Transmission of an Oxygen Availability Signal at the Salmonella enterica Serovar Typhimurium fis Promoter

Andrew D. S. Cameron¹,², Carsten Kröger¹, Heather J. Quinn¹, Isobel K. Scally¹, Anne J. Daly¹, Stefani C. Kary², Charles J. Dorman*¹

¹ Department of Microbiology, Moyne Institute of Preventive Medicine, School of Genetics and Microbiology, Trinity College Dublin, Dublin, Ireland, ² Department of Biology, University of Regina, Regina, SK, Canada

Abstract

The nucleoid-associated protein FIS is a global regulator of gene expression and chromosome structure in Escherichia coli and Salmonella enterica. Despite the importance of FIS for infection and intracellular invasion, very little is known about the regulation of S. enterica fis expression. Under standard laboratory growth conditions, fis is highly expressed during rapid growth but is then silenced as growth slows. However, if cells are cultured in non-aerated conditions, fis expression is sustained during stationary phase. This led us to test whether the redox-sensing transcription factors ArcA and FNR regulate S. enterica fis. Deletion of FNR had no detectable effect, whereas deletion of ArcA had the unexpected effect of further elevating fis expression in stationary phase. ArcA required RpoS for induction of fis expression, suggesting that ArcA indirectly affects fis expression. Other putative regulators were found to play diverse roles: FIS acted directly as an auto-repressor (as expected), whereas CRP had little direct effect on fis expression. Deleting regions of the fis promoter led to the discovery of a novel anaerobically-induced transcription start site (Pfis-2) upstream of the primary transcription start site (Pfis-1). Promoter truncation also revealed that the shortest functional fis promoter was incapable of sustained expression. Moreover, fis expression was observed to correlate directly with DNA supercoiling in non-aerated conditions. The full-length S. enterica fis promoter region may act as a topological switch that is sensitive to stress-induced duplex destabilisation and up-regulates expression in non-aerated conditions.

Introduction

The factor for inversion stimulation (FIS) is a global regulator of gene expression and chromosome compaction in Gamma-proteobacteria. In Escherichia coli and Salmonella enterica, transcription of the fis gene is very high during rapid growth, consequently FIS is one of the most abundant DNA-binding proteins during exponential growth (50,000 - 100,000 monomers/cell) [1-3]. Transcription of fis declines dramatically as cells enter stationary phase, and the FIS protein concentration drops to undetectable levels [1-3]. Control of fis expression is best understood in E. coli, where the global regulatory proteins CRP, IHF, and FIS modulate only the degree of induction, but none of them is absolutely required for fis expression in laboratory culture [4-6]. In their absence, fis continues to be induced by nutritional upshift and repressed during stationary phase [4-6]. A major determinant of growth phase-dependent fis expression is the GC-rich sequence between the −10 RNA polymerase binding site and the transcription start site, which creates a protein-independent barrier to transcription [7,8]. When DNA becomes highly supercoiled during rapid growth, the topological stress exerted on the DNA double strand facilitates melting of the GC-rich discriminator by RNA polymerase. Thus, DNA supercoiling directly controls fis expression by removing a repressive barrier [9]. The discriminator is so-called because it allows RNA polymerase to discriminate between promoters that are subject to the stringent response (like the fis promoter) and those that are not [9]. Stringently-regulated promoters respond negatively to the alarmone guanosine tetraphosphate (ppGpp) and to the protein DksA, both of which act via RNA polymerase [10,11].

FIS is an important transcription factor in natural environments where bacteria are starved for nutrients. For example, S. enterica serovar Typhimurium (S. Typhimurium)
requires FIS for pathogenicity gene expression during the initial stages of tissue invasion and later inside the macrophage vacuole [12,13]. Transcriptome analysis has revealed that S. enterica serovar Typhi fis is induced inside macrophage vacuoles despite nutrient-poor conditions and slow growth [14]. The expression of fis in intracellular environments may be explained in part by the recent finding that S. Typhimurium fis expression is elevated in stationary phase if oxygen availability is reduced, a condition referred to as “sustained expression” because the fis promoter remains active in stationary phase [13]. Sustained fis expression results in highly elevated FIS protein levels, which contribute directly to increased invasion of epithelial cells in in vitro assays [13]. It is superficially similar to the elevated expression seen with genetically altered derivatives of the E. coli fis promoter where the initiation nucleotide +1C is changed to either A or G: in these mutants fis transcription is elevated in stationary phase [15]. The activity of the native fis promoter shadows the fluctuation in the CTP pool of the cell. Our observations of sustained fis expression involve the native promoter and the goal of the study presented here was to identify the regulatory mechanisms that link oxygen availability to fis expression.

The redox sensors ArcAB and FNR regulate transitions from aerobic to anaerobic growth, making these proteins prime candidates for regulating fis expression in low oxygen environments. In the ArcAB two-component system, ArcA is a site-specific DNA binding protein that is activated by phosphorylation when ArcB senses a drop in redox potential [16]. FNR is a site-specific DNA binding protein that becomes active as a transcriptional regulator when its Fe-S cluster is reduced in the absence of oxygen [17]. ArcA and FNR regulate dozens and hundreds of genes, respectively, many of which are required for metabolism in anaerobic conditions, but neither transcription factor has been implicated in controlling fis expression.

In bacteria, DNA supercoiling changes in response to environmental conditions such as nutrient and oxygen availability [10,18]. Because DNA shape influences gene promoter activity, DNA supercoiling is a mechanism for orchestrating global cellular transcription in response to changing conditions [19]. During rapid growth, E. coli maintains high levels of DNA supercoiling and this stimulates transcription of fis along with other growth phase-dependent promoters, such as rRNA promoters [9,19,20]. Several studies have found that a minimal promoter region containing only the ς70 (RpoD) binding site is sufficient for the induction of the fis promoter during rapid growth [6,8]; this suggests that DNA supercoiling alone can activate the fis promoter in the absence of protein transcription factors, at least in laboratory conditions.

Genetic analysis revealed that neither the stringent-response-associated alarmone ppGpp nor the DksA protein were essential for the sustained expression of fis in stationary phase [21]. We examined the relative contributions made by other protein transcription factors and DNA supercoiling to the expression of S. Typhimurium fis in the non-aerated growth conditions that up-regulate the fis promoter. This led to the discovery of a second transcription start site in the S. Typhimurium fis promoter, which becomes more active as transcription from the primary transcription start site decreases in the absence of oxygen. Together the results suggest that the full-length fis promoter region activates fis expression in response to DNA supercoiling in low-aerated conditions. This topological switch may work in concert with multiple transcription start sites and the alternate sigma factor RpoS to integrate environmental and physiological signals at the S. Typhimurium fis promoter.

Materials and Methods

Bacterial strains and reporter gene constructs

Salmonella enterica serovar Typhimurium strain SL1344 and mutant derivatives were used for all experiments. Detailed descriptions of strains used in this study are provided in Table S1. Deletion mutants were constructed by the method of Datsenko and Wanner [22] using the recombineering primers listed in Table S2. Mutations were transduced into a fresh SL1344 derivative by bacteriophage P22 generalized transduction [23] and were confirmed by PCR and DNA sequencing. The pCP20 plasmid, which encodes the FLP recombinase, was used to remove the kanamycin resistance cassette from the dusB-fis-gfp<sup>102C::kan<sup>R</sup></sup> construct in the S. Typhimurium chromosome as described in [22]. After curing cells of pCP20, loss of antibiotic resistance was confirmed by screening colonies for kanamycin sensitivity using LB-plates containing kanamycin at 50 µg/ml. Construction of SL1344 Δcpr was confirmed phenotypically by sugar fermentations tests using peptone broth containing a sugar or sugar alcohol at 0.5% final concentration and bromothymol blue as an indicator. As expected SL1344 Δcpr was unable to ferment mannitol, sorbitol or maltose but retained its ability to ferment glucose.

Promoter truncates were generated by inverse PCR using the primers listed in Table S2 and pH2pcisS as template [13]. PCR amplicons were phosphorylated by T4 polynucleotide kinase and then circularised by ligation with T4 DNA ligase, followed by transformation into chemically-competent E. coli DH5α. PCR primers targeting the fis promoter region included a restriction enzyme site to facilitate confirmation of correct ligation products.

Culture growth

Well-aerated conditions were achieved by using 10 ml of LB (1% tryptone, 0.5% yeast extract, 0.5% NaCl) in a 250 ml glass conical flask, whereas non-aerated conditions used the same culture volume in a glass tube with an interior diameter 14 mm, as in [13]. All containers were loosely capped to allow air flow, and shaken at 200 RPM at 37 °C. Cultures were started by diluting cells 1/100 from well-aerated overnight cultures and grown for 22 hours to ensure that they had reached steady-state stationary phase before sampling. Anaerobic shock was administered by transferring 15 ml of well-aerated exponentially-growing cell culture into a 15 ml centrifuge tube, capping tightly, and incubating without agitation for 30 min at 37 °C. When required, antibiotics were used at the following final concentrations: carbenicillin 100 µg/ml, chloramphenicol 20 µg/ml, and kanamycin 50 µg/ml.
Flow cytometry and quantitative PCR

GFP levels in cells expressing fis-gfp^{CD} and Pfis-gfp^{+} were quantified using flow cytometry as follows: 2-30 µl of culture was fixed in 700 µl of freshly prepared phosphate buffered saline containing 2% formaldehyde. Fixed samples were stored overnight in the dark at 4 °C. The median fluorescence of 20,000 cells/sample was measured on a Dako CyAn ADP flow cytometer (PMT voltage 800-875 V). To quantify transcript abundance, RNA was isolated using TRizol as described earlier [24]. Quantitative PCR was conducted as in [25] using primers listed in Table S2.

DNA supercoiling analysis

Plasmids were isolated from cultures using the HiYield Plasmid Mini Kit (RBC Bioscience). All electrophoresis was conducted in 27-cm-long 1% agarose gels with 2x Tris Borate EDTA (TBE) as gel and running buffer. Approximately 1 µg of plasmid DNA (8–15 µl) was loaded on a gel using 4 µl of loading buffer (80% glycerol, 0.5 mg/ml bromophenol blue). The gel and buffer contained 2.5 µg/ml chloroquine, and electrophoresis was performed at 3 V/cm for 16 hours. To remove the chloroquine after electrophoresis, gels were washed by gentle rocking in large volumes of tap water for at least 3 hours; the wash water was replaced every 20–30 minutes. After washing, gels were stained by gentle rocking in water containing ethidium bromide (1 µg/ml) for at least 1 hour, then washed briefly in water, and plasmid topoisomerases were visualized with UV light. Analyses of topoisomer distributions were conducted as in [26].

WebSIDD [27] was used to predict how DNA supercoiling affects stability of the S. Typhimurium and E. coli fis promoter regions. WebSIDD uses a default 5 kbp window that slides by 500 bp. Thus the stability of each base pair, G(x), is the average of 10 calculations, where the influence of proximal bases is weighted according to how close the base pair is to the center of the sliding window.

5′ RACE and western blot analysis

5′ RACE was conducted as in [28] using the primer Pfis.expTSS.RT.Rev to reverse transcribe dusB-fis transcripts. PCR products were cloned into the linearized vector pJET (Fermentas) and transformed into E. coli strain XL-1 and at least 5 clones were DNA sequenced. Western blot analysis was conducted as in [25] using the E. coli σ^{6} (RpoS) monoclonal antibody (Neoclonie) diluted 1:5,000.

Results

Comparison of S. Typhimurium and E. coli fis promoter regions

The regulation of fis expression has been studied primarily in E. coli. As a first step to identify the regulatory elements that control S. Typhimurium fis expression, the S. Typhimurium fis region was aligned with the same region in E. coli. The bicistronic dusB-fis operon encodes FIS in both species. Because dusB has attracted very little research attention, the upstream promoter region (Pfis) bears the fis name. The dusB and fis open reading frames have 92% and 98% nucleotide identity, respectively, between E. coli and S. Typhimurium, indicating that very little change has occurred in these genes since divergence of the Escherichia and Salmonella genera. As has been noted previously, the region containing the primary transcription start site is identical between E. coli and S. Typhimurium [20], but there is significant sequence divergence upstream of position −49 (Figure 1). In E. coli there is an intergenic space of 296 bp between the dusB-fis transcription start site and the stop codon of the upstream gene, prmA. In S. Typhimurium, the prmA stop codon is 628 bp upstream, suggesting that >300 bp of non-coding DNA was inserted upstream of the ancestral promoter region. Alignment of DNA sequence from other Enterobacteriaceae species confirms that the ancestral promoter region was likely around 300 bp (not shown), making Salmonella unusual in the Enterobacteriaceae family.

To test whether the full-length intergenic region contributes to regulation, the gfp^{CD} reporter gene was fused to the S. Typhimurium dusB-fis operon in the native chromosomal location. Because FIS is autoregulatory, we avoided creating a fis null mutant by inserting gfp^{CD} downstream of the fis open reading frame, but upstream of a predicted transcriptional terminator. The kanamycin resistance marker linked to gfp^{CD} was subsequently excised to prevent the kan promoter from influencing fis expression. This chromosome-based reporter differs from the study by Ó Cróinín and Dorman that first identified sustained fis expression [13]. Ó Cróinín and Dorman used a plasmid-based reporter gene fusion containing the fis promoter region up to -298 bp, thus omitting the 330 bp of upstream non-coding sequence.

In stationary phase cultures the dusB-fis-gfp^{CD} construct demonstrated elevated expression in non-aerated conditions compared to aerated conditions (Figure 2A). This modest two-fold increase in expression corresponds to a dramatic rise in FIS protein level, which causes more invasive in a fis-dependent manner [13]. Overall, the dusB-fis-gfp^{CD} chromosomal fusion exhibited the classic expression pattern of strong induction in exponential growth in both well-aerated and non-aerated conditions (Figure 2C), followed by a rapid decrease in expression as growth slowed. Two-fold higher fluorescence was sustained from 3 to 22 hours of growth in non-aerated compared to aerated conditions (Figure 2A & C). The two-fold difference we observed with the dusB-fis-gfp^{CD} construct was less than the increase seen previously in the plasmid-based reporter system [13], and quantitative PCR confirmed that our chromosomal construct is an accurate reporter of native fis gene expression (Figure S1). The reduced intensity of sustained expression in the chromosome gfp fusion compared to the plasmid-borne reporter indicates that the plasmid system is a reliable but exaggerated reporter of sustained fis expression in non-aerated conditions.

ArcA is a repressor of S. Typhimurium fis

The redox sensors ArcAB and FNR are transcriptional activators in low oxygen conditions, making them prime candidates for activating sustained fis expression. Deletion of...
fis had no detectable effect on fis expression or on cell growth (Figure 2B, C & G). Surprisingly, deletion of arcA caused an increase in fis expression in non-aerated stationary phase (Figure 2A & D), implicating arcA as a repressor of sustained fis expression. We found that although the ΔarcA mutant had a slightly prolonged lag phase in fresh medium, its growth rate and final cell densities were very similar to wild type cells in both aerated and non-aerated conditions (Figure 2B & G). Thus, the effect of the ΔarcA mutation on fis expression is not due to altered growth in non-aerated conditions.

In E. coli arcA is a known repressor of rpoS transcription [29], and RpoS has been proposed to repress fis expression in S. Typhimurium [13], thus an S. Typhimurium ΔarcA mutant would be predicted to have reduced fis expression due to up-regulation of rpoS. This led us to test whether ArcA may act through rpoS in S. Typhimurium, perhaps by up-regulating rpoS expression as opposed to repressing it as in E. coli. First though we were surprised to discover that the chromosomal dusB-fis-gfpTCD fusion was not up-regulated in the ΔrpoS mutant (Figure 2E), which contrasts with the previously observed strong activating effect of the ΔrpoS mutation on the plasmid-based Pfis::gfp fusion [13]. Quantitative PCR confirmed that the dusB-fis-gfpTCD fusion is a reliable reporter of fis expression in non-aerated conditions (Figure S1). The ΔrpoS mutant showed an extended lag phase, but unlike the ΔarcA mutant it did not achieve wild type densities in aerated culture after 22 hours; instead it grew slightly better than wild type in non-aerated conditions (Figure 2B & G). Neither of these minor growth phenotypes had a detectable effect on fis expression.

To further address whether ArcA may act indirectly on fis through RpoS function, we constructed a ΔarcAΔrpoS double mutant. In aerated and non-aerated growth conditions, the ΔarcAΔrpoS double mutant showed consistently reduced fis expression during exponential growth (Figure 2F), unlike either single mutant. Finding an exacerbating effect of combining the two mutations suggests that ArcA acts separately from RpoS to influence fis expression in exponential growth conditions. During stationary phase in non-aerated conditions, the ΔrpoS mutation nullified the effect of the ΔarcA mutation of enhancing sustained fis expression (Figure 2A & F), implicating RpoS as a mediator of ArcA’s effect on fis expression.

The minimal fis promoter is deficient in sustained expression

Because neither FNR nor ArcA was required for sustained fis expression in non-aerated conditions, we created a series of fis promoter (Pfis) truncates to identify promoter regions required for sustained expression. Three truncates were constructed in a plasmid-based gfp reporter system. The amplification of fis expression profiles by the plasmid system provided two experimental advantages: first, the ability to create truncated promoters, and second, improved sensitivity to detect subtle changes in gene expression. Unfortunately, we were unable to clone the full intergenic region from S. Typhimurium, suggesting that an unidentified element upstream of position −300 is toxic to cells when present in multicopy. The construct Pfis(−298) contained a promoter region identical in length to the E. coli intergenic region (Figure 3A), which is the same S. Typhimurium promoter region used previously to study sustained expression [13]. The medium length construct Pfis(−198) removed sequence that in E. coli contributes to regulation (Figure 1). The shortest construct, Pfis(−49), preserved only the region of identity between S. Typhimurium and E. coli.
Figure 2. Effects of transcription factor mutations on fis expression in aerated and non-aerated conditions. A) Column graph comparing dusB-fis::gfp expression at 22 hours. All data are expressed relative to wild type at 22 hours in well-aerated conditions (dashed line). B) Average and standard deviation of four replicate culture densities after 22 hours. C-F) Time courses of dusB-fis::gfp expression in S. Typhimurium wild type and Δfnr (C), ΔarcA (D), ΔrpoS (E), and ΔarcA/ΔrpoS (F) mutants. The blue arrow in D indicates the increased sustained expression observed in the ΔarcA mutant after 22 hours in non-aerated conditions. A, C-F) Mean and standard deviation of GFP fluorescence from three or four biological replicates are plotted in arbitrary units. All data are expressed relative to wild type levels at time point 0 in well-aerated conditions. The same wild type data are presented in panels C-F. G) Growth dynamics of strains in aerated and non-aerated conditions. Smoothed curves were generated by GraphPad Prism 5.0d from the average of four or more replicate growth curves.

doi: 10.1371/journal.pone.0084382.g002
Figure 3. Defining functional regions and transcription factor input at the fis promoter. A) Schematic of the E. coli and S. Typhimurium intergenic regions. The locations of truncations in S. Typhimurium are indicated. B) Time courses of expression of plasmid-borne P_{fis}::gfp promoter truncates in aerated and non-aerated S. Typhimurium cultures. C and D) Expression of promoter truncates in S. Typhimurium transcription factor mutant backgrounds at 22 hours. In B-D, mean and standard deviation of GFP fluorescence from three or more biological replicates are plotted in arbitrary units that are reported relative to the chromosomal dusB-fis::gfpTCD fluorescence output presented in Figure 2.

doi: 10.1371/journal.pone.0084382.g003
All three constructs demonstrated the classic peak in expression during exponential growth. However, P_{Fis} (−198) and P_{Fis} (−49) showed reduced overall expression levels at all stages of growth in both aerated and non-aerated conditions (Figure 3B), indicating that the full 300-bp upstream region contributes to activation. Furthermore, the minimal RpoD-driven promoter, P_{Fis} (−49), was sufficient for strong induction during exponential growth in S. Typhimurium, as reported in E. coli [6,8]. In non-aerated conditions, sustained fis expression was lower in P_{Fis} (−198) and was even further reduced in P_{Fis} (−49) (Figure 3B), indicating that one or more regulatory elements upstream contribute to sustained expression.

Transcription factor control of S. Typhimurium fis expression

To determine which transcription factors contribute to sustained fis expression and to identify regions of the fis promoter required for transcription factor function, P_{Fis} truncates were tested in several mutant backgrounds. In E. coli, FIS represses its own promoter by binding the FIS-I and FIS-II binding sites that flank the transcription start site [4,6], and these sites are perfectly conserved between the E. coli and S. Typhimurium fis promoters (Figure 1). Up-regulation of the S. Typhimurium fis promoter in the Δfis mutant confirmed that FIS represses its own promoter, and this repression occurs in both aerated and non-aerated conditions (Figure 3C & D). The FIS-I and FIS-II sites are intact in the shortest construct, P_{Fis} (−49), allowing FIS to repress each of the three promoter truncates.

All three lengths of P_{Fis} showed elevated expression in the ΔarcA mutant in non-aerated conditions (Figure 3D). Elevated expression regardless of promoter length supports a model in which ArcA has an indirect influence on fis during stationary phase, consistent with the absence of a predicted ArcA site in the S. Typhimurium fis promoter. fis expression was slightly elevated in aerated conditions (Figure 3C), which was not detected using the chromosomal system — again indicating that the plasmid-borne system enhances or exaggerates fis promoter activity.

The ΔrpoS mutation did not have a detectable effect on the P_{Fis} (−298) and P_{Fis} (−198) truncates, as observed above with the dusB-fis-gfpTCD chromosomal fusion (Figure 3C & D). However, P_{Fis} (−49) revealed that RpoS can exert a mild repressive effect in the absence of upstream fis promoter DNA, both in aerated and non-aerated conditions.

CRP is a global-acting transcription factor that directly regulates FIS expression in E. coli [4]. We found that deletion of crp caused a slight reduction in fis expression from all three promoter truncates in both aerated and non-aerated conditions (Figure 3C & D). The absence of a DNA site matching the CRP binding site consensus (Figure 1) suggest that CRP plays an indirect regulatory role at the fis promoter in S. Typhimurium.

The S. Typhimurium fis regulatory region has a second, anaerobically-induced promoter

The reduction in fis expression caused by progressive truncation of the fis promoter could be explained by removal of one or more upstream transcription start sites (TSS). Thus, we used recently published RNA-seq data [24] to search for evidence of transcripts originating upstream of the primary TSS, which revealed a putative TSS at position −216 relative to the primary TSS (Figure 1). 5′-RACE confirmed the existence of the second TSS (Figure 4A), which we named P_{Fis} (−2) to distinguish it from the gene proximal TSS, called P_{Fis} (−1). P_{Fis} (−2) was not included in Kröger et al. [24] because it did not pass the conservative threshold used in that study.

To determine the activity of P_{Fis} (−2) in laboratory culture, quantitative PCR was used to distinguish between transcripts originating from P_{Fis} (−1) and P_{Fis} (−2). During exponential growth, less than one percent of dusB-fis transcripts originated at P_{Fis} (−2) (Figure 4B). Transcription from P_{Fis} (−2) was up-regulated two-fold in non-aerated stationary phase cultures, but this accounted for only 7±1% of total fis transcripts, indicating that P_{Fis} (−2) is not responsible for sustained fis expression. Transcription was moderately reduced at both P_{Fis} (−1) and P_{Fis} (−2) in the ΔarcA mutant (Figure 4B), suggesting that ArcA causes mild activation of fis expression during exponential growth. Deletion of rpoS revealed RpoS to be a weak repressor of P_{Fis} (−1) and a stronger repressor of P_{Fis} (−2) during exponential growth (Figure 4B).

To further test whether ArcA acts indirectly through RpoS by repressing rpoS transcription, we measured rpoS transcript abundance. No significant difference was detected in rpoS transcript levels between wild type and ΔarcA mutant cells in exponential growth (Figure 4C). RpoS is controlled primarily by post-transcriptional mechanisms; unfortunately attempts to quantify RpoS protein levels in the wild type and ΔarcA mutant in exponential growth were unsuccessful because RpoS protein levels were below the detection limit of western blot assays.

Two-fold up-regulation of P_{Fis} (−2) in non-aerated growth conditions led us to test the effects of more severe anaerobic shock, a condition that, like non-aerated growth, may imitate conditions encountered by S. Typhimurium in the intestine and in intracellular environments. Exponentially growing cells were transferred to sealed tubes to induce anaerobic shock, and this caused a significant up-regulation of P_{Fis} (−2) concomitant with 18-fold repression of P_{Fis} (−1) (Figure 4D). In these conditions, deletion of arcA caused a decrease in P_{Fis} (−1) and P_{Fis} (−2) activity, consistent with the decrease in exponential phase fis expression seen in Figure 2D. To once again test whether the ArcA effect might arise through RpoS activity, rpoS expression was measured in anaerobic shock. ArcA was found to exert a repressive effect on rpoS as revealed by higher rpoS transcript levels in the ΔarcA mutant (Figure 4E). Although higher levels of RpoS protein in the ΔarcA mutant could explain the repression of P_{Fis} (−2) observed in Figure 4C, the ΔrpoS mutant showed wild type levels of P_{Fis} (−2) expression during anaerobic shock (Figure 4D). The absence of an explicit RpoS effect in aerated, non-aerated, and anaerobic conditions (Figures 2E, 4C & D), suggests a model in which RpoS represses fis expression only when RpoS protein levels are unusually high, as in a ΔarcA mutant.
Figure 4. Characterisation of S. Typhimurium Pfis-2. A) RNA-seq and 5′-RACE identification of the Pfis-2 transcription start site. The number of sequencing reads in RNA-seq analysis of an early stationary phase S. Typhimurium culture is aligned with DNA sequencing results from 5′-RACE analysis of the same RNA sample (RNA-seq data available in [24]). B) Quantitative PCR measurement of transcripts originating from Pfis-1 and Pfis-2 during exponential growth. C) Quantitative PCR measurement of rpoS expression. D) Quantitative PCR measurement of transcripts originating from Pfis-1 and Pfis-2 during anaerobic shock. E) Quantitative PCR measurement of rpoS expression. In B-E, the mean and standard deviation from 3 to 5 biological replicates are plotted.

doi: 10.1371/journal.pone.0084382.g004
DNA supercoiling control of S. Typhimurium fis expression

DNA supercoiling is a key driver of fis expression [9], raising the intriguing question of whether DNA supercoiling and the fis promoter respond to small incremental changes in oxygen availability. Conversely, there may be specific oxygen concentrations at which regulatory control undergoes a transition that facilitates sustained fis expression in non-aerated conditions. To assess the relationship between oxygen availability, DNA supercoiling, and fis promoter activity, aeration was adjusted by growing cells in tubes with increasing culture volumes. DNA supercoiling and fis expression were measured in parallel cultures containing either pUC18 or the Pfis(-298) reporter plasmid, respectively. The small, high copy number plasmid pUC18 (2,686 bp) was ideal for quantifying supercoiling levels, whereas the larger (6,424 bp) and low copy number reporter plasmid pZec-Pfis was less reliable for resolution of topoisomers; nevertheless, we did find that pZec-Pfis had the same topological responses as pUC, as was previously confirmed for pZec [26]. In the present study, wild type cells carrying pUC18 were assayed in parallel with strains having either the plasmid-borne Pfis(-298) reporter or the chromosomal dusB-fis-gfpCT reporter.

A continuous increase in DNA supercoiling was observed as aeration decreased due to increased culture volume (Figure 5A). In these same conditions Pfis expression demonstrated a gradual increase with decreased aeration. Increased expression was observed with both the plasmid-borne and chromosomal reporters, but as expected was more pronounced in the plasmid system. Pfis expression was the same in 6 ml and the 10 ml "non-aerated" condition used in the experiments above (compare Figures 3D and 5A); thus cells appear to be severely oxygen limited in volumes above 5 ml in standard culture tubes. These results suggest that changes in DNA supercoiling during non-aerated growth may be involved in sustained fis expression.

The correlation between DNA supercoiling and sustained fis expression in non-aerated cultures prompted us to test whether increased fis expression in the ΔarcA mutant might also correlate with an increase in DNA supercoiling in this genetic background. DNA supercoiling was not elevated in the ΔarcA mutant (Figure 5B).

DNA supercoiling reduces the amount of energy required for transcription initiation by exerting torsional stress on the DNA double helix, which weakens base pairing and facilitates melting; this is referred to as stress-induced duplex destabilization (SIDD) [30]. Because DNA supercoiling stimulates fis expression we predicted that the fis promoter region would have a SIDD profile indicative of significant stress-induced denaturation, which may be focused near Pfis-1 and Pfis-2. Figure 5C plots the predicted stability of the fis promoter at the supercoiling level observed both in exponential growth and in non-aerated stationary phase (superhelix density around -0.05). This analysis suggests that the S. Typhimurium fis promoter region is particularly prone to destabilization and melting when DNA is highly supercoiled. The region immediately upstream of Pfis-1 (position -10 to -160) is predicted to be extremely destabilized, with a second smaller region of destabilization surrounding Pfis-2 (position -190 to -260). This very strong SIDD profile raises the possibility that DNA supercoiling alone is able to activate transcription. Further, the observed SIDD profile helps explain why removal of the most destabilized region makes the Pfis(-49) truncate deficient in sustained expression.

Discussion

FIS is a global regulator of gene expression, but its abundance in the cell fluctuates dramatically depending on growth phase. During periods of rapid growth in laboratory conditions the fis promoter is highly expressed, which accounts for the high levels of FIS protein during exponential growth. Activation of the fis promoter during rapid growth relies on highly supercoiled DNA and RpoD [9]. It is possible then that the fis promoter is inactive in stationary phase because DNA is in a more relaxed state and because effective concentrations of RpoD are reduced due to competition with RpoS [31]. A reduction in oxygen availability during stationary phase causes DNA to become highly supercoiled in both S. Typhimurium and E. coli [26,32,33]. Thus a simple model predicts that fis will be expressed in oxygen-limited conditions, and our results suggest that DNA supercoiling may be an important contributor to sustained fis expression. Further, our findings fit very well with the recent observation that E. coli fis expression is much more dependent on global cellular physiology than on the direct activity of transcription factors [34]; we suspect that the regulatory influence of "cellular physiology" posited by the authors is due largely to changes in DNA supercoiling, which they did not test.

The observed reduction in fis expression with progressive truncation of the promoter region suggests that the entire promoter region functions as a topological switch, and that the switch loses potency when shortened. DNA supercoiling appears to sit atop the regulatory hierarchy because promoter truncation had the same effect of reducing transcriptional output in each of the fis, arcA, rpoS, and crp regulatory mutant strains. A topological switch mechanism is further supported by the prediction of a long destabilised region upstream of the GC-rich discriminator. During rapid growth, the GC-rich discriminator is readily melted and transcription proceeds at a high rate even with a very short promoter region, as in the Pfis(-49) truncate. However, our data suggest that during the lower energy state of stationary phase, discriminator melting requires a long destabilised region to focus stress-induced melting at the fis promoter. The fis promoter is particularly striking for its large dynamic range, from very strong to silent. This makes it an ideal model for understanding how DNA supercoiling can be a dominant force in transcriptional control.

How ArcA and RpoS influence fis expression remains enigmatic. Both proteins are global regulators, and the pleiotropic effects of mutating global regulators can make it difficult to distinguish direct from indirect mechanisms of gene regulation. The simplest explanation is that ArcA indirectly influences fis expression through its activity as a repressor of rpoS expression. The expression of fis and rpoS is negatively correlated, the former being elevated in exponential growth and...
Figure 5. DNA supercoiling control of fis expression. A) Median and interquartile ranges of DNA supercoiling from four biological replicates, plotted as in Figure 4E. Fluorescence data from Pfis(-298) (green circles) and dusB-fis::gfp^{TCD} (blue diamonds) for each biological replicate is plotted on the right y-axis; units as in Figure 2. For both the DNA supercoiling and gene expression measurements, wild type cells were grown to stationary phase cells in the indicated volume of culture medium. The dashed lines are nonlinear curves fit to the expression data, with goodness-of-fit $R^2 > 0.85$ in both cases. The degree of DNA supercoiling ($\sigma$) was determined by measuring the migration of topoisomers relative to fully relaxed DNA in chloroquine gels; each topoisomer represents a change of 1 in the linking number. B) DNA supercoiling states in S. Typhimurium wildtype and ΔarcA mutant cells at 22 hours in non-aerated conditions. Medians (black bar) and interquartile ranges of pUC18 topoisomer distributions in stationary phase in well-aerated cultures. For each strain, the average interquartile range from four biological replicates is plotted. C) SIDD profile of the fis promoter region. The energy required for DNA strand separation at a base pair, $G(x)$, is a function of adjacent and distant DNA sequence [30], and $G(x)$ values below 10 indicate positions prone to SIDD. $G(x)$ values for linear DNA were calculated by WebSIDD [27] using 3,500 bp of chromosomal DNA sequence on either side of the dusB start codon (7,000 bp total); only the 550 bp region containing Pfis-1 and Pfis-2 is shown.

doi: 10.1371/journal.pone.0084382.g005
the latter in stationary phase, therefore it is unsurprising that FIS and RpoS are antagonistic at some gene promoters. For example, both proteins influence DNA supercoiling; FIS represses gyrB [35], whereas RpoS transcribes gyrB [36]. The interplay between these two regulators is particularly intriguing in conditions that promote fis expression during stationary phase, when RpoS is most active.

RpoS functions as a non-traditional repressor by (1) competing with RpoD for access to core RNA polymerase and (2) by competing with RpoD for DNA-binding sites because RpoD and RpoS bind very similar DNA sequences. RpoD depends on higher levels of DNA supercoiling than RpoS to initiate transcription [37]. For this reason, promoters can be differentially regulated by RpoD and RpoS through a mechanism in which relaxation of DNA supercoiling causes a transition from RpoD binding to RpoS binding. The dps promoter may present a useful model for understanding fis regulation by RpoS. Dps is a nucleoid-associated protein with an expression profile that is the opposite of FIS; it is absent during exponential phase growth but becomes highly abundant in stationary phase [3]. During exponential growth, RpoD and FIS bind together and remain locked at the dps promoter, thus preventing RpoS from accessing the −35 and −10 elements [38]. When FIS levels decrease as growth slows, RpoS gains access to the dps promoter and transcription is up-regulated. It may be that during stationary phase RpoS is able to gain access to and initiate transcription at the otherwise RpoD-driven fis promoter. Thus, up-regulation of rpoS in the ΔarcA mutant causes an elevation in sustained fis expression because of elevated RpoS activity at the fis promoter.

In E. coli, at least five transcription start sites have been detected in the fis promoter region [5], with the highly conserved start site (Pfis-1 in Figure 1) being chiefly responsible for fis expression in laboratory conditions [39]. We have identified a novel transcription start site, Pfis-2, in S. Typhimurium, bringing to two the number of characterised start sites in the S. Typhimurium dusB-fis operon. Pfis-2 is up-regulated in anaerobic shock and even overtakes Pfis-1 as the primary source of transcript in the absence of ArcA or RpoS. Anaerobic shock is particularly relevant to the study of S. Typhimurium pathogenesis, thus we are currently investigating whether Pfis-2 plays a significant role in infection systems.

Supporting Information

Figure S1. Quantitative PCR measurement of fis transcript in mutants. Total (Pfis-1 plus Pfis-2) fis transcript abundance, expressed relative to wild type at 22 hours in well-aerated conditions. (TIFF)

Table S1. Bacterial strains and plasmids used in this study. (DOCX)

Table S2. Oligonucleotide primers used in this study. (DOCX)

Acknowledgements

The authors wish to thank Barry Moran for assistance with flow cytometry, and the authors wish to thank anonymous reviewers for their helpful and insightful feedback.

Author Contributions

Conceived and designed the experiments: ADSC CK HJQ IKS AD SCK CJD. Performed the experiments: ADSC CK HJQ IKS AD DJ. Analyzed the data: ADSC CK HJQ IKS AD SCK CJD. Contributed reagents/materials/analysis tools: ADSC CK HJQ IKS AD SCK CJD. Wrote the manuscript: ADSC CK HJQ IKS AD SCK CJD.

References

1. Ball CA, Osuna R, Ferguson KC, Johnson RC (1992) Dramatic changes in Fis levels upon nutrient upshift in Escherichia coli. J Bacteriol 174: 8043–8056. PubMed: 1459953.
2. Osuna R, Lienau D, Hughes KT, Johnson RC (1995) Sequence, regulation, and functions of fis in Salmonella typhimurium. J Bacteriol 177: 2021–2032. PubMed: 7536730.
3. Ali Azam T, Iwata A, Nishimura A, Ueda S, Ishihama A (1999) Growth-phase-dependent variation in protein composition of the Escherichia coli nucleoid. J Bacteriol 181: 6361–6370. PubMed: 10519026.
4. Nasser W, Schneider R, Travers A, Muskhelishvili G (2001) CRP modulates fis transcription by alternate formation of activating and repressing nucleoprotein complexes. J Biol Chem 276: 17878–17886. doi: 10.1074/jbc.M100632200. PubMed: 11279109.
5. Nasser W, Rochman M, Muskhelishvili G (2002) Transcriptional regulation of fis operon involves a module of multiple coupled promoters. EMBO J 21: 715–724. doi:10.1093/emboj/21.4.715. PubMed: 11847119.
6. Pratt TS, Steiner T, Feldman LS, Walker KA, Osuna R (1997) Deletion analysis of the fis promoter region in Escherichia coli: antagonistic effects of integration host factor and Fis. J Bacteriol 179: 6367–6377. PubMed: 9335285.
7. Walker KA, Atkins CL, Osuna R (1999) Functional determinants of the Escherichia coli fis promoter: roles of −35, −10, and transcription initiation regions in the response to stringent control and growth phase-dependent regulation. J Bacteriol 181: 1269–1280. PubMed: 9973355.
8. Ninnemann O, Koch C, Kahlmann R (1992) The E.coli fis promoter is subject to stringent control and autoregulation. EMBO J 11: 1075–1083. PubMed: 1547773.
9. Schneider R, Travers A, Muskhelishvili G (2000) The expression of the Escherichia coli fis gene is strongly dependent on the superhelical density of DNA. Mol Microbiol 36: 167–175. doi:10.1046/j.1365-2958.2000.02129.x. PubMed: 11029698.
10. Travers A, Muskhelishvili G (2005) DNA supercoiling — a global transcriptional regulator for enterobacterial growth? Nat Rev Microbiol 3: 157–169. doi:10.1038/nrmicro1088.
11. Mallik P, Paul BJ, Rutherford ST, Gourse RL, Osuna R (2006) DksA is required for growth phase-dependent regulation, growth rate-dependent control, and stringent control of fis expression in Escherichia coli. J Bacteriol 188: 5775–5782. doi:10.1128/JB.00276-06. PubMed: 16885445.
12. Osborne SE, Coombes BK (2011) Transcriptional priming of Salmonella Pathogenicity Island-2 precedes cellular invasion. PLOS ONE 6: e21648. doi:10.1371/journal.pone.0021648.l001. PubMed: 21738750.
13. Crönin TÓ, Dorman CJ (2007) Expression of the Fis protein is sustained in late-exponential- and stationary-phase cultures of Salmonella enterica serovar Typhimurium grown in the absence of aeration. Mol Microbiol 66: 237–251. doi:10.1111/j.1365-2958.2007.05916.x. PubMed: 17784910.
14. Faucher SP, Porwollik S, Dozois CM, McClelland M, Daigle F (2006) Transcriptome of Salmonella enterica serovar Typhi within
macrophages revealed through the selective capture of transcribed sequences. Proc Natl Acad Sci U S A 103: 1906–1911. doi:10.1073/pnas.0509183103. PubMed: 16443683.

15. Walker KA (2004) The fis promoter is regulated by changes in the levels of its transcription initiation nucleotide CTP. J Biol Chem 279: 50818–50828. doi: 10.1074/jbc.M406285200. PubMed: 15386581.

16. Alexeeva S, Hellingwerf KJ, Teixeira de Mattos MJ (2003) Requirement of Arca for Redox Regulation in Escherichia coli under Microaerobic but Not Anaerobic or Aerobic Conditions. J Bacteriol 185: 204–209. doi: 10.1128 JB.185.1.204-209.2003. PubMed: 12486076.

17. Salmon K, Hung S-P, Mekjian K, Baldi P, Hatfield GW et al. (2003) Global gene expression profiling in Escherichia coli K12. The effects of oxygen availability and FNR. J Biol Chem 278: 29837–29855. doi: 10.1074/jbc.M213060200. PubMed: 12754220.

18. Dorman CJ (2006) DNA supercoiling and bacterial gene expression. Sci Prog 89: 151–166. doi:10.3184/003685006783238317. PubMed: 17338437.

19. Jin DJ, Caglierio C, Zhou YN (2012) Growth rate regulation in Escherichia coli. FEMS Microbiol Rev 36: 269–287. doi:10.1111/j.1365-2958.2011.01298.x. PubMed: 21569058.

20. Beach MB, Osuna R (1998) Identification and characterization of the fis operon in enteric bacteria. J Bacteriol 180: 5932–5946. PubMed: 9116582.

21. Guadarrama-Beltran S (2013) Sustained expression of fis, the gene coding for the Fis nucleoid-associated protein, during the stationary phase of growth in Salmonella enterica. University of Dublin, Trinity College.

22. Datsenko KA, Wanner BL (2000) One-step inactivation of chromosomal genes in Escherichia coli K-12 using PCR products. Proc Natl Acad Sci U S A 97: 6640–6645. doi:10.1073/pnas.120136397. PubMed: 10829079.

23. Stemberg NL, Maurer R (1991) Bacteriophage-mediated generalized transcription in Escherichia coli and Salmonella typhimurium. Methods Enzymol 204: 18–43. doi:10.1016/0076-6879(91)04004-8. PubMed: 1943777.

24. Kröger C, Dillon SC, Cameron ADS, Papenfort K, Sivasankaran SK et al. (2012) The transcriptional landscape and small RNAs of Salmonella enterica serovar Typhimurium. Proc Natl Acad Sci U S A 109: E1277–E1286. doi:10.1073/pnas.1201061109. PubMed: 22538806.

25. Cameron ADS, Dorman CJ (2012) A Fundamental Regulatory Mechanism Operating through Ompr and DNA Topology Controls Expression of Salmonella Pathogenicity Islands Spi-1 and Spi-2. PLoS Genet 8: e1002615. doi:10.1371/journal.pgen.1002615.g005. PubMed: 22457642.

26. Cameron ADS, Stoebel DM, Dorman CJ (2011) DNA supercoiling is differentially regulated by environmental factors and FIS in Escherichia coli and Salmonella enterica. Mol Microbiol 80: 85–101. doi:10.1111/j.1365-2958.2010.07580.x. PubMed: 21276095.

27. Bi C, Benham CJ (2004) WebSIDD: server for predicting stress-induced duplex destabilized (SIDD) sites in superhelical DNA. Bioinformatics 20: 1477–1479. doi:10.1093/bioinformatics/bth304. PubMed: 15130624.

28. Argaman L, Hershberg R, Vogel J, Bejerano G, Wagner EG et al. (2001) Novel small RNA-encoding genes in the intergenic regions of Escherichia coli. Curr Biol 11: 941–950. doi:10.1016/S0960-9822(01)00270-6. PubMed: 11448770.

29. Mika F, Hengge R (2005) A two-component phosphotransfer network involving ArcB, ArcA, and RsbB coordinates synthesis and proteolysis of σS (RpoS) in E. coli. Genes Dev 19: 2770–2781. doi:10.1101/gad.153705. PubMed: 16298806.

30. Wang H, Noordewier M, Benham CJ (2004) Stress-induced DNA duplex destabilization (SIDD) in the E. coli genome: SIDD sites are closely associated with promoters. Genome Res 14: 1575–1584. doi:10.1101/gr.2080004. PubMed: 15289476.

31. Farewell A, Kvint K, Nystöm T (1998) Negative regulation by RpoS: a case of sigma factor competition. Mol Microbiol 29: 1039–1051. doi:10.1046/j.1365-2958.1998.00990.x. PubMed: 9767572.

32. Dorman CJ, Barr GC, Ni Bhriain N, Higgins CF (1998) DNA supercoiling and the anaerobic and growth phase regulation of tonB gene expression. J Bacteriol 170: 2816–2826. PubMed: 2836373.

33. Hsieh LS, Burger RM, Drlica K (1991) Bacterial DNA supercoiling and [ATP]/[ADP]. Changes associated with a transition to anaerobic growth. J Mol Biol 219: 443–450. doi:10.1016/0022-2836(91)90185-9. PubMed: 1646892.

34. Berthoumieux S, de Jong H, Baptist G, Pinel C, Rancuet C et al. (2013) Shared control of gene expression in bacteria by transcription factors and global physiology of the cell. Mol Syst Biol 9: 1–11. doi:10.1038/msb.2012.70. PubMed: 23340840.

35. Keane OM, Dorman CJ (2003) The gyr genes of Salmonella enterica serovar Typhimurium are repressed by the factor for inversion stimulation, Fis. Mol Genet Genomics 270: 56–65. doi:10.1007/s00438-003-0896-1. PubMed: 12998222.

36. Macat A, Peano C, Pietrelli A, Egli T, De Bellis G et al. (2011) In vitro transcription profiling of the σ70 subunit of bacterial RNA polymerase: redefinition of the σ70 regulon and identification of σ70-specific promoter sequence elements. Nucleic Acids Res 39: 5338–5355. doi:10.1093/nar/gkr129. PubMed: 21398637.

37. Bordes P, Cortier A, Morales V, Bovier J, Kolb A et al. (2003) DNA supercoiling contributes to σ70 accumulation from σ70-dependent transcription in Escherichia coli. Mol Microbiol 48: 561–571. doi:10.1046/j.1365-2958.2003.03461.x. PubMed: 12675812.

38. Grainger DC, Goldberg MD, Lee DJ, Busby SJW (2008) Selective repression by Fis and H-NS at the Escherichia coli dps promoter. Mol Microbiol 68: 1366–1377. doi:10.1111/j.1365-2958.2008.06253.x. PubMed: 18452510.

39. Mallik P, Pratt TS, Beach MB, Bradley MD, Undammatla J et al. (2004) Growth phase-dependent regulation and stringent control of fis are conserved processes in enteric bacteria and involve a single promoter (fis P) in Escherichia coli. J Bacteriol 186: 122–135. doi:10.1128/JB.186.1.122-135.2004. PubMed: 14679232.

40. Zheng D (2004) Identification of the CRP regulon using in vitro and in vivo transcriptional profiling. Nucleic Acids Res 32: 5874–5893. doi:10.1093/nar/gkh908. PubMed: 15520470.