

# A global role for Fis in the transcriptional control of metabolism and type III secretion in *Salmonella enterica* serovar Typhimurium

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Fis is a key DNA-binding protein involved in nucleoid organization and modulation of many DNA transactions, including transcription in enteric bacteria. The regulon of genes whose expression is influenced by Fis in *Salmonella enterica* serovar Typhimurium (*S. typhimurium*) has been defined by DNA microarray analysis. These data suggest that Fis plays a central role in coordinating the expression of both metabolic and type III secretion factors. The genes that were most strongly up-regulated by Fis were those involved in virulence and located in the pathogenicity islands SPI-1, SPI-2, SPI-3 and SPI-5. Similarly, motility and flagellar genes required Fis for full expression. This was shown to be a direct effect as purified Fis protein bound to the promoter regions of representative flagella and SPI-2 genes. Genes contributing to aspects of metabolism known to assist the bacterium during survival in the mammalian gut were also Fis-regulated, usually negatively. This category included components of metabolic pathways for propanediol utilization, biotin synthesis, vitamin B<sub>12</sub> transport, fatty acids and acetate metabolism, as well as genes for the glyoxylate bypass of the tricarboxylic acid cycle. Genes found to be positively regulated by Fis included those for ethanolamine utilization. The data reported reveal the central role played by Fis in coordinating the expression of both housekeeping and virulence factors required by *S. typhimurium* during life in the gut lumen or during systemic infection of host cells.

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## INTRODUCTION

*Salmonella enterica* serovar Typhimurium (*S. typhimurium*) is the most common and best studied of the *S. enterica* serovars that infect humans (Finlay & Brumell, 2000). It is able to infect a range of animal species, including chicken, cattle and mice, and intensive study of this organism is providing important insights into key processes involved in bacterial pathogenesis. In the mouse, *S. typhimurium* is a facultative intracellular pathogen capable of invading epithelial cells and it has the ability to survive and proliferate within macrophages. The bacterium can be manipulated genetically with relative ease and the complete genome sequence is available (McClelland *et al.*, 2001), allowing a combination of genetic analysis, cell biology and animal

infection studies. This multidisciplinary approach has provided a picture of the major events involved when *S. typhimurium* infects the murine host. Following ingestion and passage through the stomach, the bacteria cross the lining of the intestine by invading the intestinal epithelium, predominantly via M cells. The Salmonellae are subsequently phagocytosed by macrophages before entering the blood stream and establishing a systemic infection (Finlay & Brumell, 2000; Galán, 2001; Groisman & Mouslim, 2000; Holden, 2002; Scherer & Miller, 2001).

*S. typhimurium* is dependent upon the products of a large number of genes (up to 200) to cause infection (Finlay & Brumell, 2000). Some of the virulence genes are located on a 90 kb pathogenicity plasmid, of which the *spv* genes are the best characterized (Holden, 2002; Libby *et al.*, 2000, 2002; Paesold *et al.*, 2002). However, most of the virulence genes are located within *Salmonella* pathogenicity islands (SPI) on the chromosome (Galán, 2001; Groisman & Ochman, 1993, 1997; Hacker & Kaper, 1999; Hensel, 2000; Hensel *et al.*, 1999) of which SPI-1 and SPI-2 have been the most

Abbreviations: FDR, false discovery rate; Fis, factor for inversion stimulation.

The complete dataset for the microarray analysis is presented as supplementary data with the online version of this paper (at <http://mic.sgmjournals.org>).

intensively studied and encode two of the three type III secretion systems of *S. typhimurium*. The Inv/Spa system is encoded by SPI-1 and exports proteins required for epithelial cell invasion (Hardt *et al.*, 1998; Mills *et al.*, 1995; Wood *et al.*, 1996). The genes of the SPI-2 island encode an alternative type III secretion system that is required for survival within the macrophage (Cirillo *et al.*, 1998; Hensel, 2000; Hensel *et al.*, 1998; Ochman *et al.*, 1996; Waterman & Holden, 2003) and for systemic infection of the mouse (Hensel *et al.*, 1995; Shea *et al.*, 1996).

The third type III secretion system in *S. typhimurium* is concerned with the production and deployment of flagella (Hirano *et al.*, 2003; McClelland *et al.*, 2001; Minamino & Macnab, 1999). In common with SPI-1 and SPI-2, the flagellar regulon is highly complex in terms of its regulation and in temporal expression (Chilcott & Hughes, 2000; Kalir *et al.*, 2001; Macnab, 1996, 2003). Several studies have reported that the expression of pathogenicity island genes is coordinated with that of genes contributing to motility (Ellermeier & Slauch, 2003; Goodier & Ahmer, 2001; Lawhon *et al.*, 2003; Lucas *et al.*, 2000). This connection between virulence gene expression and motility is not confined to *S. typhimurium* (Goodier & Ahmer, 2001; Merrell *et al.*, 2002) and probably reflects a need for the pathogen to coordinate its physical mobility with the expression of genes involved in niche invasion and adaptation. Moreover, motility is known to be required for *Salmonella* virulence (Schmitt *et al.*, 2001).

The complexity of the pathogenic phenotype is apparent from the very large number of genes involved in its expression (Eriksson *et al.*, 2003). A major challenge in this field is to understand the underlying regulatory mechanisms that control the expression of individual genes and groups of genes. Genetic studies have identified regulators that are specific to particular virulence genes. These include SpvR, a transcription factor that governs transcription of the *spv* virulence genes on the 90 kb plasmid (Grob & Guiney, 1996; Grob *et al.*, 1997; Sheehan & Dorman, 1998), the HilA protein that regulates transcription of the SPI-1 island genes (Akbar *et al.*, 2003; Bajaj *et al.*, 1996; Boddicker *et al.*, 2003) and the SsrA/SsrB two-component system that controls SPI-2 gene expression (Cirillo *et al.*, 1998; Deiwick *et al.*, 1999; Lee *et al.*, 2000; Valdivia & Falkow, 1997). In addition, several regulators concerned with house-keeping functions, such as the EnvZ/OmpR and PhoP/PhoQ two-component regulatory systems, have also been shown to influence virulence gene expression (Feng *et al.*, 2003a; Garmendia *et al.*, 2003; Groisman, 2001; Lee *et al.*, 2000).

Proteins with wide-ranging functions in bacterial gene regulation are known as global regulators and these include the nucleoid-associated proteins. Sometimes referred to as histone-like proteins, these molecules typically play roles in organizing the genetic material within the bacterial nucleoid as well as influencing transcription (for a recent review see Dorman & Deighan, 2003). The factor for inversion stimulation (Fis) is an 11.2 kDa DNA-binding protein

comprising 98 amino acids that was first identified as a stimulator of inversion of the Hin invertible DNA element in *S. typhimurium*. This is the genetic switch that is responsible for phase-variable expression of the H1 and H2 flagellar antigens (Johnson, 2002). Fis binds to an enhancer element at the switch and organizes a nucleoprotein complex that facilitates site-specific recombination by the Hin recombinase (Heichman & Johnson, 1990).

Since its discovery it has become apparent that the roles of Fis extend beyond its involvement in DNA inversion (Finkel & Johnson, 1992; Wagner, 2000). In *Escherichia coli*, Fis has been shown to modulate transcription of many genes, including those encoding stable RNA. Fis is also required for *oriC*-directed DNA replication and influences the topological state of DNA in the cell by repressing DNA gyrase and activating topoisomerase I gene expression (Gonzalez-Gil *et al.*, 1996; Ross *et al.*, 1990; Schneider *et al.*, 1999; Weinstein-Fischer *et al.*, 2000). A degenerate consensus sequence has been identified for Fis where it introduces a bend of between 40° and 90° upon binding (Hengen *et al.*, 1997). The *E. coli* Fis protein has a preference for binding sites located within regions of DNA curvature and is known to bind as a dimer (Wagner, 2000). The level of Fis in the cell is subject to complex and multifactorial control. Transcription of the *fis* gene is influenced by the stringent response, is autoregulated by Fis protein and is controlled by the intracellular concentration of cytosine triphosphate (Ball *et al.*, 1992; Walker *et al.*, 1999). The *fis* promoter is stimulated by negative supercoiling of the DNA (Schneider *et al.*, 2000). When bacteria are subcultured in fresh medium there is a dramatic burst of Fis expression producing 50 000 to 100 000 dimers per cell. Thereafter, this high level falls as the cells divide until there are fewer than 500 dimers per cell at the onset of stationary phase (Appleman *et al.*, 1998; Ball *et al.*, 1992).

Many of the Fis-related observations made in *E. coli* are also true in *S. typhimurium* (Keane & Dorman, 2003; Osuna *et al.*, 1995). Some differences in expression that have been reported reflect differences in the promoter sequence between the species (Osuna *et al.*, 1995). A *fis* mutant of *S. typhimurium* has been described as having reduced motility, although the underlying reason was not established. The same *fis* mutant had an extended lag phase in a rich growth medium and the viability of the bacterium was compromised by constitutive expression of Fis during stationary phase (Osuna *et al.*, 1995).

Recently, Fis has been implicated in the control of virulence gene expression in pathogenic strains of *E. coli* (Goldberg *et al.*, 2001; Sheikh *et al.*, 2001), in *Shigella flexneri* (Falconi *et al.*, 2001) and in *S. typhimurium*, where it has been found to influence expression of genes within the SPI-1 pathogenicity island (Schechter *et al.*, 2003; Wilson *et al.*, 2001; Yoon *et al.*, 2003). In this study, we have used DNA microarrays to investigate the extent of Fis involvement in the control of gene expression in *S. typhimurium*. We have now established that Fis regulates the expression of genes

involved with metabolism, transport, flagellar biosynthesis and invasion. We also show that Fis is required for optimal expression of the SPI-2 pathogenicity island.

## METHODS

**Bacterial strains, plasmids and growth media.** The bacterial strains used in this study are listed in Table 1. *S. typhimurium* strain SL1344 (Hoiseth & Stocker, 1981) was used throughout the work and is the same isolate used in previous studies of *S. typhimurium* gene expression (Clements *et al.*, 2002; Eriksson *et al.*, 2003). A *fis* knockout mutant, SL1344*fis::cat* (Keane & Dorman, 2003), was constructed by transducing the *fis::cat* lesion from LT-2 strain TH2285 (a gift from K. T. Hughes) to SL1344 by bacteriophage P22 generalized transduction (Sternberg & Maurer, 1991). In the mutant the *fis* gene has undergone a 150 bp deletion of the 5' end of the ORF and a chloramphenicol acetyltransferase gene has been inserted in place of the deleted *fis* DNA. The absence of the Fis protein in SL1344*fis::cat* was confirmed by Western blotting (data not shown). The promoter probe plasmid pQF50 (Table 1; Farinha & Kropinski, 1990) used to study *ssrA* and *ssaG* promoter activity has a copy number of ~10 per chromosome. Bacteria were grown routinely in Luria-Bertani (LB) broth or on LB agar plates at 37 °C (Sambrook & Russell, 2001). Motility assays were performed with swarm plates containing 1 % Bacto-Tryptone, 0.5 % NaCl and 0.3 % Bacto-Agar (Macnab, 1986). These plates were inoculated centrally with equal numbers of bacteria and incubated at 37 °C for 8 h.

**Western blot analysis.** For preparation of whole-cell proteins, 2.0 OD<sub>600</sub> units of bacteria was harvested and resuspended in lysis buffer (10 % sucrose, 50 mM Tris/HCl, pH 7.5, 100 mM NaCl, 1 mM EDTA, 5 mM dithiothreitol) with 200 µg lysozyme ml<sup>-1</sup> and subsequently freeze-thawed to ensure complete lysis. Equal volumes were added to 2 × SDS sample buffer. Proteins were resolved using 16 or 12 % SDS-polyacrylamide gels (for detection of Fis or FliC, respectively) and proteins were electroblotted onto Protran nitrocellulose membrane (Schleicher & Schuell). Equal loading and consistent transfer of protein to the nitrocellulose membrane were confirmed by staining with Ponceau S [0.2 % Ponceau S, 3 % (w/v) trichloroacetic acid, 3 % (w/v) sulphosalicylic acid] before blocking in 5 % (w/v) dried skimmed milk in PBS. Membranes were probed overnight with the anti-Fis antibody (1:1000) (Keane & Dorman, 2003) or for 1 h with the anti-FliC antibody (1:1000; Becton Dickinson) diluted in blocking solution. Membranes were washed in PBS and incubated with goat anti-rabbit horseradish peroxidase-conjugated antiserum (Cell Signalling). Membranes were treated with Supersignal chemiluminescent substrate (Pierce) and visualized on X-ray film (Kodak).

**Microarray procedures.** A microarray analysis was carried out to elucidate the *fis* regulon of *S. typhimurium* during growth in LB broth and was performed as described previously (Clements *et al.*, 2002) except that the microarrays were printed on Corning CMT-GAPS-coated slides. Each microarray contained 4414 coding sequences and was based on the *S. typhimurium* LT-2a genome sequence (McClelland *et al.*, 2001). The microarray data analysis procedures used in this study were fully MIAME compliant.

**Table 1.** Bacterial strains and plasmids

Strain/plasmid	Description/genotype	Source/reference
<b>Strain</b>		
SL1344	<i>rpsL hisG</i>	Hoiseth & Stocker (1981), Eriksson <i>et al.</i> (2003)
SL1344 <i>fis::cat</i>	SL1344 transduced to <i>fis::cat</i> by P22 lysate of TH2285	This study
RG200	14028 <i>flhD</i> <sup>+</sup> $\phi$ ( <i>flhD::lacZY</i> ) integrant	Goodier & Ahmer (2001)
RG202	14028 <i>fliA</i> <sup>+</sup> $\phi$ ( <i>fliA::lacZY</i> ) integrant	Goodier & Ahmer (2001)
RG207	14028 <i>fliC</i> <sup>+</sup> $\phi$ ( <i>fliC::lacZY</i> ) integrant	Goodier & Ahmer (2001)
RG211	14028 <i>fliE</i> <sup>+</sup> $\phi$ ( <i>fliE::lacZY</i> ) integrant	Goodier & Ahmer (2001)
RG213	14028 <i>flgA</i> <sup>+</sup> $\phi$ ( <i>flgA::lacZY</i> ) integrant	Goodier & Ahmer (2001)
AK01	SL1344 <i>flhD</i> <sup>+</sup> $\phi$ ( <i>flhD::lacZY</i> ) integrant	This study
AK02	SL1344 <i>fliA</i> <sup>+</sup> $\phi$ ( <i>fliA::lacZY</i> ) integrant	This study
AK03	SL1344 <i>fliC</i> <sup>+</sup> $\phi$ ( <i>fliC::lacZY</i> ) integrant	This study
AK04	SL1344 <i>fliE</i> <sup>+</sup> $\phi$ ( <i>fliE::lacZY</i> ) integrant	This study
AK05	SL1344 <i>flgA</i> <sup>+</sup> $\phi$ ( <i>flgA::lacZY</i> ) integrant	This study
AK06	SL1344 <i>fis::cat flhD</i> <sup>+</sup> $\phi$ ( <i>flhD::lacZY</i> ) integrant	This study
AK07	SL1344 <i>fis::cat fliA</i> <sup>+</sup> $\phi$ ( <i>fliA::lacZY</i> ) integrant	This study
AK08	SL1344 <i>fis::cat fliC</i> <sup>+</sup> $\phi$ ( <i>fliC::lacZY</i> ) integrant	This study
AK09	SL1344 <i>fis::cat fliE</i> <sup>+</sup> $\phi$ ( <i>fliE::lacZY</i> ) integrant	This study
AK10	SL1344 <i>fis::cat flgA</i> <sup>+</sup> $\phi$ ( <i>flgA::lacZY</i> ) integrant	This study
TH2285	<i>fis::cat</i>	K. T. Hughes
TH6232	$\Delta$ <i>hin5717::FRT fljBA</i> off FliC <sup>+</sup>	K. T. Hughes
TH6233	$\Delta$ <i>hin5718::FRT fljBA</i> on FliC <sup>-</sup>	K. T. Hughes
<b>Plasmid</b>		
pFis349	Ap <sup>r</sup> , pGS349 containing the <i>S. typhimurium yhdG fis</i> operon	Wilson <i>et al.</i> (2001)
pQF50	Ap <sup>r</sup> , <i>lacZ</i> reporter plasmid	Farinha & Kropinski (1990)
pQFssrA	650 bp <i>ssrA</i> promoter sequence inserted upstream of <i>lacZ</i> in pQF50	This work
pQFssaG	580 bp <i>ssaG</i> promoter sequence inserted upstream of <i>lacZ</i> in pQF50	This work

**RNA extraction.** Volumes (100 ml) of LB in 250 ml flasks were inoculated from overnight cultures of SL1344 or SL1344*fis::cat* and grown at 37 °C with shaking. At 1 and 4 h post subculture, 4·0 OD<sub>600</sub> units was harvested, transferred to 0·2 vols phenol/ethanol mix [5 % (v/v) phenol, 95 % (v/v) ethanol] and incubated on ice for at least 30 min to stabilize bacterial RNA (Tedin & Blasi, 1996). The bacteria were pelleted by centrifugation and RNA was isolated using the Promega SV total RNA purification kit as described at [www.ifr.ac.uk/safety/microarrays/protocols.html](http://www.ifr.ac.uk/safety/microarrays/protocols.html). After elution the RNA was quantified, precipitated and resuspended at a concentration of 3 µg ml<sup>-1</sup> in RNase-free water (Sigma).

**Probe preparation and scanning.** Microarray approaches have been discussed by Lucchini *et al.* (2001) and Thompson *et al.* (2001). RNA (10 µg) was fluorescently labelled during reverse transcription into cDNA. Fluorescently labelled genomic DNA (4 µg) from SL1344 was used as a reference channel in each experiment. For labelling protocols, see [www.ifr.bbsrc.ac.uk/safety/microarray/protocols.html](http://www.ifr.bbsrc.ac.uk/safety/microarray/protocols.html). Scanning and data analyses were performed as described by Eriksson *et al.* (2003). All RNA samples were hybridized to microarrays in quadruplicate and two biological replicates were performed. Only coding regions whose expression showed at least a twofold difference [false discovery rate (FDR) ≤ 0·05 %] in the absence of Fis were regarded as being affected by the *fis* mutation. The complete dataset for the microarray analysis is presented as supplementary data with the online version of this paper (at <http://mic.sgmjournals.org>).

**β-Galactosidase assays.** Chromosomal merodiploid *lacZY* transcriptional fusions to the promoters of *flhD*, *fliA*, *fliC*, *fliE* and *fliA* (Goodier & Ahmer, 2001) were transferred into SL1344 and SL1344*fis::cat* backgrounds by P22 transduction (Sternberg & Maurer, 1991) and assayed for β-galactosidase activity according to the method of Miller (1992). Plasmid derivatives of the promoter probe vector containing either the *ssrA* or the *ssaG* promoter inserted upstream of a *lacZ* reporter gene were also assayed in the SL1344 and SL1344*fis::cat* genetic backgrounds. The *ssrA* and the *ssaG* promoter fragments were amplified by PCR as 645 and 580 bp DNA fragments, respectively, and each was cloned into pQF50 that had been linearized at its multiple cloning site with *Bam*HI and *Kpn*I. The resulting *ssrA-lacZ* and *ssaG-lacZ* reporter plasmids were named pQFssrA and pQFssaG, respectively. β-Galactosidase assays were performed in duplicate and the data expressed as the means of the two measurements. Standard deviations were calculated and are indicated in each figure. Experiments were performed on at least three independent occasions and typical results are shown.

**DNA mobility shift assays.** A 723 bp fragment of the *flhDC* promoter, a 301 bp fragment of the *fliA* promoter, a 314 bp fragment of the *fliC* promoter, a 645 bp fragment of the *ssrA* promoter and a 580 bp fragment of the *ssaG* promoter were used in electrophoretic mobility shift assays. The fragments were amplified by PCR using the primer pairs, BSflhDfw and BSflhDrv, BSfliAfw and BSfliArv, BSfliCfw and BSfliCrv, ssrA\_F and ssrA\_R, and ssaG\_F and ssaG\_R (Table 2), electrophoresed through a 1·3 % agarose gel and the bands excised and extracted using the Concert Rapid Gel Extraction System (GibcoBRL). The DNA was labelled with [<sup>γ</sup>-<sup>32</sup>P]ATP (NEN). Unincorporated label was removed and the DNA was purified using the High Pure PCR Product Purification Kit (Roche Molecular Biochemicals). In each reaction, 5 ng labelled probe was added to 20 mM Tris/HCl (pH 7·5), 80 mM NaCl, 1 mM EDTA, containing 50 µg poly(dI-dC) ml<sup>-1</sup> and 300 µg BSA ml<sup>-1</sup>. Reactions therefore contained approximately 125-fold excess of non-specific synthetic competitor. Various quantities of purified Fis in Fis storage buffer (0·5 M NaCl, 20 mM Tris, pH 7·5, 0·1 mM EDTA, 50 % glycerol) were added, giving final concentrations of 0, 4, 20 or 60 ng Fis in each reaction. The reactions were then incubated at room temperature for 30 min, followed by electrophoresis on a 7 % (w/v) polyacrylamide gel in 0·5 × TBE. Radioactive fragments were visualized by autoradiography. The *S. typhimurium* *spvR* promoter was amplified using the primer pair, spvR11 and spvR14 (Table 2) and was used as a negative control.

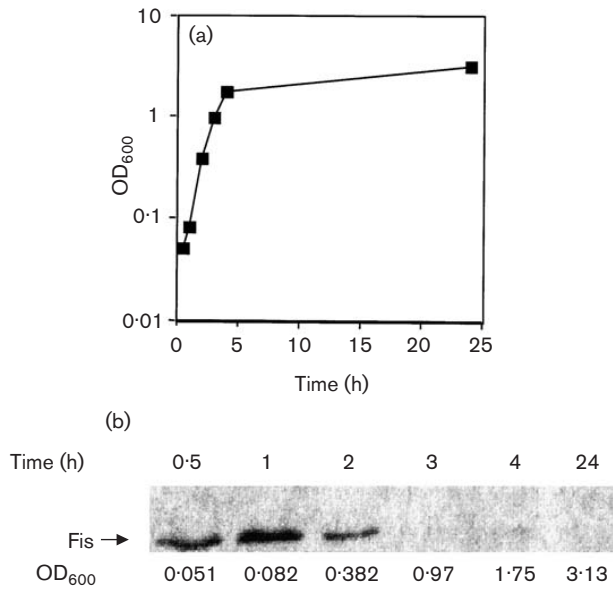
## RESULTS AND DISCUSSION

### Determination of peak Fis expression in *S. typhimurium*

To establish the optimum time points for transcriptional profiling of SL1344 and its *fis::cat* derivative, we used Western blotting analysis to monitor Fis protein in the wild-type strain throughout the growth cycle in batch culture in Luria–Bertani (LB) broth (Fig. 1). We found that peak expression of Fis protein occurred 1 h after diluting the overnight culture into fresh medium. No Fis protein was detectable by 3 h. In a parallel experiment, no Fis protein was detectable in the *fis* mutant (data not shown). We chose time points of 1 and 4 h to represent samples where the cells contained maximum and minimum levels of Fis, respectively.

**Table 2.** Primers used in this study

Primer	Sequence (5'–3')
BSflhDfw	GCGCTAATGCCACATTAATG
BSflhDrv	GTCCCATCCAGATTAACC
BSfliAfw	CGGGCCGTAAGTAACGAA
BSfliArv	GCGGTATACAGTGAATTCAC
BSfliCfw	CGGTAAGTTTGATCCCAC
BSfliCrv	TTAATGACTTGTGCCATGATC
spvR11	CCAAGCTTCAGTACTGATCTTGGGATACTG
spvR14	CCCAAGCTTCAGGTCACCGCCATCCTGTTTTGTC
SsrA_F	ATACGGATCCGAATTCGTCGACGGCAAGACAAGGCTTAGGTAAGC
SsrA_R	ATTAGGTACCGGATCCGCCTGATTACTAAAGATGTTTGC
SsaG_F	CGCGGATCCGGATTGGCCCTTGCTATTGC
SsaG_R	CGGGGTACCGGGTTGAGCAAATCATTACC



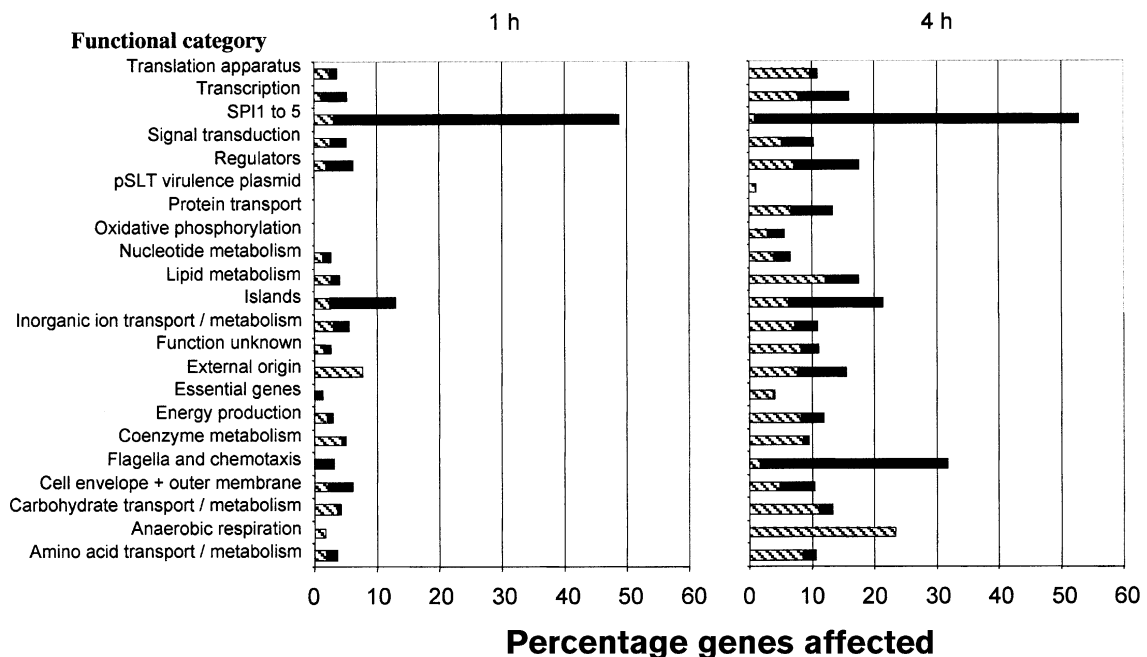
**Fig. 1.** Expression of the Fis protein in an LB culture of strain SL1344. Growth of SL1344 was monitored at 600 nm for 24 h (a). Total protein was extracted from an SL1344 culture at the time points indicated and Western blotting was used to examine Fis protein expression over a 24 h time course (b). The OD<sub>600</sub> of the culture at each time point is given below the relevant lane.

### Transcriptional profiling of SL1344 and SL1344*fis::cat*

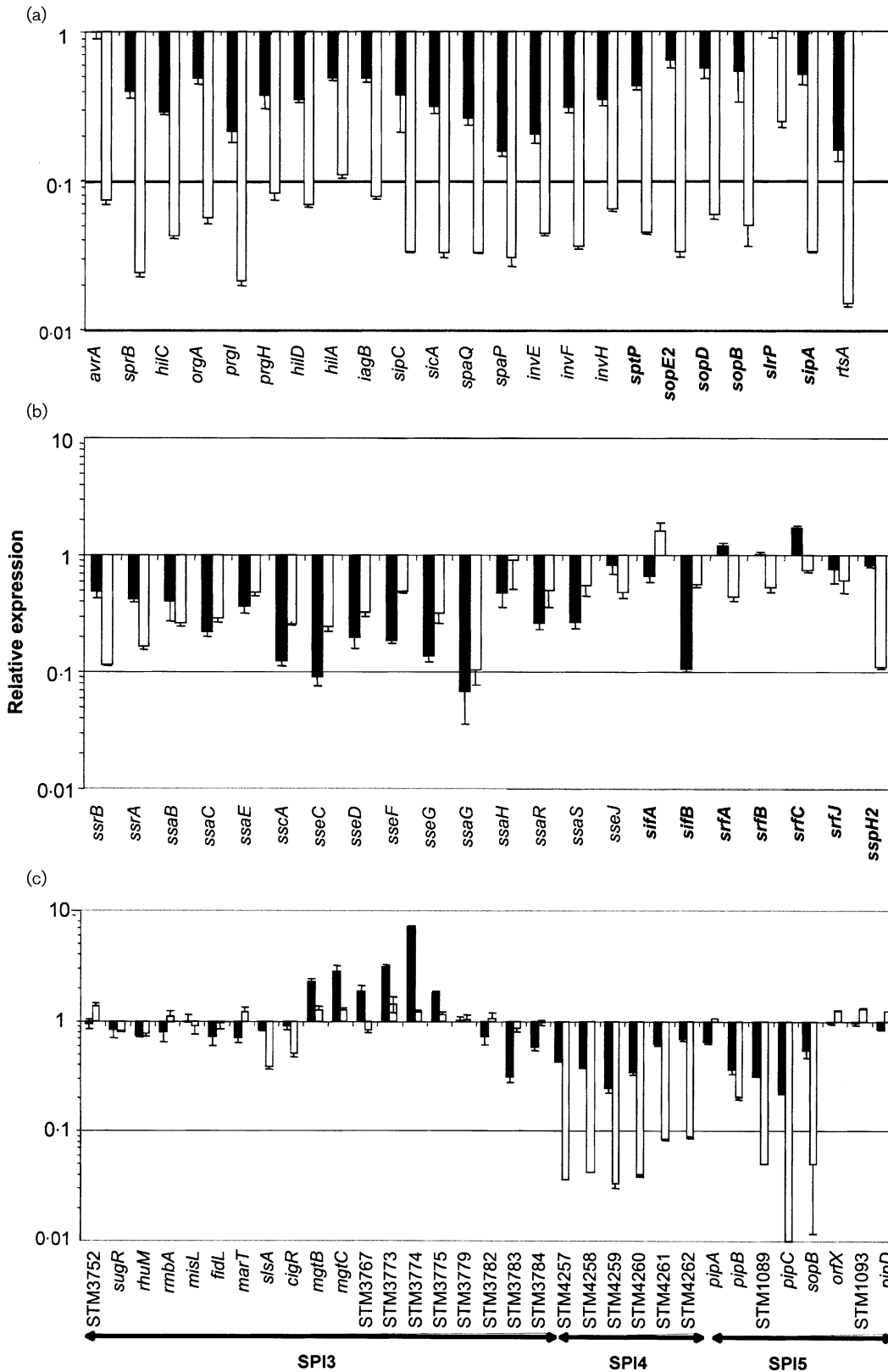
Stationary-phase overnight cultures of SL1344 and SL1344*fis::cat* were used to inoculate fresh LB and total RNA was extracted from the bacterial cultures after 1 and 4 h of growth. The RNA was used to make cDNA that was labelled and hybridized to microarrays (see Methods). Gene expression profiles were normalized to SL1344 for either the 1 or 4 h culture and expressed as the ratio of *fis* mutant to wild-type such that genes activated by Fis have a value less than one. Robust microarray data were obtained by statistical filtering with an FDR of 0.05%. Genes showing greater than a twofold change in expression between the wild-type and mutant strains were identified at the two time points.

An overview of the regulon was obtained by defining functional categories of genes based on the Kyoto Encyclopedia of Genes and Genomics (KEGG; [www.genome.ad.jp/kegg/kegg2.html](http://www.genome.ad.jp/kegg/kegg2.html)). Categories containing a high proportion of Fis-dependent genes were identified (Fig. 2). It is apparent that the majority of genes regulated by Fis are associated with virulence and motility/chemotaxis. Intriguingly, most Fis-dependent genes were observed at the 4 h time point, when we have shown Fis to be no longer detectable by Western blot analysis.

At 1 h after subinoculation, 291 of the 2041 filtered SL1344



**Fig. 2.** Categories of genes regulated by Fis. Fis-regulated genes were grouped into functional categories based on the Kyoto Encyclopedia of Genes and Genomics (KEGG). The histograms represent the percentage of genes in each category affected by the *fis* mutation at 1 and 4 h after subculture, with each functional category listed on the left. Filled bars indicate the percentage of genes more highly expressed in SL1344 than in the *fis* mutant; hatched bars represent the percentage of genes more highly expressed in the SL1344*fis::cat* mutant than in the wild-type.



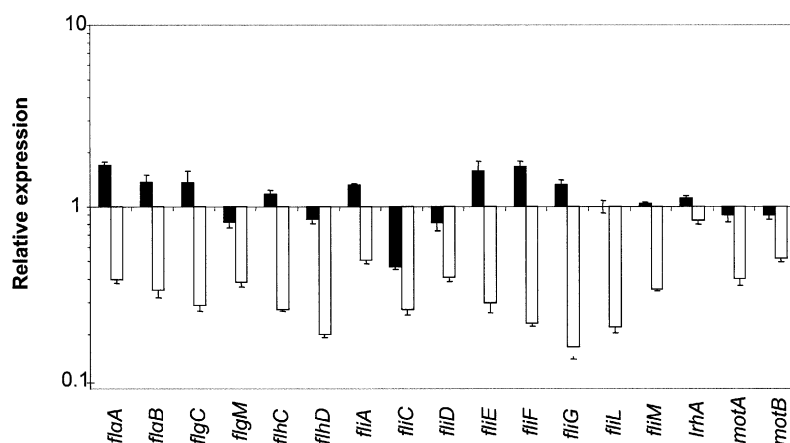
**Fig. 3.** Effect of the *fis* mutation on expression of selected virulence genes located within *S. typhimurium* pathogenicity islands. All expression data were normalized to SL1344 for the 1 (filled bars) and 4 h (open bars) time points and the ratio of the mutant/wild-type was calculated for genes within SPI-1 and SPI-1 effectors (bold) (a), SPI-2 and SPI-2 effectors (bold) (b), and SPI-3, SPI-4 and SPI-5 (c). Expression ratios less than 1.0 indicate genes normally activated by *Fis*.

coding sequences with an FDR  $\leq 0.05\%$  showed  $\geq$  twofold changes in expression. Of these differentially expressed genes, 167 showed higher levels of expression in the *fis* mutant while 124 genes showed a lower level of expression. At the 4 h time point a total of 844 genes showed statistically significant (FDR  $\leq 0.05\%$ ) changes in expression level with 356 being more highly expressed in the mutant and 488 being repressed. Of the 167 genes showing increased expression in the mutant at 1 h, 78 were downregulated at 4 h. We also found that of 124 genes showing lower expression in the *fis* mutant at 1 h, 97 had elevated expression by 4 h (see supplementary data Table S1 at <http://mic.sgmjournals.org>). Thus, for 60% of the ORFs showing an up or down response to the absence of Fis at 1 h (the time point at which the protein was most abundant in the wild-type) the response was transient. This pattern reflects the transient nature of Fis expression (Fig. 1). The fact that fewer genes were Fis-dependent at 1 h than at 4 h may reflect the involvement of additional regulators at

different stages of the growth cycle. One must also consider the possibility that Fis effects at either time point may be indirect.

### Fis and virulence gene expression

Among the most strongly Fis-activated genes were the virulence genes located within the SPI pathogenicity islands (see Fig. 2 and Fig. 3) and the chemotaxis/flagellar regulons (Fig. 4). Generally the effect of the *fis* mutation was to decrease gene expression, indicating a role for Fis as a transcription activator. The genes that were most downregulated in the *fis* mutant at 1 h were those in SPI-2 (Fig. 3b). In light of the role for SPI-2 in adaptation to the macrophage, it was interesting to observe that the macrophage-induced genes *mig-3* and *mig-14*, and a number of PhoP-PhoQ-activated genes also showed a dependency on Fis (Table 3). SPI-1 genes were also Fis-dependent, in keeping with previous findings (Wilson *et al.*, 2001); at



**Fig. 4.** Flagellar gene regulation by Fis. Expression data were normalized to SL1344 for the 1 h (filled bars) and 4 h (open bars) time points and the ratios for the mutant/wild-type were calculated. Expression ratios less than 1.0 indicate genes normally activated by Fis.

**Table 3.** Other virulence genes regulated by Fis

Gene	Function	<i>fis</i> mutant/wild-type expression ratio	
		1 h	4 h
<b>Chromosomal genes outside pathogenicity islands</b>			
<i>iagB</i>	Cell invasion protein	0.49	0.08
<i>mig-3</i>	Macrophage-induced gene	0.98	0.44
<i>mig-14</i>	Macrophage-induced gene	0.15	0.36
<i>pagC</i>	PhoPQ-regulated; macrophage survival	0.29	0.03
<i>pagD</i>	PhoPQ-regulated	0.67	0.31
<i>pagK</i>	PhoPQ-regulated	0.63	0.11
<i>pagO</i>	PhoPQ-regulated	0.53	0.24
<i>sopD</i>	Secreted; transferred to eukaryotes	0.56	0.06
<i>sopE2</i>	Type III secreted protein effector; invasion-associated	0.67	0.03
<i>virK</i>	Homologue of <i>virK</i> in <i>Shigella</i>	0.33	0.31

the 4 h time point, no other class of genes showed as strong a dependency on Fis. We found that genes within SPI-5 were regulated positively by Fis, with *pipC* showing the strongest Fis dependence. Our data identify a coordinating role for Fis in the activation of virulence genes in SPI-1, SPI-2, some in SPI-3, SPI-4 and SPI-5, and are consistent with the previously demonstrated link between expression of SPI-5 genes and those of SPI-1 and SPI-2 (Knodler *et al.*, 2002). Not all *S. typhimurium* virulence genes were regulated by Fis. For example, the *spv* genes on the 90 kb virulence plasmid were not affected by the *fis* mutation (supplementary data Tables S1 and S2 at <http://mic.sgmjournals.org>).

### The effect of the *fis* mutation on specific SPI-2 genes

Transcriptional fusions to virulence genes in the SPI-2 pathogenicity island were tested individually for Fis activation. The promoter of the *ssrA* regulatory gene and the promoter of the *ssaG* structural gene encoding part of the type III secretion apparatus were cloned upstream of the promoterless *lacZ* reporter gene in plasmid pQF50.  $\beta$ -Galactosidase expression was measured in SL1344 and in SL1344*fis* grown in LB broth. The results showed that the SPI-2 promoters were significantly less active in the absence of Fis, in agreement with the DNA microarray data. In the *fis* mutant, *ssrA-lacZ* and *ssaG-lacZ* expression was 50 and 3%, respectively, of the wild-type level.

### Motility genes

Genes contributing to flagellar biosynthesis and motility were among the most strongly downregulated in the *fis* mutant. Few of these genes were affected by the absence of Fis at the 1 h time point (Fig. 4). However, after 4 h, as the bacteria were approaching stationary phase, we detected a significant reduction in flagellar gene expression in the *fis* mutant. This presumably reflects the influence of regulatory factors additional to Fis in the late-exponential-phase culture of the wild-type. Both regulatory and structural genes involved in most aspects of flagellar expression and function were affected by the *fis* mutation and included genes from the early, middle and late stages of flagellar biosynthesis (Macnab, 1996, 2003). Also downregulated in the *fis* mutant was the lipoprotein gene *lpp* (Table 4) which affects flagellar assembly (Dailey & Macnab, 2002).

### The effect of the *fis* mutation on specific flagellar genes

Previous studies demonstrated a role for Fis in *Salmonella* motility (Osuna *et al.*, 1995; Yoon *et al.*, 2003). We used *lacZ* fusions to five different flagellar gene promoters to investigate the effects of the *fis* mutation in more detail. The genes chosen were *flhD* (the regulator of Class 2 flagellar operons), *fliA* (the sigma factor for Class 3 operon expression), *flgA* (assembly of the flagellar basal body P ring), *fliC* (phase 1 flagellin) and *fliE* (the MS ring/rod adapter in the basal body) (Macnab, 1996, 2003). In each

case the chromosomally located merodiploid *lac* fusions were transduced into SL1344 and its *fis:cat* derivative to generate strains AK01–AK10 (see Methods).

All five flagellar genes showed a similar pattern of expression in the wild-type strain (Fig. 5). Following inoculation of fresh broth, expression declined rapidly to a minimum value at approximately 2.5 h. Thereafter, there was a strong increase in flagellar gene expression leading to a peak at approximately 5 h. Expression then declined as the bacteria entered stationary phase. The effect of the *fis* knockout mutation was negative in all cases and resulted in a reduction in expression of approximately twofold (Fig. 5).

### Fis binding to flagellar and SPI-2 promoters

To examine the interaction of Fis with the flagellar and SPI-2 virulence genes in greater detail, representative promoter regions were selected for use in electrophoretic mobility shift assays. The flagellar genes selected were from the early (*flhD*), middle (*fliA*) and late (*fliC*) stages of flagellar biosynthesis (Fig. 6a). The SPI-2 genes studied were the *ssrA* regulatory and *ssaG* structural genes (Fig. 6b). Like the flagellar genes, these had already been examined individually and shown to be regulated by Fis. A DNA sequence from the promoter of the *spvR* gene, known not to be Fis-regulated (our unpublished data; see also supplementary data Tables S1 and S2 at <http://mic.sgmjournals.org>), was used as a negative control. In the case of each of the flagellar and SPI-2 genes, a shift in electrophoretic mobility was seen at the lowest concentration of Fis used (Fig. 6). In contrast, the negative control underwent only a weak shift at the highest Fis concentration. These data show that Fis interacts directly with the flagellar and SPI-2 genes.

### Fis and motility

The effect of Fis on the motility phenotype was established by tests on semi-solid agar plates. The *fis* mutant was clearly much less motile than the wild-type (Fig. 7). Moreover, full motility was restored when the *fis* lesion was complemented *in trans* using a plasmid-borne copy of the functional *fis* gene (Fig. 7).

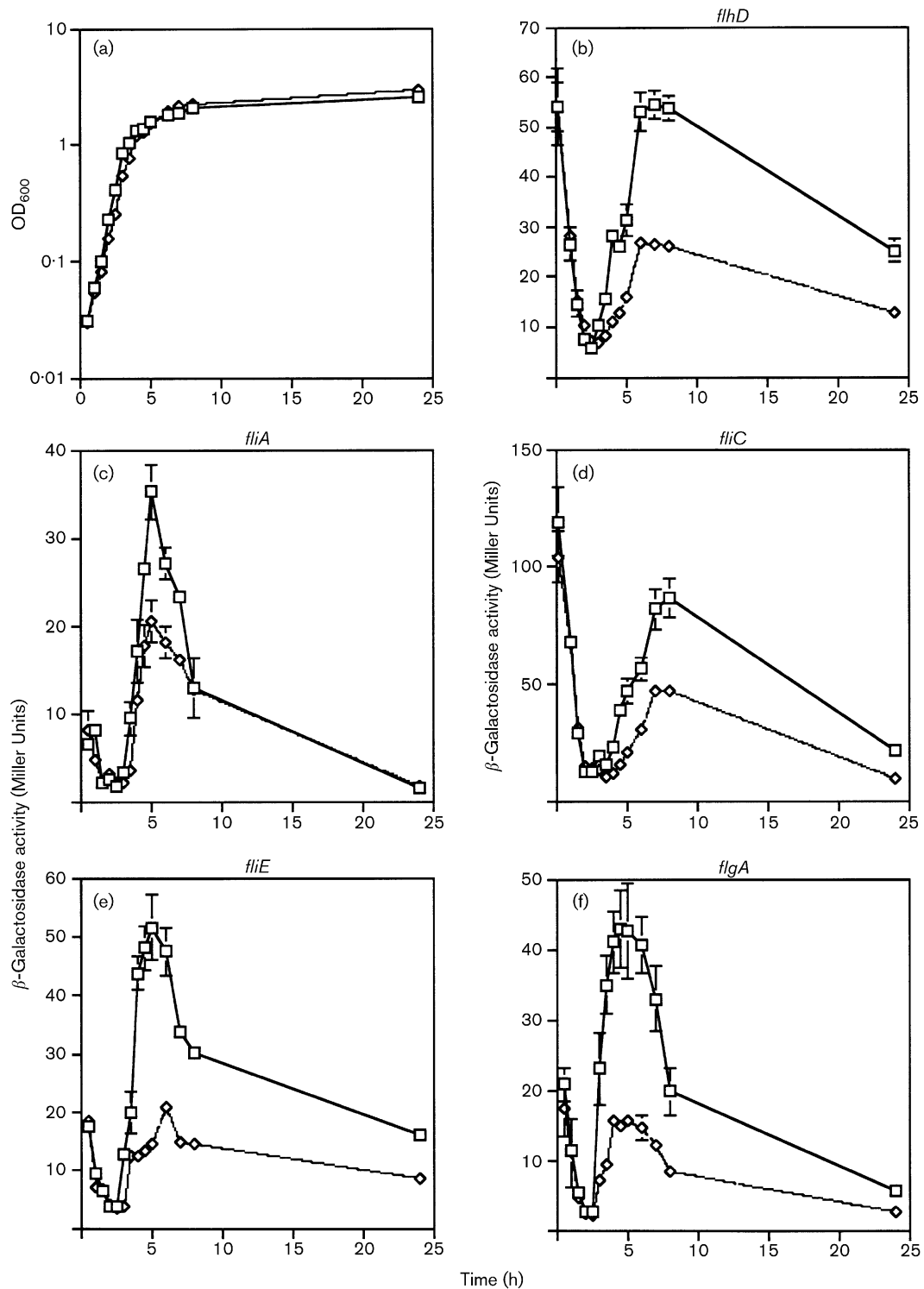
To ensure that the production of phase 1 flagellin protein was genuinely Fis-dependent, we monitored the levels of FliC by Western blotting. Total protein was isolated from wild-type and *fis* mutant cultures grown for 4 h in LB. Protein extracted from a *fliC* mutant was used as a negative control. Probing with anti-FliC antibody showed that the level of FliC was strongly repressed in the *fis* mutant (Fig. 8). This finding was fully consistent with the data from the motility assays, the  $\beta$ -galactosidase assays and the DNA microarrays.

### Genes involved in metabolism and transport

The most strongly Fis-repressed genes identified by the microarrays were involved in metabolism and transport (Table 4). This confirmed that Fis acts as a transcriptional

**Table 4.** Metabolism and transport genes regulated by *fis*

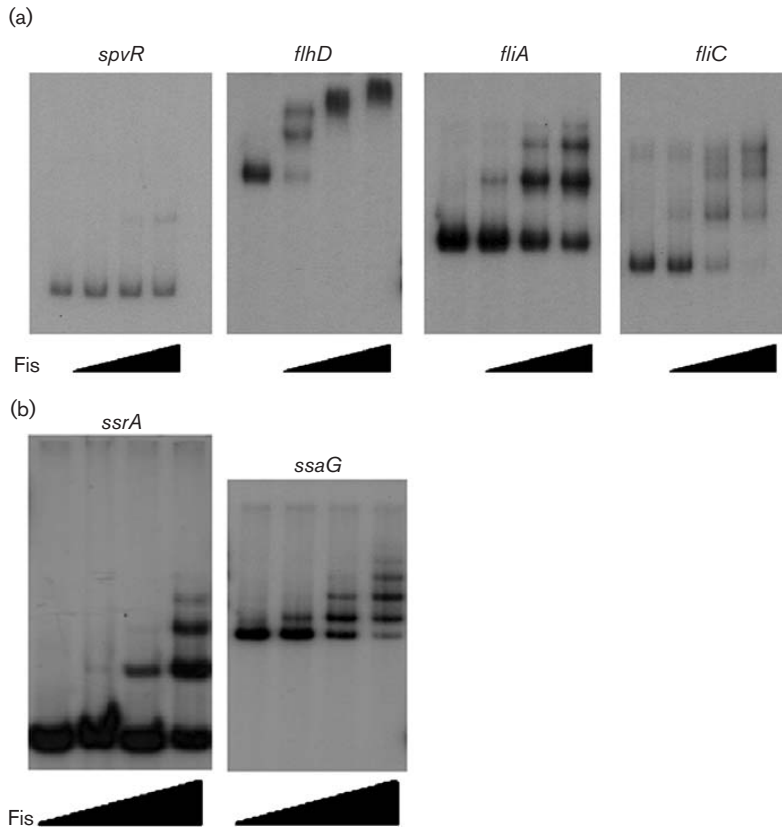
Gene	Function	<i>fis</i> mutant/wild-type expression ratio	
		1 h	4 h
<i>aceB</i>	Malate synthase A	1.52	3.3
<i>aldB</i>	Aldehyde dehydrogenase B	1.49	5.88
<i>btuB</i>	Outer-membrane receptor for vitamin B12; E colicins	1.1	3.45
<i>btuC</i>	Vitamin B12 ABC transporter	1.79	2.08
<i>cadA</i>	Lysine decarboxylase I	1.45	2.94
<i>cadB</i>	Lysine/cadaverine transport	1.22	3.45
<i>citC</i>	Citrate lyase synthetase	1.64	2.44
<i>citD</i>	Citrate lyase acyl carrier protein	1.02	2.78
<i>citF</i>	Citrate lyase alpha chain; citrate-ACP transferase	1.59	5.56
<i>citT</i>	Citrate:succinate antiporter	1.67	3.85
<i>csgF</i>	Transport and assembly of curli	1.04	2.7
<i>cysP</i>	Thiosulphate ABC transporter	1.28	3.85
<i>dadA</i>	D-Amino acid dehydrogenase	0.55	2.56
<i>eutA</i>	Chaperonin in ethanolamine utilization	1.33	0.4
<i>eutB</i>	Ethanolamine ammonia lyase; heavy chain	1.32	0.59
<i>eutC</i>	Ethanolamine ammonia lyase; light chain	1.35	0.36
<i>eutD</i>	Putative phosphotransacetylase	1.47	0.37
<i>eutE</i>	Putative aldehyde oxidoreductase	1.1	0.25
<i>eutH</i>	Putative transport protein	1.6	0.4
<i>eutJ</i>	Putative heat-shock protein	1.89	0.26
<i>eutK</i>	Putative carboxysome structural protein	1.25	0.29
<i>eutL</i>	Putative carboxysome structural protein	1.54	0.33
<i>eutM</i>	Putative detoxification protein	1.18	0.24
<i>eutN</i>	Putative detoxification protein	1.35	0.33
<i>eutP</i>	Putative ethanolamine utilization protein	1.43	0.37
<i>eutQ</i>	Putative ethanolamine utilization protein	1.2	0.41
<i>eutR</i>	Putative transcription regulator (AraC/XylS-like)	1.09	0.37
<i>eutS</i>	Putative carboxysome structural protein	0.99	0.49
<i>eutT</i>	Putative cobalamin adenosyltransferase	0.74	0.42
<i>fabB</i>	3-Oxo acyl synthase I	0.85	2.5
<i>fabD</i>	Malonyl-CoA transacylase	1.56	2.44
<i>fhuE</i>	Outer-membrane receptor for Fe III siderophores	0.88	4.55
<i>fumB</i>	Fumarase B	0.94	2.56
<i>garK</i>	Glycerate kinase	1.72	3.13
<i>glnH</i>	Glutamine high affinity ABC transporter	0.39	2.33
<i>glnP</i>	Glutamine high affinity ABC transporter	0.41	2.63
<i>gltI</i>	Glutamate/aspartate ABC transporter	0.69	2.94
<i>gltJ</i>	Glutamate/aspartate ABC transporter	1.18	2.17
<i>gltK</i>	Glutamate/aspartate ABC transporter	0.83	2.5
<i>gltS</i>	Glutamate transport protein	3.23	2.5
<i>lpp</i>	Murein lipoprotein; links inner and outer membranes	0.25	0.36
<i>marA</i>	Regulator of multiple antibiotic resistance	1.33	2.86
<i>ndk</i>	Nucleoside diphosphate kinase	0.86	4.0
<i>nupG</i>	Nucleoside transport	1.08	2.94
<i>potB</i>	Spermidine/putrescine ABC transporter	0.71	2.56
<i>potC</i>	Spermidine/putrescine ABC transporter	0.92	2.78
<i>psd</i>	Phosphatidylserine decarboxylase	0.96	2.5
<i>rbsC</i>	D-Ribose ABC transporter	1.22	2.44
<i>sbp</i>	Sulphate ABC transporter	0.62	2.94
<i>sdhC</i>	Succinate dehydrogenase; cytochrome b556	0.47	6.67
<i>sdhD</i>	Succinate dehydrogenase hydrophobic subunit	0.38	4.0
<i>speD</i>	S-Adenosylmethionine decarboxylase	1.47	3.23
<i>tctD</i>	Regulator of tricarboxylic transport	1.05	2.94



**Fig. 5.** Expression of flagellar gene fusions in the presence and absence of Fis.  $\beta$ -Galactosidase assays were used to measure expression of *lacZ* in strains harbouring fusions to a selection of flagellar genes in the presence and absence of the Fis protein. Typical growth curves are presented for SL1344 (squares) and its *fis* mutant derivative (diamonds) (a) and gene expression data throughout the growth curve are presented for *flhD* (b), *fliA* (c), *fliC* (d), *fliE* (e) and *flgA* (f).

repressor as well as an activator. A large number of these genes are required for colonization of the gut by *S.*

*typhimurium* (see below). Therefore, we suggest that Fis plays a role in coordinating the expression of house-keeping



**Fig. 6.** Binding of the Fis protein to flagellar and SPI-2 gene promoter regions *in vitro*. The interaction of the Fis protein with the transcription regulatory regions of three flagellar genes (a) and two SPI-2 genes (b) was assessed by electrophoretic mobility shift assay. The regulatory sequences were amplified by PCR, radiolabelled and incubated with 0, 4, 20 or 60 ng purified Fis protein and electrophoresed. Samples were resolved by electrophoresis in 7% polyacrylamide gels. The *spvR* promoter from the 90 kb virulence plasmid was used as a negative control.

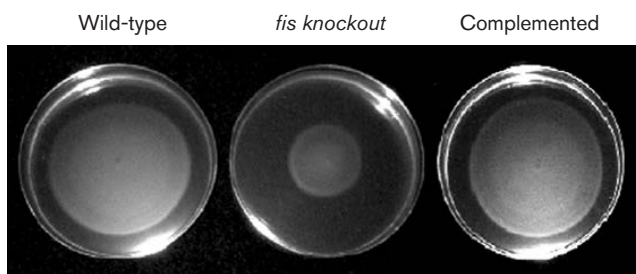
genes with that of virulence genes as part of a regulatory mechanism controlling the transition from a free-living mode in the gut lumen to an intracellular niche.

At the 1 h time point, the genes most highly up-regulated in the *fis* mutant were all involved in biotin synthesis (*bioB*, *bioC* and *bioF*) (Fig. 9). Biotin is a critical cofactor in carboxyl group transfer enzymes, such as biotin carboxylase involved in an early step of lipid biosynthesis (Cronan & Rock, 1996). Other genes from lipid biosynthesis were also found to be up-regulated in the *fis* mutant. These included

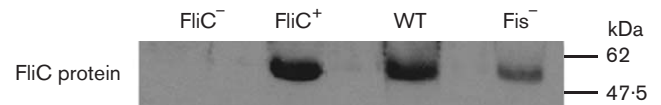
*fabB* encoding  $\beta$ -ketoacyl-ACP synthase I (KAS I), which converts malonyl-ACP to acetoacetyl-ACP, *fabD*, the gene encoding malonyl-CoA:ACP transacylase, and *psd* which encodes phosphatidylserine decarboxylase (Cronan & Rock, 1996).

Several genes concerned with carbon utilization and energy generation were found to be repressed by Fis. These include genes encoding enzymes of the citric acid cycle and its glyoxylate bypass, glycolysis and anaerobic respiration (Table 4).

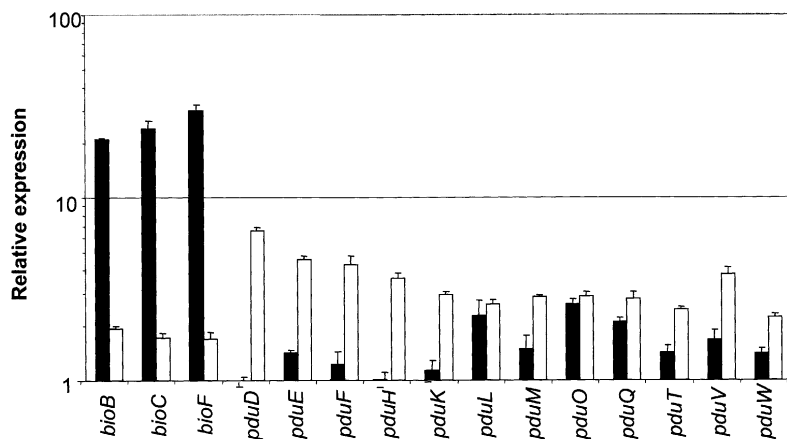
Genes involved in propanediol utilization by *S. typhimurium* were repressed by Fis. Of 18 *pdu* (propanediol utilization) genes for which data were available, 17 showed increased



**Fig. 7.** Effect of a *fis* mutation on *Salmonella* motility. The wild-type strain SL1344, the *fis* knockout mutant SL1344*fis*::*cat* and the complemented mutant SL1344*fis*::*cat* (pFis349) were compared for motility. Equal numbers of bacteria were used to inoculate the centres of semi-solid swarming agar plates and incubated at 37 °C for 8 h.



**Fig. 8.** Expression of the flagellar protein FliC in the presence or absence of Fis. Expression of the FliC protein was measured by Western blotting in wild-type strain SL1344 and its *fis* knockout derivative, SL1344*fis*::*cat* following 4 h growth in LB at 37 °C. Strains TH6233 (negative control; FliC<sup>-</sup>) and TH6232 (positive control; FliC<sup>+</sup>) were included for comparison. The migration positions of molecular mass markers are indicated.



**Fig. 9.** Effect of the *fis* mutation on expression of the *rts* regulatory genes and the *bio* and *pdu* metabolic genes. Fis is an activator for genes with a relative expression value below 1.0 and a repressor for genes with values above 1.0.

expression in the *fis* mutant at the 4 h time point (Fig. 9). Consistent with this was our finding that the *fis* mutant grew more rapidly than the wild-type in minimal medium supplemented with propanediol as carbon source (data not shown). Expression of *pdu* is dependent on the invasion gene regulator CsrA. This protein forms a regulatory link between propanediol utilization, ethanolamine utilization, vitamin B<sub>12</sub> synthesis, flagellar gene expression and SPI-1 virulence gene expression (Lawhon *et al.*, 2003). We found Fis to have a positive role in the expression of ethanolamine utilization genes (*eut*) at the 4 h time point with little or no effect at 1 h (Table 4). This was in contrast to the up-regulation of the *pdu* genes in the *fis* mutant. It was also contrary to the situation reported for CsrA which induces both *pdu* and *eut* expression (Lawhon *et al.*, 2003). The significance of this difference is unknown. Vitamin B<sub>12</sub> is required by the cell for the utilization of both propanediol and ethanolamine (Lawhon *et al.*, 2003). Although Fis was not found to affect genes involved in B<sub>12</sub> production, it did repress genes (*btuB*, *btuC*) contributing to its uptake at the 4 h time point (Table 4).

The *aldB* gene encodes aldehyde dehydrogenase, an enzyme that links propanediol and glyoxylate metabolism (Lin, 1996). Propanediol production is a consequence of L-fucose and L-rhamnose utilization, both of which occur during microbial growth in the mammalian gut (Lawhon *et al.*, 2003). The *aldB* gene was repressed by Fis (Table 4) in agreement with previous data from *E. coli* (Xu & Johnson, 1995a, b). The fact that repression was seen only at the 4 h time point may reflect the fact that *aldB* is also dependent on RpoS for transcriptional activation (Xu & Johnson, 1995a, b). Multiple regulatory inputs of this nature may underlie the differences seen at the 1 and 4 h time points for many of the Fis-regulated genes detected in this study.

Polyamines are required for optimal growth of *E. coli*, but it is unclear which systems are directly affected by them (Glansdorff, 1996). Several *S. typhimurium* genes involved in polyamine metabolism showed elevated expression in the *fis* mutant (Table 4). These encoded lysine decarboxylase (*cadA*) which is required for the conversion of lysine to

cadaverine, cadaverine transport (*cadB*), *S*-adenosylmethionine decarboxylase (*speD*) which feeds *S*-adenosylmethionine into the spermidine biosynthetic pathway, and putrescine/spermidine transport (*potB* and *potC*). The absence of the *cadA* gene from *Shigella* is important for full virulence in that pathogen (Maurelli *et al.*, 1998). While it may be tempting to speculate that repression of *cadA* transcription by Fis may represent a step in the expression of virulence in *S. typhimurium*, it is not known if lysine decarboxylase activity plays any role in *S. typhimurium* virulence. However, it is known that *cadA* contributes to acid tolerance in *S. typhimurium* (Park *et al.*, 1996) and this may point to a role for Fis in adaptation to pH stress.

Fis repressed transcription of *ndk*, the gene that encodes nucleoside diphosphate kinase (Table 4). The involvement of Fis in negative regulation of *ndk* was of interest given its similar role in the expression of the *nupG* and *rbsC* nucleoside transport genes, suggesting that Fis coordinates the expression of genes involved in pyrimidine metabolism. Furthermore, the *ndk* gene product catalyses the inter-conversion of GDP and GTP, a key step in regulating the size of the pppGpp and ppGpp pools that underlie the stringent response (Cashel *et al.*, 1996). This response regulates many important genes in the cell, including the *fis* gene itself. This effect on *ndk* expression may reflect yet another route through which the Fis protein autoregulates *fis* gene expression.

### Stress response genes and global regulators

Few classical stress response genes were Fis-dependent at the 1 h time point. However, by 4 h several genes known to be involved in adaptation to stress were Fis-activated (Table 5). These included the *htrA* heat-shock and *cspC* cold-shock genes, together with the *proV* and *proX* genes of the *proU* osmotic stress response locus. Also found to be Fis-dependent were the *sodC* gene, encoding the Cu–Zn-containing superoxide dismutase, the *sodA* and *sodB* genes, encoding the Mn- and Fe-containing superoxide dismutases, respectively, and the *dsbA* gene encoding the periplasmic protein disulphide isomerase (Table 5).

**Table 5.** Stress response genes regulated by Fis

Gene	Function	<i>fis</i> mutant/wild-type expression ratio	
		1 h	4 h
<i>cspC</i>	Cold-shock protein	0.56	0.42
<i>dsbA</i>	Periplasmic protein disulphide isomerase I	0.77	0.34
<i>htrA</i>	Periplasmic heat-shock protein; serine protease	0.77	0.36
<i>katE</i>	Hydroperoxidase HP11; catalase	1.0	0.49
<i>osmE</i>	Osmotic stress; activator of <i>ntnL</i> transcription	0.77	0.42
<i>osmY</i>	Osmotic stress; periplasmic protein	1.05	0.17
<i>proV</i>	Osmotic stress response	0.59	0.3
<i>proX</i>	Osmotic stress response	0.63	0.36
<i>psiF</i>	Phosphate starvation-induced gene	0.67	0.43
<i>sodA</i>	Superoxide dismutase (Mn)	0.43	0.42
<i>sodB</i>	Superoxide dismutase (Fe)	0.63	0.4
<i>sodC</i>	Superoxide dismutase (Cu-Zn)	0.71	0.36

A number of genes encoding nucleoid-associated proteins with global regulatory roles were affected by the absence of Fis in the mutant (supplementary data Tables S1 and S2 at <http://mic.sgmjournals.org>). These included the cold-shock-responsive *hns* gene previously shown to be activated by Fis (Dersch *et al.*, 1994; Falconi *et al.*, 1996), the *hha* gene whose product can form heteromeric complexes with H-NS and (like H-NS) regulates several virulence genes in response to temperature (Madrid *et al.*, 2002; Nieto *et al.*, 2002), and the *stpA* gene that encodes a paralogue of H-NS and can also form heteromers with it (Deighan *et al.*, 2003; Free *et al.*, 2001; Johansson *et al.*, 2001; Williams *et al.*, 1996). It has been reported previously that Fis has no effect on *stpA* gene expression in *E. coli* at 30 min following subinoculation (Free & Dorman, 1997). Here, no effect of the *fis* mutation on *stpA* expression was detected at 1 h, although *stpA* expression was dependent on Fis when wild-type and mutant were compared at 4 h. The *hupA* and *hupB* genes encode the subunits of the heterodimeric DNA-binding protein HU (Hillyard *et al.*, 1990; Oberto *et al.*, 1994). In addition to its role in nucleoid organization, HU contributes to the osmotic stress response of the cell and normal regulation of the *proU* osmotic stress response operon (Manna & Gowrishankar, 1994). Expression of the *hupA* gene showed a strong requirement for Fis at 1 h and has been described previously as being activated by Fis in *E. coli* (Claret & Rouvière-Yaniv, 1996) (supplementary data Tables S1 and S2 at <http://mic.sgmjournals.org>). The repressive effect of Fis on *hupB* expression described in *E. coli* (Claret & Rouvière-Yaniv, 1996) was not detected under the conditions used in our study.

The RtsA and RtsB proteins have widespread effects on gene expression in *S. typhimurium* (Ellermeier & Slauch, 2003). RtsA shows homology to AraC-like proteins, while RtsB possesses a helix–turn–helix motif that is characteristic of DNA-binding proteins. These proteins are known to coordinate the expression of SPI-1 pathogenicity island genes and the genes of the flagellar regulon. Specifically,

RtsA binds to the *hilA* regulatory gene promoter in SPI-1 while RtsB binds to the *flhDC* regulatory operon promoter in the flagellar regulon (Ellermeier & Slauch, 2003). Interestingly, the genes encoding these proteins, STM4315 (*rtsA*) and STM4314 (*rtsB*) were among the most strongly Fis-activated genes detected in our microarray study (supplementary data Tables S1 and S2 at <http://mic.sgmjournals.org>). This shows that Fis can act at multiple levels within a regulatory hierarchy. For example, RtsB expression depends on Fis (our data), RtsB interacts with the *flhDC* promoter (Ellermeier & Slauch, 2003) as does Fis, which also interacts with promoters at lower levels in the flagellar gene regulatory hierarchy (Fig. 5).

### Concluding remarks

The data presented in this paper show that Fis exerts wide-ranging effects on gene expression in *S. typhimurium*, fully justifying its description as a global regulator. However, the major effects of Fis are confined largely to specific classes of genes (see Fig. 2). In particular, Fis regulates those genes encoding the type III secretion machinery and cognate effectors required by the bacterium for invasion of host epithelial cells, for survival in macrophage and for the deployment of flagella for motility. Therefore, this is not a general effect on all promoters arising from the ability of Fis to influence DNA topology. Our discovery that Fis regulates the expression of all three type III secretion systems in *Salmonella* is in keeping with other studies that have pointed to regulatory overlaps between virulence genes and flagella in *Salmonella* (Eichelberg & Galan, 2000; Ellermeier & Slauch, 2003; Lawhon *et al.*, 2003) and other bacteria (Goodier & Ahmer, 2001; Grant *et al.*, 2003). The effect of Fis on murein lipoprotein expression is also relevant here, since the *lpp* gene product is a structural component of the cell envelope. It is attractive to consider that Fis coordinates expression of *lpp* with that of type III secretion systems that require cell surface integrity for function.

The *Salmonella* pathogenicity islands are regarded as having

been acquired by horizontal gene transfer, possibly from outside the enteric group of Gram-negative bacteria (Galán, 2001; Groisman & Ochman, 1993, 1997; Hacker & Kaper, 1999; Hensel, 2000; Hensel *et al.*, 1999). The degeneracy associated with the binding site used by Fis may have aided its recruitment as a regulator of these horizontally acquired genes. Perhaps this represents a selective pressure acting on Fis to maintain its ability to bind to sites with a high degree of DNA sequence diversity.

None of the Fis-responsive genes found in this study is regulated by Fis alone. Each has at least one, and frequently more than one, additional regulator. By co-operating with or antagonizing the action of the other regulators, Fis appears to modulate and fine-tune gene expression in ways that benefit the cell during growth and adaptation to environmental change.

It is apparent that many genes of unknown function showed a positive or a negative response to Fis (supplementary data Tables S1 and S2 at <http://mic.sgmjournals.org>). Furthermore, our analyses involved *S. typhimurium* strain SL1344 coupled with microarrays which were based on the genome sequence of the strain LT2. The sequence of SL1344 is incomplete ([www.sanger.ac.uk/Projects/Salmonella/](http://www.sanger.ac.uk/Projects/Salmonella/)), but it is already clear that this strain contains a number of genes not found in LT2. This means that knowledge of the full Fis regulon remains incomplete at the global level. At a local level, much must be done to unravel the detail of the contributions made by Fis at specific promoters to allow the regulon to be appreciated more fully. This will contribute in a significant way to a deepening of our appreciation of the gene regulatory circuits used by bacteria, leading to a much more complete understanding of the workings of the cell.

We found that Fis acts to modulate expression of genes involved in aspects of metabolism and transport that are relevant to *S. typhimurium* during life in the gut. These are genes involved in propanediol utilization, ethanolamine utilization, acetate and fatty acid utilization (Table 4; Fig. 9). This points to an even wider role for this protein in coordinating the gene expression programme of the bacterium. It is intuitively appealing that *S. typhimurium* can benefit from coordinating the expression of its major virulence factors with its metabolism and motility, and that it should use a global regulator such as Fis to accomplish this. In particular Fis seems to be well placed to coordinate the expression of genes involved in the transition from a free-living life in the gut lumen to the intracellular niche.

A striking feature of the Fis regulon is the fact that considerably more Fis-dependent genes were expressed or repressed in late, rather than early, exponential phase. This phenomenon is reminiscent of observations made in enteropathogenic *E. coli* where several Fis-dependent virulence genes encoded by the Locus for Enterocyte Effacement (LEE) were found to be maximally expressed in late stationary phase (Goldberg *et al.*, 2001). Similarly, the effect of a *fis* mutation on *gyr* gene transcription is most

acute in stationary phase in both *S. typhimurium* (Keane & Dorman, 2003) and *E. coli* (Schneider *et al.*, 1999). This seems paradoxical since Fis protein levels peak 1 h after diluting stationary-phase cultures into fresh medium (Fig. 1). The levels of Fis declined rapidly such that by 3–4 h the protein was no longer detectable by Western blotting. A number of factors may explain this phenomenon. First, we speculate that the tolerance shown by Fis for degeneracy in the sequence of its binding site allows it to bind to a range of sites with different affinities and that high-affinity sites will continue to be occupied even as Fis protein levels decline. Promoters with such high affinity sites may be regarded as privileged in that they continue to be occupied by Fis when lower affinity sites become vacant. Second, the involvement of additional growth-phase-dependent factors may assist Fis in widening the range of its effects at later stages of the growth cycle. This may involve cooperativity between the additional factors and the remaining Fis molecules to target Fis to the promoters. Third, the absence of Fis is known to alter the structural dynamics of the genome, even at late time points when Fis levels in wild-type cells are very low (Schneider *et al.*, 1999). Therefore, variations in local DNA topology may contribute to differences in the gene expression patterns of the wild-type and *fis* mutant, even at the 4 h time point. Finally, it should also be borne in mind that many of the effects of the *fis* mutation may be indirect, regardless of the stage of growth. These complex issues will be important topics for future research.

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