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

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ABSTRACT

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 hours post-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 the 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 to the wild type. In contrast, inactivation of just the *hupB* gene resulted in increased fitness and correlated with the up-regulation 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 genes, and contributes to the success of *S. enterica* as a pathogen.
INTRODUCTION

The HU DNA-binding protein is one of approximately twelve 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, HUα and HUβ (Guo & Adhya, 2007) and can exist in three forms, the HUαβ heterodimer and the HUα2 and HUβ2 homodimers. The relative abundances of the three forms vary with the phase of growth (Claret & Rouvière-Yaniv, 1997). The αβ heterodimer is the dominant form of the protein at most stages of growth, except at the earliest stage of the exponential phase when the α2 form predominates; the β2 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 difference
concerns the abilities of the proteins to introduce negative supercoils into DNA in the presence of topoisomerase I: the αβ and α₂ forms can do this, the β₂ 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. 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 SPI1 island 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 the SPI2 island 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 nucleoid-associated proteins (NAPs) act as global regulators that contribute to the coordinated expression of all three TTSS (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; 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

*Escherichia coli* K-12 strain DH5α 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 μg ml⁻¹), chloramphenicol (20 μg ml⁻¹), kanamycin (50 μg ml⁻¹), and nalidixic acid (50 μg 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 previously described (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, Gaithersburg, MD) according to the manufacturer’s instructions. Details of the oligonucleotides used in this study are presented in *Supplementary material* (Table S1).

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 the polymerase chain reaction (PCR) using 50 ng of plasmid DNA as template, in the presence of 2.5 μM dNTP, 2.5mM MgCl₂, and 50 μM of the appropriate oligonucleotide primer. The PCR reaction was carried out using the Phusion polymerase and conditions recommended by the manufacturer (Finnzymes: Espoo, Finland). 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.cm 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 λRed 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. 100 μl aliquots of competent *S. Typhimurium* CH607(pKD46) were then transformed by electroporation with either purified PCR product 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, Gaithersburg, MD), and digested with EcoRI. The \textit{hup} gene allelic replacements were confirmed using Southern blot analysis (Sambrook & Russell, 2001). Bacteriophage P22 transducing lysates of strains CH6\textit{07hupA} and CH6\textit{07hupB} were prepared (Sternberg and Maurer, 1991), and used to transfer the mutated \textit{hup} genes individually to \textit{S}. Typhimurium strain SL1344. Double \textit{hupA}\textit{hupB} mutants were then prepared by transducing either of the single \textit{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 at 1:100 25 ml of fresh antibiotic-free LB in a 250 ml flask which was then incubated with shaking at 250 rpm 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 h, 4 h 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 \textit{et al}., 2004; Hinton \textit{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 \textit{et al}., 2004). RNA concentrations were determined by absorbance at 260 nm, and RNA quality was assessed using the Bioanalyzer 2100 (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 labeled cDNA as described in Eriksson \textit{et al.} (2003). Hybridization, microarray scanning and data analysis were carried out as described previously (Kelly \textit{et al.}, 2004). The microarray data are provided in Table S2, 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 (Table S1). Primers were designed \textit{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. 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, 55°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 \textit{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 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, SL1344hupA::kan, SL1344hupB::cat, SL1344hupA::kan hupB::cat as test strains and the comparator strain S. Typhimurium SL1344 Nalr, 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 $10^5$ cfu 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 composition 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 (Madison, WI) 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 and 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, re-suspended in pre-warmed culture medium and added to CHO or J774.1 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 2 h to allow invasion (epithelial cells) or phagocytosis (macrophage) to occur. The mammalian cells were then washed and incubated with fresh medium containing 100 µg 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 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 using a plasmid purification kit as directed by the manufacturer (Qiagen) and electrophoresed on a 0.8% agarose gel containing 2.5 μg ml⁻¹ chloroquine. 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; \( \mu_{\text{max}} = 0.047 \) h\(^{-1}\)), the β subunit (hupB, CJD2004; \( \mu_{\text{max}} = 0.04 \) h\(^{-1}\)), or both subunits (CJD2005; \( \mu_{\text{max}} = 0.1 \) h\(^{-1}\)) (Table 1). The single mutants had growth profiles that were similar to that of the wild type (\( \mu_{\text{max}} = 0.05 \) h\(^{-1}\)) when growing 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 optical density similar to that of the wild type by end of exponential growth (Fig. 1). DNA microarrays were used to analyze the S. Typhimurium transcriptome in the presence and absence of the hup genes at three time points (1 h, 4 h, and 6 h) in cultures growing in LB liquid medium at 37°C. The numbers of genes (ranging from 50 to 482) that were up- or down-regulated 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 up- or down-regulated in the hupB single mutant at 1 h and 6 h but this mutant had the largest number of genes up-regulated 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 down-regulated at all three time points (Figs. 3 & 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 up-regulate the SPI1 type III secretion apparatus genes and the effector protein genes. The genes hilA, hilC and hilD were down-regulated 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 down-regulated in the hupB mutant at the 1-h and 4-h time points, but were up-regulated at 6 hours (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 down-regulated in each of the mutants at all of the time points, with the exception of the hupB mutant at 6 h where each of the three genes was up-regulated (Fig. 3). Similarly, the SPI1 structural genes sipA, sipC and sipD showed a similar response to the absence of the HU proteins (Fig. 3). 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 \textit{hup} genes alter invasiveness in epithelial cells

The \textit{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 colony-forming units (cfu) were determined (Table 3). Loss of HU $\beta$ 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 \textit{et al.}, 2000). Inactivation of \textit{hupA} also resulted in a small reduction in invasion that was not statistically significant. On the other hand, the \textit{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 \textit{hupA} and \textit{hupB} genes resulted in down regulation of the SPI1 virulence genes whereas the \textit{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 type III secretion system and its cognate effector proteins that enable S. Typhimurium to survive in the hostile environment of the macrophage. SPI2 genes were also down-regulated in the \textit{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 down-regulated, as were
those coding for the type III secretion apparatus (ssa). In contrast, the effector protein
genes (sse) showed only minor or no reductions in expression at the 1-h time point,
although they too were strongly down-regulated at later stages of growth.

The single hupA mutant displayed reduced expression of the ssrA ssrB
regulatory genes, especially at the 1 and 4-h time points; these genes were down-
regulated in the hupB mutant only at the 1-h time point. The ssa secretion apparatus
genes were down-regulated in the hupA mutant at the 1 and 4-h time points; at 6 hours
they were up-regulated 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 hours; at the 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 type III secretion system, 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. 5). For example, genes involved in flagellar biosynthesis, such as flgK, were up-regulated in the absence of hupB at all three time points but down-regulated in the hupA hupB double mutant at the same three time points. The hupB mutation resulted in the up regulation 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 down regulation 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 (Fig. S2). 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 up-regulated 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 to 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* (Fig. S2). The *nrf* genes, coding for nitrite reductase, were up-regulated in the *hupA* mutant (Fig. S2) in agreement with data for *E. coli* (Oberto *et al.*, 2009). The *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 down-regulated 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 (Fig. S4). Here, loss of HU α expression resulted in up regulation of the *pdu* operon whereas loss of both HU subunits caused these genes to be down-regulated. The mutation in *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 (Fig. S3).

The 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 up regulation of the gal regulon genes, in keeping with the role of HU as a co-repressor (Table S2). 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 posttranscriptional 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 (Fig. S5).

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; 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 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 integration host factor (IHF) are encoded by the ihfA and ihfB genes which were among the most severely down-regulated in the hupA hupB double mutant at all three time points (Fig. S1; 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 up-regulated 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 up-regulates the transcription of ihfA and ihfB. It is not known whether the regulatory effects are direct, i.e. if each nucleoid associated protein (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, 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 down-regulated in the absence of either HU or IHF. However, SPI1 regulatory genes are upregulated in
the \textit{ihfA} \textit{ihfB} double mutant at the 6-h time point whereas they are down-regulated in
the \textit{hupA} \textit{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 \textit{ssrA} \textit{ssrB} regulatory genes are down-regulated in the absence of either HU or IHF,
as are the genes coding for the secretion apparatus and the effector proteins. The
motility (\textit{mot}) and chemotaxis (\textit{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 up-regulated in the IHF-deficient strain and down-regulated in the
absence of HU. It is important to recall that the loss of HU results in strong down
regulation of \textit{ihf} gene expression whereas the loss of IHF causes the genes coding for
HU to be up-regulated. For this reason knockout mutations in \textit{ihf} and in \textit{hup} are not
equivalent.

\textbf{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 \textit{S.}
Typhimurium has not one but three HU regulons since inactivating the two \textit{hup} genes
individually and together results in distinct transcriptomic effects. In fact, in several
cases (e.g. within the RpoS regulon), the loss of \textit{hupA} or \textit{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 (Fig S6). 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*. 
REFERENCES


off of multicopy plasmid replicons and activate chromosomal events that favor evolution of new transposons. *Genes Dev* 9, 1123-1136.


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Table 1. Bacterial strains and plasmids used in this study

<table>
<thead>
<tr>
<th>Strain/plasmid</th>
<th>Relevant details</th>
<th>Reference/source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Escherichia coli K-12 DH5 α</td>
<td>(\text{endA1 hsdR17(rk'mk) glnV44 thi-1 recA1 gyrA(Nal') relA1 Δ(lacIZYA-argF) U169 deoR (φ80 ΔlacZM15)})</td>
<td>Promega</td>
</tr>
<tr>
<td>C600 (JO2057)</td>
<td>(\text{thr-I leu86 thi-1 lacY glnV44} ) C600 (\text{hupA::cat hupB::kan})</td>
<td>Oberto et al. (2009)</td>
</tr>
<tr>
<td>JO3020</td>
<td></td>
<td>Oberto et al. (2009)</td>
</tr>
<tr>
<td>S. Typhimurium</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CH607</td>
<td>(\text{polA-2 zig204::Tn10})</td>
<td>Lab stocks</td>
</tr>
<tr>
<td>CJD2000</td>
<td>CH607 (\text{hupA::cat})</td>
<td>This study</td>
</tr>
<tr>
<td>CJD2001</td>
<td>CH607 (\text{hupB::kan})</td>
<td>This study</td>
</tr>
<tr>
<td>CJD2003</td>
<td>SL1344 (\text{hupA::cat})</td>
<td>This study</td>
</tr>
<tr>
<td>CJD2004</td>
<td>SL1344 (\text{hupB::kan})</td>
<td>This study</td>
</tr>
<tr>
<td>CJD2005</td>
<td>SL1344 (\text{hupA::cat hupB::kan})</td>
<td>This study</td>
</tr>
<tr>
<td>SL1344</td>
<td>(\text{hisG})</td>
<td>Hoiseth &amp; Stocker (1981)</td>
</tr>
<tr>
<td>SL1344 Nal'</td>
<td>(\text{hisG nald})</td>
<td>Doyle et al. (2007)</td>
</tr>
<tr>
<td>TH6232</td>
<td>LT2 (\text{Δhin7517::FRT fliBA-OFF fliC-ON})</td>
<td>K.T. Hughes</td>
</tr>
<tr>
<td>TH6233</td>
<td>LT2 (\text{Δhin7517::FRT fliBA-ON fliC-OFF})</td>
<td>K.T. Hughes</td>
</tr>
<tr>
<td>Plasmids</td>
<td>Template for (\text{kan}) cassette</td>
<td>Datsenko &amp; Wanner (2000)</td>
</tr>
<tr>
<td>pKD3</td>
<td>Inducible (\text{λredBCD}) under (\text{P}_{\text{BAD}}) control</td>
<td>Datsenko &amp; Wanner (2000)</td>
</tr>
<tr>
<td>pKD46</td>
<td>Template for (\text{cat}) cassette</td>
<td>Ussau et al. (2001)</td>
</tr>
<tr>
<td>pSU311</td>
<td>(\text{oriPMB1, Ap'}})</td>
<td>Yanisch-Perron et al. (1985)</td>
</tr>
</tbody>
</table>
Table 2. Numbers of genes in the HU regulons

<table>
<thead>
<tr>
<th>Strain</th>
<th>Time (h)</th>
<th>1 h (up)</th>
<th>1 h (down)</th>
<th>4 h (up)</th>
<th>4 h (down)</th>
<th>6 h (up)</th>
<th>6 h (down)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1 h</td>
<td>4 h</td>
<td>6 h</td>
<td>6 h</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>(up)</td>
<td>(down)</td>
<td>(up)</td>
<td>(down)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>hupA</td>
<td>277</td>
<td>180</td>
<td>323</td>
<td>319</td>
<td>283</td>
<td>299</td>
<td></td>
</tr>
<tr>
<td>hupB</td>
<td>50</td>
<td>75</td>
<td>482</td>
<td>275</td>
<td>54</td>
<td>50</td>
<td></td>
</tr>
<tr>
<td>hupA hupB</td>
<td>248</td>
<td>134</td>
<td>263</td>
<td>241</td>
<td>208</td>
<td>206</td>
<td></td>
</tr>
</tbody>
</table>

The table summarizes the numbers of genes whose expression increased (up) or decreased (down) compared to the SL1344 wild type in the hupA and hupB single mutants and in the hupA hupB double mutant at the 1-h, 4-h and 6-h timepoints.
Table 3. Comparison of phenotypes of HU strains.

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>Comparator</th>
<th>SL1344 hupA</th>
<th>SL1344 hupB</th>
<th>SL1344 hupA hupB</th>
</tr>
</thead>
<tbody>
<tr>
<td>Epithelial cell invasion</td>
<td>SL1344</td>
<td>53.5% (± 30%)</td>
<td>64% (± 33%)</td>
<td>24 (± 6%)*</td>
</tr>
<tr>
<td>Survival following macrophage</td>
<td>SL1344</td>
<td>88.7 (± 14%)</td>
<td>111 (± 15%)</td>
<td>22 (± 3%)*</td>
</tr>
<tr>
<td>uptake</td>
<td>SL1344Nař</td>
<td>64% (± 6%)</td>
<td>110 (± 4%)</td>
<td>61% (± 6%)</td>
</tr>
<tr>
<td>Relative competitive fitness</td>
<td>SL1344</td>
<td>88% (± 3%)*</td>
<td>50% (± 2%)*</td>
<td>38% (± 2%)*</td>
</tr>
</tbody>
</table>

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 hours. The patterns established at 2 hour for the invasion and survival phenotypes were typical of those seen at 4 hours and 8 hours (data not shown). The relative competitive fitness of strains lacking HU subunits was measured by co-culture for 18 hours with SL1344 Nař, 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 (Materials and Methods). In all cases, data (± standard deviations) are shown relative to the indicated comparator strain (100%).

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

Fig. 1. Growth curves of parental strain SL1344 and its three hup derivatives

Growth curves are shown for the parental strain, SL1344 and its hup mutant derivatives: hupA (open squares), hupB (open triangles) and the double mutant (inverted filled triangles) growing in LB with aeration at 37°C. The vertical arrows indicate the three time points (1 h, 4 h and 6 h) at which RNA was extracted for the transcriptomic analyses.

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 percentages of genes up regulated or down regulated at each time point is shown by the bars in the histograms. Down regulation is shown by bars to the left and up regulation 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).

Fig. 3. Effect of the hup mutations on gene expression within the SPI1 pathogenicity island. Data are presented for SPI1 regulatory genes (top) and SPI1 invasion genes (centre) 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. RT-PCR data for the hilA transcript as expressed in each of the hup mutants relative to the wild type are provided in the bottom panel.
**Fig. 4.** Effect of the *hup* mutations on SPI2 gene expression. Expression data are presented for the SPI2 regulatory genes *ssrA* and *ssrB* (top), representative SPI2 type III secretion system apparatus genes (middle) and representative SPI2 effector protein genes (bottom) 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.

**Fig. 5.** Motility, chemotaxis and flagellar gene expression in the *hup* mutants. Data are presented for the motility and chemotaxis genes (top) and representative flagellar genes (bottom) 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.

**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.
1 h

ATP synthesis
TCA Cycle
Fatty acid metabolism
Glutathione metabolism
Peptidoglycan biosynthesis
Protein export
Phospholipid degradation
Nucleotide metabolism
SPI1 regulatory genes

Relative expression

hilA (A)  hilA (B)  hilA (AB)  hilC (A)  hilC (B)  hilC (AB)  hilD (A)  hilD (B)  hilD (AB)

hilA  hilC  hilC
SPI2 regulatory genes

Relative expression

ssrA (A)  ssrA (B)  ssrA (AB)  ssrB (A)  ssrB (B)  ssrB (AB)

ssrA

ssrB
SPI2 type III effector protein genes

Relative expression

sseC (A)  sseC (B)  sseC (AB)  sseF (A)  sseF (B)  sseF (AB)  sseG (A)  sseG (B)  sseG (AB)

sseC  sseF  sseG
Motility and chemotaxis genes

Relative expression

motA (A)  motA (B)  motA (AB)  motB(A)  motB(B)  motB(AB)  cheA (A)  cheA (B)  cheA(AB)  cheB (A)  cheB (B)  cheB(AB)  cheR (A)  cheR (B)  cheR (AB)  cheZ (A)  cheZ (B)  cheZ (AB)

motA  motB  cheA  cheB  cheR  cheZ