Nucleoid-associated protein HU controls three regulons that coordinate virulence, response to stress and general physiology in *Salmonella enterica* serovar Typhimurium

Michael W. Mangan,¹ Sacha Lucchini,² Tadhg Ó Cróinín,^{1†}
Stephen Fitzgerald,¹ Jay C. D. Hinton^{1,2} and Charles J. Dorman¹

Correspondence
Charles J. Dorman
cjdorman@tcd.ie

¹Department of Microbiology, School of Genetics and Microbiology, Moyné Institute of Preventive Medicine, Trinity College, Dublin 2, Ireland

²Department of Molecular Microbiology, Institute for Food Research, Colney, Norwich NR4 7UA, UK

The role of the HU nucleoid-associated proteins in gene regulation was examined in *Salmonella enterica* serovar Typhimurium. The dimeric HU protein consists of different combinations of its α and β subunits. Transcriptomic analysis was performed with cultures growing at 37 °C at 1, 4 and 6 h after inoculation with mutants that lack combinations of HU α and HU β . Distinct but overlapping patterns of gene expression were detected at each time point for each of the three mutants, revealing not one but three regulons of genes controlled by the HU proteins. Mutations in the *hup* genes altered the expression of regulatory and structural genes in both the SPI1 and SPI2 pathogenicity islands. The *hupA hupB* double mutant was defective in invasion of epithelial cell lines and in its ability to survive in macrophages. The double mutant also had defective swarming activity and a competitive fitness disadvantage compared with the wild-type. In contrast, inactivation of just the *hupB* gene resulted in increased fitness and correlated with the upregulation of members of the RpoS regulon in exponential-phase cultures. Our data show that HU coordinates the expression of genes involved in central metabolism and virulence and contributes to the success of *S. enterica* as a pathogen.

Received 22 October 2010
Revised 17 December 2010
Accepted 3 January 2011

INTRODUCTION

The HU DNA-binding protein is one of approximately 12 nucleoid-associated proteins (NAPs) that have been described in Gram-negative bacteria such as *Escherichia coli* and *Salmonella enterica*. NAPs have the potential to influence the expression of large numbers of genes in bacteria and help to organize the nucleoid (Azam & Ishihama, 1999; Dillon & Dorman, 2010; Dorman & Deighan, 2003). In *Salmonella*, NAPs have been shown to influence virulence gene expression (Harrison *et al.*, 1994; O'Byrne & Dorman, 1994a, b; Lucchini *et al.*, 2006; Marshall

et al., 1999; Navarre *et al.*, 2006; Schechter *et al.*, 2003). HU binds to DNA relatively non-specifically and influences many DNA-based transactions, including replication, transcription, site-specific-, general and illegitimate recombination, transposition and DNA repair (Kamashev *et al.*, 2008; Li & Waters, 1998; Merickel & Johnson, 2004; Ryan *et al.*, 2002; Semsey *et al.*, 2004; Shanado *et al.*, 1998; Signon & Kleckner, 1995). It also has RNA-binding activity (Balandina *et al.*, 2001) and a preference for binding to unusual conformations in DNA such as four-way junctions and extruded cruciform structures (Kamashev *et al.*, 1999; Pontiggia *et al.*, 1993; Swinger & Rice, 2004).

HU is a dimer composed of two closely related subunits, HU α and HU β (Guo & Adhya, 2007) and can exist in three forms, the HU $\alpha\beta$ heterodimer and the HU α_2 and HU β_2 homodimers. The relative abundances of the three forms vary with the phase of growth (Claret & Rouvière-Yaniv, 1997). The $\alpha\beta$ heterodimer is the dominant form of the protein at most stages of growth, except at the earliest stage of the exponential phase when the α_2 form predominates; the β_2 homodimer is detectable principally in late stationary phase. The presence of three forms of HU raises the question of whether each form has unique biological properties. A key

[†]Present address: School of Biomolecular and Biomedical Science, Room C140, Health Science Centre, University College Dublin, Belfield, Dublin 4, Ireland.

Abbreviations: IHF, integration host factor; NAP, nucleoid-associated protein; SPI, salmonella pathogenicity island; TTSS, type three secretion system.

The microarray data reported in this paper have been submitted to the gene expression omnibus (GEO) database with the accession number GSE22860.

Six supplementary figures and seven supplementary tables are available with the online version of this paper.

difference concerns the abilities of the proteins to introduce negative supercoils into DNA in the presence of topoisomerase I: the $\alpha\beta$ and α_2 forms can do this, the β_2 form cannot (Claret & Rouvière-Yaniv, 1997). Double mutants of *E. coli* that have knockout mutations in both of the genes coding for the α (*hupA*) and β (*hupB*) subunits have growth defects but remain viable (Huisman *et al.*, 1989).

The role of HU in global gene regulation has not been determined previously by transcriptomic analysis in a bacterial pathogen. In the commensal organism *E. coli* K-12, HU influences, *inter alia*, the expression of genes involved in anaerobic respiration, the response to osmotic stress, the acid stress response and the response to DNA damage (Kar *et al.*, 2005; Oberto *et al.*, 2009). *S. enterica* serovar Typhimurium (*S. Typhimurium*) is an attractive subject for studies of global gene regulatory mechanisms in a pathogen because it has been characterized in great detail at the genetic and physiological levels. *S. Typhimurium* infects mice, where it causes a typhoid-like disease that is a valuable model for human typhoid. It can also infect other animals, such as pigs, cattle and chickens. The bacterium infects the host orally and must traverse the alimentary tract en route to the site of host cell invasion in the small intestine. This journey exposes the bacterium to a number of severe environmental stresses (e.g. the strongly acidic environment of the stomach) and *S. Typhimurium* must adjust its gene expression profile in response to each stress if it is to survive. More than 200 genes contribute to the virulence of this pathogen, with some of the best characterized being located in the chromosome within the major pathogenicity islands (Finlay & Brumell, 2000; Groisman & Mouslim, 2000; Groisman & Ochman, 1997). The *Salmonella* pathogenicity island (SPI)1 harbours the genes that encode the Inv/Spa type III secretion system (TTSS) and its effector proteins (Galán, 2001; Hardt *et al.*, 1998; Hensel, 2000; Mills *et al.*, 1995; Wood *et al.*, 1996). This TTSS is required for epithelial cell invasion while an alternative TTSS encoded by SPI2 is required for survival of *S. Typhimurium* in macrophages (Cirillo *et al.*, 1998; Hensel, 2000; Holden, 2002; Waterman & Holden, 2003). A third TTSS is responsible for the expression of a flagellum-dependent motile phenotype (Macnab, 2003). Previous studies have shown that NAPs act as global regulators that contribute to the coordinated expression of all three TTSSs (Dillon *et al.*, 2010; Kelly *et al.*, 2004; Mangan *et al.*, 2006). The expression of many housekeeping genes is also coordinated by the gene regulatory mechanisms that operate as *S. Typhimurium* navigates the host (Eriksson *et al.*, 2003; Hautefort *et al.*, 2008; Hensel *et al.*, 1995; Mahan *et al.*, 1993; Rhen & Dorman, 2005; Valdivia & Falkow, 1997). Understanding how these regulatory circuits are composed and operate represents a significant challenge. We have previously determined the membership of the regulons of genes whose expression is influenced by the NAPs Fis, H-NS, IHF, Sfh and StpA in *S. Typhimurium* (Dillon *et al.*, 2010; Dorman, 2007; Kelly *et al.*, 2004; Lucchini *et al.*, 2006, 2009; Mangan *et al.*,

2006). In this study we used DNA-microarray-based transcriptomics analysis to identify those genes in *S. Typhimurium* whose expression changed in the presence of mutations in *hupA* or *hupB* or both *hupA* and *hupB*.

METHODS

Bacterial strains and culture conditions. *E. coli* K-12 strain DH5 α was used as the host for cloning experiments, and strain C600 was used to assess the effect of *hup* gene knockouts on RpoS sigma factor expression (Oberto *et al.*, 2009). *S. Typhimurium* strain SL1344, used in previous transcriptomic studies (Eriksson *et al.*, 2003; Kelly *et al.*, 2004; Mangan *et al.*, 2006), was the host background for the construction of the mutant strains for transcriptome profiling. These and other strains used in this study are listed in Table 1. Bacteria were cultured in LB medium, pH 7.0 (Sambrook & Russell, 2001) at 37 °C unless indicated otherwise. Where required, antibiotics were used at the following concentrations: carbenicillin (100 $\mu\text{g ml}^{-1}$), chloramphenicol (20 $\mu\text{g ml}^{-1}$), kanamycin (50 $\mu\text{g ml}^{-1}$) and nalidixic acid (50 $\mu\text{g ml}^{-1}$). Bacterial 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. The diameter of the disc-shaped growth zone was then measured. Microbial growth in LB medium was modelled using Baranyi–Roberts approximation parameters as described previously (Perni *et al.*, 2005).

Strain construction and DNA manipulations. Routine recombinant DNA manipulations were carried out as described by Sambrook & Russell (2001). The plasmids used in this study are described in Table 1. Strains harbouring knockout mutations in the various genes were constructed as follows. Total genomic DNA was isolated from *S. Typhimurium* strain SL1344 using the bacterial genomic DNA purification kit (EdgeBiosystems) according to the manufacturer's instructions. Details of the oligonucleotides used in this study are presented in Supplementary Table S1 (available with the online version of this paper).

The *hupA* and *hupB* genes were knocked out by allelic exchange using the method of Datsenko & Wanner (2000). Linear DNA for the exchange was generated by PCR using 50 ng plasmid DNA as template, in the presence of 2.5 μM dNTP, 2.5 mM MgCl₂, and 50 μM of the appropriate oligonucleotide primer. The PCR was carried out using the Phusion polymerase and conditions recommended by the manufacturer (Finnzymes). The *hupA* gene was replaced on the chromosome with a kanamycin resistance cassette amplified using *hupA*.k.o.kan oligonucleotide primers and plasmid pSU311 as template (Uzzau *et al.*, 2001). The *hupB* gene was replaced with a chloramphenicol resistance cassette generated using the combination of *hupB*.k.o.cat primers and plasmid pKD3 as the template (Datsenko & Wanner, 2000). *S. Typhimurium* CH607 was transformed with plasmid pKD46 and carbenicillin-resistant transformants were grown in LB broth supplemented with carbenicillin overnight at 30 °C with agitation. Fresh LB broth containing carbenicillin was inoculated 1 : 50 from this culture and incubated at 30 °C with agitation for 90 min, before the addition of 0.02 % (w/v) arabinose to induce expression of the λ Red recombination genes. Cells were grown to OD₆₀₀ 0.4, and then rapidly chilled on ice water for 10 min followed by two washes with 10 ml sterile ice-cold water. The cell pellet was then resuspended in 1 ml sterile ice-cold water. Aliquots (100 μl) of competent *S. Typhimurium* CH607(pKD46) were transformed by electroporation with one of the purified PCR products for *hup* gene replacement; the cells were plated to select the desired allelic replacement at 37 °C. Genomic DNA was purified from candidate mutants using the bacterial genomic DNA purification kit (EdgeBiosystems), and

Table 1. Bacterial strains and plasmids used in this study

Strain/plasmid	Relevant details	Reference/source
<i>E. coli</i> K-12		
DH5 α	<i>endA1 hsdR17(r_K⁻m_K⁺) glnV44 thi-1 recA1 gyrA(Nal^r) relA1 Δ(lacIZYA-argF) U169 deoR (ϕ80ΔlacZM15)</i>	Promega
C600 (JO2057)	<i>thr-1 leu86 thi-1 lacY glnV44</i>	Oberto <i>et al.</i> (2009)
JO3020	C600 <i>hupA::cat hupB::kan</i>	Oberto <i>et al.</i> (2009)
<i>S. Typhimurium</i>		
CH607	LT2 <i>polA-2 zig204::Tn10</i>	Lab stocks
CJD2000	CH607 <i>hupA::cat</i>	This study
CJD2001	CH607 <i>hupB::kan</i>	This study
CJD2003	SL1344 <i>hupA::cat</i>	This study
CJD2004	SL1344 <i>hupB::kan</i>	This study
CJD2005	SL1344 <i>hupA::cat hupB::kan</i>	This study
SL1344	<i>hisG</i>	Hoiseth & Stocker (1981)
SL1344 Nal ^r	<i>hisG nal</i>	Doyle <i>et al.</i> (2007)
TH6232	LT2 Δ <i>hin7517::FRT fliBA-OFF fliC-ON</i>	K.T. Hughes, University of Fribourg, Switzerland
TH6233	LT2 Δ <i>hin7517::FRT fliBA-ON fliC-OFF</i>	K.T. Hughes
Plasmids		
pKD3	Template for <i>kan</i> cassette	Datsenko & Wanner (2000)
pKD46	Inducible λ <i>redBCD</i> under P _{BAD} control	Datsenko & Wanner (2000)
pSU311	Template for <i>cat</i> cassette	Uzzau <i>et al.</i> (2001)
pUC19	<i>ori</i> pMB1, Ap ^r	Yanisch-Perron <i>et al.</i> (1985)

digested with *EcoRI*. The *hup* gene allelic replacements were confirmed by using Southern blot analysis (Sambrook & Russell, 2001). Bacteriophage P22 transducing lysates of strains CH607*hupA* and CH607*hupB* were prepared (Sternberg & Maurer, 1991), and used to transfer the mutated *hup* genes individually to *S. Typhimurium* strain SL1344. Double *hupA hupB* mutants were prepared by transducing either of the single *hup* knockouts with lysate prepared from strains lacking the alternative subunit. Serial passage of transducants on green agar plates was used to ascertain that SL1344 strains were free of P22 (Sternberg & Maurer, 1991).

RNA isolation, microarray procedures and data analysis. To prepare cells for RNA extraction, an overnight culture grown under antibiotic selection was used to inoculate 25 ml of fresh antibiotic-free LB in a 250 ml flask at 1:100, which was then incubated with shaking at 250 r.p.m. in a New Brunswick Innova 3100 waterbath at 37 °C. Two biological replicates were performed for each strain and samples were removed for RNA extraction at 1, 4 and 6 h after inoculation (Fig. 1). 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 concentrations were determined from A₂₆₀, and RNA quality was assessed by using the Bioanalyser 2100 (Agilent Technologies) as directed by the manufacturer. Total RNA was diluted to 0.4 µg µl⁻¹, and 4 µg total RNA was used to make labelled cDNA as described by Eriksson *et al.* (2003). Hybridization, microarray scanning and data analysis were carried out as described previously (Kelly *et al.*, 2004). The microarray data are provided in Supplementary Tables S2–S7, available with the online version of this paper, and are in MIAME-compliant format. The data have been submitted to the GEO database (accession number GSE22860).

Corroboration of microarray data. RT-PCR analyses were performed on transcripts using gene-specific primer pairs (Supplementary Table S1). Primers were designed *in silico* (<http://fokker.wi.mit.edu/primer3/>) to minimize primer–primer complementarity and to yield predicted amplicons in the 150–250 bp range. Total RNA was isolated using the SV40 total RNA isolation kit (Promega), and reverse transcribed in 50 µl Stratascript first-strand buffer in the presence of 0.4 mM dNTPs, 300 ng random hexamers and 40 U Stratascript

Fig. 1. Growth curves of parental strain SL1344 (●) and its three *hup* derivatives: *hupA* (□), *hupB* (△) and the double mutant (▼) growing in LB with aeration at 37 °C. The vertical arrows indicate the three time points (1, 4 and 6 h) at which RNA was extracted for the transcriptomic analyses.

(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, 55 °C for 1 min and 72 °C for 1 min, followed by a final extension at 72 °C for 10 min.

Sampling of PCRs throughout the RT-PCR cycle was performed according to Grifantini *et al.* (2003), and products were resolved by electrophoresis on a 2 % 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 by using the image capture facility, and densitometry was performed using ImageJ (NIH) software. 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 STM3400 *yrdB* gene did not respond to the absence of the HU proteins and was used as a negative control in the RT-PCR experiments (data not shown).

Measurement of competitive fitness. The competitive fitness of each of the strains was established by competitive growth assays using *S. Typhimurium* SL1344, SL1344*hupA::kan*, SL1344*hupB::cat*, SL1344 *hupA::kan hupB::cat* as test strains and the comparator strain SL1344 *Nal^r*, a spontaneously nalidixic-acid-resistant mutant, the mutation for which does not significantly affect the competitive fitness of the strain (Doyle *et al.*, 2007). Briefly, each strain was grown overnight in LB, pH 7.0, and overnight cultures were used to establish initial (founder) populations of 10^5 c.f.u. ml⁻¹ in fresh LB broth, pre-warmed to 37 °C. Cultures were gently mixed, and then samples taken and serially diluted on fresh LB agar with selection appropriate to each of the strains under study. The culture was grown at 37 °C with agitation for 18 h, and the compositions of the final populations were determined by serial dilution of the 18 h samples on fresh LB agar supplemented with appropriate selective agents. Competitive fitness was established as described previously (Doyle *et al.*, 2007). Measurements were conducted in triplicate on at least three independent occasions, and each datum point presented is the average of at least nine measurements.

Western blotting. The RpoS sigma factor was detected in equivalent amounts of total protein (Bradford, 1976) from wild-type and *hup* mutant strains (grown in LB for 6 h) by Western blotting using an antibody from NeoClone as described previously (Mangan *et al.*, 2006).

Macrophage infection and epithelial cell invasion. Macrophage infection assays were performed on the J774.1 macrophage cell line (American type Culture Collection) maintained in Dulbecco's modified Eagle's medium with 10 % fetal bovine serum. Epithelial cell invasion assays were performed with CHO 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. Macrophage survival assays were performed with J774.1 macrophage-like cells. Both assays were carried out essentially as described previously (Marshall *et al.*, 2000). Overnight bacterial broth cultures were diluted 1:100 and incubated with shaking at 37 °C overnight. The bacteria were harvested, resuspended in pre-warmed culture medium and added to CHO or J774.1 cells grown in 12-well plates at a m.o.i. of 100:1. Plates were spun at 600 g for 5 min and then incubated at 37 °C in 5 % CO₂ for 2 h to allow invasion (epithelial cells) or phagocytosis (macrophages) to occur. The mammalian cells were then washed and incubated with fresh medium containing 100 µg gentamicin ml⁻¹ 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 c.f.u. recovered was determined following serial dilution and plating on LB. The presence of the *hup* mutations had no effect on the sensitivity of the bacteria to Triton X-100 (data not shown). Experiments were repeated three times and typical data were reported.

Separation of plasmid topoisomers by gel electrophoresis. To investigate changes in DNA supercoiling, *S. Typhimurium* SL1344, CJD2003 (SL1344*hupA::kan*), CJD2004 (SL1344*hupB::cat*) and CJD2005 (SL1344 *hupA::kan hupB::cat*) were transformed with multicopy plasmid pUC19 by electroporation, and selection on carbenicillin. Strains were grown with carbenicillin selection in LB overnight, and subsequently inoculated into fresh LB 1:100, maintaining selection. Samples were harvested after growth for 18 h. Plasmid DNA was recovered by using a plasmid purification kit as directed by the manufacturer (Qiagen) and separated by electrophoresis on a 0.8 % agarose gel containing 2.5 µg chloroquine ml⁻¹. Under these conditions topoisomers that are more negatively supercoiled migrate faster in the gel than more relaxed topoisomers (Higgins *et al.*, 1988). The chloroquine gel was washed for a minimum of 5 h in water to remove chloroquine before staining with ethidium bromide to visualize the topoisomer bands.

RESULTS AND DISCUSSION

Identification of three HU regulons

Mutants of the *S. Typhimurium* strain SL1344 were constructed with knockout mutations in the genes coding for the α subunit of HU (*hupA*, CJD2003; $\mu_{\max}$ 0.047 min⁻¹), the β subunit (*hupB*, CJD2004; $\mu_{\max}$ 0.04 min⁻¹), or both subunits (CJD2005; $\mu_{\max}$ 0.1 min⁻¹) (Table 1). The single mutants had growth profiles that were similar to that of the wild-type ($\mu_{\max}$ 0.05 min⁻¹) when grown in LB broth under aerobic conditions at 37 °C; the double mutant initially showed a reduced growth rate but on entering the exponential phase of growth it grew rapidly, eventually attaining a culture OD similar to that of the wild-type by the end of exponential growth (Fig. 1). DNA microarrays were used to analyse the *S. Typhimurium* transcriptome in the presence and absence of the *hup* genes at three time points (1, 4 and 6 h) in cultures growing in LB liquid medium at 37 °C.

The numbers of genes (ranging from 50 to 482) that were upregulated or downregulated differed for each of the HU mutants at each of the time points, yielding a total of 18 data points (Table 2). The mutations in just *hupA* or in both *hupA* and *hupB* affected approximately similar (but not identical) numbers of genes. Far fewer genes were upregulated or downregulated in the *hupB* single mutant at

Table 2. Numbers of genes in the HU regulons

The table summarizes the numbers of genes whose expression increased (up) or decreased (down) compared with the SL1344 wild-type in the *hupA* and *hupB* single mutants and in the *hupA hupB* double mutant at the 1, 4 and 6 h time points.

Strain	1 h		4 h		6 h	
	Up	Down	Up	Down	Up	Down
<i>hupA</i>	277	180	323	319	283	299
<i>hupB</i>	50	75	482	275	54	50
<i>hupA hupB</i>	248	134	263	241	208	206

Fig. 2. Functional groups of genes influenced by HU. Data are presented for named functional groups from the Kyoto Encyclopedia of Genes and Genomics (KEGG) at 1, 4 and 6 h post-inoculation. The percentage of genes upregulated or downregulated at each time point is shown by the bars in the histograms. Downregulation is shown by bars to the left and upregulation by bars to the right of zero. Data are presented for the *hupA* mutant (grey bars), the *hupB* mutant (white bars) and the *hupA hupB* double mutant (black bars).

1 and 6 h but this mutant had the largest number of genes upregulated at 4 h. These differences revealed distinct regulons of genes whose expression was influenced by the three forms of HU, and are apparent when the effects of the *hup* mutations on different functional groups of genes are considered; a data summary is shown in Fig. 2 where it can be seen that mutations in *hupA*, *hupB* and in both *hup* genes influenced a variety of metabolic processes such as ATP synthesis, the TCA cycle and fatty acid metabolism. Among horizontally acquired genetic elements, the virulence genes in the pathogenicity islands SPI1 and SPI2 were downregulated at all three time points (Figs 3 and 4). However, the effects differed between the three *hup* mutants and at the different time points.

HU and SPI1 gene expression

A complex regulatory cascade involving genes located within the SPI1 pathogenicity island and other regulatory genes lying elsewhere on the chromosome controls the transcription of

the invasion genes in SPI1 (Rhen & Dorman, 2005). Three SPI1 regulatory genes encode the AraC-like transcription factors HilA, HilC and HilD that upregulate the SPI1 type III secretion apparatus genes and the effector protein genes. The genes *hilA*, *hilC* and *hilD* were downregulated in the *hupA* and *hupA hupB* mutants at all three time points in the microarray data (Fig. 3). This was confirmed by RT-PCR and sample data are provided for *hilA* (Fig. 3). These transcriptomic data agreed with previous findings that knockout mutations in *hupA* or *hupA* and *hupB* resulted in decreased expression of these three *hil* regulatory genes (Schechter *et al.*, 2003). We found that *hilA*, *hilC* and *hilD* were also downregulated in the *hupB* mutant at the 1 and 4 h time points, but were upregulated at 6 h (Fig. 3). This may account for an earlier observation that a mutation in *hupB* has a positive effect on the expression of *hilA* (Fahlen *et al.*, 2000). The target promoters of *hilA*, *hilC* and *hilD* displayed a similar expression pattern. The structural genes *invA*, *invF* and *invH* were downregulated in each of the mutants at all of

Fig. 3. Effect of the *hup* mutations on gene expression within the SPI1 pathogenicity island. Data are presented for SPI1 regulatory genes (a) and SPI1 invasion genes (b) in mutants deficient in *hupA* (A), *hupB* (B) and *hupA hupB* (AB) at each of the three time points (1, 4 and 6 h). In each case, the gene expression data were normalized to SL1344 for the 1 (black), 4 (white) and 6 (grey) h time points and the ratio of the mutant : wild-type was calculated. Expression ratios less than 1 indicate genes normally activated by HU at that time point while ratios greater than 1 show where and when HU acts negatively. (c) RT-PCR data for the *hilA* transcript as expressed in each of the *hup* mutants relative to the wild-type. Error bars, SEM.

the time points, with the exception of the *hupB* mutant at 6 h where each of the three genes was upregulated (Fig. 3). Similarly, the SPI1 structural genes *sipA*, *sipC* and *sipD* showed a similar response to the absence of the HU proteins (Supplementary Table S3). Our results reveal an important role for each subunit of HU in modulating the expression of the SPI1-encoded virulence factors throughout bacterial growth. This led us to predict that mutations in the genes coding for HU influence the invasiveness of *S. Typhimurium*.

Mutations in the *hup* genes alter invasiveness in epithelial cells

The *hup* mutants were compared with the parental strain SL1344 for their ability to invade and proliferate in cultured epithelial cells. The invasion assays were performed as described in Methods. Bacteria were recovered from the mammalian cells after 2 h and the numbers of c.f.u. were determined (Table 3). Loss of HU β had only a minor effect on the survival of *S. Typhimurium* growing intracellularly and was not statistically significant, in agreement with results from an earlier study (Fahlen *et al.*, 2000). Inactivation of

hupA also resulted in a small reduction in invasion that was not statistically significant. On the other hand, the *hupA hupB* double mutant showed a marked reduction in epithelial cell invasion (Table 3). These data were consistent with those showing that inactivation of both the *hupA* and *hupB* genes resulted in downregulation of the SPI1 virulence genes whereas the *hupB* mutation had a less severe negative effect and even had a positive influence on transcription at the 6 h time point (Fig. 3).

SPI2 gene expression, macrophage survival and the HU regulon

The SPI2 pathogenicity island contains genes coding for a second TTSS and its cognate effector proteins that enable *S. Typhimurium* to survive in the hostile environment of the macrophage. SPI2 genes were also downregulated in the *hupA hupB* double mutant at all three time points (Fig. 4). In particular, the genes coding for the SsrA/B two-component master regulator were downregulated, as were those coding for the type III secretion apparatus (*ssa*). In contrast, the effector protein genes (*sse*) showed only minor or no

Fig. 4. Effect of the *hup* mutations on SPI2 gene expression. Expression data are presented for the SPI2 regulatory genes *ssrA* and *ssrB* (a), representative SPI2 TTSS apparatus genes (b) and representative SPI2 effector protein genes (c) in mutants deficient in *hupA* (A), *hupB* (B) and *hupA hupB* (AB) at each of the three time points (1, 4 and 6 h). In each case, the gene expression data were normalized to SL1344 for the 1 (black), 4 (white) and 6 (grey) h time points and the ratio of the mutant : wild-type was calculated. Expression ratios less than 1 indicate genes normally activated by HU at that time point while ratios greater than 1 show where and when HU acts negatively. Error bars, SEM.

reductions in expression at the 1 h time point, although they too were strongly downregulated at later stages of growth.

The single *hupA* mutant displayed reduced expression of the *ssrA* and *ssrB* regulatory genes, especially at the 1 and 4 h time points; these genes were downregulated in the *hupB* mutant only at the 1 h time point. The *ssa* secretion apparatus genes

were downregulated in the *hupA* mutant at the 1 and 4 h time points; at 6 h they were upregulated in the absence of the HU α protein. The knockout mutation in the *hupB* gene had little or no effect on *ssa* gene expression at any time point.

The *sse* genes coding for the effector proteins showed reduced expression in the *hupA* mutant at 1 and 4 h; at the

Table 3. Comparison of phenotypes of HU strains

Strains lacking HU subunits as indicated were compared with the SL1344 wild-type for their ability to invade epithelial cells and survive macrophage uptake after 2 h. The patterns established at 2 h for the invasion and survival phenotypes were typical of those seen at 4 and 8 h (data not shown). The relative competitive fitness of strains lacking HU subunits was measured by co-culture for 18 h with SL1344 Nal^r, a strain harbouring a neutral selectable genetic marker (resistance to nalidixic acid; Table 1). The motility of the *hup* mutants was compared with the SL1344 wild-type strain on motility agar plates (see Methods). In all cases, data (% $\pm$ SD) are shown relative to the indicated comparator strain (100 %).

Phenotype	Comparator	SL1344		
		<i>hupA</i>	<i>hupB</i>	<i>hupA hupB</i>
Epithelial cell invasion	SL1344	53.5 $\pm$ 30	64 $\pm$ 33	24 $\pm$ 6*
Survival following macrophage uptake	SL1344	88.7 $\pm$ 14	111 $\pm$ 15	22 $\pm$ 3*
Relative competitive fitness	SL1344 Nal ^r	64 $\pm$ 6	110 $\pm$ 4	61 $\pm$ 6
Relative motility	SL1344	88 $\pm$ 3*	50 $\pm$ 2*	38 $\pm$ 2*

*P-values were calculated by paired *t*-test. Significance is denoted as $P < 0.05$.

6 h time point there was no detectable effect on their transcription. Inactivation of just *hupB* had little effect on *sse* gene expression at any of the three time points.

The mutants were compared with the parental strain SL1344 for their ability to survive in J774.1 macrophages (Table 3). The presence of single mutations in the *hupA* or *hupB* genes did not have a statistically significant effect on the survival of the bacteria compared with strain SL1344. However, the *hupA hupB* double mutant survived much less well than the parental strain. Our results showed that HU contributes to the regulation of virulence genes in the two major pathogenicity islands of *S. Typhimurium*. This bacterium has a third TTSS, responsible for the secretion of flagella. We next investigated the possibility that genes involved in motility might be influenced by mutations in the *hup* genes.

Flagella gene expression and motility are HU-dependent

Flagellar gene expression involves a large regulon of genes that is controlled by a complex regulatory network (Brown *et al.*, 2008; Macnab, 2003). Once the bacterial propulsion system is assembled, its activity is controlled through signals that influence the direction of flagellar motor rotation. These signals flow through a chemosensory signal transduction pathway involving reversible protein phosphorylation. Genes coding for the flagellum, its motor or the signal transduction pathway proteins showed altered expression in the *hup* mutants.

The effect of the *hup* mutations on the flagellar genes was complex and varied with the mutation and the time point (Fig. 5b). For example, genes involved in flagellar biosynthesis, such as *flgE*, were upregulated in the absence of *hupB* at all three time points but downregulated in the *hupA hupB* double mutant at the same three time points. The *hupB*

mutation resulted in the upregulation of most of the flagellar genes tested at least at some time points; in contrast, the mutation in *hupA* (and the double mutation) resulted in downregulation in almost all cases.

Motility assays on soft agar plates were used to compare the different *hup* mutants and the SL1344 parent strain (Table 3). The *hupA* mutation had the mildest effect on motility, followed by the mutation in *hupB* and the double *hupA hupB* lesions. The data did not show a clear correlation between motility as assessed by the agar plate assay and the pattern of expression of flagellar and other motility genes (Fig. 5).

Respiration and metabolism

The most dramatic effects on the expression of genes involved in respiration and metabolism occurred at the 4 h time point (see Supplementary Fig. S1, available with the online version of this paper). Genes involved in aerobic and in anaerobic respiration showed altered expression in the *hup* mutants. In particular, the *cyo* genes coding for cytochrome *o* ubiquinol oxidase were strongly upregulated at the 4 h time point in the *hupB* and *hupA hupB* mutants. Inactivation of *hupA* alone did not alter their expression. This was in contrast with the situation in *E. coli*, where these genes are positively regulated by HU (Oberto *et al.*, 2009). The *narH* gene, coding for nitrate reductase A, was very strongly upregulated at the 4 h time point, but only in the *hupA* mutant. The *narH* gene is required for anaerobic respiration in the presence of nitrate and would normally be repressed in mid-exponential-phase cultures growing under aerated conditions. HU has been reported to regulate *narH* positively in *E. coli* (Oberto *et al.*, 2009) whereas *hupA* acts negatively on *narH* transcription in *Salmonella* (Supplementary Fig. S1). The *nrf* genes, coding for nitrite reductase, were upregulated in the *hupA* mutant (Supplementary Fig. S1) in agreement with data for *E. coli* (Oberto *et al.*, 2009). The

Fig. 5. Motility, chemotaxis and flagellar gene expression in the *hup* mutants. Data are presented for the motility and chemotaxis genes (a) and representative flagellar genes (b) in mutants deficient in *hupA* (A), *hupB* (B) and *hupA hupB* (AB) at each of the three time points (1, 4 and 6 h). In each case the gene expression data were normalized to SL1344 for the 1 (black), 4 (white) and 6 h (grey) time points and the ratio of the mutant : wild-type was calculated. Expression ratios less than 1 indicate genes normally activated by HU at that time point while ratios greater than 1 show where and when HU acts negatively. Error bars, SEM.

nrf genes are usually expressed in bacteria growing in the absence of oxygen and in the presence of nitrite (Clarke *et al.*, 2008). They were downregulated in the *hupB* and the *hupA hupB* mutants. These data show that HU α and HU β play distinct roles in organizing the gene expression patterns of the cell under aerobic and anaerobic growth conditions and that the detail of their contributions is not always equivalent in *E. coli* and *Salmonella*.

Propanediol utilization is an important feature of *S. Typhimurium* growth in the lumen of the mammalian gut (Bobik *et al.*, 1999; Cheng & Bobik, 2010; Lawhon *et al.*, 2003). The horizontally acquired *pdu* genes showed a strong response to *hup* mutations at the 4 h time point (see Supplementary Fig. S2, available with the online version of this paper). Here, loss of HU α expression resulted in upregulation of the *pdu* operon whereas loss of both HU subunits caused these genes to be downregulated. Mutation of *hupB* alone had only a minor influence on *pdu* transcription.

ABC transporters play key roles in nutrient uptake and scavenging (Davidson *et al.*, 2008). The expression of genes coding for different transporters responded strongly to the loss of HU protein expression. However, the details of the response varied from system to system: e.g. expression of the dipeptide permease operon *dpp* was most affected in the *hupB* mutant, while the oligopeptide permease operon *opp* was affected most strongly in the *hupA* strain and *liv* gene expression was affected most in the *hupA hupB* double mutant (see Supplementary Fig. S3, available with the online version of this paper).

gal operon and regulon

The role of the HU protein in the regulation of transcription has been worked out in most detail in the case of the galactose regulon, where the GalR DNA-binding protein represses the expression of genes involved in galactose uptake and utilization and in the transport and metabolism of other carbon sources. HU acts in the *gal* repressosome to facilitate the action of the GalR protein in association with DNA looping and supercoiling (Lewis *et al.*, 2010, 1999; Semsey *et al.*, 2004). In *S. Typhimurium*, inactivation of *hupA* resulted in the upregulation of the *gal* regulon genes, in keeping with the role of HU as a co-repressor (see Supplementary Table S4). However, the effect was seen only at the 1 h time point and only the *hupA* mutant showed a significant effect. These data suggest that in *S. Typhimurium*, HU β acts to modulate the action of HU α in the Gal repressosome.

HU and competitive fitness

Measuring the fitness of different bacterial strains in competition with a common competitor is a useful method of assessing the impact of mutations on the overall physiology of the organisms. The competitive fitness of the SL1344 parental strain and those of its *hup* mutant derivatives were compared as described in Methods. Our results showed that the *hupA* mutation diminished the fitness of SL1344

whereas the *hupB* lesion had a positive influence (Table 3). Combining both mutations produced a fitness value similar to that seen when the *hupA* lesion alone was present. The negative effect of the *hupA* mutation on fitness was in line with our expectations given the number of genes whose expression was altered in its presence. Also, the absence of a negative effect on fitness due to the *hupB* mutation alone was consistent with the large body of data in this study showing that *hupA* and *hupB* knockout mutations have distinct impacts on cellular physiology.

HU and the RpoS regulon

A previous study established a role for HU in the post-transcriptional control of the RpoS stress and stationary phase sigma factor in *E. coli* K-12 strain C600 (Balandina *et al.*, 2001). We found that in *Salmonella*, RpoS protein expression was also reduced by the inactivation of both *hup* genes and the earlier data for *E. coli* C600 (Balandina *et al.*, 2001) were confirmed here (see Supplementary Fig. S4, available with the online version of this paper).

In our transcriptomic analysis, few members of the RpoS regulon (Lacour & Landini, 2004; Patten *et al.*, 2004; Weber *et al.*, 2005) were found to respond to the presence of knockout mutations in the *hup* genes, when present either individually or together, with the exception of the 4 h time point (Fig. 6; Supplementary Tables S2–S7). This time point represented the transition from exponential growth to stationary phase in the wild-type and the two single *hup* mutants (Fig. 1) and was equivalent to the ‘transition phase’ described in an earlier study of HU in *E. coli* (Oberto *et al.*, 2009). Here, inactivation of *hupA* resulted in the down-regulation of many RpoS-dependent genes while the *hupB* mutation caused the same genes to be expressed at a higher level. The effect of the double *hupA hupB* mutation varied from gene to gene (Fig. 6). This pattern differed from that reported previously for *E. coli hup* mutants, where the mutations had either a mild or a negligible effect on the expression of the RpoS regulon (Oberto *et al.*, 2009).

HU and integration host factor (IHF)

The HU and IHF proteins are closely related at the level of amino acid sequence but have distinct interactions with DNA (Swinger & Rice, 2004). The α and β subunits of the IHF are encoded by the *ihfA* and *ihfB* genes which were among the most severely downregulated in the *hupA hupB* double mutant at all three time points (see Supplementary Fig. S5 and Supplementary Tables S2–S7). We have previously determined the regulon of IHF in *S. Typhimurium* strain SL1344 and discovered that it includes the *hupA* and *hupB* genes, both of which are upregulated in an *ihfA ihfB* double mutant (Mangan *et al.*, 2006). These results revealed a reciprocal regulatory relationship between HU and IHF at the level of transcription in which IHF represses *hup* gene transcription and HU upregulates the transcription of *ihfA* and *ihfB*. It is not known whether the regulatory effects are

Fig. 6. Altered expression of RpoS regulon members in the *hup* mutants. Data are presented for representative members of the RpoS regulon in mutants deficient in *hupA* (A), *hupB* (B) and *hupA hupB* (AB) at the 4 h time point. In each case, the gene expression data were normalized to SL1344 and the ratio of the mutant: wild-type was calculated. Expression ratios less than 1 indicate genes normally activated by HU at that time point while ratios greater than 1 show where HU acts negatively. Error bars, SEM.

direct, i.e. if each NAP binds at the promoters of the genes coding for the other. Our finding that HU positively regulates IHF expression may reflect some overlap between the regulons of genes under the control of these two NAPs. Certainly, the classes of genes that show altered expression in the absence of HU and IHF show a considerable overlap: the SPI1 and SPI2 virulence genes, and the chemotaxis and motility genes discussed above are HU-dependent and are also influenced by IHF (Mangan *et al.*, 2006). However, the details of the responses vary. For example, most SPI1 virulence genes are downregulated in the absence of either HU or IHF. However, SPI1 regulatory genes are upregulated in the *ihfA ihfB* double mutant at the 6 h time point, whereas they are downregulated in the *hupA hupB* mutant at all time points. There is a much closer correspondence between the effects of HU and IHF among the genes of the SPI2 pathogenicity island: the *ssrA ssrB* regulatory genes are downregulated in the absence of either HU or IHF, as are the genes coding for the secretion apparatus and the effector proteins. The motility (*mot*) and chemotaxis (*che*) genes showed similar responses to the loss of either HU or IHF at all time points but the flagellar genes showed opposite responses at 6 h, being upregulated in the IHF-deficient strain and downregulated in the absence of HU. It is important to recall that the loss of HU results in strong downregulation of *ihf* gene expression, whereas the loss of IHF causes the genes coding for HU to be upregulated. For this reason knockout mutations in *ihf* and in *hup* are not equivalent.

Conclusion

This transcriptomic analysis has revealed wide-ranging roles for the HU NAP in controlling the expression of genes involved in adaptation to stress, changes in growth phase, in motility and chemotaxis and in virulence. The study shows that *S. Typhimurium* has not one but three HU regulons since inactivating the two *hup* genes individually and together results in distinct transcriptomic effects. In fact, in several cases (e.g. within the RpoS regulon), the loss of *hupA* or *hupB* has opposite effects on the expression of the same gene (Fig. 6). Presumably this reflects a highly dynamic

relationship between HU composition and the HU-dependent genes. It is not known how many of the effects of HU on transcription reported here are direct. The complication that arises due to the influence of HU on the expression of IHF, another global regulator, has been discussed already. HU is also known to influence DNA supercoiling in *E. coli* (Claret & Rouvière-Yaniv, 1997; Guo & Adhya, 2007), and we found that the level of DNA supercoiling of a reporter plasmid was altered in each of the *hup* mutants used in this study (see Supplementary Fig S6, available with the online version of this paper). Thus, HU may exert effects at promoters through changes in DNA topology rather than through directly acting as a transcription factor or by influencing the expression of another transcription factor. Furthermore, the differences between the transcriptomes of *E. coli* and *Salmonella* mutants deficient in HU may reflect, in part, the fact that DNA in these bacteria is supercoiled to different levels (Champion & Higgins, 2007).

Identifying the membership of the three HU regulons in *S. Typhimurium* has shown the breadth of influence of this NAP on gene expression: it is a true global regulator that coordinates the expression of central metabolism and virulence genes in *S. Typhimurium*.

ACKNOWLEDGEMENTS

We thank Gary Sawers for valuable help and insights into HU purification, Yvette Wormstone and Matthew Rolfe for printing the microarrays used in this study and Marie Doyle for advice on competitive fitness assays. Ishita Mukerji, Roger L. McMacken, and Remus Thei Dame are thanked for valuable help and insights into HU purification. We are grateful to Therese Hall for secretarial assistance and to Mike Eddington for computational support. This work was supported by a Science Foundation of Ireland Investigator Programme Grant (to C.J.D.) and a BBSRC Core Strategic Grant (to J.C.D.H.).

REFERENCES

- Azam, T. A. & Ishihama, A. (1999). Twelve species of the nucleoid-associated protein from *Escherichia coli*. Sequence recognition specificity and DNA binding affinity. *J Biol Chem* **274**, 33105–33113.

- Balandina, A., Claret, L., Hengge-Aronis, R. & Rouvière-Yaniv, J. (2001). The *Escherichia coli* histone-like protein HU regulates *rpoS* translation. *Mol Microbiol* **39**, 1069–1079.
- Bobik, T. A., Havemann, G. D., Busch, R. J., Williams, D. S. & Aldrich, H. C. (1999). The propanediol utilization (*pdu*) operon of *Salmonella enterica* serovar Typhimurium LT2 includes genes necessary for formation of polyhedral organelles involved in coenzyme B₁₂-dependent 1, 2-propanediol degradation. *J Bacteriol* **181**, 5967–5975.
- Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding. *Anal Biochem* **72**, 248–254.
- Brown, J. D., Saini, S., Aldridge, C., Herbert, J., Rao, C. V. & Aldridge, P. D. (2008). The rate of protein secretion dictates the temporal dynamics of flagellar gene expression. *Mol Microbiol* **70**, 924–937.
- Champion, K. & Higgins, N. P. (2007). Growth rate toxicity phenotypes and homeostatic supercoil control differentiate *Escherichia coli* from *Salmonella enterica* serovar Typhimurium. *J Bacteriol* **189**, 5839–5849.
- Cheng, S. & Bobik, T. A. (2010). Characterization of the PduS cobalamin reductase of *Salmonella enterica* and its role in the Pdu microcompartment. *J Bacteriol* **192**, 5071–5080.
- Cirillo, D. M., Valdivia, R. H., Monack, D. M. & Falkow, S. (1998). Macrophage-dependent induction of the *Salmonella* pathogenicity island 2 type III secretion system and its role in intracellular survival. *Mol Microbiol* **30**, 175–188.
- Claret, L. & Rouvière-Yaniv, J. (1997). Variation in HU composition during growth of *Escherichia coli*: the heterodimer is required for long term survival. *J Mol Biol* **273**, 93–104.
- Clarke, T. A., Mills, P. C., Poock, S. R., Butt, J. N., Cheesman, M. R., Cole, J. A., Hinton, J. C., Hemmings, A. M., Kemp, G. & other authors (2008). *Escherichia coli* cytochrome *c* nitrite reductase NrfA. *Methods Enzymol* **437**, 63–77.
- Datsenko, K. A. & Wanner, B. L. (2000). One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. *Proc Natl Acad Sci U S A* **97**, 6640–6645.
- Davidson, A. L., Dassa, E., Orelle, C. & Chen, J. (2008). Structure, function, and evolution of bacterial ATP-binding cassette systems. *Microbiol Mol Biol Rev* **72**, 317–364.
- Dillon, S. C. & Dorman, C. J. (2010). Bacterial nucleoid-associated proteins, nucleoid structure and gene expression. *Nat Rev Microbiol* **8**, 185–195.
- Dillon, S. C., Cameron, A. D. S., Hokamp, K., Lucchini, S., Hinton, J. C. D. & Dorman, C. J. (2010). Genome-wide analysis of the H-NS and Sfh regulatory networks in *Salmonella* Typhimurium identifies a plasmid-encoded transcription silencing mechanism. *Mol Microbiol* **76**, 1250–1265.
- Dorman, C. J. (2007). H-NS, the genome sentinel. *Nat Rev Microbiol* **5**, 157–161.
- Dorman, C. J. & Deighan, P. (2003). Regulation of gene expression by histone-like proteins in bacteria. *Curr Opin Genet Dev* **13**, 179–184.
- Doyle, M., Fookes, M., Ivens, A., Mangan, M. W., Wain, J. & Dorman, C. J. (2007). An H-NS-like stealth protein aids horizontal DNA transmission in bacteria. *Science* **315**, 251–252.
- Eriksson, S., Lucchini, S., Thompson, A., Rhen, M. & Hinton, J. C. (2003). Unravelling the biology of macrophage infection by gene expression profiling of intracellular *Salmonella enterica*. *Mol Microbiol* **47**, 103–118.
- Fahlen, T. F., Mathur, N. & Jones, B. D. (2000). Identification and characterization of mutants with increased expression of *hila*, the invasion gene transcriptional activator of *Salmonella typhimurium*. *FEMS Immunol Med Microbiol* **28**, 25–35.
- Finlay, B. B. & Brumell, J. H. (2000). *Salmonella* interactions with host cells: *in vitro* to *in vivo*. *Philos Trans R Soc Lond B Biol Sci* **355**, 623–631.
- Galán, J. E. (2001). *Salmonella* interactions with host cells: type III secretion at work. *Annu Rev Cell Dev Biol* **17**, 53–86.
- Grifantini, R., Sebastian, S., Frigimelica, E., Draghi, M., Bartolini, E., Muzzi, A., Rappuoli, R., Grandi, G. & Genco, C. A. (2003). Identification of iron-activated and -repressed Fur-dependent genes by transcriptome analysis of *Neisseria meningitidis* group B. *Proc Natl Acad Sci U S A* **100**, 9542–9547.
- Groisman, E. A. & Mouslim, C. (2000). Molecular mechanisms of *Salmonella* pathogenesis. *Curr Opin Infect Dis* **13**, 519–522.
- Groisman, E. A. & Ochman, H. (1997). How *Salmonella* became a pathogen. *Trends Microbiol* **5**, 343–349.
- Guo, F. & Adhya, S. (2007). Spiral structure of *Escherichia coli* HU $\alpha\beta$ provides foundation for DNA supercoiling. *Proc Natl Acad Sci U S A* **104**, 4309–4314.
- Hardt, W. D., Chen, L. M., Schuebel, K. E., Bustelo, X. R. & Galán, J. E. (1998). *S. typhimurium* encodes an activator of Rho GTPases that induces membrane ruffling and nuclear responses in host cells. *Cell* **93**, 815–826.
- Harrison, J. A., Pickard, D., Higgins, C. F., Khan, A., Chatfield, S. N., Ali, T., Dorman, C. J., Hormaeche, C. E. & Dougan, G. (1994). Role of *hns* in the virulence phenotype of pathogenic salmonellae. *Mol Microbiol* **13**, 133–140.
- Hautefort, I., Thompson, A., Eriksson-Ygberg, S., Parker, M. L., Lucchini, S., Danino, V., Bongaerts, R. J., Ahmad, N., Rhen, M. & Hinton, J. C. (2008). During infection of epithelial cells *Salmonella enterica* serovar Typhimurium undergoes a time-dependent transcriptional adaptation that results in simultaneous expression of three type 3 secretion systems. *Cell Microbiol* **10**, 958–984.
- Hensel, M. (2000). *Salmonella* pathogenicity island 2. *Mol Microbiol* **36**, 1015–1023.
- Hensel, M., Shea, J. E., Gleeson, C., Jones, M. D., Dalton, E. & Holden, D. W. (1995). Simultaneous identification of bacterial virulence genes by negative selection. *Science* **269**, 400–403.
- Higgins, C. F., Dorman, C. J., Stirling, D. A., Waddell, L., Booth, I. R., May, G. & Bremer, E. (1988). A physiological role for DNA supercoiling in the osmotic regulation of gene expression in *S. typhimurium* and *E. coli*. *Cell* **52**, 569–584.
- Hinton, J. C. D., Hautefort, I., Eriksson, S., Thompson, A. & Rhen, M. (2004). Benefits and pitfalls of using microarrays to monitor bacterial gene expression during infection. *Curr Opin Microbiol* **7**, 277–282.
- Hoise, S. K. & Stocker, B. A. (1981). Aromatic-dependent *Salmonella typhimurium* are non-virulent and effective as live vaccines. *Nature* **291**, 238–239.
- Holden, D. W. (2002). Trafficking of the *Salmonella* vacuole in macrophages. *Traffic* **3**, 161–169.
- Huisman, O., Faelen, M., Girard, D., Jaffé, A., Toussaint, A. & Rouvière-Yaniv, J. (1989). Multiple defects in *Escherichia coli* mutants lacking HU protein. *J Bacteriol* **171**, 3704–3712.
- Kamashev, D., Balandina, A. & Rouvière-Yaniv, J. (1999). The binding motif recognized by HU on both nicked and cruciform DNA. *EMBO J* **18**, 5434–5444.
- Kamashev, D., Balandina, A., Mazur, A. K., Arimondo, P. B. & Rouvière-Yaniv, J. (2008). HU binds and folds single-stranded DNA. *Nucleic Acids Res* **36**, 1026–1036.

- Kar, S., Edgar, R. & Adhya, S. (2005). Nucleoid remodeling by an altered HU protein: reorganization of the transcription program. *Proc Natl Acad Sci U S A* **102**, 16397–16402.
- Kelly, A., Goldberg, M. D., Carroll, R. K., Danino, V., Hinton, J. C. D. & Dorman, C. J. (2004). A global role for Fis in the transcriptional control of metabolism and type III secretion in *Salmonella enterica* serovar Typhimurium. *Microbiology* **150**, 2037–2053.
- Lacour, S. & Landini, P. (2004). σ^S -dependent gene expression at the onset of stationary phase in *Escherichia coli*: function of σ^S -dependent genes and identification of their promoter sequences. *J Bacteriol* **186**, 7186–7195.
- Lawhon, S. D., Frye, J. G., Suyemoto, M., Porwollik, S., McClelland, M. & Altier, C. (2003). Global regulation by CsrA in *Salmonella typhimurium*. *Mol Microbiol* **48**, 1633–1645.
- Lewis, D. E., Geanacopoulos, M. & Adhya, S. (1999). Role of HU and DNA supercoiling in transcription repression: specialized nucleoprotein repression complex at *gal* promoters in *Escherichia coli*. *Mol Microbiol* **31**, 451–461.
- Lewis, D. E. A., Lee, S. J. & Adhya, S. (2010). Role of HU in regulation of *gal* promoters. In *Bacterial Chromatin*, pp. 395–417. Edited by R. T. Dame & C. J. Dorman. Heidelberg: Springer.
- Li, S. & Waters, R. (1998). *Escherichia coli* strains lacking protein HU are UV sensitive due to a role for HU in homologous recombination. *J Bacteriol* **180**, 3750–3756.
- Lucchini, S., Rowley, G., Goldberg, M. D., Hurd, D., Harrison, M. & Hinton, J. C. D. (2006). H-NS mediates the silencing of laterally acquired genes in bacteria. *PLoS Pathog* **2**, e81.
- Lucchini, S., McDermott, P., Thompson, A. & Hinton, J. C. (2009). The H-NS-like protein StpA represses the RpoS (σ^{38}) regulon during exponential growth of *Salmonella* Typhimurium. *Mol Microbiol* **74**, 1169–1186.
- Macnab, R. M. (1986). Proton-driven bacterial flagellar motor. *Methods Enzymol* **125**, 563–581.
- Macnab, R. M. (2003). How bacteria assemble flagella. *Annu Rev Microbiol* **57**, 77–100.
- Mahan, M. J., Slauch, J. M. & Mekalanos, J. J. (1993). Selection of bacterial virulence genes that are specifically induced in host tissues. *Science* **259**, 686–688.
- Mangan, M. W., Lucchini, S., Danino, V., Ó Cróinín, T., Hinton, J. C. D. & Dorman, C. J. (2006). The integration host factor (IHF) integrates stationary-phase and virulence gene expression in *Salmonella enterica* serovar Typhimurium. *Mol Microbiol* **59**, 1831–1847.
- Marshall, D. G., Sheehan, B. J. & Dorman, C. J. (1999). A role for the leucine-responsive regulatory protein and integration host factor in the regulation of the *Salmonella* plasmid virulence (*spv*) locus in *Salmonella typhimurium*. *Mol Microbiol* **34**, 134–145.
- Marshall, D. G., Bowe, F., Hale, C., Dougan, G. & Dorman, C. J. (2000). DNA topology and adaptation of *Salmonella typhimurium* to an intracellular environment. *Philos Trans R Soc Lond B Biol Sci* **355**, 565–574.
- Merickel, S. K. & Johnson, R. C. (2004). Topological analysis of Hincatylised DNA recombination *in vivo* and *in vitro*. *Mol Microbiol* **51**, 1143–1154.
- Mills, D. M., Bajaj, V. & Lee, C. A. (1995). A 40 kb chromosomal fragment encoding *Salmonella typhimurium* invasion genes is absent from the corresponding region of the *Escherichia coli* K-12 chromosome. *Mol Microbiol* **15**, 749–759.
- Navarre, W. W., Porwollik, S., Wang, Y., McClelland, M., Rosen, H., Libby, S. J. & Fang, F. C. (2006). Selective silencing of foreign DNA with low GC content by the H-NS protein in *Salmonella*. *Science* **313**, 236–238.
- O'Byrne, C. P. & Dorman, C. J. (1994a). The *spv* virulence operon of *Salmonella typhimurium* LT2 is regulated negatively by the cyclic AMP (cAMP)–cAMP receptor protein system. *J Bacteriol* **176**, 905–912.
- O'Byrne, C. P. & Dorman, C. J. (1994b). Transcription of the *Salmonella typhimurium spv* virulence locus is regulated negatively by the nucleoid-associated protein H-NS. *FEMS Microbiol Lett* **121**, 99–105.
- Oberto, J., Nabti, S., Jooste, V., Mignot, H. & Rouvière-Yaniv, J. (2009). The HU regulon is composed of genes responding to anaerobiosis, acid stress, high osmolarity and SOS induction. *PLoS ONE* **4**, e4367.
- Patten, C. L., Kirchhof, M. G., Schertzberg, M. R., Morton, R. A. & Schellhorn, H. E. (2004). Microarray analysis of RpoS-mediated gene expression in *Escherichia coli* K-12. *Mol Genet Genomics* **272**, 580–591.
- Perni, S., Andrew, P. W. & Shama, G. (2005). Estimating the maximum growth rate from microbial growth curves: definition is everything. *Food Microbiol* **22**, 491–495.
- Pontiggia, A., Negri, A., Beltrame, M. & Bianchi, M. E. (1993). Protein HU binds specifically to kinked DNA. *Mol Microbiol* **7**, 343–350.
- Rhen, M. & Dorman, C. J. (2005). Hierarchical gene regulators adapt *Salmonella enterica* to its host milieu. *Int J Med Microbiol* **294**, 487–502.
- Ryan, V. T., Grimwade, J. E., Nievera, C. J. & Leonard, A. C. (2002). IHF and HU stimulate assembly of pre-replication complexes at *Escherichia coli oriC* by two different mechanisms. *Mol Microbiol* **46**, 113–124.
- Sambrook, J. & Russell, D. (2001). *Molecular Cloning: a Laboratory Manual*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Schechter, L. M., Jain, S., Akbar, S. & Lee, C. A. (2003). The small nucleoid-binding proteins H-NS, HU, and Fis affect *hlyA* expression in *Salmonella enterica* serovar Typhimurium. *Infect Immun* **71**, 5432–5435.
- Semsey, S., Tolstorukov, M. Y., Virnik, K., Zhurkin, V. B. & Adhya, S. (2004). DNA trajectory in the Gal repressosome. *Genes Dev* **18**, 1898–1907.
- Shanado, Y., Kato, J. & Ikeda, H. (1998). *Escherichia coli* HU protein suppresses DNA-gyrase-mediated illegitimate recombination and SOS induction. *Genes Cells* **3**, 511–520.
- Signon, L. & Kleckner, N. (1995). Negative and positive regulation of Tn10/IS10-promoted recombination by IHF: two distinguishable processes inhibit transposition off of multicopy plasmid replicons and activate chromosomal events that favor evolution of new transposons. *Genes Dev* **9**, 1123–1136.
- Sternberg, N. L. & Maurer, R. (1991). Bacteriophage-mediated generalized transduction in *Escherichia coli* and *Salmonella typhimurium*. *Methods Enzymol* **204**, 18–43.
- Swinger, K. K. & Rice, P. A. (2004). IHF and HU: flexible architects of bent DNA. *Curr Opin Struct Biol* **14**, 28–35.
- Uzzau, S., Figueroa-Bossi, N., Rubino, S. & Bossi, L. (2001). Epitope tagging of chromosomal genes in *Salmonella*. *Proc Natl Acad Sci U S A* **98**, 15264–15269.
- Valdivia, R. H. & Falkow, S. (1997). Fluorescence-based isolation of bacterial genes expressed within host cells. *Science* **277**, 2007–2011.
- Waterman, S. R. & Holden, D. W. (2003). Functions and effectors of the *Salmonella* pathogenicity island 2 type III secretion system. *Cell Microbiol* **5**, 501–511.
- Weber, H., Polen, T., Heuveling, J., Wendisch, V. F. & Hengge, R. (2005). Genome-wide analysis of the general stress response network in *Escherichia coli*: σ^S -dependent genes, promoters, and sigma factor selectivity. *J Bacteriol* **187**, 1591–1603.

Wood, M. W., Rosqvist, R., Mullan, P. B., Edwards, M. H. & Galyov, E. E. (1996). SopE, a secreted protein of *Salmonella dublin*, is translocated into the target eukaryotic cell via a *sip*-dependent mechanism and promotes bacterial entry. *Mol Microbiol* **22**, 327–338.

Yanisch-Perron, C., Vieira, J. & Messing, J. (1985). Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. *Gene* **33**, 103–119.

Edited by: D. W. Ussery