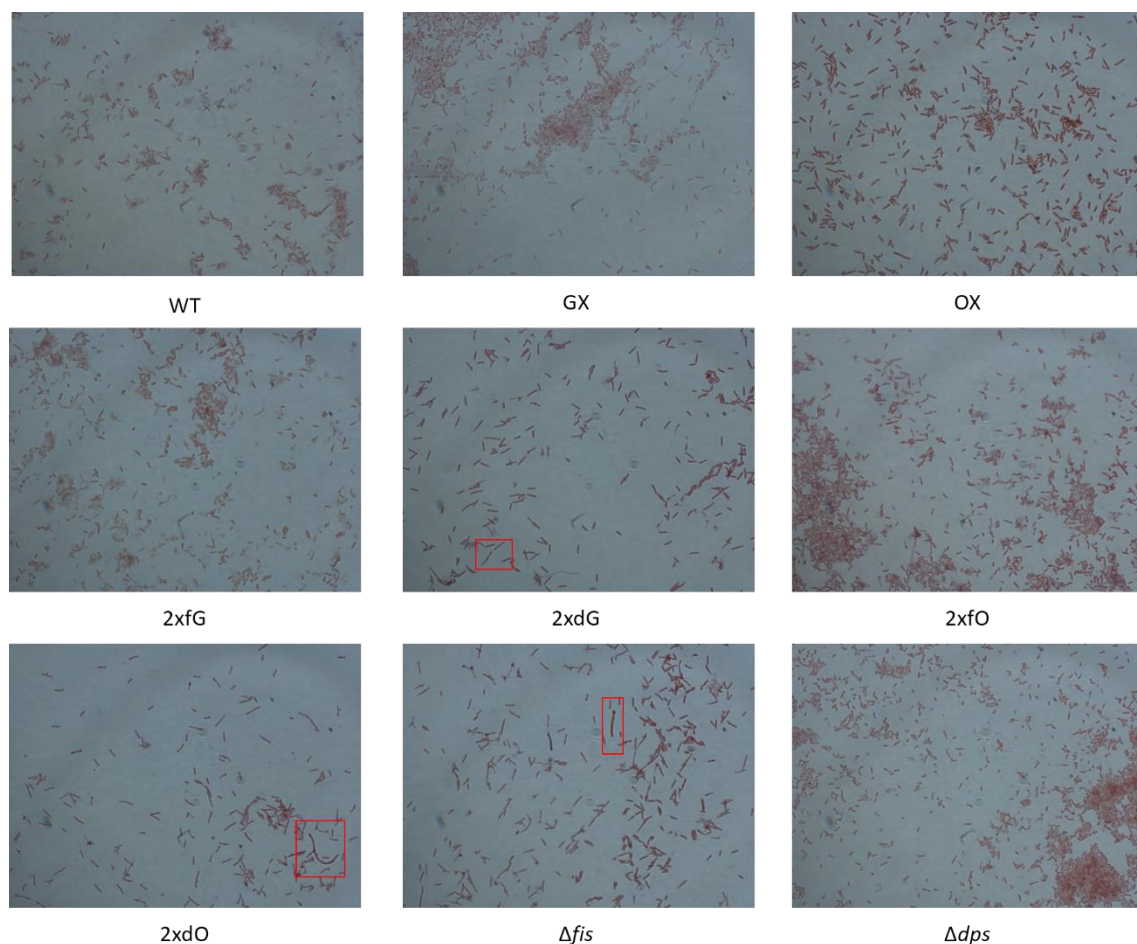


Fig 5.6. Cell morphology during early stationary phase. Bacteria were grown to early stationary phase, harvested, washed with PBS and stained with safranin. Filamentous cells were observed in 2xdG, 2xdO and Δfis (indicated with red boxes). No morphological changes were observed in any other strains compared to WT. Images are representative of 3 biological replicates.

5.2.5 Fis as a regulator of motility

Fis is known to regulate the expression of flagellar genes in *Salmonella* and flagellar biosynthesis genes are among those downregulated in *fis* mutants (Kelly *et al.*, 2004, Wang *et al.*, 2013). Further, Fis binds directly to the promoters of early, middle and late genes of the flagellar biosynthesis pathway (Kelly *et al.*, 2004). Therefore, the swimming motility of the rewired and 2x strains was compared with the WT on 0.3% soft agar over a 5-h period (Fig 5.7). The *hns-1* mutant, containing a truncated version of H-NS, is non-motile and was used as a negative control.

In agreement with previous studies, strains lacking *fis* (2xdG, 2xdO and Δfis) had, on average, a 1.9-fold decrease in the diameter of swimming motility compared to the WT (Fig 5.7). A 1.9-fold decrease was also observed in OX, and a 1.5-fold decrease was observed in 2xfO. No other changes in motility were observed.

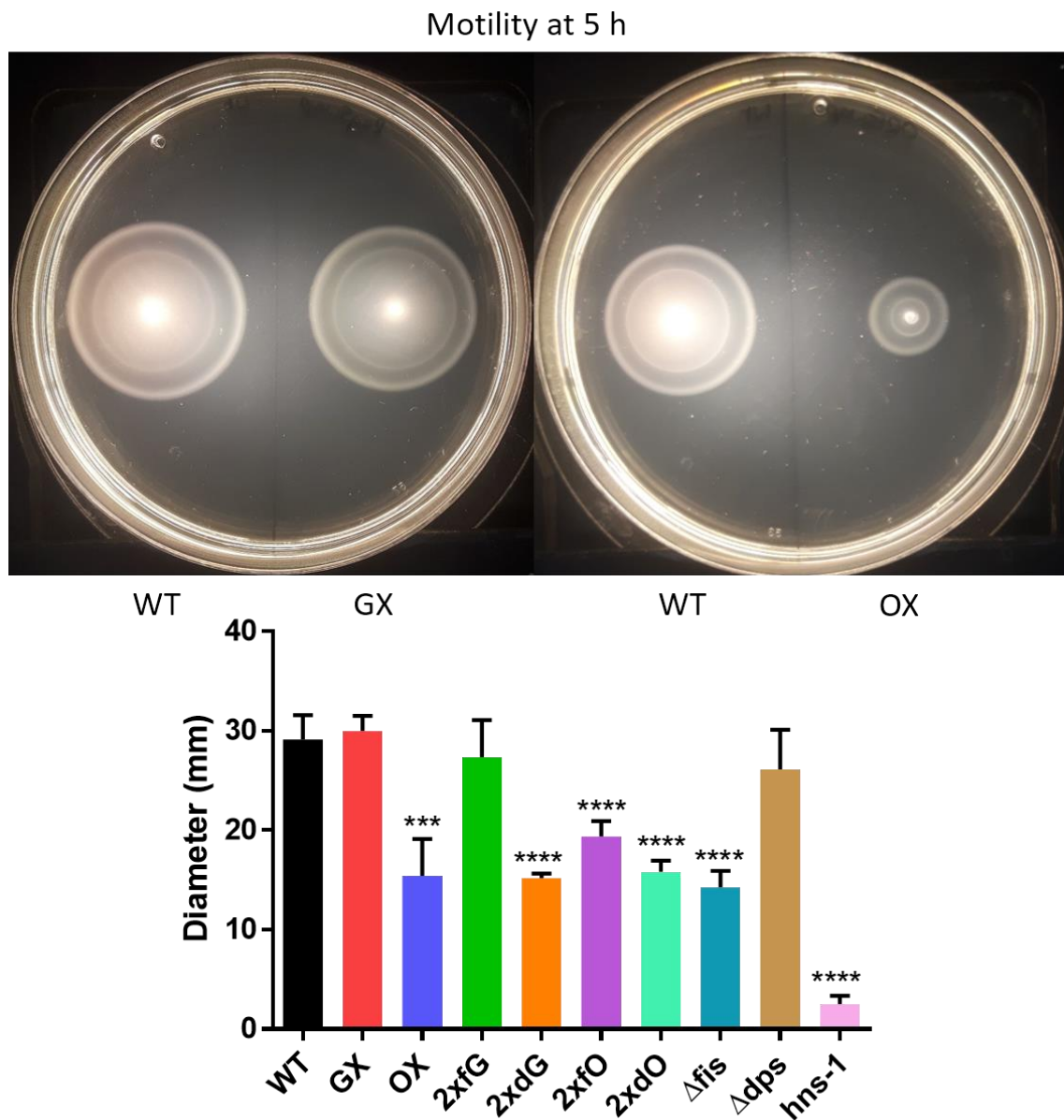


Fig 5.7. Motility defect observed in OX, 2xfO and *fis* deletion strains. Motility was determined by measuring the swimming diameter after a 5 h incubation at 37 °C. Top: Motility of GX and OX compared to WT at 5-h post-innoculation. Bottom: Decreases in motility observed in OX, 2xdG, 2xfO, 2xdO, Δfis and *hns-1* ($p = 0.0007$ for OX and $p < 0.0001$ for 2xdG, 2xfO, 2xdO, Δfis and *hns-1*). No other changes in motility were observed. Error bars represent standard deviation of at least 3 biological replicates. Statistical significance was determined by the student's t-test.

5.2.6 Motility defects are related to extended lag phase

A motility defect was observed in all strains which had an extended lag phase of 1 h (Section 5.2.1). To determine whether this motility defect was related to the early reduced growth rate, these strains were incubated for a further 1 h and compared to WT at 5 h post-inoculation (Fig 5.8). A 1.3-fold reduction in motility was still observed in *fis* deletion strains, while OX and 2xfO reached the same diameter of swimming motility as WT. These data indicate that the motility defects observed in OX and 2xfO in Section 5.2.5 are directly related to the extended lag phase in these strains, rather than the true motility defects of *fis* deletion strains.

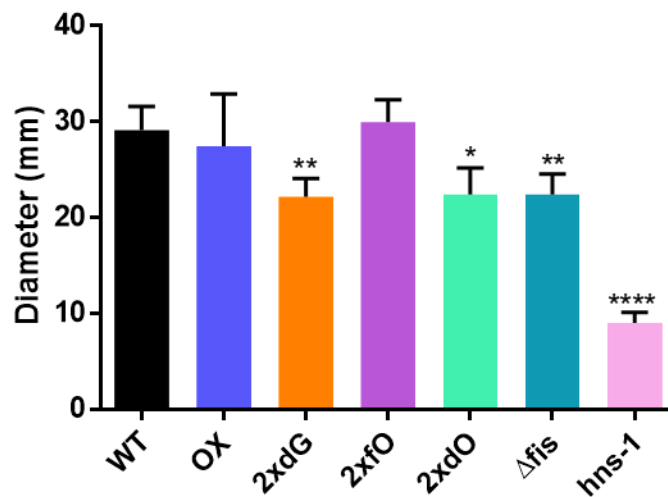


Fig 5.8. The motility defect in OX and 2xfO is related to an extended lag phase. Strains that had a lag phase extension of 1-h were assayed for a further 1-h and their diameter of swimming motility was compared to WT at 5 h. Decreases in motility are observed in 2xdG, 2xdO, Δfis and *hns-1* ($p = 0.006$, 0.01 , 0.008 and <0.0001 respectively). No changes in motility were observed in OX or 2xfO compared to WT. Error bars represent standard deviation of 3 biological replicates. Statistical significance was determined by the student's t-test.

5.2.7 The Effect of *fis* and *dps* on *Salmonella* Pathogenicity Island (SPI) gene expression

Fis is a known positive regulator of SPI-1 and SPI-2 gene expression and *fis* deletion mutants have a reduced ability to invade epithelial cells or to replicate within murine macrophages (Wang *et al.*, 2013). To examine the effect of *fis* and *dps* rearrangements on SPI-1 and SPI-2 expression, *gfp* reporter fusions to *prgH* (SPI-1) and *ssaG* (SPI-2) were created and fluorescence at 510 nm was monitored throughout growth in Luria-Bertani media (Ibarra *et al.*, 2010). Under the growth conditions used, SPI-1 expression rises during exponential growth and peaks in early stationary phase, while SPI-2 is expressed from stationary phase onwards (Sridhar and Steele-Mortimer, 2016).

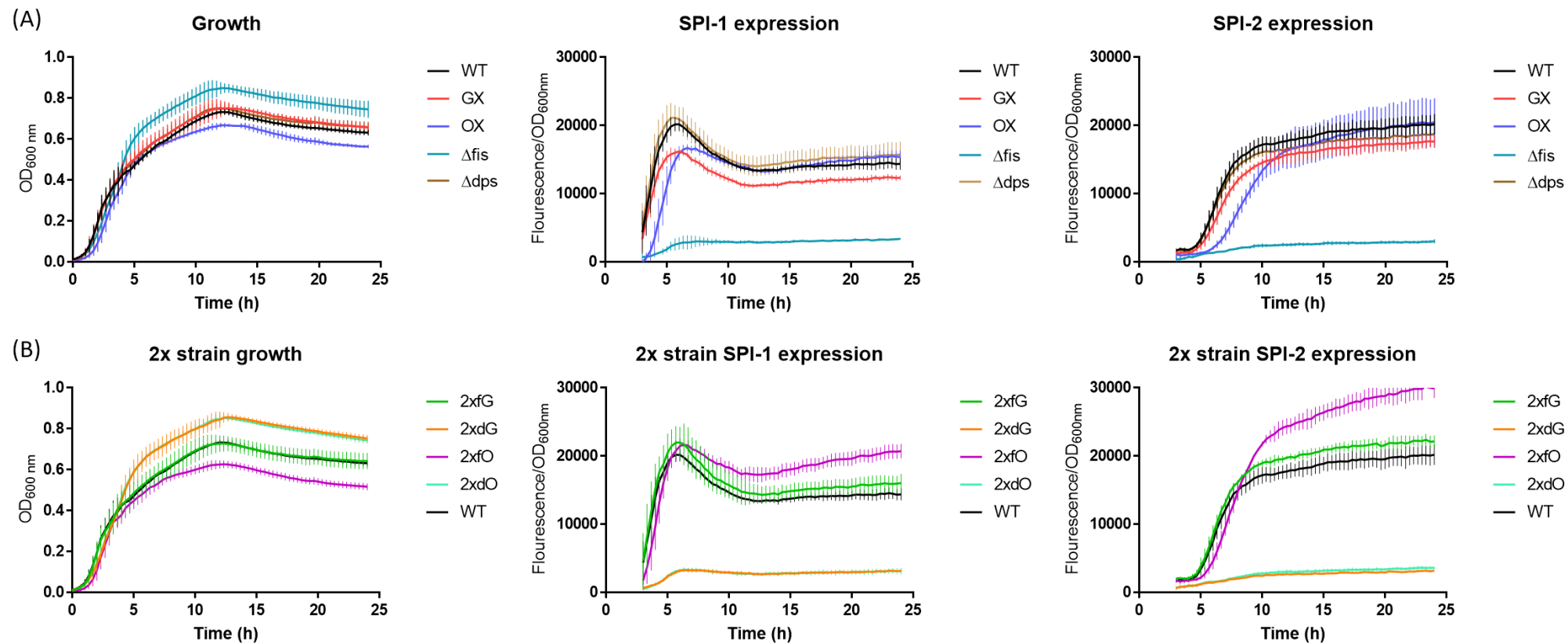
SPI-1 expression is reduced in both GX and OX strains compared to WT (Fig 5.9 (A)). In particular, both SPI-1 and SPI-2 expression is delayed by 1-h and 2-h, respectively, in OX compared to WT. This delay in expression may be related to the change in *fis* expression patterns and overall a reduced Fis concentration during exponential growth (Fig 3.6). By 7-h and 10-h, SPI-1 and SPI-2 expression, respectively, reached similar levels to that of WT.

In GX, SPI-1 expression was reduced from 5-h onwards, while SPI-2 expression was reduced between 7 and 10-h (Fig 5.9(A)). Similarly the reduction in SPI-1 and SPI-2 expression in GX may be related to reduced *fis* gene copy number and to Fis protein concentration during exponential growth.

In line with previous observations, SPI-1 and SPI-2 expression is greatly reduced in *fis* deletion strains (2xdG, 2xdO and Δfis) while Dps has no impact on SPI expression (Fig 5.9 (B)) (Kelly *et al.*, 2004, Wang *et al.*, 2013). Interestingly, an increase in SPI-1 and SPI-2 expression was observed in 2xfO from 7-h onwards. In 2xfG, SPI-2 expression was also higher between 11 and 13-h compared to WT (Fig 5.9 (B)). These strains have 2 copies of the *fis* gene and the increase in expression could be related to gene copy number.

Additionally, strains lacking the *fis* gene grew to a higher maximum OD_{600 nm} value than that of WT. As this assay was performed in a microtiter plate, these data suggest that Δfis has a growth advantage over WT in reduced aerobic conditions.

Fig 5.9. Expression of SPI-1 and SPI-2 monitored using GFP+ reporters. (A) Growth and expression of SPI-1 and SPI-2 of WT, GX, OX, Δfis and Δdps . For GX, there was a statistically significant decrease in SPI-1 and SPI-2 expression from 5-h onwards, and between 7 and 10-h respectively. For OX, there was a statistically significant decrease in SPI-1 and SPI-2 expression between 3-h and 7-h, and 3-h and 10-h respectively. For Δfis , there was a statistically significant decrease in SPI-1 and SPI-2 expression at all timepoints. There was no statistically significant change in SPI-1 and SPI-2 expression in Δdps (B) Growth and expression of SPI-1 and SPI-2 of 2xfG, 2xdG, 2xfO and 2xdO. There was no statistically significant change in SPI-1 expression in 2xfG, SPI-2 expression was increased between 11 and 13-h. For 2xfO, there was a statistically significant increase in SPI-1 and SPI-2 expression from 7-h and 9-h onwards, respectively. For 2xdG and 2xdO, there was a statistically significant decrease in SPI-1 and SPI-2 expression at all points in growth. Error bars represent standard deviation of 3 biological replicates. Statistical significance was determined by the student's t-test.



5.2.8 The Exchange of *fis* and *dps* affects bacterial competitive fitness

To examine whether the changes in *fis* and *dps* gene positions affected overall bacterial fitness, two strains were co-cultured in a 1:1 ratio and bacterial counts were obtained at 0 h and 24 h post-inoculation. Prior to this, the OD₆₀₀ nm values to CFU/ml conversions of overnight cultures were calculated to ensure an equal starting inoculum of 1×10^8 cells per strain. To differentiate between the two strains, a chloramphenicol resistance cassette was inserted into the pseudogene, *SL1483*. This allowed the strains to be distinguished on the basis of chloramphenicol resistance/sensitivity and the chloramphenicol resistance marker had a neutral effect on competitive fitness (pers. comms, Carsten Kroeger). Each strain was competed against its chloramphenicol resistant (cmr) counterpart to ensure that this was so. For the three strains, WT, GX and OX, this led to a total of nine competition experiments; three controls (WT v WTcmr, GX v GXcmr and OX v OXcmr) and two sets of three experimental competitions (WT v GX, WT v OX and GX v OX).

In this method, the growth rate can be used to calculate the fitness index (F.I.) as previously described (Fitzgerald *et al.*, 2015). Initial plate counts at 0 h showed no significant difference in starting inoculum (Fig 5.10), and from calculations of the F.I. no significant difference between the fitness of the strains and their cmr counterparts were observed (Fig 5.11). A significant decrease in fitness in OX was observed compared to WT and GX (Fig 5.11). This observation was only made with OXcmr. On the other hand, a statistically significant increase in fitness was observed in GX compared to WT (Fig. 5.11).

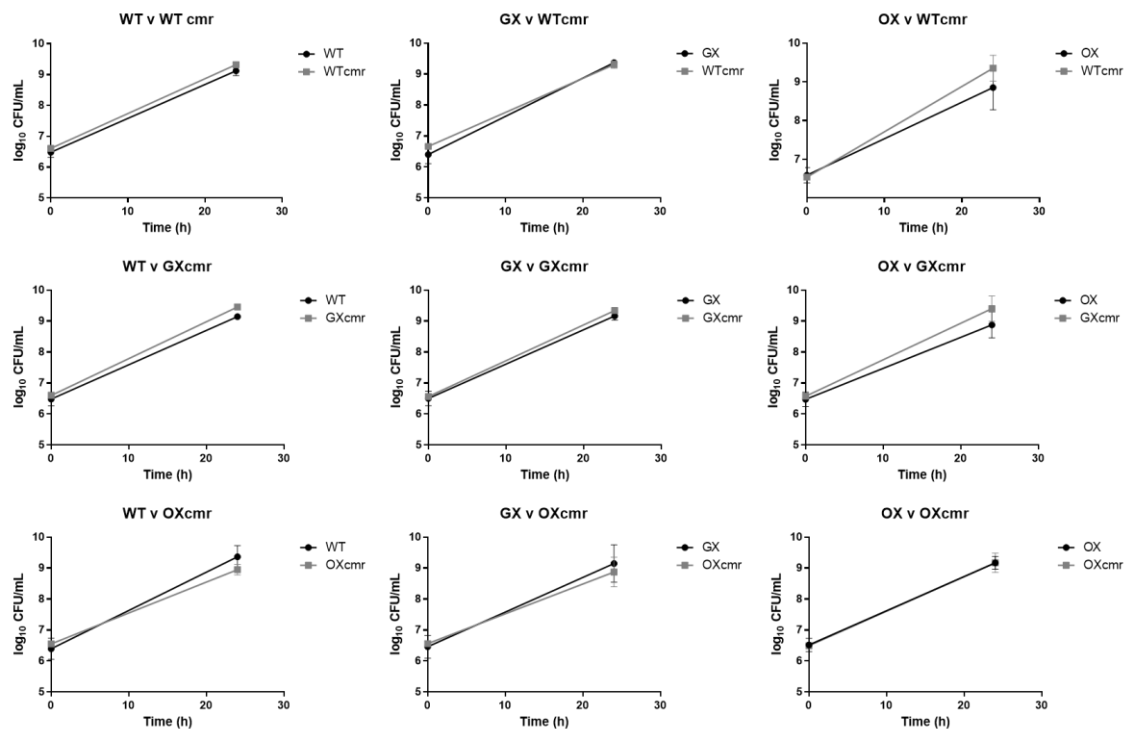


Fig 5.10 Relative competitive fitnesses of the WT, GX and OX strains. Bacteria were co-cultured in a 1:1 ratio and grown for 24 h. Initial and final plate counts were taken and plotted. These graphs confirm that the inocula contained equal numbers of bacterial cells. Error bars represent standard deviation of at least 4 biological replicates.

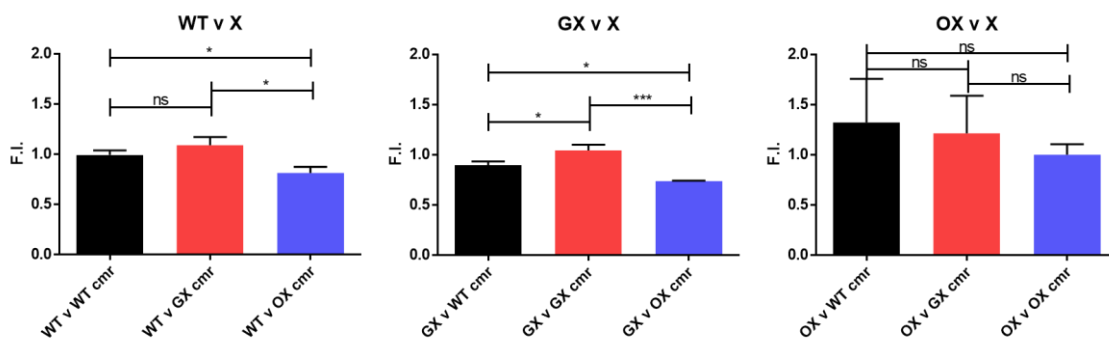


Fig 5.11 Fitness indices calculated from competition assays. A fitness index of 1 was seen when strains were competed against their chloramphenicol-resistant counterpart (WT v WTcmr, GX v GX cmr and OX v OX cmr). WT was competitively fitter than OX (for WT v OXcmr $p = 0.03$). GX was competitively fitter than either WTcmr or OXcmr (for GX v WTcmr $p = 0.02$ and for GX v OXcmr $p = 0.0003$). Error bars represent standard deviation of at least 4 biological replicates. Statistical significance was calculated by the student's t-test.

5.2.9 Competitive fitness in late stationary phase

It was possible that the increase in fitness in GX was due to increased survival in stationary phase arising from increased Dps production. To test this hypothesis, competitive fitness assays were carried out over a 6-day period. Again, each strain tested was competed against its own chloramphenicol resistant counterpart, and monitored over a 6-day period. During these competitions it was noted that there was a downward trend in the viability on the chloramphenicol resistant counterpart in the WT v WTcmr and GX v GXcmr competitions (Fig 5.12). This downward trend seemed to affect the trends in competitive fitness between WT and GX, with a larger difference in bacterial counts in the GX v WTcmr competition, which was not observed in the WT v GXcmr competition. These results indicate that this method of comparing bacterial competitive fitness is not suitable for extended stationary phase assays.

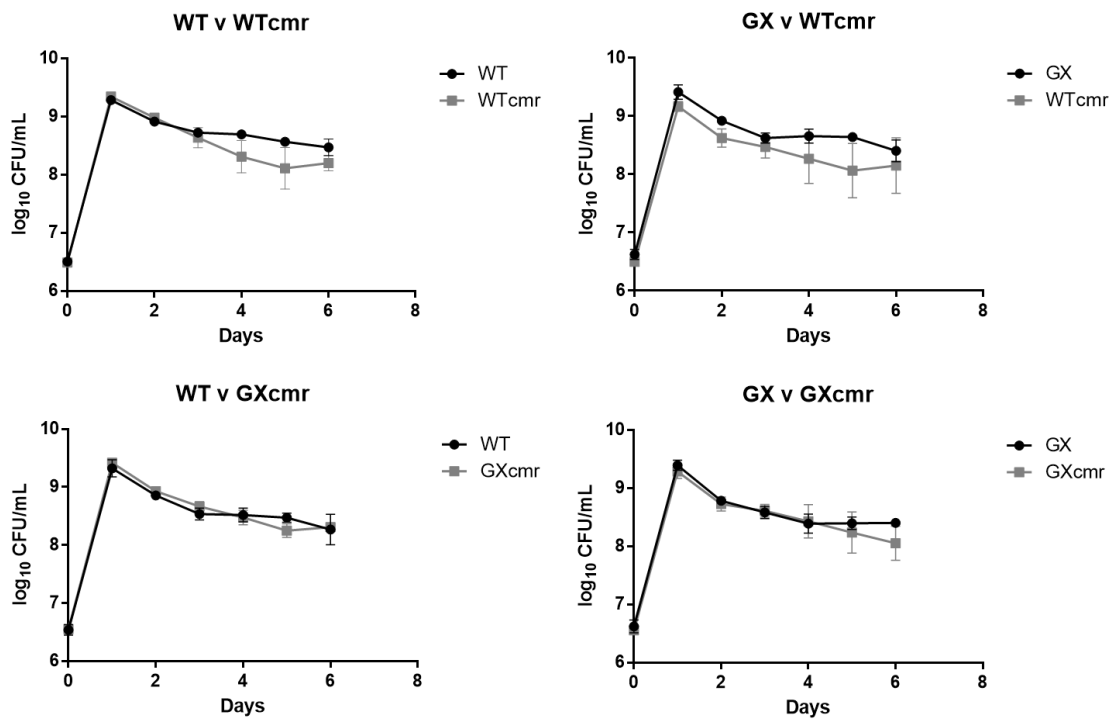


Fig 5.12 Extended stationary phase competitions. Bacteria were co-cultured in a 1:1 ratio and plate counts of each strain were carried out daily. The downward trend in CFU/ml of the cmr strain observed in the WT v WTcmr and GX v GXcmr competitions indicate that the method used was not suitable for extended stationary phase assays. Error bars represent standard deviation of 3 biological replicates.

5.3 Discussion

To test the effects of gene location and regulation on bacterial physiology, *Salmonella* strains were created in which the *fis* and *dps* genes, including their regulatory regions (GX), or the *fis* and *dps* ORFs alone (OX), were exchanged. Specific phenotypes relating to either Fis or Dps were examined to investigate the effect of these genetic exchanges on the bacterium. The intermediate “2x” strains, together with *fis* and *dps* deletion strains, were also examined to provide insight into any altered phenotypes of the exchanged strains. The phenotypes relating to Dps included altered stationary phase viability and oxidative stress resistance, and phenotypes related Fis included altered growth kinetics, motility, cell morphology and SPI gene expression.

The exchange of the *fis* and *dps* genes, and of their regulatory regions, had a minimal impact on bacterial physiology. Of the phenotypes tested, only a decrease in SPI gene expression and an increase in bacterial competitive fitness were detected (Fig 5.9 and Fig 5.11). On the other hand, the exchange of *fis* and *dps* ORFs (OX) greatly impacted bacterial physiology. Under normal growth conditions, an extended lag phase was detected (Fig 5.1 and Fig 5.2). OX was also less resistant to oxidative stress, had delayed SPI gene expression and was competitively weaker than the WT (Fig 5.4, Fig 5.9 and Fig 5.11).

Fis has a direct positive role in DNA replication initiation by its interaction with the origin of replication, *oriC* (Flatten and Skarstad, 2013). Further, Fis is a regulator of genes encoding proteins involved in chromosome replication initiation, such as DnaA (Froelich *et al.*, 1996). The importance of Fis for the timely initiation of DNA replication increases when bacteria are grown in rich media, and delays in replication initiation have been observed in *fis* mutants under these conditions (Flatten and Skarstad, 2013). Therefore, it is unsurprising that an extended lag phase was observed in the *fis* deletion strains (2xdG, 2xdO and Δfis) in LB media (Fig 5.1 and Fig 5.2). This extended lag phase was also observed in OX and 2xfO and both of these strains have the *fis* ORF under the control of the *cis*-acting regulatory sequences of *dps* (Fig 5.1 and Fig 5.2). In the case of OX, Fis production increases in early stationary phase, and the concentration of Fis may still be high when entering nutrient upshift (Fig 3.6). This increase in Fis

production later in the growth cycle may also be a reason for how OX has a greater growth rate than WT later in the growth cycle (Fig 3.3 and Fig 5.1). However, a previous study has shown that overexpression of Fis has negative effects on cell growth (Richins and Chen, 2001). As the same phenotype was observed in both OX and 2xfO, these data suggest that expression of *fis* in stationary phase has negative effects on bacterial growth rate upon nutrient upshift. Further, in the case of OX, Dps is produced upon entry into exponential growth and studies have shown that Dps prevents DnaA-dependent unwinding of *oriC*, and DNA replication, which would have implications for the growth rate (Chodavarapu *et al.*, 2008). This same phenotype is not observed in 2xfG where *fis* is under the control of its own *cis*-acting regulatory sequences in both positions. In this strain, *fis* expression is under the control of its own negative feedback loop which may prevent Fis concentrations reaching the same level as in the OX strain in stationary phase. To further examine the effects of the *fis-dps* exchanges on growth rate, it would have been of interest to observe growth under slower growing conditions, such as minimal media, which would extend the bacterial doubling time.

Upon entry into stationary phase bacteria are faced with multiple challenges such as starvation and interactions with DNA-damaging agents. Dps has been shown to be important in protecting DNA from these damaging or mutagenic agents in stationary phase (Almiron *et al.*, 1992, Martinez and Kolter, 1997, Karas *et al.*, 2015). In agreement with this, a reduced viability was observed in *dps* deletion strains (2xfG, 2xfO and Δdps) (Fig 5.3). Surprisingly, an increase in viability was observed in 2xdO. This increase in survival was not observed in the oxidative stress resistance assays (Fig 5.4), which may indicate that it is related to the second *dps* ORF under the *fis* *cis*-acting regulatory sequences.

As Dps' role in oxidative stress resistance is well characterised it is unsurprising that *dps* deletion strains had reduced survival rates when challenged with 0.5 and 1 mM H₂O₂ (Fig 5.4) (Almiron *et al.*, 1992, Halsey *et al.*, 2004). This reduced survival was particularly severe in OX and 2xfO and is likely to be related to the untimely expression of *fis* under the *cis*-acting regulatory sequences of *dps*. Indeed, previous studies have reported the negative effects of Fis overexpression on cell growth (Richins and Chen, 2001).

Previous studies have reported a filamentous phenotype in *fis* deletion strains, with the number of filamentous cells increasing with increases in temperature (Filutowicz *et al.*, 1992, Flatten and Skarstad, 2013, Osuna *et al.*, 1995). The same filamentous phenotype was observed only in *fis* deletion strains. Flatten and Skarstad (2013) found that these filamentous cells were non-replicating and only present in nutrient rich conditions, highlighting the importance of Fis on the cell cycle.

Fis' positive influence on motility is well documented and several studies have observed a motility defect in *fis* mutants (Kelly *et al.*, 2004, Yoon *et al.*, 2003, Wang *et al.*, 2013). Flagellar biosynthesis is a tightly controlled process, with the transcription factors FlhDC serving as master regulators of the structural and regulatory components of the flagellar machinery (Fitzgerald *et al.*, 2014). The ChIP-seq experiments from this study (Section 4.2.7) reported binding of Fis at the *flhD* promoter. Further, Kelly *et al.* (2004) reported binding of Fis at several promoters of genes in flagellar biosynthesis, and downregulation of these genes in a *fis* deletion strain (Kelly *et al.*, 2004). Consistent with these findings, a motility defect was observed in all *fis* deletion strains in this study, and initially it was thought that OX and 2xfO had a motility defect (Fig 5.7). However, to compensate for the extended lag phase in these strains, the assay was lengthened by a further 1 h. While the *fis* deletion strains maintained their motility defect, OX and 2xfO had a similar diameter of swimming motility compared to that of WT (Fig 5.8). This indicates that these strains maintain sufficient Fis levels to activate genes involved in flagellar biosynthesis, and the apparent motility defect observed in Fig. 5.7 is directly related to the extended lag phase.

The ability of *Salmonella* to invade and survive within macrophages is generally attributed to SPI-1 and SPI-2 respectively (O Cróinín *et al.*, 2006). Genes of these 2 pathogenicity islands are downregulated in the absence of Fis, and the ability of *Salmonella* to invade or survive within the macrophage is also greatly reduced (Kelly *et al.*, 2004, Wang *et al.*, 2013). In agreement with these studies, SPI-1 and SPI-2 expression was greatly reduced in the *fis* deletion strains in this study, and Dps appeared to have no impact on SPI gene expression. The reduction of SPI-1 and SPI-2 gene expression in GX (Fig 5.9 (A)) may be directly related to the reduced *fis* copy number and Fis production in this strain (Fig 3.4 and Fig

3.6). The similar reduction of SPI-1 expression observed in OX (Fig 5.9(A)) may also be related to reduced Fis concentrations at this point in growth. Interestingly, SPI-2 and SPI-1 gene expression increased in 2xfG and 2xfO respectively (Fig 5.9 (B)). Although Fis concentrations in these strains are yet to be determined, these findings suggest a Fis concentration-dependent activation of SPI-1 and SPI-2 transcription.

Despite the few changes in global gene expression and Fis binding, GX is a competitively fitter strain than both WT and OX (Fig 5.11). This is in contrast to other studies which moved *fis* to a more Ori-distal position in *E. coli* (Gerganova *et al.*, 2015). One possibility is that the increase in *dps* copy number and expression gave GX a fitness advantage over WT. An increase in Dps production could possibly increase the difference in fitness index as the co-culture progressed later into stationary phase. However, the 6 day competition involving WT v WTcmr and GX v GXcmr revealed a downward trend in viability of the chloramphenicol resistant counterpart (Fig 5.12). Such trends would skew the differences in fitness index, and therefore this method is not suitable for assaying in late stationary phase. On the other hand, given the growth defect, and global changes in gene expression in OX, it is not surprising that it is a competitively weaker strain than both WT and GX.

In order to understand how bacterial physiology was affected by the changes in *fis* and *dps* position and regulation, phenotypes directly related to Fis and Dps were examined. These phenotypes included growth, bacterial viability, oxidative stress resistance, cell morphology, motility, SPI expression and competitive fitness (Nilsson *et al.*, 1992, Halsey *et al.*, 2004, Kelly *et al.*, 2004, Flatten and Skarstad, 2013). Out of the strains tested, the one which exchanged *fis*, *dps* and their regulatory regions (GX) was phenotypically most similar to WT. Despite the changes in *fis* copy number and Fis production, only SPI gene expression and competitive fitness were affected out of the phenotypes examined. As expected, the strains which were phenotypically most different were exhibited by the *fis* and *dps* deletion strains. OX, in which *fis* and *dps* expression patterns are reversed, showed some similar phenotypes to the deletion strains. In particular phenotypes which required a strong induction of either Fis or Dps were the most similar, such as growth and oxidative stress resistance (Fig 5.1, Fig 5.2 and Fig 5.4). These

results suggest that placing either *fis* or *dps* under different *cis*-acting regulatory sequences has negative consequences for bacterial physiology. Bacteria are quite tolerant of subtle changes in Fis and Dps production, and in some cases it is beneficial to have these changes, such as seen in bacterial competitive fitness (Fig 5.11). Together, these data highlight the robustness of *Salmonella* gene regulatory networks, and the resilience of *Salmonella* to changes in NAP gene position and expression.

Chapter 6:

General Discussion

6.1 Discussion

In bacteria, DNA replication of circular chromosomes occurs bidirectionally, beginning at *oriC* and terminating at Ter (Reyes-Lamothe and Sherratt, 2019). In nutrient rich conditions, multiple rounds of DNA replication are ongoing, and consequently DNA in the proximity to *oriC* is present in higher copy number than DNA in the proximity of Ter (Katayama, 2017). Throughout the cell cycle macrodomains occupy distinct positions. In newborn cells, Ori and Ter positioned mid-cell and the Right and Left macrodomains extended towards the poles (Dame *et al.*, 2011). As the DNA replication and the cell cycle progresses, newly replicated Ori-proximal DNA moves towards the poles, and DNA replication termination occurs mid-cell.

The region in the bacterial cell in which DNA occupies is termed the nucleoid. It is highly organised, and has evolved in a way that allows it to adapt to changes in environment in a timely manner. There are many factors which influence the nucleoid structure, and each of these factors are highly-regulated in a connective way that maintains the homeostatic balance (the ability of systems to remain stable in response to stimuli) within the bacterial cell. Along with DNA replication-specific proteins, the bacterial cell cycle is regulated through the combined contributions of DNA topology and NAPs (Kasho *et al.*, 2014, Chodavarapu *et al.*, 2008, Schwartzman *et al.*, 2013). The expression of some NAP- and topoisomerase-encoding genes is regulated by DNA topology, and by proteins involved with responding to environmental stimuli (Cameron *et al.*, 2013, Altuvia *et al.*, 1994, Cameron *et al.*, 2011, Travers *et al.*, 2001). Therefore, it is not surprising that variations in NAP composition and DNA topology of the nucleoid are some of the governing factors in determining gene expression (Dorman and Dorman, 2016).

With the recent advances in synthetic biology, the concept of designing a bacterium for a specific purpose is foreseeable in the near future. However, there are still many outstanding problems to solve before this can be realised. For instance, what is the optimal spacing between a given gene on a chromosome and *oriC*?

This study sought to develop our understanding of the observable synteny in NAP-encoding genes by exchanging the position of two of these genes, and examining how *Salmonella* physiology was affected by these changes. In contrast to previous studies which examined exchanging two orthologous NAP-encoding genes, two NAP-encoding genes which differ in function were chosen to be exchanged (Fitzgerald *et al.*, 2015, Colgan *et al.*, unpublished). Fis and Dps are produced in exponential and stationary phases, respectively (Ali Azam *et al.*, 1999). The *fis* and *dps* genes are located on opposite replichores, with the *fis* gene located in NS-Left, and the *dps* gene located in the Right macrodomain. In one exchange the *fis* and *dps* genes and their *cis*-acting regulatory sequences were exchanged, this strain was named GX (Gene Exchange). In the other exchange, the *fis* and *dps* ORFs were exchanged, and this strain was named OX (ORF Exchange). During construction of these exchange strains, “2x” intermediate strains were also constructed (Fig 3.2).

Whole genome sequencing was used to confirm the success of these exchanges, and identified SNPs in OX, 2xdO, 2xfO and 2xdG (Table 3.1). The majority of the SNPs identified were either in genes encoding hypothetical proteins or in close proximity to *fis* or *dps*. As none of these genes are linked to the Fis or Dps regulon, repeating the construction of the strains seemed unnecessary.

In bacteria, a gene dosage gradient exists from Ori to Ter, which is dependent on the growth rate (Khedkar and Seshasayee, 2016). This means that at certain points through the cell cycle DNA in proximity to Ori are present in greater copy number than Ter-proximal regions (Fig 1.10). Changes in growth rate will therefore affect the gene dosage gradient throughout growth. While the growth patterns of GX remained similar to those of WT, there was an extended lag of 1 h in OX (Fig 5.1 and Fig 5.2). Consequently, the gene dosage gradient was affected in OX (Fig 3.3).

By exchanging the *fis* and *dps* genes, the distance between either gene and *oriC* was affected, influencing the dosage of each gene throughout the cell cycle. In the GX strain, the copy number of *fis* was reduced, while the copy number of *dps* was increased (Fig 3.4). This is to be expected as the native *fis* gene is located in the NS-Left region, and *dps* is located in the Right macrodomain. Previously,

the *fis* gene has been moved to a Ter-proximal region in *E. coli*, and a reduction in *fis* copy number was observed (Gerganova *et al.*, 2015). The same effect was not seen in the same extent in the OX strain, and the copy number of *fis* was unaffected during exponential growth (Fig 3.4). The cause of this is likely due to the change in gene dosage gradient, and the quantity of Ori-proximal DNA throughout growth was lower in OX than in WT (Fig 3.3). This change in gene dosage gradient was caused by the change in growth rate (Fig 3.3 and Fig 5.1).

The changes in *fis* and *dps* expression in GX and OX are caused by the changes in gene dosage and *cis*-acting regulatory sequences. In GX there was a decrease in *fis* expression, while *dps* expression was increased (Fig 3.5). These changes are in agreement with the changes in gene dosage in this strain, and with previous studies which observed changes in gene expression related to changes in gene copy number (Gerganova *et al.*, 2015, Bryant *et al.*, 2014). Conversely, there was a reversal of *fis* and *dps* expression patterns in OX, with *dps* expression occurring during exponential growth, and *fis* expression increased upon entry into stationary phase (Fig 3.5). These changes in *fis* and *dps* expression patterns reflect the changes in *cis*-acting regulatory sequences; *fis* is under the control of the *dps* promoter and *vice versa* (Fig 3.2). Exchanging two ORFs does not always result in each ORF adopting the expression patterns of the other ORF. For example, a study which exchanged *hns* and *stpA* ORFs resulted in no *stpA* expression, which was compensated by an increase in *hns* expression (Fitzgerald *et al.*, 2015).

In both *Salmonella* and *E. coli*, the expression of *fis* is characterised by rapid induction upon nutrient upshift (Ali Azam *et al.*, 1999, Yuan *et al.*, 1991). In OX, nutrient upshift results in *dps* expression in the place of *fis* expression, and expression of *fis* occurs later in the growth cycle (Fig 3.5). This may be the reason for the 1-h extension in lag phase (Fig 5.1), as the Fis regulon encompasses genes required for rapid growth, such as metabolism genes and *rrn* operons, and during exponential growth, these genes are deprived of the activating role of Fis on gene expression (Kelly *et al.*, 2004).

When examining the effect of the *fis-dps* exchanges on global gene expression, OX was again more affected than GX (Fig 4.5). Despite the statistically significant

changes in Fis production in GX (Fig 3.6), only 11 and 17 genes were differentially expressed in exponential and early stationary phases, respectively (Table 4.1). The few transcriptomic changes in GX indicate that bacteria are resilient to small changes in intracellular Fis concentrations, such as the 1.2-fold reduction in Fis concentration observed in this strain (Fig 3.6).

In contrast to GX, 1,207 and 1,543 genes were differentially expressed in OX in exponential and early stationary phases, respectively (Fig 4.5). As Dps is not a regulator of gene expression, these transcriptomic changes are directly related to changes in Fis production (Janissen *et al.*, 2018). Despite Fis directly binding to the promoters of topoisomerase genes, there was no statistically significant change in expression of *gyrA*, *gyrB* or *topA*, encoding the subunits of DNA gyrase (*gyrA* and *gyrB*), and topoisomerase I (*topA*) (Keane and Dorman, 2003, Weinstein-Fischer and Altuvia, 2007).

The changes in expression of genes involved in metabolism, motility, and virulence, highlight Fis' central role in the physiology and pathogenic lifestyle of *Salmonella*, and these three factors are intrinsically linked to one another by regulatory proteins. The regulatory proteins, RtsA and RtsB regulate *hilA* and *flhDC*, respectively, and *rtsAB* was differentially expressed in a manner which indicated Fis-dependence in OX (Ellermeier and Slauch, 2003). Additionally, a repressor of *hilA* expression, HilD, is a positive regulator of *flhDC*, while the class II flagellar protein, FlhZ, post-translationally regulates HilD to induce *hilA* expression (Singer *et al.*, 2014, Chubiz *et al.*, 2010). SPI-1 expression is linked to carbon metabolism by cAMP-CRP, which post-transcriptionally regulates *hild* by binding to the 3' UTR (Fig 6.1) (El Mouali *et al.*, 2018).

In agreement with previous findings, genes from early, middle and late gene classes of the flagellar biosynthesis pathway were differentially expressed (Kelly *et al.*, 2004). In general, flagellar genes were downregulated during exponential growth and upregulated in early stationary phase in a manner which reflects the changes in Fis production in OX (Fig 4.6). Besides the extended lag phase in this strain, this downregulation of flagellar genes during exponential growth may be a reason for the observed motility defect at 5 h, which disappears after a further hour of the motility assay (Fig 5.7 and Fig 5.8). A further investigation into the

motility of OX should be a priority for the future, and this could be done on a single cell level using digital holographic microscopy (Vater *et al.*, 2014).

The propanediol utilization pathway is a member of the Fis regulon, and the expression of 20 out of 21 *pdu* genes was elevated in early stationary phase in OX (Kelly *et al.*, 2004). These genes are a direct link between metabolism and virulence, as they play a role in growth within host tissues, and *pdu* mutants display reduced virulence (Fig 6.1) (Heithoff *et al.*, 1999, Conner *et al.*, 1998).

Genes encoded on SPI-1 and SPI-2 were also differentially expressed in OX. The differential expression of SPI-1 genes followed the same trend as those involved in flagellar biosynthesis, where these genes were generally downregulated during exponential growth, and upregulated in early stationary phase (Fig 4.7). SPI-2 gene expression was downregulated in early stationary phase, and unaffected in exponential phase in OX. These results were confirmed by measuring fluorescence, generated from chromosomal *gfp* fusions to the promoters of a SPI-1 (*PprgH*) gene and a SPI-2 (*PssaG*) gene (Fig 5.9 (A)) (Ibarra *et al.*, 2010). In OX, SPI-1 and SPI-2 expression was delayed by 1-h and 2-h, respectively, but the fluorescence reached the same level as the WT's in stationary phase. Surprisingly, this assay indicated that SPI-1 and SPI-2 expression was reduced in GX, which was not observed in the RNA-seq experiment. This discrepancy may be explained by the lowered concentration of Fis in this strain (Fig 3.6). While there were few statistically significant changes in gene expression, a larger sample size could have elucidated minute changes in gene expression, which would have knock-on effects for the proteome. A proteomic study would have also contributed to this knowledge on the effects of *fis* gene position on bacterial physiology. Although, the influences of sRNA and mRNA stability would also need to be assessed. Indeed, 35 sRNAs were differentially expressed in OX (Appendix I), and these sRNAs may directly influence the expression of their targets at a posttranscriptional level.

The SPI-1 and SPI-2 reporter fusions also highlighted the dose-dependency of Fis on SPI-1 and SPI-2 expression (Fig 5.9). In the absence of Fis (strains Δfis , 2xdG and 2xdO), expression of *prgH* and *ssaG* was eliminated. In strains which harboured two copies of the *fis* gene, SPI expression increased compared to the

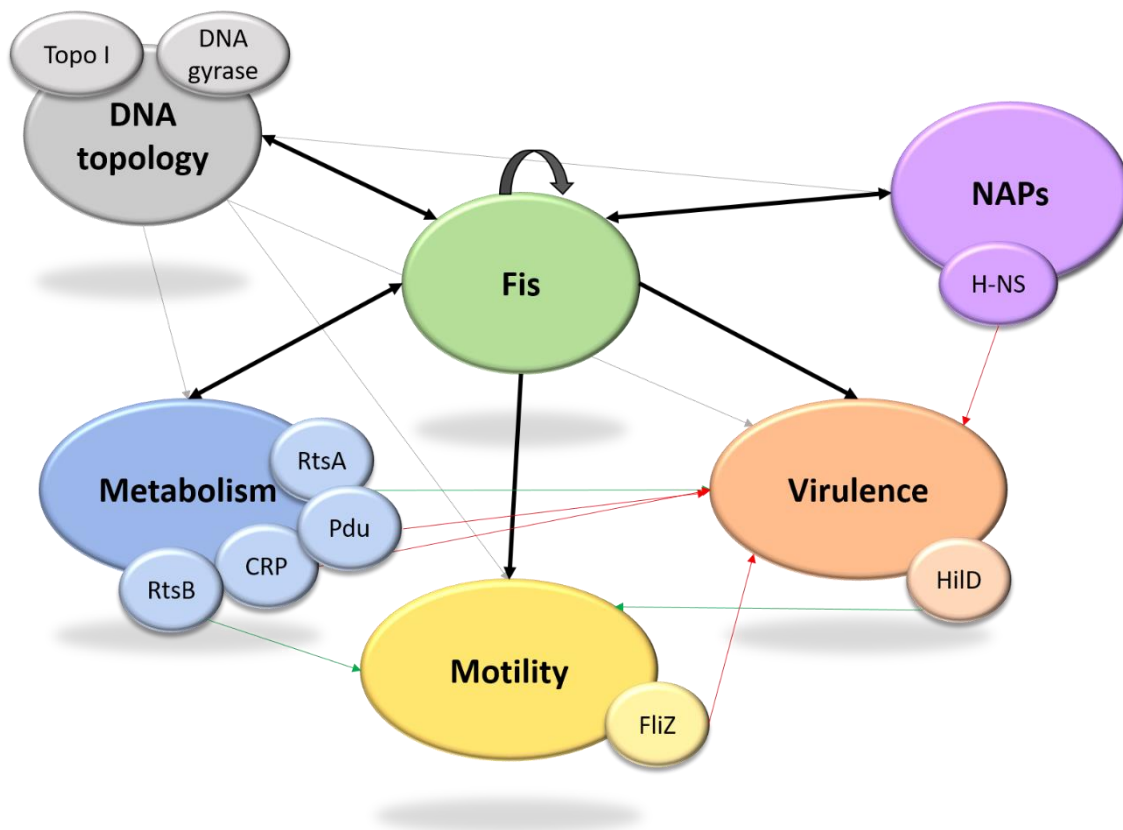


Fig 6.1. The central role of Fis in gene regulatory networks. Fis is a regulator of genes involved in DNA topology, metabolism, motility, virulence and NAP-encoding genes (indicated by black arrows). Fis negatively autoregulates itself. Specific proteins involved in crosstalk between functional categories are shown in small bubbles, and their effect on other categories are indicated by green (positive influence), and red (negative influence) arrows. DNA topology effects genes in all categories, indicated by light grey arrows.

WT, and this was particularly striking in the 2xfO strain (Fig 5.9 (B)). Dose-dependent activation of transcription by Fis has been observed before. An example of this was seen when Brandi *et al.* (2016) observed dose-dependent activation of *cspA* expression by Fis, and once Fis concentration fell below a certain level, it was no longer able to prevent repression of *cspA* by H-NS. H-NS is a repressor of SPI expression, and acting together, OmpR and Fis are able to relieve repression by H-NS at SPI promoters (Lucchini *et al.*, 2006, Cameron *et al.*, 2013).

ChIP-seq was used to detect changes in Fis binding during exponential growth (Fig 4.14). The relocation of the *fis* gene with its *cis*-acting regulatory sequences to the *dps* locus reduced the expression of *fis* (Fig 3.5) in a manner that was related to a change in gene dosage (Fig 3.4). This was reflected in the ChIP-seq assay, where Fis had a reduced binding intensity throughout the genome. While gene movement studies have previously been performed, none have investigated alterations in protein binding (Gerganova *et al.*, 2015, Fitzgerald *et al.*, 2015). The effect on Fis binding throughout the genome was starker when the *fis* and *dps* ORFs were exchanged reciprocally in OX (Fig 4.14). This strain has lower levels of Fis than GX and WT during exponential growth, and Fis binding intensity was lower than in either the WT or the GX strain. When examining differential Fis binding patterns across the genome in GX and OX, there was a noticeable skew in binding intensity with chromosomal position, with an increase in binding intensity around the Ter region (Fig 4.14). This effect was greater in OX, and may be directly related to the change in gene position, and the change in intracellular Fis concentration (Fig 3.6). Although they both may contribute to this effect, at this time, the available data cannot be used to determine which is the greater contributing factor.

Bacterial competitive fitness was affected in both GX and OX, where increased fitness was observed in GX when competing against the WT, and OX was less fit than the WT (Fig 5.11). The reduced fitness in OX was unsurprising, as this strain has an extended lag phase, which would give the competitor strain a “head start” in replicating and utilization of nutrients. Additionally, the OX strain had a large change in global gene expression patterns, including genes involved in metabolism, motility and chemotaxis, which may put the strain at a competitive

disadvantage. Interestingly, despite the few changes in global gene expression, GX was competitively fitter than WT (Fig 5.11). One possible reason for this may have been the increase in Dps production in this strain (Fig 3.7), which would improve the survival rates in stationary phase (Nair and Finkel, 2004). It was hypothesised that if this was the reason for the increase in competitive fitness, then the fitness index would increase as the competition progressed into late stationary phase (Fig 5.12). These competition assays involve the insertion of a chloramphenicol resistance marker into the pseudogene *SL1483*, and after three days there was a downward trend in the viable counts of the chloramphenicol resistant strains, and this method of differentiating between competitors was deemed unsuitable for late stationary phase competitions. Regardless, increase in survival in stationary phase was not observed in GX (Fig 5.3). Another possible reason is related to the downregulation of SPI-1 and SPI-2 as reported by *gfp* fusions (Fig 5.9). Production of Type III secretion systems are energetically expensive to express and produce. Previous studies have shown that producing Type III secretion systems slows growth of *Salmonella*, and SPI-1 and SPI-2 genes both encode type III secretion systems (Sturm *et al.*, 2011, Kelly *et al.*, 2004). Therefore, a reduction in SPI expression, could give a fitness advantage to GX.

Treatment of *Salmonella* with hydrogen peroxide results in the induction of thirty proteins whose genes are regulated by OxyR, and includes proteins involved in peroxide metabolism, redox balance and peroxide protection (Storz *et al.*, 1990). One such protein involved in peroxide protection is Dps, and *dps* mutants have reduced resistance to oxidative stress (Karas *et al.*, 2015, Almiron *et al.*, 1992). Oxidative stress resistance was examined in the *fis-dps* exchange and “2x” strains. Consistent with previous findings, *dps* knockout strains had reduced survival rates when challenged with 0.5 and 1 mM hydrogen peroxide (Fig 5.4) (Halsey *et al.*, 2004). No change in oxidative stress resistance was detected in GX, suggesting that Dps induction by OxyR occurs in this strain, even though *dps* and its *cis*-acting regulatory sequences are located at the native *fis* locus. Conversely, OX was one of the most severely affected when challenged with hydrogen peroxide, and its survival rate was lower than that a *dps* knockout. In OX, *dps* is located in the NS-Left region, but it is under the control of *fis*' *cis*-acting

regulatory signals, while the *fis* ORF is in the Right macrodomain, under the control of *dps*' *cis*-acting regulatory sequences (Fig 3.2). Theoretically, OxyR activation would induce expression of *fis* in this strain.

When examining cell morphology, a filamentous phenotype was observed in *fis* knockout strains (2xdG, 2xdO and Δfis) (Fig 5.5 and Fig 5.6). This observation is consistent with previous studies, and highlights Fis' involvement in the cell cycle (Filutowicz *et al.*, 1992, Osuna *et al.*, 1995, Flatten and Skarstad, 2013). Although it is unknown why exactly these cells are filamentous, these cells have lower *ori/ter* ratios, indicative of slower- or non-replicating chromosomes, and are related to the stimulating role Fis has on the cell cycle (Flatten and Skarstad, 2013). Indeed, no cellular morphology changes were observed in strains harbouring the *fis* gene (Fig 5.5 and Fig 5.6).

This study focused on the effect of exchanging *fis* and *dps* on bacterial physiology predominantly in Luria Bertani media at 37°C. This is a nutrient-rich environment, which is favourable for rapid growth, and therefore phenotypes relating to Fis. However, it would be of interest to examine growth rates in more stressful environments in which Dps is an important factor, such as thermal stress, high-osmolarity, and late stationary phase (Karas *et al.*, 2015). Additionally, many genes involved in metabolism were differentially expressed in OX as determined by the RNA-seq experiment, and it would be of key interest to further investigate how these changes affected growth on defined media supplemented with various nutrient sources, such as propanediol, propionate or glutamate (Table 4.4 and Fig 4.10). Further, infection assays in murine macrophages would have been of interest to investigate, as both Fis and Dps are required for virulence in *Salmonella*, where Fis is an activator of SPI expression, and Dps promotes survival within the macrophage (Wang *et al.*, 2013, Halsey *et al.*, 2004, Kelly *et al.*, 2004). This list of experiments should be made priority for future work.

Studies involving the alterations in gene position have been performed before (Schmid and Roth, 1987, Sousa *et al.*, 1997). Bryant *et al.* (2014), inserted a *gfp* reporter fused to the *lac* promoter at various positions throughout the *E. coli* chromosome. By measuring fluorescence, they observed that gene dosage was not the only factor determining expression at different sites on the chromosome.

A study which moved the *dusB-fis* operon to regions in the Ter macrodomains in *E. coli* observed more drastic phenotypic changes than GX, with reductions in bacterial competitive fitness, and in hydrogen peroxide resistance (Gerganova *et al.*, 2015). Further, Gerganova *et al.* (2015) reported an increase in *fis* expression to compensate for the reduction in *fis* gene dosage, and this was not observed in GX, again highlighting that gene dosage is not the only contributing factor to gene expression at a given position.

Fitzgerald *et al.* (2015) exchanged the *hns* and *stpA* ORFs in *S. Typhimurium*, and observed osmotic- and temperature-dependent changes in bacterial competitive fitness. Despite the large regulons of H-NS and StpA, this study identified only 26 differentially expressed genes in the exchange strain, this may be due to the similarities in H-NS and StpA function. The *fis-dps* ORF exchange strain in this thesis, exchanged two genes whose products differ in function, and a much larger number of genes was differentially expressed. The study outlined in this thesis is most similar to that of Fitzgerald *et al.* (2015), in that gene positions of NAP-encoding genes were exchanged. One limitation of this approach is that there is a potential for difficulty in determining whether a phenotype is related to a specific exchanged gene, or whether both exchanged genes were responsible for the phenotype. In this study, Δfis , Δdps and “2x” strains were used in an attempt to elucidate the cause of phenotypic changes. However, strains in which *fis* was moved to a locus near in the vicinity of *dps* or *vice versa*, could also have been used as determinants for phenotypic changes.

Additionally, in the case of OX, there were multiple factors to consider in evaluating this strain. By exchanging the *fis* and *dps* ORFs alone, the gene dosages of these genes, and the *cis*-acting regulatory sequences were affected, which could lead to difficulties in determining which was the greater factor for any observed changes. Therefore, it would be of interest to make an examination of strains in which the *fis* and *dps* promoters alone has been exchanged, as this would remove the possibilities of changes in *fis* and *dps* gene dosages, while maintaining the changes in *cis*-acting regulatory sequences.

This study detailed is novel in that two exchange strains were made, using genes whose products differ markedly in function. An exchange of the *fis* and *dps* genes

with their *cis*-acting regulatory sequences produced changes in *fis* and *dps* expression, which were related to gene dosage. Despite the change in Fis production, and the overall reduction in Fis binding intensity, there were few changes in global gene expression. Consequently, there were few effects on bacterial physiology under the conditions tested, and there were only changes in SPI expression and an increase in bacterial competitive fitness. These results highlight the resilience of bacterial regulatory networks to changes in Fis concentrations. Conversely, an exchange of *fis* and *dps* ORFs had many negative consequences for bacterial physiology, and gene regulatory networks were drastically affected. This is the first study which examined the effects of producing Fis in early stationary phase, and demonstrates the role of Fis under fast-growing conditions. These findings extend significantly our knowledge of the Fis regulatory network, and of the effect of changing *fis* gene position and *fis cis*-acting regulatory sequences on bacterial physiology.

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