

The integration host factor (IHF) integrates stationary-phase and virulence gene expression in *Salmonella enterica* serovar Typhimurium

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Summary

The integration host factor (IHF) is a DNA-binding and -bending protein with roles in local DNA structural organization and transcriptional regulation in Gram-negative bacteria. This heterodimeric protein is composed of the two highly homologous subunits IHF α and IHF β . DNA microarray analysis was used to define the regulon of genes subject to IHF control in *Salmonella enterica* serovar Typhimurium (*S. Typhimurium*). The transcription profile of the wild type was compared with those of mutants deficient in IHF α , IHF β , or both IHF α and IHF β . Our data reveal a new connection between IHF and the expression of genes required by the bacterium to undergo the physiological changes associated with the transition from exponential growth to stationary phase. When a mutant lacking IHF entered stationary phase, it displayed downregulated expression of classic stationary-phase genes in the absence of any concomitant change in expression of the RpoS sigma factor. Purified IHF was found to bind to the regulatory regions of stationary-phase genes indicating an auxiliary and direct role for IHF in RpoS-dependent gene activation. Loss of IHF also had a profound influence on expression of the major virulence genes and epithelial cell invasion, indicating a role in co-ordinating regulation of the pathogenic traits with adaptation to stationary phase. Although the three mutants showed considerable overlaps in the genes affected by the *ihf* lesions,

the observed patterns were not identical, showing that *S. Typhimurium* has not one but three overlapping IHF regulons.

Introduction

The integration host factor (IHF) was discovered in *Escherichia coli* as an essential protein for efficient site-specific integration and excision of bacteriophage lambda (Miller *et al.*, 1979; Miller and Friedman, 1980). IHF is a member of a disparate group of approximately 12 different proteins that have been described as histone-like or nucleoid-associated and are found in *E. coli*, *Salmonella enterica* and related bacteria (Ali Azam and Ishihama, 1999; Dorman and Deighan, 2003). IHF contributes to genome organization (Oppenheim *et al.*, 1993; Pettijohn, 1996) and the control of DNA transactions such as transcription (Goosen and van de Putte, 1995), replication (Ryan *et al.*, 2002), site-specific recombination (Bushman *et al.*, 1985; Dorman and Higgins, 1987; Eisenstein *et al.*, 1987; Esposito *et al.*, 2001) and transposition (Makris *et al.*, 1990; Crellin *et al.*, 2004). IHF binds to a conserved sequence in DNA and it bends the DNA by angles of up to 180° (Rice *et al.*, 1996; Rice, 1997). This DNA-bending activity is critical to the role it plays in several systems due to its ability to promote long-range interactions (Goosen and van de Putte, 1995). The influence of IHF on local DNA structure is critical to its contribution to transcription control. In some cases, it has been shown to enhance the formation of open complexes at promoters by transferring DNA twist from upstream regions of A+T-rich DNA to the promoter through its DNA-bending activity (Parekh and Hatfield, 1996; Parekh *et al.*, 1996; Sheridan *et al.*, 1998). In *S. enterica*, IHF controls expression of the *pnt* operon (O'Brien *et al.*, 1992; Palacios and Escalante-Semerena, 2000) and the *cysJ/H* genes (Sirko *et al.*, 1998). IHF can also regulate gene expression by modulation of a methylation site in the control region of the *carAB* operon (Charlier *et al.*, 1995). The intracellular concentration of IHF is growth phase dependent and most studies agree that IHF concentration increases with the onset of the stationary phase (Bushman *et al.*, 1985; Aviv *et al.*, 1994; Ditto *et al.*, 1994; Weglenska *et al.*, 1996; Murtin *et al.*, 1998; Ali Azam *et al.*, 1999).

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In *S. enterica* and *E. coli* IHF is a heterodimeric protein whose subunits are encoded by the *ihfA* and *ihfB* genes. These genes are found at distinct locations in the chromosome and are subject to independent regulatory influences (Aviv *et al.*, 1994). Although IHF is usually considered as a heterodimer, its subunits are capable of forming homodimers and these have DNA-binding activity (Werner *et al.*, 1994; Zulianello *et al.*, 1994; Zablewska and Kur, 1995; Hiszczynska-Sawicka and Kur, 1997).

Salmonella enterica serovar Typhimurium (*S. Typhimurium*) infects a range of animal species, including cows, chickens and pigs. In mice *S. Typhimurium* causes a typhoid-like disease that has served as a model for human typhoid. Following ingestion by the mouse, it passes through the stomach before invading gut epithelial cells, predominantly through M cells. It is then phagocytosed by macrophage in which it is capable of surviving and proliferating before entering the vascular system and establishing a systemic infection (Finlay and Brumell, 2000; Groisman and Mouslim, 2000; Galán, 2001; Scherer and Miller, 2001; Holden, 2002). The bacterium must adapt to a series of dynamic environments as it traverses the infection route. To do this successfully the bacterium must adapt its gene expression profile to meet the demands imposed by the changing environmental conditions. *S. Typhimurium* is dependent on the products of a large number of genes (up to 200) to cause infection (Finlay and Brumell, 2000). Some of the virulence genes are located on a 90 kb pathogenicity plasmid, of which the *spv* genes are the best characterized (Libby *et al.*, 2000; 2002; Holden, 2002). However, most of the virulence genes are located on the chromosome within *Salmonella* pathogenicity islands (SPI) (Groisman and Ochman, 1993; 1997; Hacker and Kaper, 1999; Hensel *et al.*, 1999; Hensel, 2000; Galán, 2001) of which SPI-1 and SPI-2 have been the most intensively studied. These encode two of the three type III secretion systems (TTSS) of *S. Typhimurium*, the third TTSS being involved in flagellum expression and bacterial motility. The Inv/Spa system encoded by SPI-1 exports proteins required for epithelial cell invasion (Mills *et al.*, 1995; Hardt *et al.*, 1998; Wood *et al.*, 1996). The genes of the SPI-2 island encode an alternative TTSS that is required for survival within the macrophage (Ochman *et al.*, 1996; Cirillo *et al.*, 1998; Hensel *et al.*, 1998; Hensel, 2000; Waterman and Holden, 2003) and for systemic infection of the mouse (Hensel *et al.*, 1995; Shea *et al.*, 1996). It is clear that many housekeeping genes involved in core metabolism and cellular homeostasis are also needed in addition to the dedicated virulence genes for full pathogenicity (Mahan *et al.*, 1993; Hensel *et al.*, 1995; Valdivia and Falkow, 1997; Eriksson *et al.*, 2003); co-ordinating the expression of this large assortment of genes represents a significant challenge. To meet this challenge the

bacterium has evolved the ability to control gene expression in a hierarchical manner, using global control elements to organize the background levels of expression against which more specific regulators operate (Martinez-Antonio and Collado-Vides, 2003; Rhen and Dorman, 2005).

Despite the role played by IHF in pathogenicity (Mahan *et al.*, 1993; Marshall *et al.*, 1999), until now the details of its contributions to gene expression at a global level have been lacking. In our investigation we have used DNA microarrays to define the IHF regulon in *S. Typhimurium*. Unlike a previous study carried out in *E. coli* that only investigated an *ihfA* mutant at mid-logarithmic growth (Arfin *et al.*, 2000), we have compared a virulent wild-type strain of *S. Typhimurium* with mutants deficient in either IHF α or IHF β , and both IHF α and IHF β at early, late logarithmic and the early stationary phases of growth. The result is the first comprehensive picture of the role of IHF in global gene regulation during growth of a facultative intracellular pathogen.

Results and discussion

Determination of the optimal time points for RNA recovery

Mutant derivatives of *S. Typhimurium* strain SL1344 deficient in expression of IHF α , IHF β , or both IHF α and IHF β were constructed by bacteriophage P22 transduction (*Experimental procedures*) and following verification of the mutations, growth of the mutant and the wild-type strains was examined in Luria–Bertani (LB) broth at 37°C. All of the strains showed similar growth curves, indicating that the absence of either or both of the IHF subunits did not affect the growth characteristics of the culture under the conditions used (Fig. 1). In order to obtain a representative picture of the role of the IHF proteins in influencing gene expression throughout growth, we isolated RNA from cultures at early logarithmic, late logarithmic, and early stationary phases of growth, corresponding, respectively, to time points at 1 h, 4 h and 6 h after inoculation. Subsequently, cDNA was synthesized, labelled and hybridized to microarrays (see *Experimental procedures*). Gene expression profiles were normalized to wild-type SL1344 for each of the three time points and expressed as a ratio of mutant to wild type such that genes activated by IHF had a value less than one. Robust microarray data were obtained by statistical filtering with a false discovery rate (FDR) of 0.05%. Genes showing greater than a two-fold change in expression between the wild type and mutant were identified at all three time points.

The total number of coding sequences with an FDR $\leq 0.05\%$ that showed ≥ 2 -fold changes in expression is summarized in Table 1. Comparing the wild-type strain SL1344 and its three mutant derivatives at three time

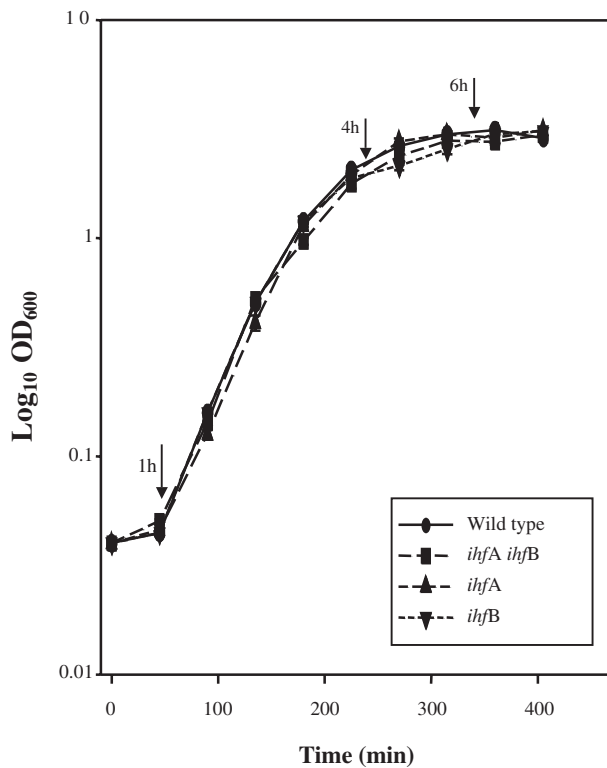


Fig. 1. Growth curves of the wild-type and *ihf* mutant strains. Overnight cultures of the wild-type strain SL1344, the *ihfA* and *ihfB* single mutants and the *ihfA ihfB* double mutant were used to inoculate fresh LB broth. These were grown with aeration and the optical density was measured at fixed time points at A_{600} . Each datum point is the average of two measurements and error bars represent standard deviations. The vertical arrows indicate the 1 h, 4 h and 6 h time points at which samples were removed for RNA extraction.

points generated very large data sets comprising more than 50 000 data points. For convenience, in this report we concentrate primarily on the comparison between the *ihfA ihfB* double mutant and the wild type, making reference to the single mutant data where appropriate. All of the data from the study are presented in *Supplementary material* (Table S1).

Stationary phase

The transition into stationary phase is accompanied by profound changes in the gene expression profile of the bacterial cell (Huisman *et al.*, 1996; Selinger *et al.*, 2000). It is also the phase of growth in which IHF protein levels peak (Bushman *et al.*, 1985; Aviv *et al.*, 1994; Ditto *et al.*, 1994; Weglenska *et al.*, 1996; Murtin *et al.*, 1998; Ali Azam *et al.*, 1999). Our data revealed that the *ihfA ihfB* mutant had very low expression levels of many classical stationary-phase genes at 6 h, the point in the growth curve at which their upregulation would normally be anticipated (Fig. 2). All of these genes depend on the RpoS

stationary-phase sigma factor (*cbpA*, Yamashino *et al.*, 1994; *cfa*, Kim *et al.*, 2005; *csgA*, Olsen *et al.*, 1993; *dps*, Altuvia *et al.*, 1994; *fic*, Hiratsu *et al.*, 1995; *glgS*, Hengge-Aronis *et al.*, 1993; *katE*, Ibanez-Ruiz *et al.*, 2000; Hengge-Aronis *et al.*, 1993; *poxB*, Chang *et al.*, 1994; *spvA*, Heiskanen *et al.*, 1994; *treA*, Hengge-Aronis *et al.*, 1991). The microarray data showed that transcription of the *rpoS* gene was not altered by the loss of IHF and this was confirmed by reverse transcription polymerase chain reaction (RT-PCR) analysis (Fig. 2B). Because most *rpoS* regulation occurs post-transcriptionally (Hengge-Aronis, 2002), we examined the level of RpoS protein and found that it was slightly increased in the mutants compared with the wild type (Fig. 2B). These results demonstrated that the downregulation of the RpoS-dependent genes in the *ihfA ihfB* mutant was not caused by a reduction in the level of the RpoS sigma factor.

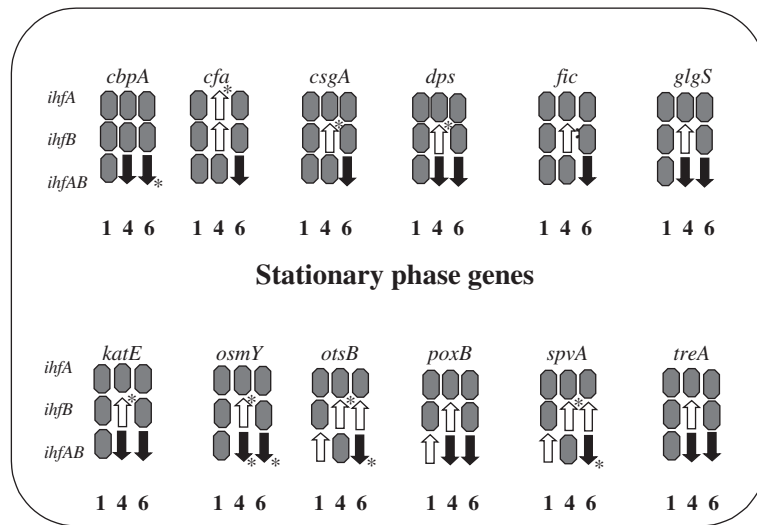
Previous data have implicated IHF in the regulation of some *E. coli* stationary-phase genes, including *csg* curli genes (Gerstel *et al.*, 2003), *dps* (Altuvia *et al.*, 1994) and *osmY* (Colland *et al.*, 2000). IHF has been described as a repressor of *osmY* transcription in minimal medium cultures of *E. coli* in work performed with an *ihfB* mutant (Colland *et al.*, 2000). Our data show that IHF β_2 is a repressor of *osmY* in LB cultures of *Salmonella* in late exponential growth, but that IHF $\alpha\beta$ is a co-activator with RpoS in stationary phase (Fig. 2A).

To our knowledge, the *cfa* and *poxB* genes have not been tested previously for direct interaction with IHF. We found that purified IHF $\alpha\beta$ bound to the promoter proximal regions of each gene, but not to DNA from regions located upstream and distal to their promoters (Fig. 3). The shifted regions contained DNA sequence motifs that were good matches to the consensus for IHF binding sites (Fig. 3; Goodrich *et al.*, 1990; Steffen *et al.*, 2002). These data were consistent with a direct role for IHF as an adjunct to RpoS in the upregulation of stationary-phase genes. However, in keeping with the complex pattern of stationary-phase gene regulation seen in the transcriptomic data (summarized in Fig. 2A), the *cfa* and *poxB* regulatory regions differed in the detail of their interactions with IHF:

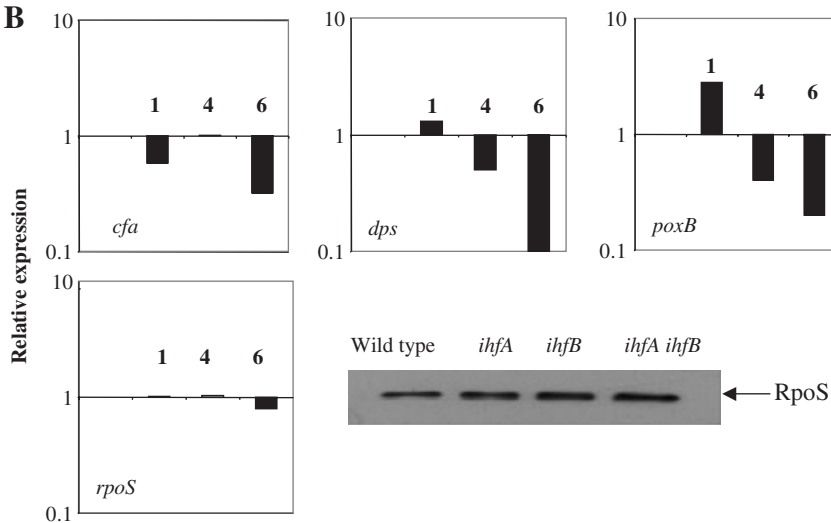
Table 1. Number of genes showing altered expression in the three *ihf* mutant genetic backgrounds.

Strain	Number of genes showing ≥ 2 -fold change in expression (FDR ≤ 0.05)					
	1 h		4 h		6 h	
	Up	Down	Up	Down	Up	Down
<i>ihfA</i>	149	81	189	146	21	29
<i>ihfB</i>	8	38	222	144	155	108
<i>ihfA ihfB</i>	652	236	504	391	278	446

A



B



while all three forms of IHF bound *in vitro* to the *poxB* regulatory region, only IHF $\alpha\beta$ and IHF β_2 did so at *cfa* (Fig. 3).

Motility and chemotaxis

The *che* genes code for the chemotaxis proteins involved in the phosphorelay signalling system that regulates flagellar motor rotation and showed reduced expression in the *ihfA ihfB* mutant at all time points (Fig. 4A); this reveals a positive role for IHF in the transcriptional control of the chemotactic signal transduction system. A strong negative effect was seen at 1 h and 4 h among all flagellar genes and a strong positive effect was recorded at 6 h for a subset of these genes (Fig. 4B). The expression pattern at 6 h correlated with the functions of the gene products. Genes coding for the flagellar TTSS were upregulated in

Fig. 2. The effect of IHF on stationary-phase gene expression.

A. Data are presented for genes previously described as being induced on entry to the stationary phase of growth (Hengge-Aronis, 2002). The numbers 1, 4 and 6 refer to the three time points (h) at which RNA was extracted. Downward (black) and upward (white) arrows represent reductions and elevations, respectively, of at least twofold in gene expression in each *ihf* mutant compared with the wild type. The grey octagonal symbol indicates that no significant change was recorded. Mutant identity is given at the left of each row. (Asterisk: genes changing more than twofold in each replicate but with an FDR > 0.05).

B. RT-PCR data showing expression of selected stationary-phase genes at each time point in the *ihfA ihfB* mutant relative to expression of the same gene in the wild type. Western analysis of RpoS protein levels in the wild type and *ihf* mutants at 6 h is also shown at lower right.

the *ihfA ihfB* mutant whereas those coding for the flagellum or hook assembly proteins, their chaperones or factors that determine flagellum length, were all downregulated.

The IHF protein stimulates expression of the *fis* gene whose product is required for efficient inversion of the *hin* genetic switch that controls FliC-FliB flagellin protein phase variation in *Salmonella* (Beach and Osuna, 1998). In addition, IHF suppresses the inhibitory effect of the H-NS protein on the same genetic switch (Goshima *et al.*, 1994). Therefore, we anticipated that loss of IHF expression would result in bacterial populations expressing exclusively FliC or FliB due to strongly reduced rates of flagellar switching. PCR analysis of *hin* switch inversion rates showed that the normal operation of the switch was indeed inhibited in all three *ihf* mutants (data not shown) and we also examined this phenomenon at the phenotypic level.

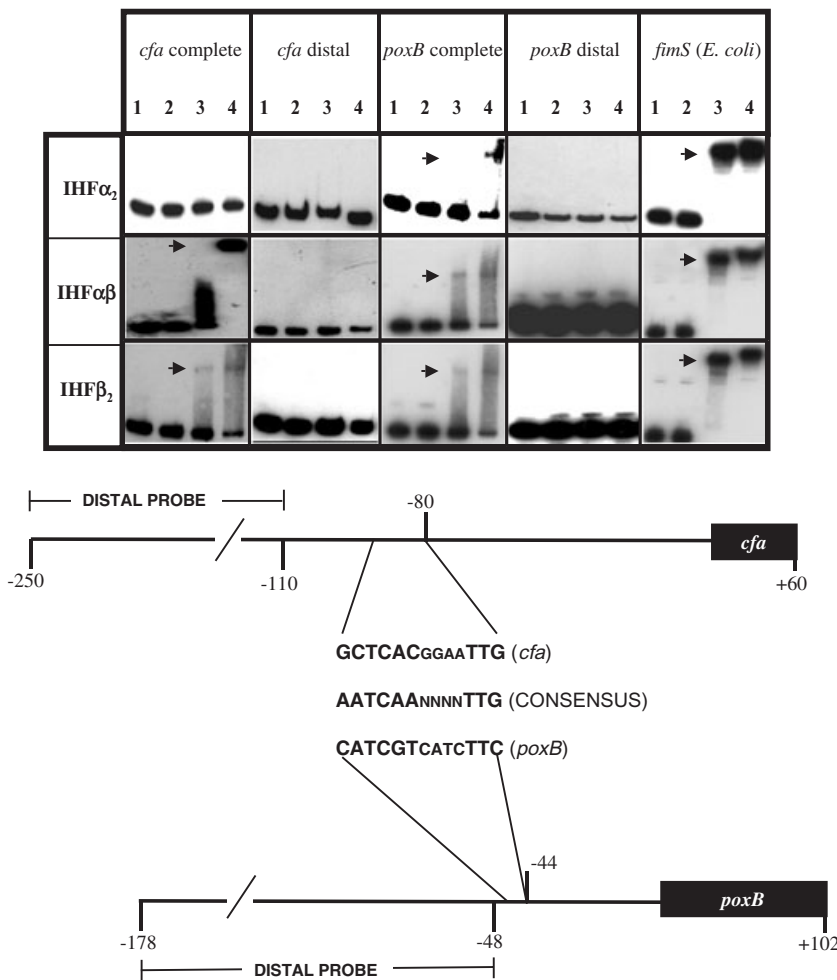


Fig. 3. Interaction of IHF α , IHF β and IHF $\alpha\beta$ with the regulatory regions of two stationary phase-inducible genes. The upper portion of the figure shows electrophoretic mobility shift assays in which labelled DNA probes corresponding to the regulatory regions of the *cfa* or *poxB* stationary-phase genes were incubated with increasing concentrations of IHF protein. The arrows indicate IHF–DNA complexes detected following electrophoresis. The *fimS* sequence from *E. coli* which contains one well-characterized IHF binding site was used as a positive control. Material loaded in each lane had been incubated with 0 (lanes 1), 100 (lanes 2), 200 (lanes 3) or 300 (lanes 4) ng of purified IHF. The form of IHF used (IHF α_2 , IHF $\alpha\beta$ or IHF β_2) is shown at the left. The lower portion of the figure summarizes the structures of the DNA probes used. The complete probes for *cfa* (+60 to –250) and *poxB* (+102 to –178) were calibrated with respect to the first base of the translation initiation codon (designated +1). The positions of the distal probes are also shown. The filled rectangles represent the 5' portions of the open reading frames of the genes. The matches to the IHF consensus sequence (Goodrich *et al.*, 1990; Steffen *et al.*, 2002) identified in the *cfa* and *poxB* regulatory regions are shown in the centre of the figure. The diagram is not to scale.

The FliC flagellum protein was undetectable by Western blotting in the *ihfA ihfB* mutant at 6 h; however, the mutant did express the alternative FliJ flagellum protein, although at lower levels than an IHF⁺ control that was phase-locked ON for FliJ expression (Fig. 4C). Similarly, a mutant that was only deficient in IHF α expressed just FliJ, and it did so to a level only slightly below that of an FliJ⁺-locked strain. In keeping with this near-wild-type level of flagellin protein expression, the *ihfA* mutant showed only a modest decrease in motility compared with the wild type (Fig. 4C). The *ihfB* single mutant expressed only FliC and did so to levels below those of the FliC⁺-locked control. It also showed a moderate decrease in motility (Fig. 4C). Thus, in addition to the anticipated phase locking phenotype, the loss of IHF also had negative effects on motility due to reductions in the transcription of key motility genes.

Previous work has shown a role for IHF as a positive regulator of flagellar gene expression in *E. coli* K-12 (Yona-Nadler *et al.*, 2003) and *Caulobacter* (Marques and Gober, 1995) but as a repressor in enteropathogenic and enterohaemorrhagic *E. coli* (Yona-Nadler *et al.*, 2003; Li

et al., 2004). Flagellar rotation requires proton motive force (pmf; Gabel and Berg, 2003; Macnab, 2003), and we found IHF to regulate genes (e.g. *atp*, *nuo*) involved in the generation of proton motive force. However, inactivating the *ihfA ihfB* genes at stationary phase resulted in upregulation of genes involved in pmf production suggesting that this would not be a limiting factor (Table S1). Therefore, the most likely explanation for the loss of motility seen in the *ihfA ihfB* mutant is downregulation of the genes involved in the final stages of flagellum production.

Virulence genes

We discovered that loss of IHF resulted in specific effects among the major virulence gene clusters of *S. Typhimurium*. SPI-1 pathogenicity island genes exhibited strong negative effects in the *ihfA ihfB* mutant at the 1 h and 4 h time points (Fig. 5A). The genes coding for components of the TTSS and the effector proteins exhibited distinct responses at the 6 h time point. Genes contributing to the assembly of the type III secretion

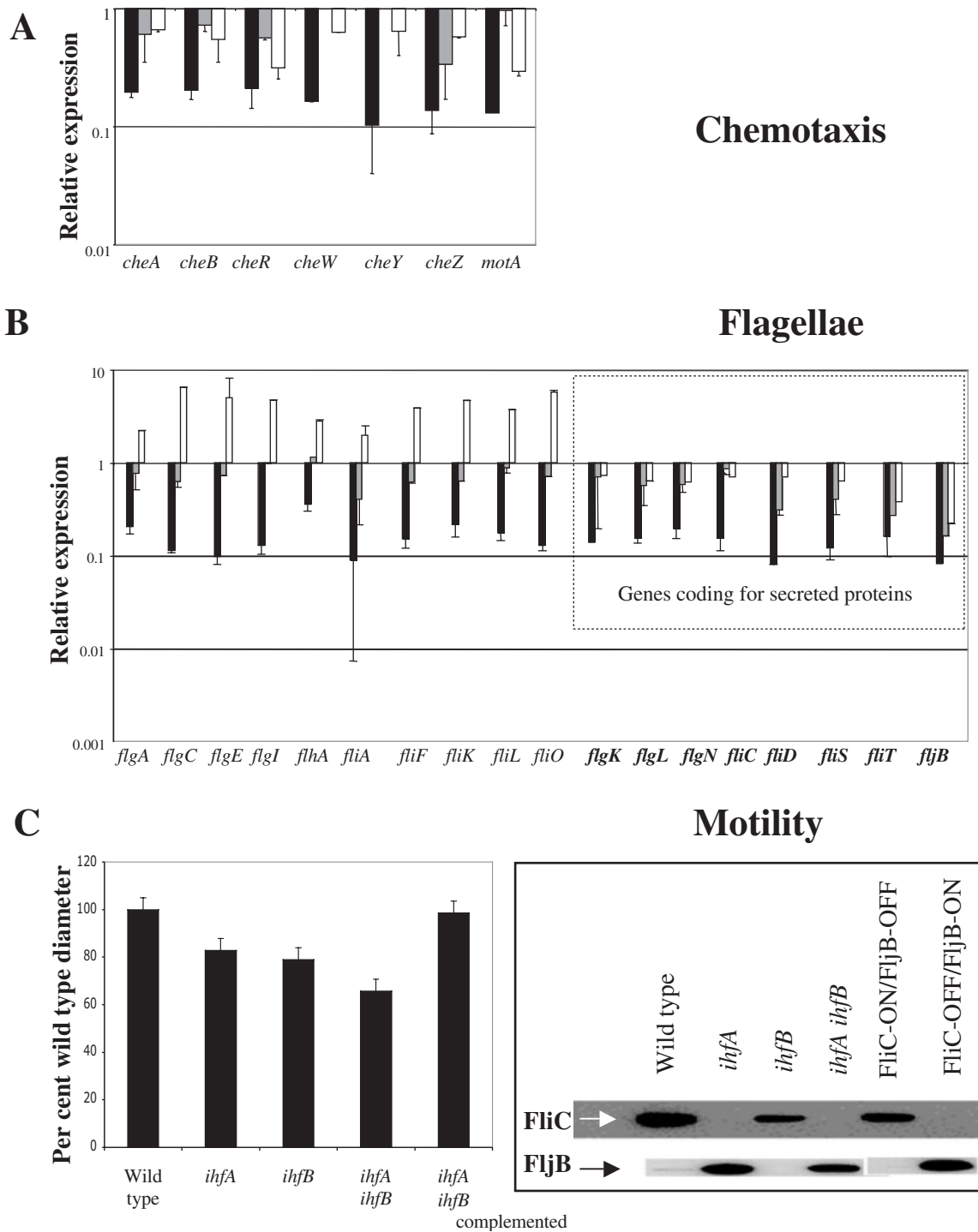


Fig. 4. Effect of *ihfA ihfB* mutations on expression of representative flagellar genes, chemotaxis genes and bacterial motility. A and B. Chemotaxis (A) and flagellar (B) gene expression data were normalized to SL1344 for the 1 (black), 4 (grey) and 6 h (white) time points and the ratio of the mutant/wild type was calculated. Expression ratios less than 1 indicate genes normally activated by IHF at that time point while ratios greater than 1 show where and when IHF normally acts negatively. The effect of IHF loss on the transcription pattern in the flagellar regulon (B) is illustrated using genes from the following classes: regulators (*fliA*); components of the type III secretion apparatus and basal body (*flgA*, *flgC*, *flgE*, *flgI*, *flhA*, *fliF*, *fliK*, *fliL* and *fliO*); hook and flagellum components and their accessory factors (shown in bold type on figure: *flgK*, *flgL*, *flgN*, *fliB*, *fliC*, *fliD*, *fliS*, *fliT* and *fliB*). C. The effects of the *ihfA*, the *ihfB* and the *ihfA ihfB* mutations on bacterial motility were measured using motility plates and reported as percentages of wild-type motility. FliC and FliB flagellar proteins were detected in wild type and *ihf* mutants by Western blotting. Phase-locked FliC-ON/FliB-OFF (TH6232) and FliC-OFF/FliB-ON (TH6233) mutants were used as controls.

apparatus, including the needle complex, were upregulated at 6 h whereas the genes coding for the effector proteins and their accessory factors were downregulated at this time point (Fig. 5 and Table S1). This was strongly reminiscent of the pattern seen in the flagellar regulon where genes coding for the type III secretion apparatus were upregulated at 6 h, while those encoding the hook and flagellum filament components which are secreted through the apparatus were downregulated (compare Figs 4 and 5). This is particularly interesting given the observation that flagellin stimulates inflammation and assists translocation of the SPI-1-secreted SopE2 effector protein into host cells (Huang *et al.*, 2004). The *sopE2* gene lies outside SPI-1 and was downregulated by more than 10-fold compared with the control at the 6 h time point (Fig. 5A). This gene is located within a bacteriophage remnant and codes for an activator of the mammalian signalling and G-binding protein Cdc42 (Ehrbar and Hardt, 2005). Our data show that while genes inside and outside SPI-1 that code for SPI-1-secreted proteins may be regulated collectively and in the same direction by IHF, the degree to which they respond to the loss of this protein is not identical. We reasoned that a strong reduction in transcription of the *sopE2* gene, together with negative effects on the expression of other effector protein genes, might be expected to compromise the ability of *S. Typhimurium* to invade mammalian epithelial cells. In keeping with this hypothesis, the *ihf* mutants were found to be strongly impaired in their ability to invade cultured epithelial cell lines (Table 2). Recovery and enumeration of bacteria from the epithelial cells showed that this was not due simply to enhanced intracellular killing of the mutants over time (data not shown).

The absence of IHF correlated with strong downregulation of all classes of SPI-2 genes at all time points, with the greatest effect seen at 6 h (Fig. 5B). Satellite genes that are under SPI-2 control but located outside the island showed distinct responses. For example, the *srf* gene cluster, located at a distance from SPI-2 but regulated by the SPI-2 SsrAB regulatory proteins (Worley *et al.*, 2000), showed a positive response at 6 h. The *sspH 2* gene is also under SsrAB control but is located in a prophage

outside SPI-2 (Miao and Miller, 2000). Its expression matched that of the SPI-2 genes at 6 h but showed a distinct pattern at the earlier time points.

Different genes within SPI-5 have been described previously as being co-regulated with SPI-1 (e.g. *sopB*) or with SPI-2 (e.g. *pipB*) (Knodler *et al.*, 2002). The response of the SPI-5 genes to the *ihfA ihfB* mutations corresponded with their regulatory relationships to SPI-1 or SPI-2. Genes within the poorly defined SPI-3 island showed a variety of responses to the *ihfA ihfB* lesions but were mainly downregulated at 6 h. SPI-4 genes showed a uniform response that mirrored the pattern seen in SPI-1 (Fig. 5C). The *spv* virulence genes on the 90 kb virulence plasmid showed a response (Fig. 5C) that was characteristic of RpoS-dependent stationary-phase genes (Fig. 2A). Principally, this involved strong downregulation at the 6 h time point, a feature shared with SPI-2 genes. This last observation is in agreement with data from IHF protein–DNA interaction studies at the *spvR* transcription activator gene promoter (Marshall *et al.*, 1999). Interestingly, *spv* also shares with the SPI-2 genes the property of being induced within macrophage (Ochman *et al.*, 1996; Cirillo *et al.*, 1998; Hensel *et al.*, 1998; Hensel, 2000; Marshall *et al.*, 2000; Waterman and Holden, 2003).

Overall, our data indicate that IHF plays a positive regulatory role in the expression of SPI-1 genes in the early to late exponential phase of growth. At the onset of stationary phase, IHF is required for expression of the genes coding for the secreted effector proteins while inhibiting expression of the genes coding for the TTSS. Alternatively, downregulation of the effector genes in the *ihfA ihfB* mutant may cause upregulation of the SPI-1 structural genes. This suggests a regulatory role for IHF in the timing of SPI-1 TTSS assembly and the expression of the virulence factors that will traverse it. In the case of SPI-2, IHF serves to inhibit transcription of the TTSS and its SPI-2-located effector genes, especially as the culture approaches stationary phase. Our data reveal a role for IHF in co-ordinating the gene expression programmes of the two virulence-associated TTSS of *S. Typhimurium* and of the TTSS that is required for expression of flagella. In this context it is interesting to note that IHF is also required for the expression of the locus of enterocyte effacement-encoded effector proteins of enteropathogenic *E. coli* (Friedberg *et al.*, 1999; Li *et al.*, 2004), stationary-phase expression of the VirB-dependent TTSS virulence regulon in *Shigella flexneri* (Porter and Dorman, 1997) and the *virB* operon of *Brucella abortus* (Sieira *et al.*, 2004).

Single and double *ihf* mutations do not have equivalent effects

To determine the relative contributions of *ihfA* and *ihfB* to

Table 2. Epithelial cell invasion ability is reduced in the *ihf* mutants.

Strain	Percentage invasion activity	
	CHO cells	CACO-2 cells
Wild type	100	100
<i>ihfA</i>	12.2 (±1.3)	17.1 (±9.7)
<i>ihfB</i>	6.9 (±2.5)	13.4 (±4.8)
<i>ihfA ihfB</i>	6.9 (±1.9)	7.1 (±2.4)

Data are the average of three biological replicates. Standard errors are shown in parentheses.

Fig. 5. Effect of *ihfA ihfB* mutations on virulence gene expression within the pathogenicity islands and on the virulence plasmid. All expression data for representative genes in SPI-1 (A), SPI-2 (B), and SPI-3, SPI-4, SPI-5 and *spv* (C) were normalized to SL1344 for the 1 (black), 4 (grey) and 6 h (white) time points and the ratio of the mutant/wild type was calculated. Expression ratios less than 1 indicate genes normally activated by IHF at that time point while ratios greater than 1 show where and when IHF normally acts negatively. The effect of IHF loss on the transcription pattern in SPI-1 (A) is illustrated using genes from the following classes: regulators (*hilA, hilC, hilD*); components of the type III secretion apparatus (*iagB, invA, invG, invI, invJ*); effectors (shown in bold type on figure: *sicA, sicP, sipD, sopB, sopD, sopE2*). The *sopE2* gene is located in a bacteriophage fragment that lies outside the SPI-1 pathogenicity island (Ehrbar and Hardt, 2005). In SPI-2, the response to the absence of IHF is shown for the following gene classes: regulators (*ssrA, ssrB*); components of the type III secretion system (*ssaB, ssaC*); effectors and their accessory factors (*sscB, sseA, sseF*); satellite genes located outside SPI-2 that are regulated by the SPI-2 SsrAB two-component regulator (*sspH 2, srfABC*). DNA microarray data were corroborated by RT-PCR analysis for the SPI-1 genes *hilC* and *sipD* (A) and the SPI-4 gene STM4259 (C). Expression data for *ssaB* and *sseA* (B) were corroborated by β -galactosidase assays using *lacZ* reporter fusions to the promoters of these genes.

the control of gene expression, we performed DNA microarray analyses in mutants deficient in individual *ihf* genes. The results obtained showed strong similarities to those recorded with the double *ihfA ihfB* mutant but they were not completely equivalent. Far fewer genes were affected in the single mutants and the effects of the mutations in individual *ihf* genes varied with time (Fig. 6; Table S1). Generally, the genes that were upregulated or downregulated in the single mutants were regulated in a similar way in the *ihfA ihfB* double mutant. Loss of just IHF α had more widespread effects at 1 h than at 6 h, and the reverse was true when just IHF β was absent. Moreover, certain categories of genes that showed strong responses to the double mutation were unaffected by the loss of just individual IHF subunits. For example, the loss of IHF β did not alter the expression of the virulence genes in the SPI-2 pathogenicity island. On the other hand, these genes did respond to the loss of just IHF α , and in a manner that was similar to the response to the absence of both subunits (Table S1). The absence of IHF β did not affect genes involved in ATP synthesis at the 1 h time point but had a negative effect at 4 h and 6 h (Fig. 6). Expression of neither peptidoglycan nor lipopolysaccharide synthesis genes was affected by the *ihfB* mutation at 6 h whereas loss of just *ihfA* or both *ihfA* and *ihfB* caused increased expression at this time point. The *ihfA* single mutation produced effects on peptidoglycan synthesis genes that were opposite to those seen in the *ihfA ihfB* double mutant at 1 h and 4 h. However, by 6 h the effects seen were similar in both mutants (Fig. 6).

The single and double mutants also showed phenotypic differences. When their abilities to survive acid stress or oxidative stress were measured, the *ihfB* mutant was the most impaired in surviving H₂O₂ stress whereas the *ihfA* mutant was the most acid-sensitive (Fig. 7). These data are entirely consistent with a key role for IHF in stationary phase-associated phenotypes that contribute to stress resistance, and the differing effects of the *ihf* mutations on stationary gene expression are clear (Figs 2 and 3). What is the explanation for this very dynamic picture? It is important to recall that IHF rarely acts alone to influence gene expression. In the case of well-characterized

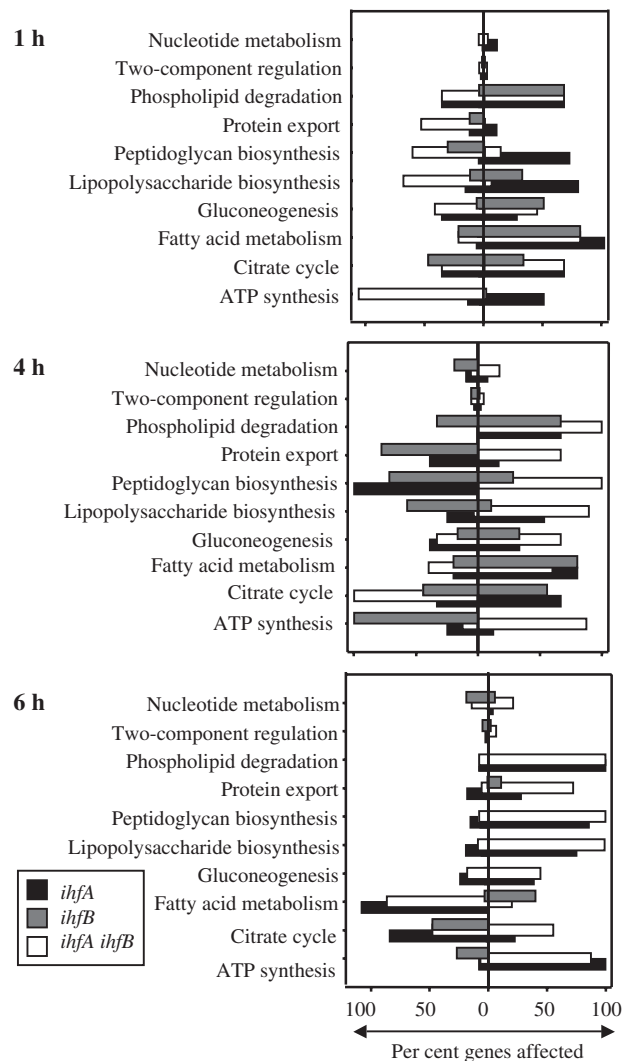


Fig. 6. Functional categories of genes affected by the *ihf* mutations. Genes showing altered expression in the three mutants are grouped according to the functional categories in the Kyoto Encyclopedia of Genes and Genomics (KEGG). The histogram represents the percentage of genes in each category affected by the mutations at the three time points. Percentage values to the left of zero indicate genes that are normally under positive control by IHF.

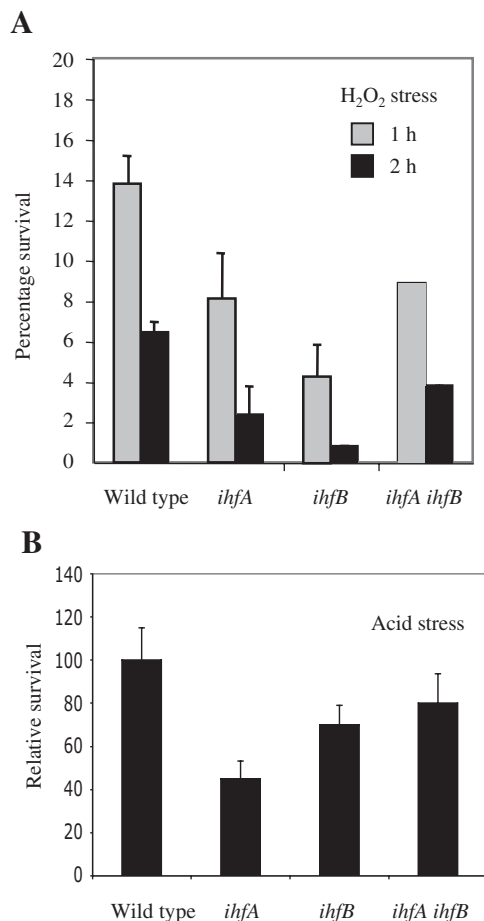


Fig. 7. Distinct phenotypes associated with single and double *ihf* mutations. The response of the wild type and *ihf* mutants (A) to hydrogen peroxide stress (for 1 h and 2 h) and (B) to acid stress is shown. See *Experimental procedures* for details.

stationary-phase genes such as *osmY* this protein collaborates with cAMP-CRP and Lrp to modulate the response of the *osmY* promoter to the onset of stationary phase (Colland *et al.*, 2000). The same regulatory proteins perform directly analogous functions in the case of the RpoS-dependent *spv* virulence genes (Marshall *et al.*, 1999). The complex regulation of these co-regulators, the influence of globally acting repressive factors such as H-NS, together with variations of DNA template topology as bacteria move from one of phase of growth to the next all make for a highly dynamic situation. This is made more complex by our finding that single and double *ihf* knockout mutations are not always equivalent, an insight that has implications for studies that rely on single mutations to investigate the role of IHF in the global control of gene regulation. Our discovery that there is not one but three overlapping IHF regulons is not entirely surprising when one takes into account the fact that each IHF subunit is capable of forming homodimers and that these are func-

tional in DNA binding (Werner *et al.*, 1994; Zuilianello *et al.*, 1994; Zablewska and Kur, 1995; Hiszczynska-Sawicka and Kur, 1997). The recent finding that IHF subunits (and other nucleoid-associated proteins) can interact with several unrelated proteins hints at additional layers of complexity (Butland *et al.*, 2005).

Perspective/conclusion

We have identified a role for IHF in managing the co-ordinate expression of major classes of genes in *S. Typhimurium*. In particular, IHF is required for the normal expression of genes involved in the expression of all three TTSS and their effector proteins, and the genes that assist the bacterium in adjusting to the stationary phase of growth. In the absence of IHF, the stationary-phase regulon fails to become fully activated and the energy-generating and energy-consuming cellular processes fail to shut down. Our findings point to a key role for IHF in the adaptation of the bacterium to the cessation of growth and suggest that bacteria that lack this protein may face considerable disadvantages compared with the wild type, especially in surviving starvation. Previous work showing that an *ihf* mutant dies off more rapidly than the wild type during extended periods in stationary phase is consistent with this hypothesis (Nyström, 1995).

Invasion of host cells can be regarded as a mechanism for avoiding stress by providing access to an environment without competing bacterial species that contains plentiful growth resources. Co-ordinating the expression of the virulence genes that allow *S. Typhimurium* to invade mammalian cells with those concerned with motility and the management of its responses to stress makes ecological sense. This study has established a critical role for IHF in this co-regulatory process.

Experimental procedures

Bacterial strains

Escherichia coli K-12 strain DH5 α was used as the host for cloning experiments. *S. Typhimurium* strain SL1344 was the host background for the construction of the mutant strains used in transcriptome profiling and is the same isolate used in previous microarray studies (Clements *et al.*, 2002; Eriksson *et al.*, 2003; Kelly *et al.*, 2004; Lloyd *et al.*, 2004). These and other strains used in this study are listed in Table 3. Bacteria were cultured in LB medium, pH 7.0 (Sambrook and Russell, 2001) at 37°C unless indicated otherwise. Where necessary, antibiotics were used at the following concentrations: carbenicillin (50 μ g ml⁻¹), erythromycin (200 μ g ml⁻¹), kanamycin (50 μ g ml⁻¹) and tetracycline (12 μ g ml⁻¹). Motility assays were performed using swarm plates containing (w/v) 1% Bacto-Tryptone, 0.5% NaCl and 0.3% Bacto-Agar (Macnab, 1986). The centre of each plate was inoculated with equal numbers of bacteria and incubated at 37°C for 8 h.

Table 3. Bacterial strains and plasmids.

Strain/plasmid	Relevant details	Reference/source
<i>Escherichia coli</i> K-12		
DH5 α	<i>endA1</i> hsdR17(<i>r_k-m_k+</i>) <i>glnV44 thi-1 recA1 gyrA(Nal^r) relA1 Δ(<i>lacZYA-argF</i>) U169 deoR [ϕ80dLac(<i>lacZ</i>)M15] <i>fimA-lacZ</i></i>	Promega
VL386		Lab stocks
<i>S. Typhimurium</i>		
CH607	LT2 <i>polA-2 zig204::Tn10</i>	Lab stocks
CJD2902	CH607 <i>ihfA::erm</i>	This study
CJD2903	CH607 <i>ihfB::tet</i>	This study
CJD2910	SL1344 <i>ihfA::erm</i>	This study
CJD2911	SL1344 <i>ihfB::tet</i>	This study
CJD2912	SL1344 <i>ihfA::erm ihfB::tet</i>	This study
CJD2913	CJD2912 (pHX3-8)	This study
CJD2926	CJD2910 (pQFsseA)	This study
CJD2927	CJD2910 (pQFssaB)	This study
CJD2928	CJD2911 (pQFsseA)	This study
CJD2929	CJD2911 (pQFssaB)	This study
CJD2930	CJD2912 (pQFsseA)	This study
CJD2931	CJD2912 (pQFssaB)	This study
SL1344	<i>hisG</i>	Hoiseht and Stocker (1981)
TH6232	LT2 Δ <i>hin7517::FRT flhBA-OFF flhC-OFF</i>	K.T. Hughes
TH6233	LT2 Δ <i>hin7517::FRT flhBA-ON flhC-OFF</i>	K.T. Hughes
Plasmids		
pACYC184	<i>ori</i> p15A, Tet ^r	Chang and Cohen (1978)
pAM434	<i>ori</i> pMB1, Erm ^r	Flannagan and Clewell (2002)
pBluescriptKSII+	<i>ori</i> pMB1, Amp ^r	Stratagene
pHX3-8	<i>ihfA</i> , <i>ihfB</i> complementation plasmid	Lee <i>et al.</i> (1991)
pIHFA	<i>ihfA</i> gene cloned in pBluescript KSII ⁺	This study
pIHFB	<i>ihfB</i> gene cloned in pBluescript KSII ⁺	This study
pIHFA::erm	pIHFA harbouring Erm ^r <i>trans</i> -posome insert in <i>ihfA</i>	This study
pIHFB::tet	pIHFB harbouring Tet ^r <i>trans</i> -posome insert in <i>ihfB</i>	This study
pMOD3	<i>ori</i> pMB1, Amp ^r , transposase substrate	Epilcentre
pQF50	promoterless <i>lacZ</i> vector Amp ^r	Farinha and Kropinski (1990)
pQssaB	<i>ssaB</i> promoter cloned in pQF50	Carroll (2003)
pQFsseA	<i>sseA</i> promoter cloned in pQF50	Carroll (2003)
pTnErm	<i>erm</i> PCR product cloned in pMOD3	This study
pTnTet	<i>tet</i> PCR product cloned in pMOD3	This study

Strain construction and DNA manipulations

Recombinant DNA techniques were carried out as previously described (Sambrook and Russell, 2001). The plasmids used in this study are described in Table 2. Strains harbouring knockout mutations in the *ihf* genes were constructed as follows. Total genomic DNA was isolated from *S. Typhimurium* strain SL1344 using the Bacterial Genomic DNA purification kit (EdgeBiosystems, Gaithersburg, MD) according to the manufacturer's instructions. Details of the oligonucleotides used in this study are presented in *Supplementary material* (Table S2). The oligonucleotide primers (MWG-Biotech, Ebersburg-bei-München, Germany) *ihfA*-mutf, *ihfA*-mutr, *ihfB*-mutf and *ihfB*-mutr (Table S2) were used to amplify regions of approximately 1500 bp centred on the *ihfA* and *ihfB* genes of *S. Typhimurium* by the PCR using polymerase PwoI as directed by the manufacturers (Roche). The PCR products were cloned in pBluescript KS II+ (Promega) digested with EcoRV, generating plasmids pIHFA and pIHFB. The integrity of the cloned chromosomal DNA fragments was confirmed by DNA sequencing (GATC Biotech, Konstanz, Germany).

PwoI was used to amplify the *tet* (tetracycline resistance) gene of pACYC184 using the primers tetfor and tetrev, and

the erythromycin cassette of pAM434 using the primers ermfor and ermrev. PCR reactions were incubated at 94°C for 2 min followed by 35 cycles of 94°C for 1 min, 50°C for 1 min and 72°C for 2 min, followed by a 7 min incubation at 72°C. The resistance gene cassettes were cloned into the Klenow polymerase blunted EcoRI site of pMOD3 (Epilcentre) generating pTnTet and pTnErm respectively.

The cloned copies of the *ihfA* and *ihfB* genes were inactivated by *in vitro* mutagenesis using a transposome kit (Epilcentre, Madison, WI). Transposomes conferring erythromycin and tetracycline resistance were prepared by PCR amplification of pTnErm and pTnTet with the PCR primers FP-1 and RP-1 generating TnErm and TnTet respectively. The amplicon mixtures were digested with restriction endonuclease DpnI to degrade template DNA. TnErm was incubated with both pIHFA and EZ::TN transposase, as recommended by the manufacturers (Epilcentre), and TnTet was incubated with both pIHFB and EZ::TN transposase, thereby introducing an erythromycin cassette into pIHFA, and a tetracycline cassette into pIHFB. Insertion mutants were selected with appropriate antibiotic selection following transformation of *E. coli* DH5 α , and recombinant plasmids were screened by restriction fragment digestion. Plasmids in which transposome insertion had occurred close to the transcription

start site of the cloned *ihf* gene were detected and two (pIHFA::erm and pIHFB::tet, Table 3) were retained for further work.

Plasmid pIHFA::erm was used to transform *S. Typhimurium* strain CH607 to both carbenicillin and erythromycin resistance, and pIHFB::tet was used to transform CH607 to carbenicillin and tetracycline resistance. CH607 lacks a functional copy of the *polA* gene, preventing replication of ColE1 plasmids (Helinski *et al.*, 1996). Antibiotic-resistant colonies arise as a result of homologous recombination between the plasmids harbouring the IHF disruptions and the host genome at sites flanking the relevant IHF locus. P22 transduction was used to transfer carbenicillin and erythromycin/tetracycline resistance to *S. Typhimurium* strain SL1344. The resulting transductants were then screened for spontaneous loss of carbenicillin resistance. The carbenicillin-sensitive bacteria had lost the cloning vector, leaving the inactivated *ihf* gene on the chromosome in place of the wild-type copy.

Mutations at the *ihf* loci were verified by PCR analysis. The insertion in *ihfA* was within codon 11 of the open reading frame while that in *ihfB* was in codon 4. The oligonucleotide primers exihfAfor, exihfArev, exihfBfor and exihfBrev (MWG-Biotech, Ebersburg-bei-München, Germany) correspondingly overlapped the 5' and 3' termini of the *ihfA* and *ihfB* open reading frames respectively. The DNA fragment size polymorphisms detected when these primers were used in PCR reactions performed on template DNA purified from wild type and each of the isogenic single *ihf* mutants confirmed the correct insertion of the relevant antibiotic cassettes (data not shown). Immediately before the microarray experiments were carried out, the inactivated *ihf* gene copies were transduced to a fresh SL1344 genetic background by P22 transduction (Sternberg and Maurer, 1991).

RNA isolation, microarray procedures and data analysis

To prepare cells for RNA extraction, 25 ml of fresh antibiotic-free LB was inoculated 1:100 from an overnight culture and grown under antibiotic selection in a 250 ml flask incubated with shaking at 250 r.p.m. in a New Brunswick Innova 3100 waterbath at 37°C. Two biological replicates were pre-formed for each strain and samples were removed for RNA extraction at 1 h, 4 h and 6 h after inoculation. Twenty per cent v/v ice-cold RNA stabilization solution (5% v/v phenol: 95% ethanol) was added with mixing and the cultures were immediately incubated on ice for 30 min (Kelly *et al.*, 2004; Hinton *et al.*, 2004). The cultures were pelleted by centrifugation (3100 g, 30 min, 4°C) and pellets were stored at -80°C until required. RNA was extracted using a Promega SV total RNA purification kit as described previously (Kelly *et al.*, 2004). RNA concentration was determined by absorbance at 260 nm, and RNA quality was assessed using the RNA Laboratory-on-a-Chip (Agilent Technologies, Palo Alto, CA) as directed by the manufacturers. Total RNA was diluted to a concentration of 0.4 µg µl⁻¹, and 4 µg of total RNA was used to make labelled cDNA as described in Eriksson *et al.* (2003). The method reports on the abundance of RNA at particular time points during the growth of a batch culture. While it cannot discriminate between effects on transcription initiation and mRNA turnover, as IHF has not been shown previously to influence the half-lives of bacterial mRNA, our data most likely reflect

the influence of IHF on transcription. Hybridization, microarray scanning and data analysis were carried out as described previously (Kelly *et al.*, 2004). The microarray data are provided in Table S1, and are in MIAME-compliant format.

Corroboration of microarray data

RT-PCR analyses were performed on transcripts using gene-specific primer pairs (Table S2). Primers were designed *in silico* (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) to minimize primer-primer complementarity and to yield predicted amplicons in the 150–250 bp range, except for the *dps* gene, where the oligonucleotide primers amplified the entire reading frame (approximately 500 bp). Total RNA was isolated using the SV40 total RNA isolation Kit (Promega), and reverse transcribed in 50 µl of Stratascript first-strand buffer in the presence of 0.4 mM dNTPs, 300 ng of random hexamers and 40 U Stratascript (Stratagene) according to the manufacturer's protocol. Reactions were subjected to one cycle of 94°C for 3 min, followed by 20–40 cycles of 94°C for 1 min, 50°C for 1 min and 72°C for 1 min, followed by a final extension of 72°C for 10 min. Sampling of PCR reactions throughout the RT-PCR cycle was performed according to Grifantini *et al.* (2003), and products were resolved by electrophoresis on a 1.5% TAE agarose gel allowing PCR regimes for the transcripts of interest to be optimized at given time points. PCR product intensities on the resulting gels were measured using a Bio-Rad Densitometer (Bio-Rad, Hercules, CA) with the Quantity-One densitometry package (Bio-Rad). The expression of a gene at any time point in a given mutant background was recorded as a multiple of the value for the cognate wild-type sample obtained using the same cycling parameters. The *rplT* gene did not respond to the absence of the IHF proteins and was used as a negative control in the RT-PCR experiments (data not shown).

Phenotypic analyses

To investigate responses to inorganic acid stress, overnight cultures of *S. Typhimurium* SL1344, SL1344*ihfA::erm*, SL1344*ihfB::tet* and SL1344 *ihfA::erm ihfB::tet* were grown in LB, pH 7.0, and used to inoculate 1:10 000 fresh LB, pH 2.5 with HCl and LB, pH 7.0, as described by Lin *et al.* (1995). Bacteria were cultured with agitation for 1 h at 37°C, serial 10-fold dilutions of each culture prepared, and spread on LB agar plates. Colony-forming units were enumerated and the relative survival of each strain was determined relative to the pH 7.0 control for that strain.

The response of each strain to 1 mM hydrogen peroxide was measured as described previously (Halsey *et al.*, 2004). Bacterial strains were split into four aliquots, pelleted by centrifugation, and the bacterial pellets were resuspended in PBS and in PBS containing 1 mM H₂O₂. Bacterial suspensions were incubated at 37°C for both 1 h and 2 h at 37°C, and the percentage survival relative to that for a PBS control was determined as above. Each stress test was carried out at least in duplicate on biological duplicates, and typical results are shown.

The FlhC flagellum subunit was detected in equivalent amounts of total protein (Bradford, 1976) from wild-type and

ihf mutant strains (grown in LB for 6 h) by Western blotting using a commercially supplied antibody (Difco catalogue number 228241) as described previously (Kelly *et al.*, 2004). The FljB subunit was detected in an identical manner using commercially supplied antibody (Difco catalogue number 224741). Antiserum to the RpoS sigma factor was a gift from R. Hengge.

The activities of *ssaB-lacZ* and *sseA-lacZ* fusions in plasmid pQF50 were assayed in *S. Typhimurium* SL1344 and SL1344*ihfA::erm*, SL1344*ihfB::tet* and SL1344 *ihfA::erm ihfB::tet* backgrounds for β -galactosidase activity according to the method of Miller (1992). β -Galactosidase assays were performed in triplicate on three independent cultures and the data expressed as the means of the nine measurements.

Cell invasion assays

Invasion assays were performed with CHO-K1 and CACO II epithelial cell lines (American Type Culture Collection) maintained in Dulbecco's modified Eagle's medium with F12 Nutrient mix supplemented with 10% fetal bovine serum. Infections with *S. Typhimurium* bacteria were carried out essentially as described previously (Marshall *et al.*, 2000). Overnight bacterial broth cultures were diluted 1:100 and incubated at 37°C in a rotating waterbath for 6 h. The bacteria were harvested, resuspended in pre-warmed culture medium and added to either CHO or CACO II cells grown in 12-well plates at a multiplicity of infection of 100:1. Plates were spun at 600 g for 5 min and then incubated at 37°C in 5% CO₂ for 1 h to allow invasion to occur. The epithelial cells were then washed and incubated with fresh medium containing 100 μ g ml⁻¹ gentamicin for 1 h to kill extracellular bacteria. The monolayers were washed and lysed by the addition of PBS containing 0.5% (v/v) Triton X-100 to release the intracellular bacteria. The number of bacterial colony-forming units recovered was determined following serial dilution and plating on LB. The presence of the *ihf* mutations had no effect on the sensitivity of the bacteria to Triton X-100. Experiments were repeated three times and typical data reported.

Electrophoretic mobility shift assays

Probes for electrophoretic mobility shift assays (EMSA) were prepared by amplification of DNA segments upstream of encompassing the 5' end of the open reading frames of the *cfa* and *poxB* genes. Using the 'A' of each translation start codon as a reference point (position +1), oligonucleotide primers Shftcfaf1 and Shftcfar amplified the +60 to -250 region of *cfa*, primers Shftcfaf2 and Shftcfar amplified -110 to -250 of the same gene, while primers shftpoxBf1 and shftpoxBr amplified the +102 to -178 region of *poxB* and shftpoxBf2 and shftpoxBr amplified the -48 to -178 region of the same gene (Table S2). The *fimS* DNA element of *E. coli* contains a well-characterized IHF binding site (Blomfield *et al.*, 1997) and this was used as a positive control in the EMSA. It was amplified from the chromosome of *E. coli* K-12 strain VL386 using the primer pair *fimS*for and *fimS*rev (Table S2). PCR reactions were performed using Pfu polymerase according to the supplier's instructions (Promega). The primers were biotinylated at the 5' end and PCR reac-

tions contained 10 mM dNTPs and 100 pM biotinylated primers. Reactions consisted of one cycle at 95°C for 3 min, 30 cycles at 95°C for 1 min each, one at 45°C for 1 min and one at 72°C for 30 s, followed by one cycle at 72°C for 7 min. EMSA reactions contained 0–400 nM protein, 20–40 fM DNA and were performed in a buffer supplied for the purpose in the Chemiluminescent Nucleic Acid Detection Module (Pierce). Binding reactions were optimized by the addition of 50 ng of poly dI.dC, 0.05% Nonident P40, 5 mM DTT, 10 mM KCl. Protein–DNA binding reactions were carried out for 10 min at room temperature and then electrophoresed on a 6% DNA retardation gel (Invitrogen). DNA–protein complexes were transferred to nylon membranes (Pall) using the DNA Blot Module (Invitrogen) and detected with the Chemiluminescent Nucleic Acid Detection Module following the manufacturers' directions.

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Supplementary material

The following supplementary material is available for this article online:

Table S1. Gene expression ratios of the *ihfA*, *ihfB* single mutants and the *ihfA ihfB* double mutant of *S. Typhimurium* SL1344 are compared with the wild type at three stages of growth (designated 1 h, 4 h and 6 h after inoculation). Data shown in **bold** are considered to show statistically significant changes of more than twofold (FDR \leq 0.05).

Table S2. Oligonucleotides used in this study.

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