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1 The nucleoid-associated protein HU controls three regulons that coordinate virulence,
2 response to stress and general physiology in *Salmonella enterica* serovar
3 Typhimurium.
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18

19 **ABSTRACT**

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

36

## 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, HU $\alpha$ and HU $\beta$ (Guo & Adhya, 2007) and can exist in three forms, the HU $\alpha\beta$ heterodimer and the HU $\alpha_2$ and HU $\beta_2$ 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 $\alpha_2$ form predominates; the $\beta_2$ 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 difference

62 concerns the abilities of the proteins to introduce negative supercoils into DNA in the
63 presence of topoisomerase I: the $\alpha\beta$ and $\alpha_2$ forms can do this, the $\beta_2$ form cannot
64 (Claret & Rouvière-Yaniv, 1997). Double mutants of *E. coli* that have knockout
65 mutations in both of the genes coding for the $\alpha$ (*hupA*) and $\beta$ (*hupB*) subunits have
66 growth defects but remain viable (Huisman *et al.,* 1989).

67 The role of HU in global gene regulation has not been determined previously
68 by transcriptomic analysis in a bacterial pathogen. In the commensal organism *E. coli*
69 K-12, HU influences, *inter alia,* the expression of genes involved in anaerobic
70 respiration, the response to osmotic stress, the acid stress response and the response to
71 DNA damage (Kar *et al.,* 2005; Oberto *et al.,* 2009). *S.* Typhimurium is an attractive
72 subject for studies of global gene regulatory mechanisms in a pathogen because it has
73 been characterized in great detail at the genetic and physiological levels. *S.*
74 Typhimurium infects mice where it causes a typhoid-like disease that is a valuable
75 model for human typhoid. It can also infect other animals, such as pigs, cattle and
76 chickens. The bacterium infects the host orally and must traverse the alimentary tract
77 en route to the site of host cell invasion in the small intestine. This journey exposes
78 the bacterium to a number of severe environmental stresses (e.g. the strongly acidic
79 environment of the stomach) and *S.* Typhimurium must adjust its gene expression
80 profile in response to each stress if it is to survive. More than 200 genes contribute to
81 the virulence of this pathogen, with some of the best characterized being located in
82 the chromosome within the major pathogenicity islands (Finlay & Brumell, 2000;
83 Groisman & Mouslim, 2000; Groisman & Ochman, 1997). The SPI1 island harbours
84 the genes that encode the Inv/Spa type III secretion system (TTSS) and its effector
85 proteins (Galán, 2001; Hardt *et al.,* 1998; Hensel, 2000; Mills *et al.,* 1995; Wood *et*
86 *al.,* 1996). This TTSS is required for epithelial cell invasion while an alternative

87 TTSS encoded by the SPI2 island is required for survival of *S.* Typhimurium in
88 macrophages (Cirillo *et al.,* 1998; Hensel, 2000; Holden, 2002; Waterman & Holden,
89 2003). A third TTSS is responsible for the expression of a flagellum-dependent motile
90 phenotype (Macnab, 2003). Previous studies have shown that nucleoid-associated
91 proteins (NAPs) act as global regulators that contribute to the coordinated expression
92 of all three TTSS (Dillon et al., 2010; Kelly *et al.,* 2004; Mangan *et al.,* 2006). The
93 expression of many housekeeping genes is also coordinated by the gene regulatory
94 mechanisms that operate as *S.* Typhimurium navigates the host (Eriksson *et al.,* 2003;
95 Hautefort *et al.,* 2008; Hensel *et al.,* 1995; Mahan *et al.,* 1993; Rhen & Dorman,
96 2005; Valdivia & Falkow, 1997). Understanding how these regulatory circuits are
97 composed and operate represents a significant challenge. We have previously
98 determined the membership of the regulons of genes whose expression is influenced
99 by the NAPs Fis, H-NS, IHF, Sfh, and StpA in *S.* Typhimurium (Dillon *et al.,* 2010;
100 Kelly *et al.,* 2004; Lucchini *et al.,* 2006; 2009; Mangan *et al.,* 2006). In this study we
101 used DNA microarray-based transcriptomics analysis to identify those genes in *S.*
102 Typhimurium whose expression changed in the presence of mutations in *hupA,* or
103 *hupB* or both *hupA* and *hupB*.
104

105
106
107 **METHODS**

108

109 **Bacterial strains and culture conditions**

110 *Escherichia coli* K-12 strain DH5α was used as the host for cloning experiments and
111 strain C600 was used to assess the effect of *hup* gene knockouts on RpoS sigma factor
112 expression (Oberto *et al.,* 2009). *S.* Typhimurium strain SL1344, used in previous
113 transcriptomic studies (Eriksson *et al*., 2003; Kelly *et al*., 2004; Mangan *et al,* 2006)
114 was the host background for the construction of the mutant strains for transcriptome
115 profiling. These and other strains used in this study are listed in Table 1. Bacteria
116 were cultured in LB medium, pH 7.0 (Sambrook & Russell, 2001) at 37C unless
117 indicated otherwise. Where required, antibiotics were used at the following
118 concentrations: carbenicillin (100 μg $ml^{-1}$), chloramphenicol (20 μg $ml^{-1}$), kanamycin
119 (50 μg $ml^{-1}$), and nalidixic acid (50 μg $ml^{-1}$). Bacterial motility assays were performed
120 using swarm plates containing (w/v) 1% Bacto-Tryptone, 0.5% NaCl and 0.3% Bacto-
121 Agar (Macnab, 1986). The centre of each plate was inoculated with equal numbers of
122 bacteria and incubated at 37$^o$C for 8 h. The diameter of the disc-shaped growth zone
123 was then measured. Microbial growth in LB medium was modelled using Baranyi-
124 Roberts approximation parameters as described previously (Perni *et al.,* 2005).

125

126 **Strain construction and DNA manipulations**

127 Routine recombinant DNA manipulations were carried out as previously described
128 (Sambrook & Russell, 2001). The plasmids used in this study are described in Table
129 1. Strains harbouring knockout mutations in the various genes were constructed as
130 follows. Total genomic DNA was isolated from *S.* Typhimurium strain SL1344 using

131 the Bacterial Genomic DNA purification kit (EdgeBiosystems, Gaithersburg, MD)
132 according to the manufacturer's instructions. Details of the oligonucleotides used in
133 this study are presented in *Supplementary material* (Table S1).

134 The *hupA* and *hupB* genes were knocked out by allelic exchange using the
135 method of Datsenko & Wanner (2000). Linear DNA for the exchange was generated
136 by the polymerase chain reaction (PCR) using 50 ng of plasmid DNA as template, in
137 the presence of 2.5 μM dNTP, 2.5mM $MgCl_2$, and 50 μM of the appropriate
138 oligonucleotide primer. The PCR reaction was carried out using the Phusion
139 polymerase and conditions recommended by the manufacturer (Finnzymes: Espoo,
140 Finland). The *hupA* gene was replaced on the chromosome with a kanamycin
141 resistance cassette amplified using hupA.k.o.kan oligonucleotide primers and plasmid
142 pSU311 as template (Uzzau *et al.,* 2001). The *hupB* gene was replaced with a
143 chloramphenicol resistance cassette generated using the combination of hupB.k.o.cm
144 primers and plasmid pKD3 as the template (Datsenko & Wanner, 2000). *S.*
145 Typhimurium CH607 was transformed with plasmid pKD46 and carbenicillin
146 resistant transformants were grown in LB broth supplemented with carbenicillin
147 overnight at 30$^o$C with agitation. Fresh LB broth containing carbenicillin was
148 inoculated 1:50 from this culture and incubated at 30$^o$C with agitation for 90 min,
149 before the addition of 0.02% w/v arabinose to induce expression of the λRed
150 recombination genes. Cells were grown to $OD_{600}$ = 0.4, and then rapidly chilled on
151 ice water for 10 min followed by two washes with 10 ml sterile ice-cold water. The
152 cell pellet was then resuspended in 1 ml sterile ice-cold water. 100 μl aliquots of
153 competent *S*. Typhimurium CH607(pKD46) were then transformed by electroporation
154 with either purified PCR product for *hup* gene replacement, the cells were plated to
155 select the desired allelic replacement at 37˚C. Genomic DNA was purifed from

156 candidate mutants using the Bacterial Genomic DNA purification kit
157 (EdgeBiosystems, Gaithersburg, MD), and digested with EcoRI. The *hup* gene alleleic
158 replacements were confirmed using Southern blot analysis (Sambrook & Russell,
159 2001). Bacteriophage P22 transducing lysates of strains CH607*hupA* and CH607*hupB*
160 were prepared (Sternberg and Maurer, 1991), and used to transfer the mutated *hup*
161 genes individually to *S*. Typhimurium strain SL1344. Double *hupAhupB* mutants
162 were then prepared by transducing either of the single *hup* knockouts with lysate
163 prepared from strains lacking the alternative subunit. Serial passage of transducants
164 on green agar plates was used to ascertain that SL1344 strains were free of P22
165 (Sternberg & Maurer, 1991).
166

167 **RNA isolation, microarray procedures and data analysis**

168 To prepare cells for RNA extraction, an overnight culture grown under antibiotic
169 selection was used to inoculate at 1:100 25 ml of fresh antibiotic-free LB in a 250 ml
170 flask which was then incubated with shaking at 250 rpm in a New Brunswick Innova
171 3100 waterbath at 37°C. Two biological replicates were performed for each strain and
172 samples were removed for RNA extraction at 1 h, 4 h and 6 h after inoculation (Fig.
173 1). Twenty per cent v/v ice-cold RNA stabilization solution (5% v/v phenol: 95%
174 ethanol) was added with mixing and the cultures were immediately incubated on ice
175 for 30 min (Kelly *et al*., 2004; Hinton *et al*., 2004). The cultures were pelleted by
176 centrifugation (3100 *g*, 30 min, 4°C) and pellets were stored at −80°C until required.

177 RNA was extracted using a Promega SV total RNA purification kit as
178 described previously (Kelly *et al*., 2004). RNA concentrations were determined by
179 absorbance at 260 nm, and RNA quality was assessed using the Bioanalyzer 2100
180 (Agilent Technologies, Palo Alto, CA) as directed by the manufacturers. Total RNA

181 was diluted to a concentration of 0.4 μg $\mu l^{-1}$, and 4 μg of total RNA was used to make
182 labeled cDNA as described in Eriksson *et al*. (2003). Hybridization, microarray
183 scanning and data analysis were carried out as described previously (Kelly *et al*.,
184 2004). The microarray data are provided in Table S2, and are in MIAME-compliant
185 format. The data have been submitted to the GEO database (accession number
186 GSE22860).
187
188 **Corroboration of microarray data**
189 RT-PCR analyses were performed on transcripts using gene-specific primer pairs
190 (Table S1). Primers were designed *in silico* (http://frodo.wi.mit.edu/cgi-
191 bin/primer3/primer3_www.cgi) to minimize primer–primer complementarity and to
192 yield predicted amplicons in the 150–250 bp range. Total RNA was isolated using the
193 SV40 total RNA isolation Kit (Promega), and reverse transcribed in 50 μl of
194 Stratascript first-strand buffer in the presence of 0.4 mM dNTPs, 300 ng of random
195 hexamers and 40 U Stratascript (Stratagene) according to the manufacturer's protocol.
196 Reactions were subjected to one cycle of 94$^{o}$C for 3 min, followed by 20–40 cycles of
197 94$^{o}$C for 1 min, 55$^{o}$C for 1 min and 72$^{o}$C for 1 min, followed by a final extension of
198 72$^{o}$C for 10 min.
199 Sampling of PCR reactions throughout the RT-PCR cycle was performed
200 according to Grifantini *et al*. (2003), and products were resolved by electrophoresis on
201 a 2% TAE agarose gel allowing PCR regimes for the transcripts of interest to be
202 optimized at given time points. PCR product intensities on the resulting gels were
203 measured using the image capture facility and densitometry was performed using
204 ImageJ (NIH) software. The expression of a gene at any time point in a given mutant
205 background was recorded as a multiple of the value for the cognate wild-type sample

206 obtained using the same cycling parameters. The STM3400 *yrdB* gene did not
207 respond to the absence of the HU proteins and was used as a negative control in the
208 RT-PCR experiments (data not shown).
209
210 **Measurement of competitive fitness**
211 The competitive fitness of each of the strains was established by competitive growth
212 assays using *S.* Typhimurium SL1344, SL1344*hupA*::*kan*, SL1344*hupB::cat,* SL1344
213 *hupA*::*kan hupB::cat* as test strains and the comparator strain *S.* Typhimurium SL1344
214 $Nal^r$, a spontaneously nalidixic acid resistant mutant, the mutation for which does not
215 significantly affect the competitive fitness of the strain (Doyle *et al.*, 2007). Briefly,
216 each strain was grown overnight in LB, pH 7.0, and overnight cultures were used to
217 establish initial (founder) populations of $10^5$ cfu $ml^{-1}$ in fresh LB broth, pre-warmed to
218 $37^oC$. Cultures were gently mixed, and then samples taken and serially diluted on
219 fresh LB agar with selection appropriate to each of the strains under study. The
220 culture was grown at 37˚C with agitation for 18 h, and the composition of the final
221 populations were determined by serial dilution of the 18-h samples on fresh LB agar
222 supplemented with appropriate selective agents. Competitive fitness was established
223 as described previously (Doyle *et al.*, 2007). Measurements were conducted in
224 triplicate on at least three independent occasions, and each datum point presented is
225 the average of at least nine measurements.
226
227 **Western blotting**
228 The RpoS sigma factor was detected in equivalent amounts of total protein (Bradford,
229 1976) from wild-type and *hup* mutant strains (grown in LB for 6 h) by Western

230 blotting using an antibody from NeoClone (Madison, WI) as described previously
231 (Mangan *et al*., 2006).
232
233 **Macrophage infection and epithelial cell invasion**
234 Macrophage infection assays were performed on the J774.1 macrophage cell line
235 (American Type Culture Collection) maintained in Dulbecco's modified Eagle's
236 medium with 10% fetal bovine serum. Epithelial cell invasion assays were performed
237 with and CHO epithelial cell lines (American Type Culture Collection) maintained in
238 Dulbecco's modified Eagle's medium with F12 Nutrient mix supplemented with 10%
239 fetal bovine serum. Macrophage survival assays were performed with J774.1
240 macrophage-like cells. Both assays were carried out essentially as described
241 previously (Marshall *et al.,* 2000). Overnight bacterial broth cultures were diluted
242 1:100 and incubated with shaking at 37°C overnight. The bacteria were harvested, re-
243 suspended in pre-warmed culture medium and added to CHO or J774.1 cells grown in
244 12-well plates at a multiplicity of infection of 100:1. Plates were spun at 600 g for
245 5 min and then incubated at 37°C in 5% $CO_2$ for 2 h to allow invasion (epithelial
246 cells) or phagocytosis (macrophage) to occur. The mammalian cells were then washed
247 and incubated with fresh medium containing 100 μg $ml^{-1}$ gentamicin for 1 h to kill
248 extracellular bacteria. The monolayers were washed and lysed by the addition of PBS
249 containing 0.5% (v/v) Triton X-100 to release the intracellular bacteria. The number
250 of bacterial colony-forming units recovered was determined following serial dilution
251 and plating on LB. The presence of the *hup* mutations had no effect on the sensitivity
252 of the bacteria to Triton X-100 (data not shown). Experiments were repeated three
253 times and typical data were reported.
254

255 **Separation of plasmid topoisomers by gel electrophoresis**

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

268

269

270 **RESULTS AND DISCUSSION**

271

272 **Identification of three HU regulons**

273 Mutants of the *S.* Typhimurium strain SL1344 were constructed with knockout

274 mutations in the genes coding for the $\alpha$ subunit of HU (*hupA,* CJD2003; $\mu_{max}$ = 0.047

275 $h^{-1}$), the $\beta$ subunit (*hupB,* CJD2004; $\mu_{max}$ = 0.04 $h^{-1}$), or both subunits (CJD2005; $\mu_{max}$

276 = 0.1 $h^{-1}$) (Table 1). The single mutants had growth profiles that were similar to that

277 of the wild type ($\mu_{max}$ = 0.05 $h^{-1}$) when growing in LB broth under aerobic conditions

278 at 37˚C; the double mutant initially showed a reduced growth rate but on entering the

279 exponential phase of growth it grew rapidly eventually attaining a culture optical

280 density similar to that of the wild type by end of exponential growth (Fig. 1). DNA

281 microarrays were used to analyze the *S.* Typhimurium transcriptome in the presence

282 and absence of the *hup* genes at three time points (1 h, 4 h, and 6 h) in cultures

283 growing in LB liquid medium at 37˚C.

284 The numbers of genes (ranging from 50 to 482) that were up- or down-

285 regulated differed for each of the HU mutants at each of the time points, yielding a

286 total of 18 data points (Table 2). The mutations in just *hupA* or in both *hupA* and *hupB*

287 affected approximately similar (but not identical) numbers of genes. Far fewer genes

288 were up- or down-regulated in the *hupB* single mutant at 1 h and 6 h but this mutant

289 had the largest number of genes up-regulated at 4 h. These differences revealed

290 distinct regulons of genes whose expression was influenced by the three forms of HU,

291 and are apparent when the effects of the *hup* mutations on different functional groups

292 of genes are considered; a data summary is shown in Fig. 2 where it can be seen that

293 mutations in *hupA, hupB* and in both *hup* genes influenced a variety of metabolic

294 processes such as ATP synthesis, the TCA cycle and fatty acid metabolism. Among

295 horizontally-acquired genetic elements, the virulence genes in the pathogenicity
296 islands SPI1 and SPI2 were down-regulated at all three time points (Figs. 3 & 4).
297 However, the effects differed between the three *hup* mutants and at the different time
298 points.
299
300 **HU and SPI1 gene expression**
301 A complex regulatory cascade involving genes located within the SPI1 pathogenicity
302 island and other regulatory genes lying elsewhere on the chromosome controls the
303 transcription of the invasion genes in SPI1 (Rhen & Dorman, 2005). Three SPI1
304 regulatory genes encode the AraC-like transcription factors HilA, HilC and HilD that
305 up-regulate the SPI1 type III secretion apparatus genes and the effector protein genes.
306 The genes *hilA, hilC* and *hilD* were down-regulated in the *hupA* and *hupA hupB*
307 mutants at all three time points in the microarray data (Fig. 3). This was confirmed by
308 RT-PCR and sample data are provided for *hilA* (Fig. 3). These transcriptomic data
309 agreed with previous findings that knockout mutations in *hupA* or *hupA* and *hupB*
310 resulted in decreased expression of these three *hil* regulatory genes (Schechter *et al.,*
311 2003). We found that *hilA, hilC* and *hilD* were also down-regulated in the *hupB*
312 mutant at the 1-h and 4-h time points, but were up-regulated at 6 hours (Fig. 3). This
313 may account for an earlier observation that a mutation in *hupB* has a positive effect on
314 the expression of *hilA* (Fahlen *et al.,* 2000). The target promoters of *hilA, hilC* and
315 *hilD* displayed a similar expression pattern. The structural genes *invA, invF,* and *invH*
316 were down-regulated in each of the mutants at all of the time points, with the
317 exception of the *hupB* mutant at 6 h where each of the three genes was up-regulated
318 (Fig. 3). Similarly, the SPI1 structural genes *sipA, sipC* and *sipD* showed a similar
319 response to the absence of the HU proteins (Fig. 3). Our results reveal an important

320 role for each subunit of HU in modulating the expression of the SPI1-encoded
321 virulence factors throughout bacterial growth. This led us to predict that mutations in
322 the genes coding for HU influence the invasiveness of *S.* Typhimurium.
323
324 **Mutations in the *hup* genes alter invasiveness in epithelial cells**
325 The *hup* mutants were compared with the parental strain SL1344 for their ability to
326 invade and proliferate in cultured epithelial cells. The invasion assays were performed
327 as described in Methods. Bacteria were recovered from the mammalian cells after 2 h
328 and the numbers of colony-forming units (cfu) were determined (Table 3). Loss of
329 HU β had only a minor effect on the survival of *S.* Typhimurium growing
330 intracellularly and was not statistically significant, in agreement with results from an
331 earlier study (Fahlen *et al.,* 2000). Inactivation of *hupA* also resulted in a small
332 reduction in invasion that was not statistically significant. On the other hand, the *hupA*
333 *hupB* double mutant showed a marked reduction in epithelial cell invasion (Table 3).
334 These data were consistent with those showing that inactivation of both the *hupA* and
335 *hupB* genes resulted in down regulation of the SPI1 virulence genes whereas the *hupB*
336 mutation had a less severe negative effect and even had a positive influence on
337 transcription at the 6-h time point (Fig. 3).
338
339 **SPI2 gene expression, macrophage survival and the HU regulon**
340 The SPI2 pathogenicity island contains genes coding for a second type III secretion
341 system and its cognate effector proteins that enable *S.* Typhimurium to survive in the
342 hostile environment of the macrophage. SPI2 genes were also down-regulated in the
343 *hupA hupB* double mutant at all three time points (Fig. 4). In particular, the genes
344 coding for the SsrA/B two-component master regulator were down-regulated, as were

345 those coding for the type III secretion apparatus (*ssa*). In contrast, the effector protein
346 genes (*sse*) showed only minor or no reductions in expression at the 1-h time point,
347 although they too were strongly down-regulated at later stages of growth.

348 The single *hupA* mutant displayed reduced expression of the *ssrA ssrB*
349 regulatory genes, especially at the 1 and 4-h time points; these genes were down-
350 regulated in the *hupB* mutant only at the 1-h time point. The *ssa* secretion apparatus
351 genes were down-regulated in the *hupA* mutant at the 1 and 4-h time points; at 6 hours
352 they were up-regulated in the absence of the HU $\alpha$ protein. The knockout mutation in
353 the *hupB* gene had little or no effect on *ssa* gene expression at any time point.

354 The *sse* genes coding for the effector proteins showed reduced expression in
355 the *hupA* mutant at 1 and 4 hours; at the 6-h time point there was no detectable effect
356 on their transcription. Inactivation of just *hupB* had little effect on *sse* gene expression
357 at any of the three time points.

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

367

368 **Flagella gene expression and motility are HU-dependent**

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

376 The effect of the *hup* mutations on the flagellar genes was complex and varied
377 with the mutation and the time point (Fig. 5). For example, genes involved in flagellar
378 biosynthesis, such as *flgK,* were up-regulated in the absence of *hupB* at all three time
379 points but down-regulated in the *hupA hupB* double mutant at the same three time
380 points. The *hupB* mutation resulted in the up regulation of most of the flagellar genes
381 tested at least at some time points; in contrast the mutation in *hupA* (and the double
382 mutation) resulted in down regulation in almost all cases.

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

389

390 **Respiration and metabolism**

391 The most dramatic effects on the expression of genes involved in respiration and
392 metabolism occurred at the 4-h time point (Fig. S2). Genes involved in aerobic and in

393 anaerobic respiration showed altered expression in the *hup* mutants. In particular, the
394 *cyo* genes coding for cytochrome o ubiquinol oxidase were strongly up-regulated at
395 the 4-h time point in the *hupB* and *hupA hupB* mutants. Inactivation of *hupA* alone did
396 not alter their expression. This was in contrast to the situation in *E. coli,* where these
397 genes are positively regulated by HU (Oberto *et al.,* 2009). The *narH* gene, coding for
398 nitrate reductase A, was very strongly upregulated at the 4-h time point, but only in
399 the *hupA* mutant. The *narH* gene is required for anaerobic respiration in the presence
400 of nitrate and would normally be repressed in mid-exponential phase cultures growing
401 under aerated conditions. HU has been reported to regulate *narH* positively in *E. coli*
402 (Oberto *et al.,* 2009) whereas *hupA* acts negatively on *narH* transcription in
403 *Salmonella* (Fig. S2). The *nrf* genes, coding for nitrite reductase, were up-regulated in
404 the *hupA* mutant (Fig. S2) in agreement with data for *E. coli* (Oberto *et al.,* 2009). The
405 *nrf* genes are usually expressed in bacteria growing in the absence of oxygen and in
406 the presence of nitrite (Clarke *et al.,* 2008). They were down-regulated in the *hupB*
407 and the *hupA hupB* mutants. These data show that HU α and HU β play distinct roles
408 in organizing the gene expression patterns of the cell under aerobic and anaerobic
409 growth conditions and that the detail of their contributions is not always equivalent in
410 *E. coli* and *Salmonella*.

411 Propanediol utilization is an important feature of *S.* Typhimurium growth in
412 the lumen of the mammalian gut (Bobik *et al.,* 1999; Cheng & Bobik, 2010; Lawhon
413 *et al.,* 2003). The horizontally-acquired *pdu* genes showed a strong response to *hup*
414 mutations at the 4-h time point (Fig. S4). Here, loss of HU α expression resulted in up
415 regulation of the *pdu* operon whereas loss of both HU subunits caused these genes to
416 be down-regulated. The mutation in *hupB* alone had only a minor influence on *pdu*
417 transcription.

418 ABC transporters play key roles in nutrient uptake and scavenging (Davidson
419 *et al.,* 2008). The expression of genes coding for different transporters responded
420 strongly to the loss of HU protein expression. However, the details of the response
421 varied from system to system: e.g. expression of the dipeptide permease operon *dpp*
422 was most affected in the *hupB* mutant, while the oligopeptide permease operon *opp*
423 was affected most strongly in the *hupA* strain and *liv* gene expression was affected
424 most in the *hupA hupB* double mutant (Fig. S3).
425

426 **The *gal* operon and regulon**

427 The role of the HU protein in the regulation of transcription has been worked out in
428 most detail in the case of the galactose regulon, where the GalR DNA binding protein
429 represses the expression of genes involved in galactose uptake and utilization and in
430 the transport and metabolism of other carbon sources. HU acts in the *gal*
431 repressosome to facilitate the action of the GalR protein in association with DNA
432 looping and supercoiling (Lewis *et al.,* 2010; 1999; Semsey *et al.,* 2004). In *S.*
433 Typhimurium, inactivation of *hupA* resulted in the up regulation of the *gal* regulon
434 genes, in keeping with the role of HU as a co-repressor (Table S2). However, the
435 effect was seen only at the 1-h time point and only the *hupA* mutant showed a
436 significant effect. These data suggest that in *S.* Typhimurium, HU β acts to modulate
437 the action of HU α in the Gal repressosome.
438

439 **HU and competitive fitness**

440 Measuring the fitness of different bacterial strains in competition with a common
441 competitor is a useful method of assessing the impact of mutations on the overall
442 physiology of the organisms. The competitive fitness of the SL1344 parental strain

443 and those of its *hup* mutant derivatives were compared as described in Methods. Our
444 results showed that the *hupA* mutation diminished the fitness of SL1344 whereas the
445 *hupB* lesion had a positive influence (Table 3). Combining both mutations produced a
446 fitness value similar to that seen when the *hupA* lesion alone was present. The
447 negative effect of the *hupA* mutation on fitness was in line with our expectations
448 given the number of genes whose expression was altered in its presence. Also, the
449 absence of a negative effect on fitness due to the *hupB* mutation alone was consistent
450 with the large body of data in this study showing that *hupA* and *hupB* knockout
451 mutations have distinct impacts on cellular physiology.
452
453 **HU and the RpoS regulon**
454 A previous study established a role for HU in the posttranscriptional control of the
455 RpoS stress and stationary phase sigma factor in *E. coli* K-12 strain C600 (Balandina
456 *et al.,* 2001). We found that in *Salmonella,* RpoS protein expression was also reduced
457 by the inactivation of both *hup* genes and the earlier data for *E. coli* C600 (Balandina
458 *et al.*, 2001) were confirmed here (Fig. S5).
459 In our transcriptomic analysis, few members of the RpoS regulon (Lacour &
460 Landini, 2004; Patten *et al.,* 2004; Weber *et al.*, 2005) were found to respond to the
461 presence of knockout mutations in the *hup* genes, when present either individually or
462 together, with the exception of the 4-h time point (Fig. 6; Tables S2-S7). This time
463 point represented the transition from exponential growth to stationary phase in the
464 wild type and the two single *hup* mutants (Fig. 1) and was equivalent to the 'transition
465 phase' described in an earlier study of HU in *E. coli* (Oberto *et al.,* 2009). Here,
466 inactivation of *hupA* resulted in the down regulation of many RpoS-dependent genes
467 while the *hupB* mutation caused the same genes to be expressed at a higher level. The

468 effect of the double *hupA hupB* mutation varied from gene to gene (Fig. 6). This
469 pattern differed from that reported previously for *E. coli hup* mutants, where the
470 mutations had either a mild or a negligible effect on the expression of the RpoS
471 regulon (Oberto *et al.,* 2009).
472
473 **HU and IHF**
474 The HU and IHF proteins are closely related at the level of amino acid sequence but
475 have distinct interactions with DNA (Swinger & Rice, 2004). The α and β subunits of
476 the integration host factor (IHF) are encoded by the *ihfA* and *ihfB* genes which were
477 among the most severely down-regulated in the *hupA hupB* double mutant at all three
478 time points (Fig. S1; Tables S2-S7). We have previously determined the regulon of
479 IHF in *S.* Typhimurium strain SL1344 and discovered that it includes the *hupA* and
480 *hupB* genes, both of which are up-regulated in an *ihfA ihfB* double mutant (Mangan *et*
481 *al.,* 2006). These results revealed a reciprocal regulatory relationship between HU and
482 IHF at the level of transcription in which IHF represses *hup* gene transcription and
483 HU up-regulates the transcription of *ihfA* and *ihfB*. It is not known whether the
484 regulatory effects are direct, i.e. if each nucleoid associated protein (NAP) binds at the
485 promoters of the genes coding for the other. Our finding that HU positively regulates
486 IHF expression may reflect some overlap between the regulons of genes under the
487 control of these two NAPs. Certainly, the classes of genes that show altered
488 expression in the absence of HU and IHF show a considerable overlap: the SPI1 and
489 SPI2 virulence genes, the chemotaxis and motility genes discussed above are HU-
490 dependent and are also influenced by IHF (Mangan *et al.,* 2006). However, the details
491 of the responses vary. For example, most SPI1 virulence genes are down-regulated in
492 the absence of either HU or IHF. However, SPI1 regulatory genes are upregulated in

493 the *ihfA ihfB* double mutant at the 6-h time point whereas they are down-regulated in
494 the *hupA hupB* mutant at all time points. There is a much closer correspondence
495 between the effects of HU and IHF among the genes of the SPI2 pathogenicity island:
496 the *ssrA ssrB* regulatory genes are down-regulated in the absence of either HU or IHF,
497 as are the genes coding for the secretion apparatus and the effector proteins. The
498 motility (*mot*) and chemotaxis (*che*) genes showed similar responses to the loss of
499 either HU or IHF at all time points but the flagellar genes showed opposite responses
500 at 6 h, being up-regulated in the IHF-deficient strain and down-regulated in the
501 absence of HU. It is important to recall that the loss of HU results in strong down
502 regulation of *ihf* gene expression whereas the loss of IHF causes the genes coding for
503 HU to be up-regulated. For this reason knockout mutations in *ihf* and in *hup* are not
504 equivalent.
505
506 **Conclusion**
507 This transcriptomic analysis has revealed wide-ranging roles for the HU NAP in
508 controlling the expression of genes involved in adaptation to stress, changes in growth
509 phase, in motility and chemotaxis and in virulence. The study shows that *S.*
510 Typhimurium has not one but three HU regulons since inactivating the two *hup* genes
511 individually and together results in distinct transcriptomic effects. In fact, in several
512 cases (e.g. within the RpoS regulon), the loss of *hupA* or *hupB* has opposite effects on
513 the expression of the same gene (Fig. 6). Presumably this reflects a highly dynamic
514 relationship between HU composition and the HU-dependent genes. It is not known
515 how many of the effects of HU on transcription reported here are direct. The
516 complication that arises due to the influence of HU on the expression of IHF, another
517 global regulator, has been discussed already. HU is also known to influence DNA

518 supercoiling in *E. coli* (Claret & Rouvière-Yaniv, 1997; Guo & Adhya, 2007), and we
519 found that the level of DNA supercoiling of a reporter plasmid was altered in each of
520 the *hup* mutants used in this study (Fig S6). Thus HU may exert effects at promoters
521 through changes in DNA topology rather than through directly acting as a
522 transcription factor or by influencing the expression of another transcription factor.
523 Furthermore, the differences between the transcriptomes of *E. coli* and *Salmonella*
524 mutants deficient in HU may reflect, in part, the fact that DNA in these bacteria is
525 supercoiled to different levels (Champion & Higgins, 2007).
526 Identifying the membership of the three HU regulons in *S.* Typhimurium has
527 shown the breadth of influence of this NAP on gene expression: it is a true global
528 regulator that coordinates the expression of central metabolism and virulence genes in
529 *S.* Typhimurium.

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758

759 **Table 1.** Bacterial strains and plasmids used in this study

| Strain/plasmid | Relevant details | Reference/source |
|---|---|---|
| *Escherichia coli* K-12 | | |
| DH5 α | *endA1 hsdR17(rk⁻mk) glnV44 thi-1 recA1 gyrA*(Nal$^r$) *relA1* Δ(*lacIZYA-argF*) *U169 deoR* (ϕ80*ΔlacZM15*) | Promega |
| C600 (JO2057) | *thr-1 leu86 thi-1 lacY glnV44* | Oberto *et al*. (2009) |
| JO3020 | C600 *hupA*::*cat hupB*::*kan* | Oberto *et al*. (2009) |
| *S*. Typhimurium | | |
| CH607 | *polA-2 zig204::*Tn*10* | Lab stocks |
| CJD2000 | CH607 *hupA*::*cat* | This study |
| CJD2001 | CH607 *hupB*::*kan* | This study |
| CJD2003 | SL1344 *hupA*::*cat* | This study |
| CJD2004 | SL1344 *hupB*::*kan* | This study |
| CJD2005 | SL1344 *hupA*:*cat hupB::kan* | This study |
| SL1344 | *hisG* | Hoiseth & Stocker (1981) |
| SL1344 Nal$^r$ | *hisG nal* | Doyle *et al*. (2007) |
| TH6232 | LT2 Δ*hin7517*::FRT *fliBA*-OFF *fliC*-ON | K.T. Hughes |
| TH6233 | LT2 Δ*hin7517*::FRT *fliBA*-ON *fliC*-OFF | K.T. Hughes |
| Plasmids | | |
| pKD3 | Template for *kan* cassette | Datsenko & Wanner (2000) |
| pKD46 | Inducible λ*redBCD* under $P_{BAD}$ control | Datsenko & Wanner (2000) |
| pSU311 | Template for *cat* cassette | Ussau *et al*. (2001) |
| pUC19 | *ori*pMB1, Ap$^r$ | Yanisch-Perron *et al.* (1985) |

760
761
762

763 **Table 2.** Numbers of genes in the HU regulons

| Time (h) / Strain | 1 h (up) | 1 h (down) | 4 h (up) | 4 h (down) | 6 h (up) | 6 h (down) |
|---|---|---|---|---|---|---|
| *hupA* | 277 | 180 | 323 | 319 | 283 | 299 |
| *hupB* | 50 | 75 | 482 | 275 | 54 | 50 |
| *hupA hupB* | 248 | 134 | 263 | 241 | 208 | 206 |

764
765 The table summarizes the numbers of genes whose expression increased (up) or
766 decreased (down) compared to the SL1344 wild type in the *hupA* and *hupB* single
767 mutants and in the *hupA hupB* double mutant at the 1-h, 4-h and 6-h timepoints.

768 **Table 3.** Comparison of phenotypes of HU strains.

| Phenotype | Comparator | SL1344 *hupA* | SL1344 *hupB* | SL1344 *hupA hupB* |
|---|---|---|---|---|
| Epithelial cell invasion | SL1344 | 53.5% (± 30%) | 64% (± 33%) | 24 (± 6%)* |
| Survival following macrophage uptake | SL1344 | 88.7(± 14%) | 111 (± 15%) | 22 (± 3%)* |
| Relative competitive fitness | SL1344Nal$^r$ | 64% (± 6%) | 110 (± 4%) | 61% (± 6%) |
| Relative motility | SL1344 | 88% (± 3%)* | 50% (± 2%)* | 38% (± 2%)* |

769

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

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

781 **Figure legends**

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

783 Growth curves are shown for the parental strain, SL1344 and its *hup* mutant

784 derivatives: *hupA* (open squares), *hupB* (open triangles) and the double mutant

785 (inverted filled triangles) growing in LB with aeration at 37˚C. The vertical arrows

786 indicate the three time points (1 h, 4 h and 6 h) at which RNA was extracted for the

787 transcriptomic analyses.

788

789 **Fig. 2.** Functional groups of genes influenced by HU. Data are presented for named

790 functional groups from the Kyoto Encyclopedia of Genes and Genomics (KEGG) at

791 1, 4 and 6 h post inoculation. The percentages of genes up regulated or down

792 regulated at each time point is shown by the bars in the histograms. Down regulation

793 is shown by bars to the left and up regulation by bars to the right of zero. Data are

794 presented for the *hupA* mutant (grey bars), the *hupB* mutant (white bars) and the *hupA*

795 *hupB* double mutant (black bars).

796

797 **Fig. 3.** Effect of the *hup* mutations on gene expression within the SPI1 pathogenicity

798 island. Data are presented for SPI1 regulatory genes (top) and SPI1 invasion genes

799 (centre) in mutants deficient in *hupA* (A), *hupB* (B) and *hupA hupB* (AB) at each of

800 the three time points (1, 4 and 6 h). In each case the gene expression data were

801 normalized to SL1344 for the 1 (black), 4 (white) and 6 h (grey) time points and the

802 ratio of the mutant/wild type was calculated. Expression ratios less than 1 indicate

803 genes normally activated by HU at that time point while ratios greater than 1 show

804 where and when HU acts negatively. RT-PCR data for the *hilA* transcript as expressed

805 in each of the *hup* mutants relative to the wild type are provided in the bottom panel.

806

807 **Fig. 4.** Effect of the *hup* mutations on SPI2 gene expression. Expression data are
808 presented for the SPI2 regulatory genes *ssrA* and *ssrB* (top), representative SPI2 type
809 III secretion system apparatus genes (middle) and representative SPI2 effector protein
810 genes (bottom) in mutants deficient in *hupA* (A), *hupB* (B) and *hupA hupB* (AB) at
811 each of the three time points (1, 4 and 6 h). In each case the gene expression data were
812 normalized to SL1344 for the 1 (black), 4 (white) and 6 h (grey) time points and the
813 ratio of the mutant/wild type was calculated. Expression ratios less than 1 indicate
814 genes normally activated by HU at that time point while ratios greater than 1 show
815 where and when HU acts negatively.

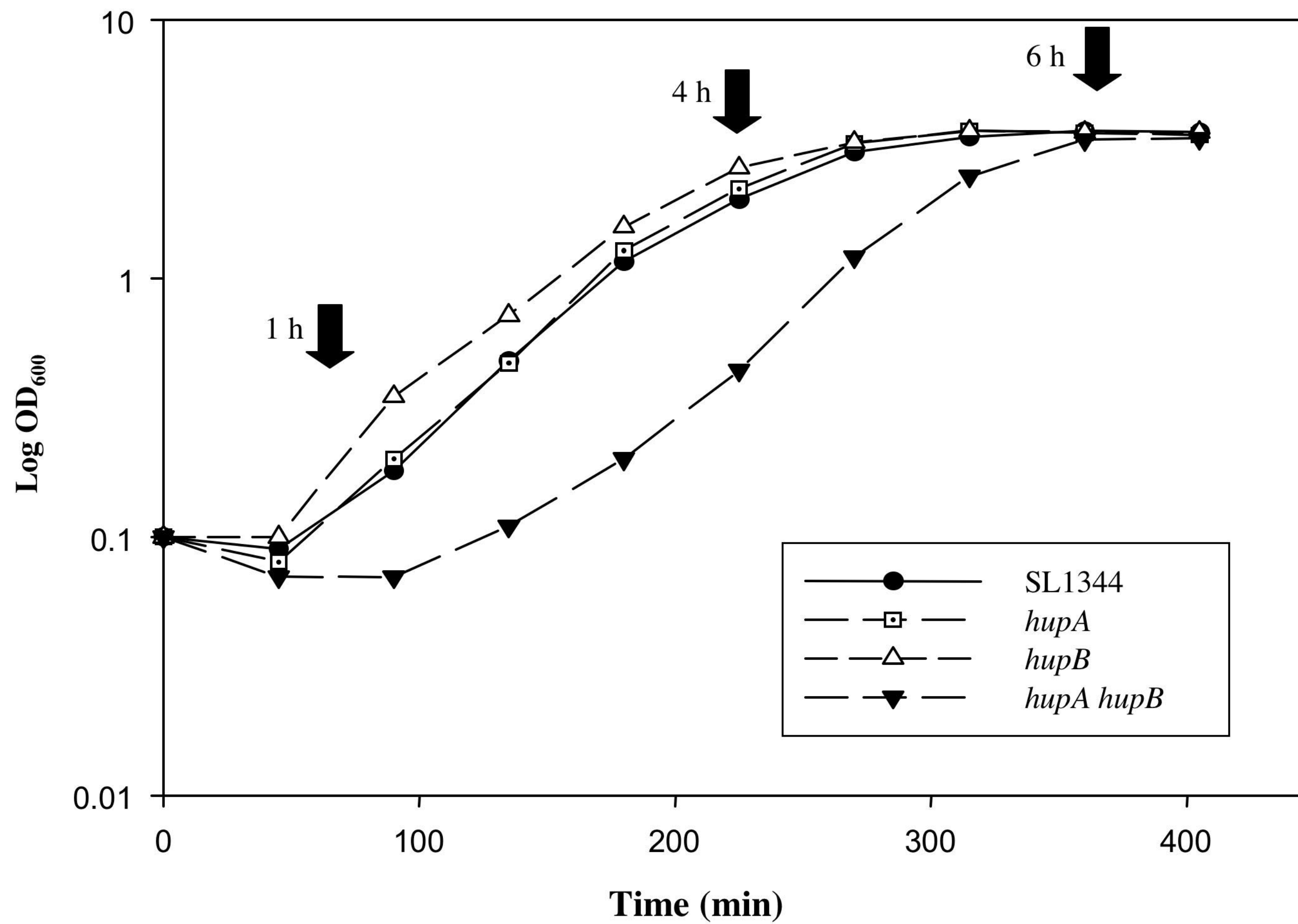
816

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

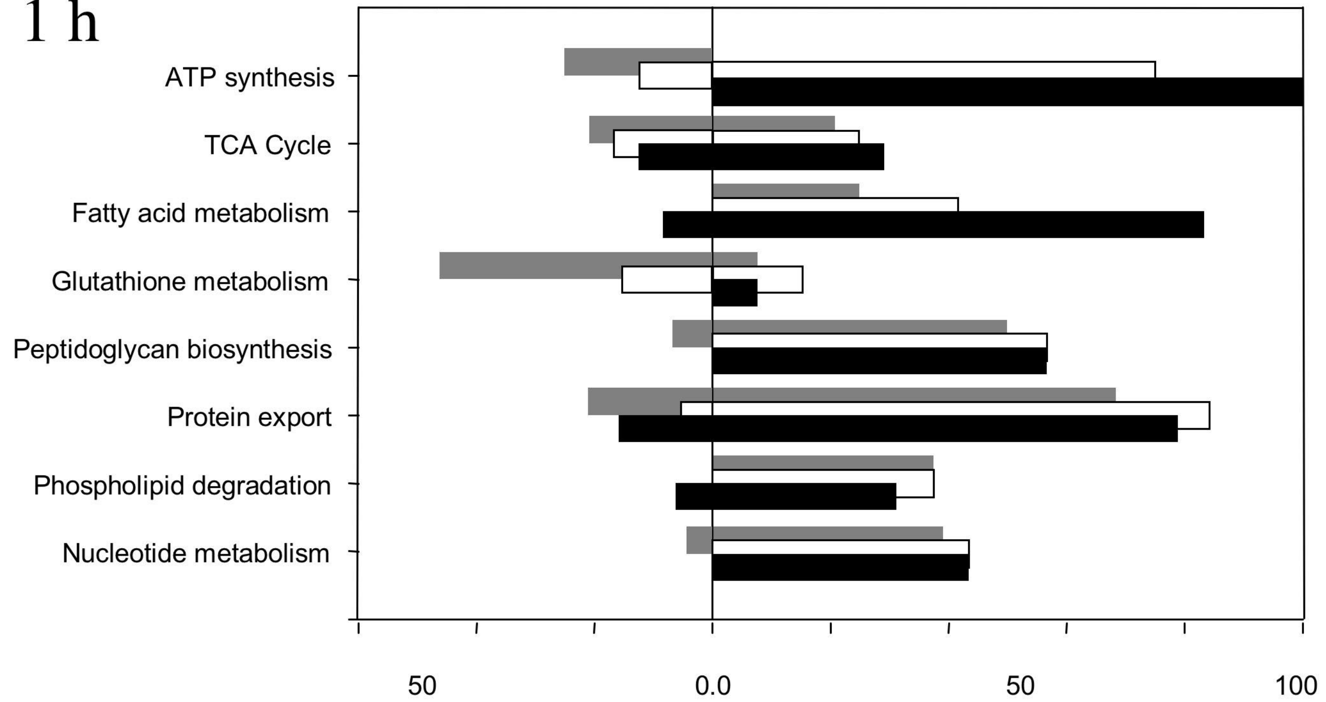
825

826 **Fig. 6.** Altered expression of RpoS regulon members in the *hup* mutants. Data are
827 presented for representative members of the RpoS regulon in mutants deficient in
828 *hupA* (A), *hupB* (B) and *hupA hupB* (AB) at the 4-h time point. In each case the gene
829 expression data were normalized to SL1344 and the ratio of the mutant/wild type was
830 calculated. Expression ratios less than 1 indicate genes normally activated by HU at

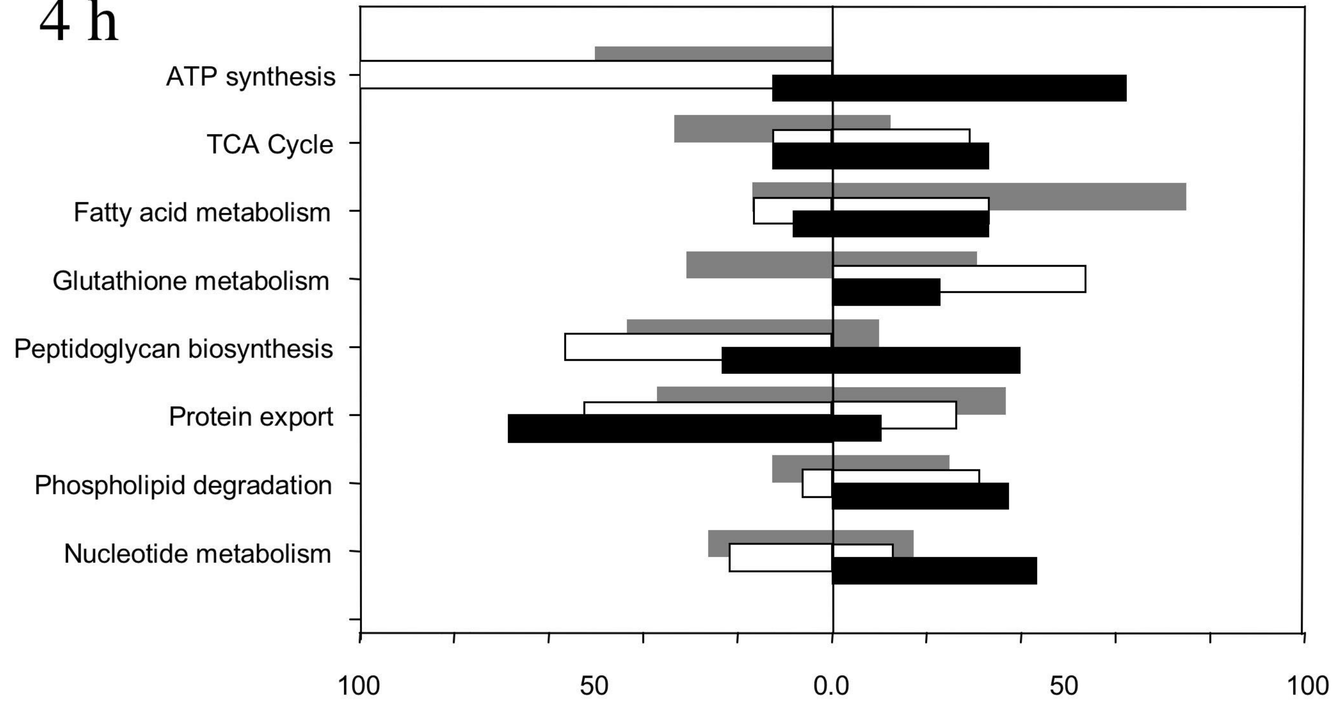
831 that time point while ratios greater than 1 show where HU acts negatively.

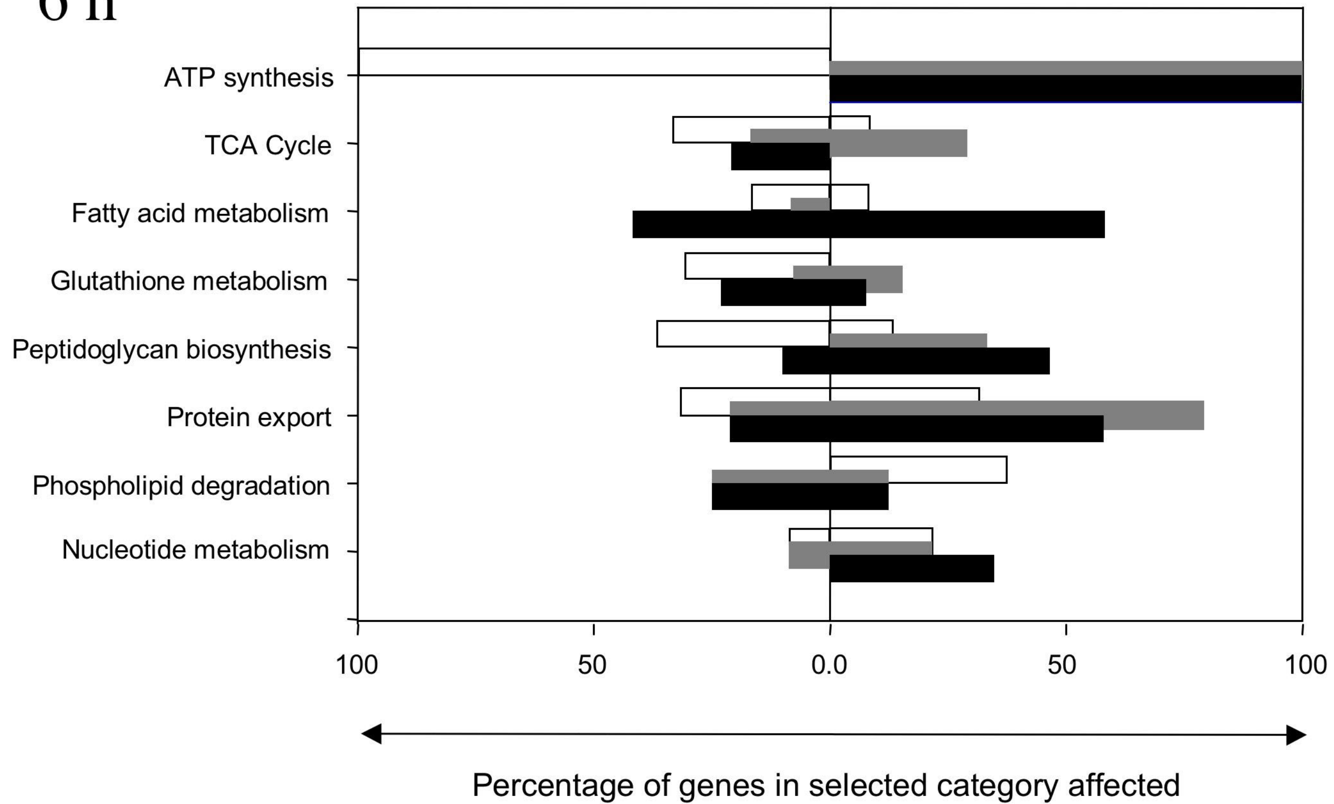


10
1
0.1
0.01
Log OD600
0
100
200
300
400
Time (min)
1 h
4 h
6 h
SL1344
hupA
hupB
hupA hupB

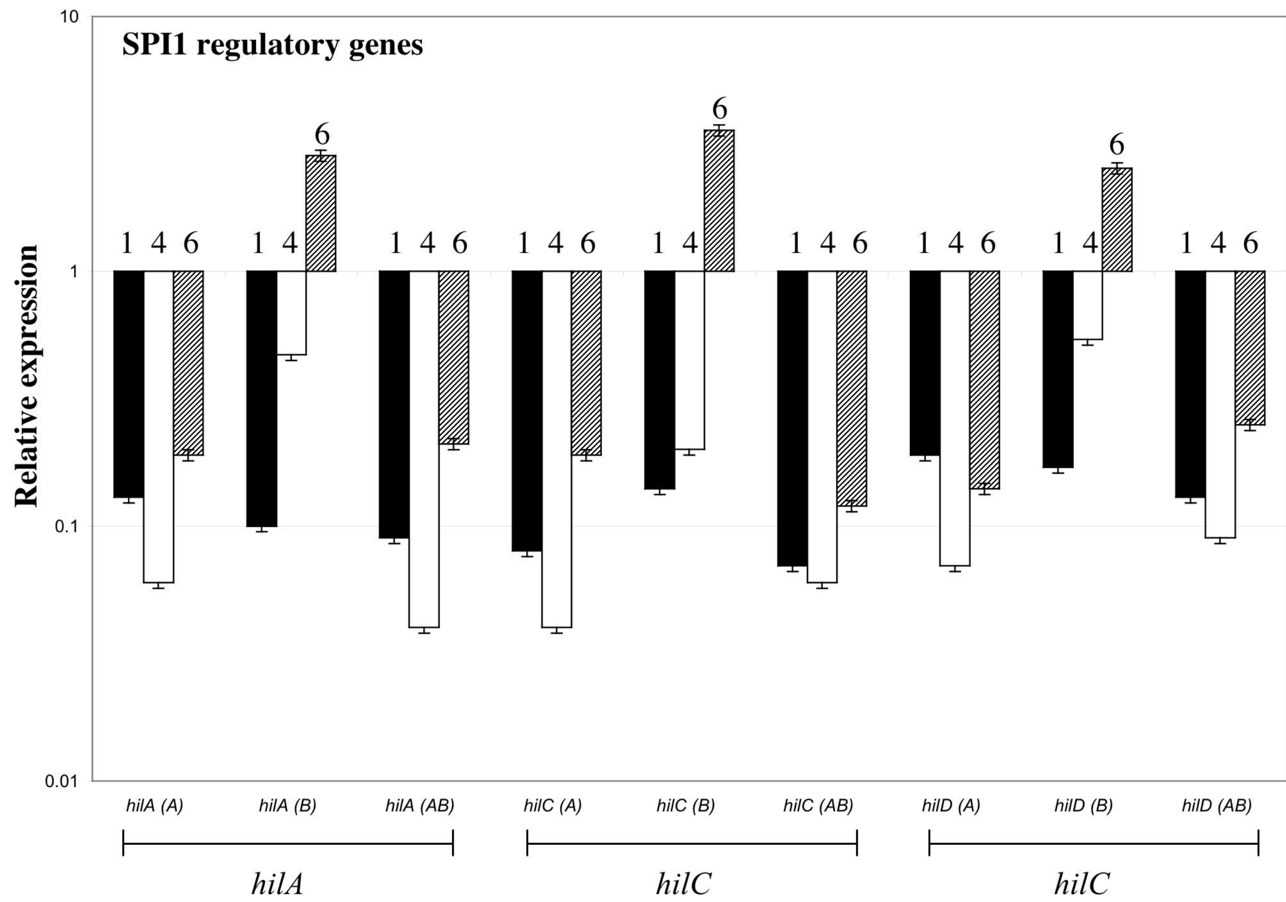


1 h
ATP synthesis
TCA Cycle
Fatty acid metabolism
Glutathione metabolism
Peptidoglycan biosynthesis
Protein export
Phospholipid degradation
Nucleotide metabolism
50
0.0
50
100

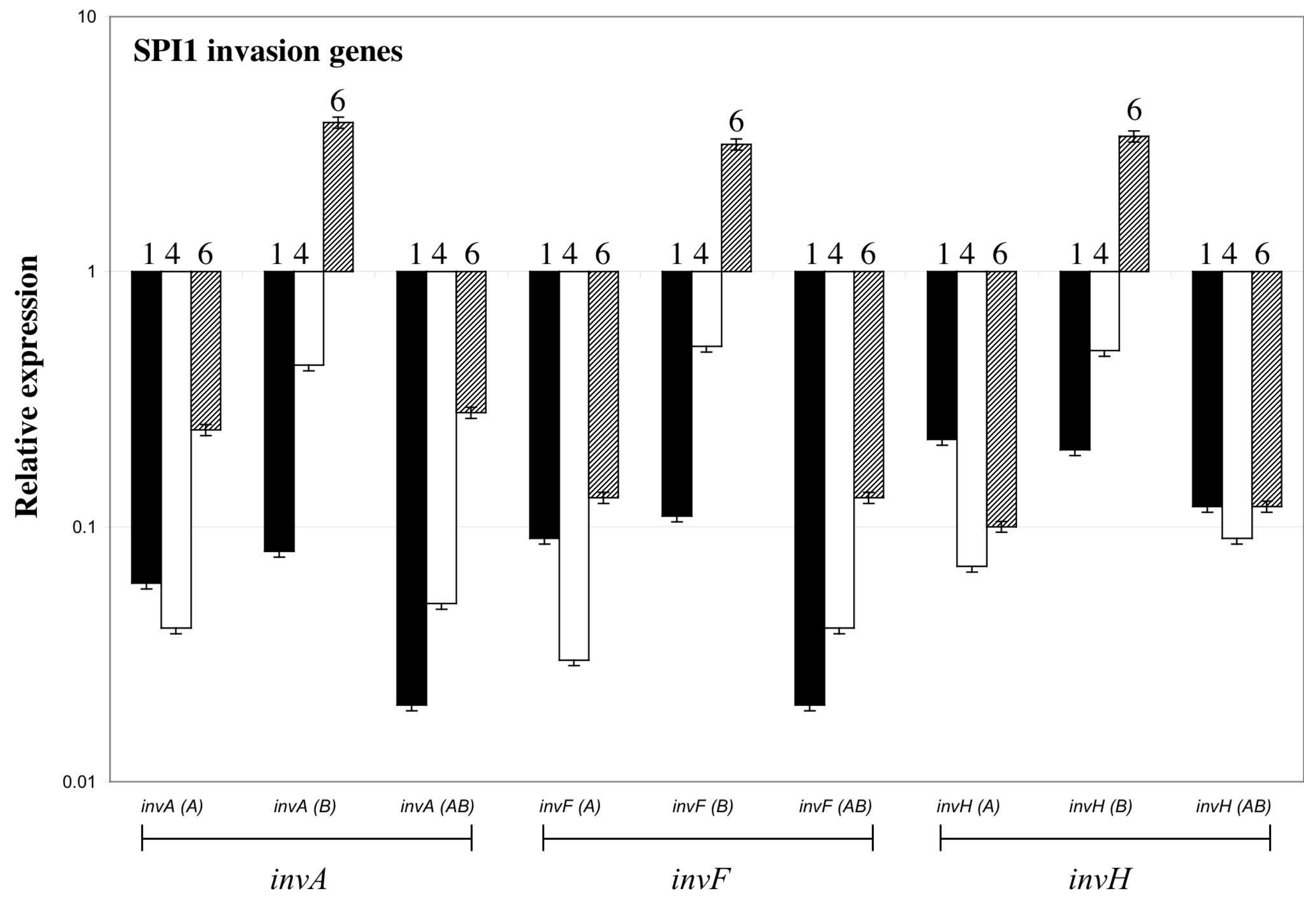

4 h
ATP synthesis
TCA Cycle
Fatty acid metabolism
Glutathione metabolism
Peptidoglycan biosynthesis
Protein export
Phospholipid degradation
Nucleotide metabolism
100
50
0.0
50
100

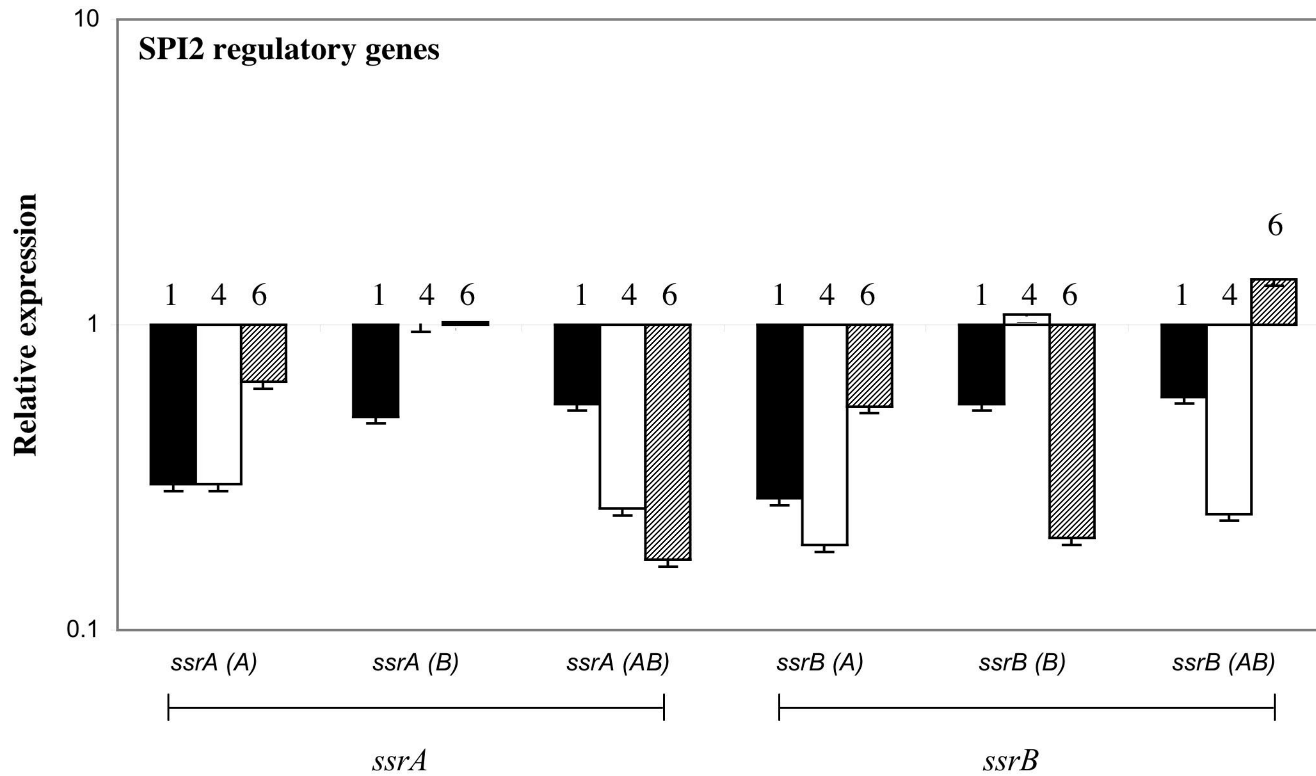


6 h
ATP synthesis
TCA Cycle
Fatty acid metabolism
Glutathione metabolism
Peptidoglycan biosynthesis
Protein export
Phospholipid degradation
Nucleotide metabolism
100
50
0.0
50
100
Percentage of genes in selected category affected

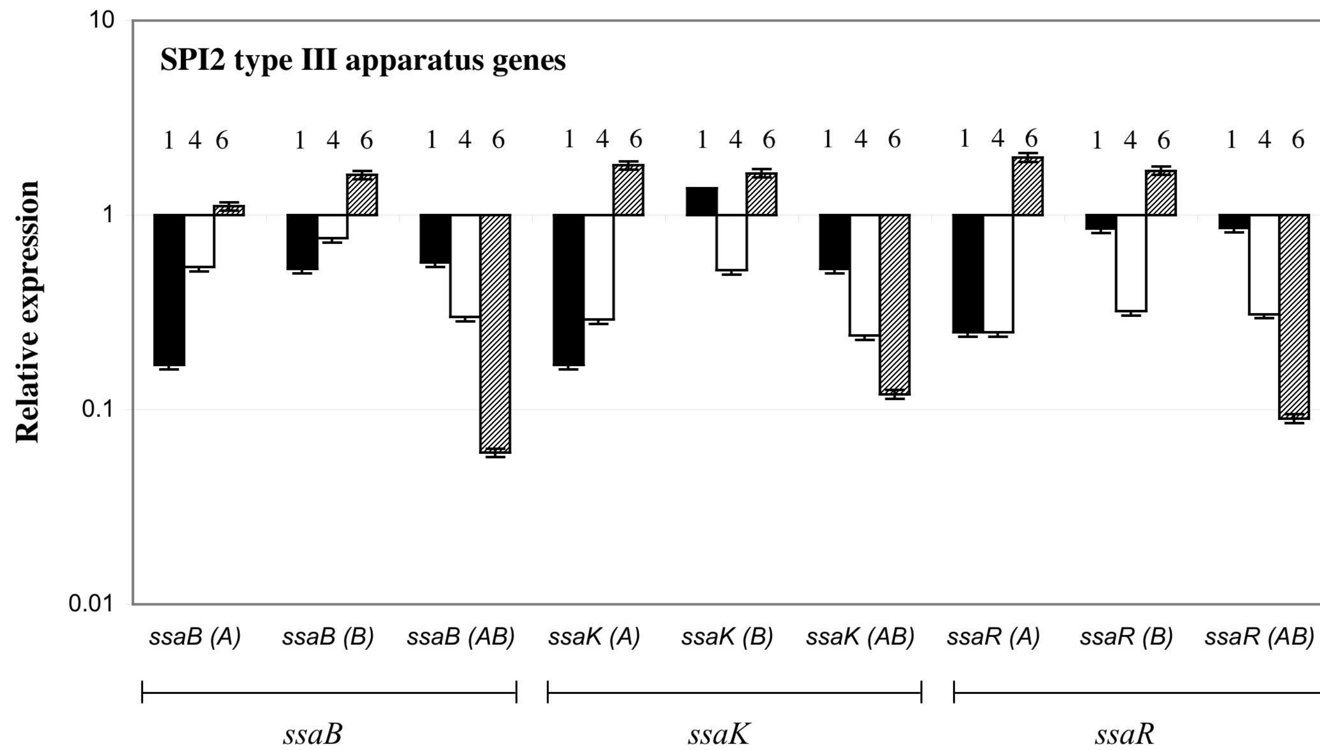


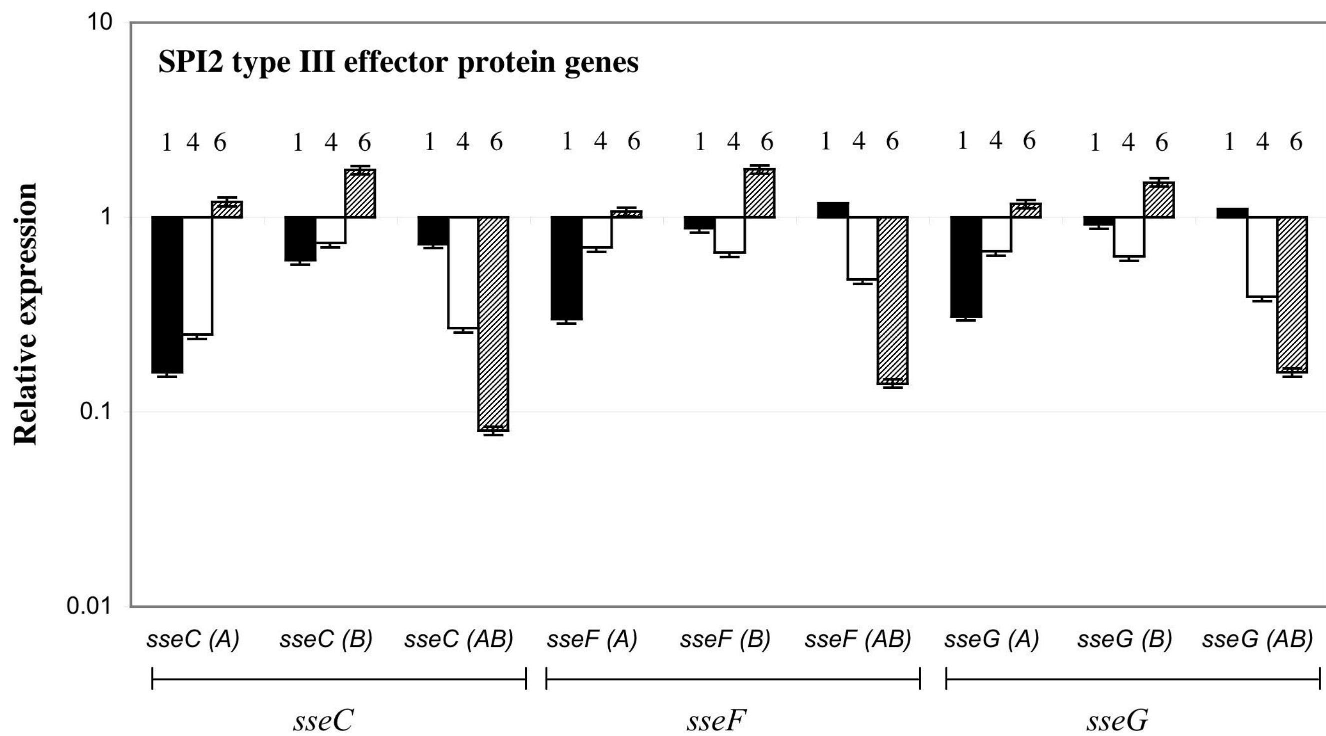
SPI1 regulatory genes
Relative expression
10
1
0.1
0.01
6
6
6
1 4 6
1 4
1 4 6
1 4 6
1 4
1 4 6
1 4 6
1 4
1 4 6
hilA (A)
hilA (B)
hilA (AB)
hilC (A)
hilC (B)
hilC (AB)
hilD (A)
hilD (B)
hilD (AB)
hilA
hilC
hilC


SPI1 invasion genes
Relative expression
10
1
0.1
0.01
1 4 6
1 4
6
invA (A)
invA (B)
invA (AB)
invF (A)
invF (B)
invF (AB)
invH (A)
invH (B)
invH (AB)
invA
invF
invH

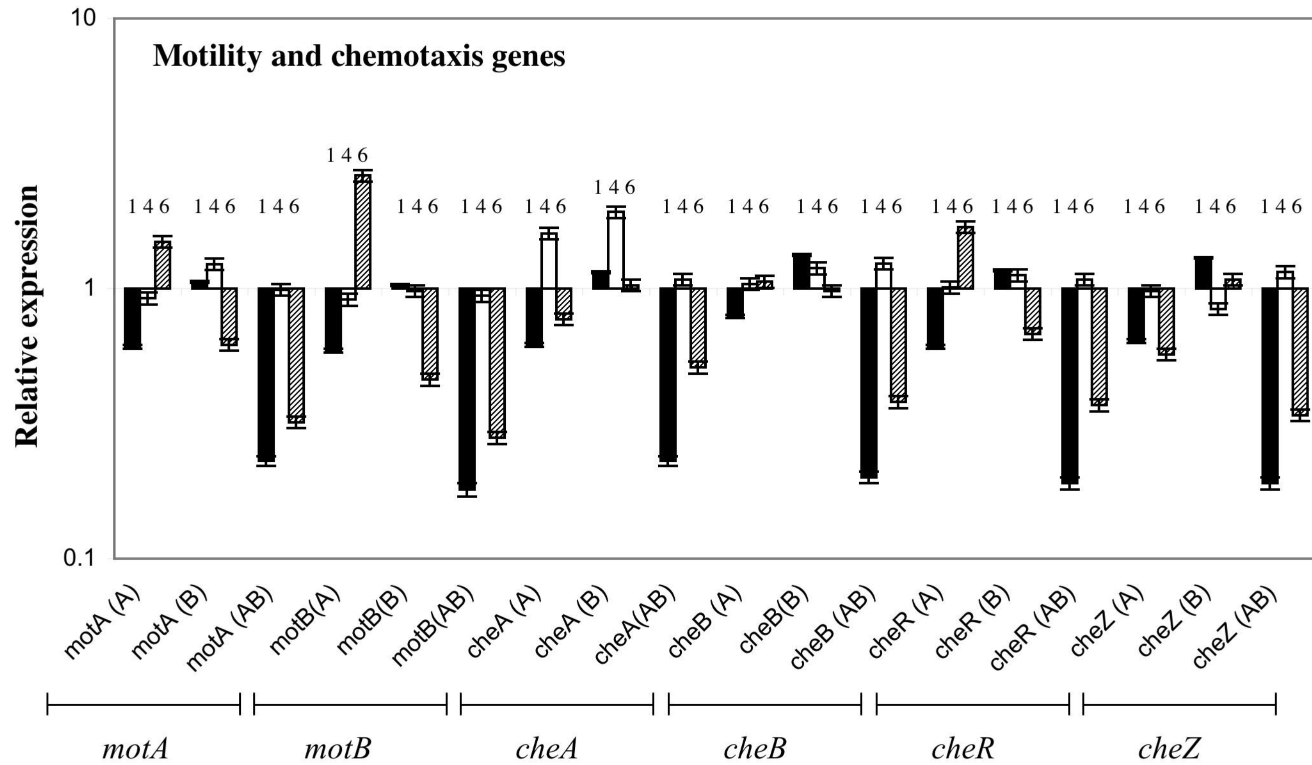


SPI2 regulatory genes
Relative expression
10
1
0.1
1 4 6
1 4 6
1 4 6
1 4 6
1 4 6
1 4 6
ssrA (A)
ssrA (B)
ssrA (AB)
ssrB (A)
ssrB (B)
ssrB (AB)
ssrA
ssrB

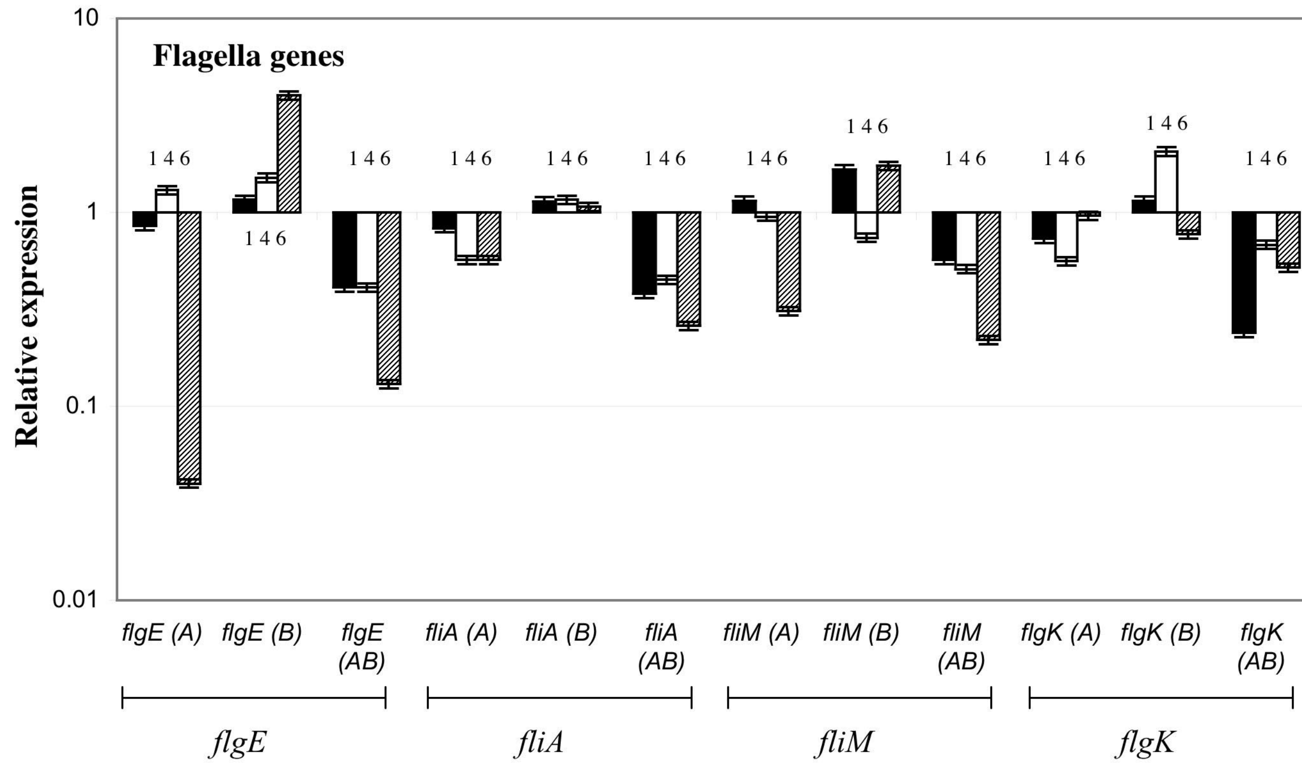

SPI2 type III apparatus genes
Relative expression
10
1
0.1
0.01
1 4 6
ssaB (A)
ssaB (B)
ssaB (AB)
ssaK (A)
ssaK (B)
ssaK (AB)
ssaR (A)
ssaR (B)
ssaR (AB)
ssaB
ssaK
ssaR



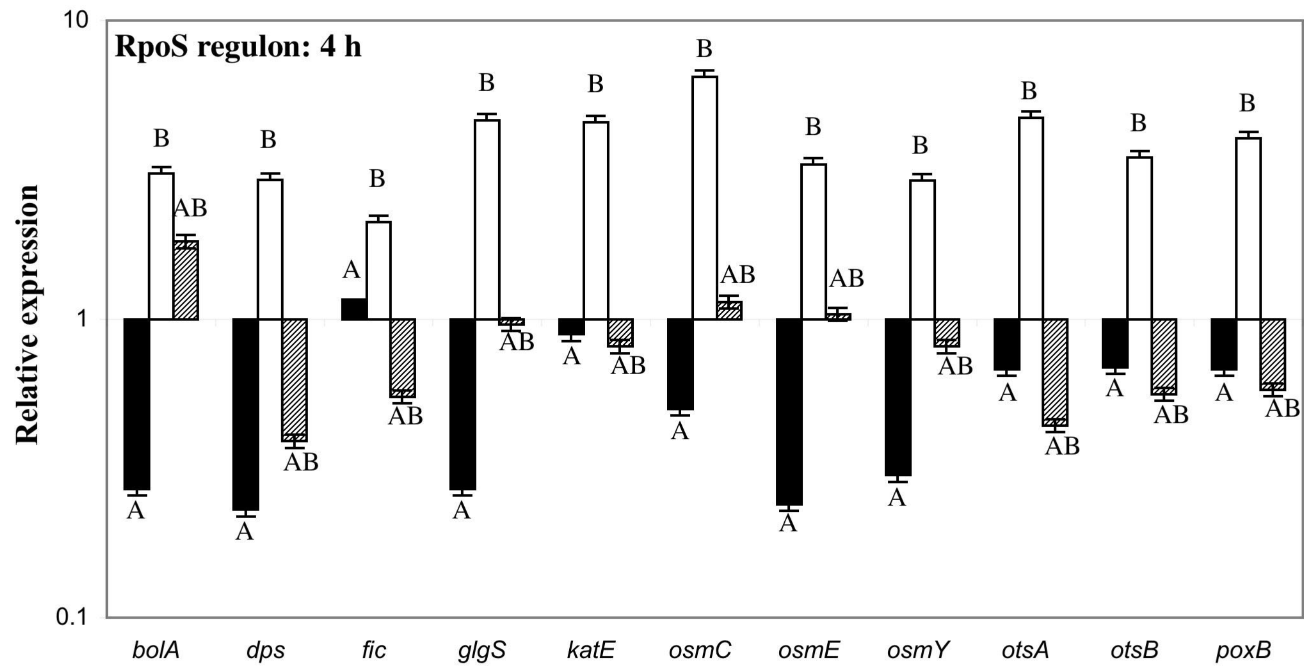
SPI2 type III effector protein genes
Relative expression
10
1
0.1
0.01
1 4 6
sseC (A)
sseC (B)
sseC (AB)
sseF (A)
sseF (B)
sseF (AB)
sseG (A)
sseG (B)
sseG (AB)
sseC
sseF
sseG



Motility and chemotaxis genes
Relative expression
10
1
0.1
1 4 6
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



Flagella genes
Relative expression
10
1
0.1
0.01
1 4 6
flgE (A)
flgE (B)
flgE (AB)
fliA (A)
fliA (B)
fliA (AB)
fliM (A)
fliM (B)
fliM (AB)
flgK (A)
flgK (B)
flgK (AB)
flgE
fliA
fliM
flgK



RpoS regulon: 4 h
Relative expression
10
1
0.1
bolA
dps
fic
glgS
katE
osmC
osmE
osmY
otsA
otsB
poxB
A
B
AB