Integration host factor alleviates H-NS silencing of the *Salmonella enterica* serovar Typhimurium master regulator of SPI1, *hilA*

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Coordination of the expression of *Salmonella enterica* invasion genes on *Salmonella* pathogenicity island 1 (SPI1) depends on a complex circuit involving several regulators that converge on expression of the *hilA* gene, which encodes a transcriptional activator (HilA) that modulates expression of the SPI1 virulence genes. Two of the global regulators that influence *hilA* expression are the nucleoid-associated proteins Hha and H-NS. They interact and form a complex that modulates gene expression. A chromosomal transcriptional fusion was constructed to assess the effects of these modulators on *hilA* transcription under several environmental conditions as well as at different stages of growth. The results obtained showed that these proteins play a role in silencing *hilA* expression at both low temperature and low osmolarity, irrespective of the growth phase. H-NS accounts for the main repressor activity. At high temperature and osmolarity, H-NS-mediated silencing completely ceases when cells enter the stationary phase, and *hilA* expression is induced. Mutants lacking IHF did not induce *hilA* in cells entering the stationary phase, and this lack of induction was dependent on the presence of H-NS. Band-shift assays and *in vitro* transcription data showed that for *hilA* induction under certain growth conditions, IHF is required to alleviate H-NS-mediated silencing.

**INTRODUCTION**

*Salmonella enterica* serovars are able to colonize and cause disease in a wide range of hosts. These bacteria are facultative intracellular pathogens that are typically ingested from contaminated food or water. Inside the host, they can invade and survive in epithelial cells and macrophages. Therefore, invasion of the host intestinal cells is critical for the initiation of salmonellosis. Several genetic elements responsible for the invasive phenotype of *S. enterica* serovar Typhimurium (*S. Typhimurium*) map to a 40 kb region of the chromosome at centrisome 63, termed *Salmonella* pathogenicity island 1 (SPI1). Pathogenicity islands are genetic elements of the chromosomes of several bacterial pathogens (Schmidt & Hensel, 2004) and accommodate clusters of genes that contribute to a particular virulence phenotype. *Salmonella* SPI1 encodes various components of a type III secretion system (T3SS), including its regulators and its specific effectors (Collazo & Galán, 1997; Marcus et al., 2000).

The ability of a bacterial pathogen to cause infection and disease requires not only the display of several virulence determinants, but a precise control of their expression, such that each is expressed at the appropriate time and place in the host. A good example of this is SPI1 expression. Several environmental factors and regulators have been shown to affect SPI1 expression (Altier, 2005). SPI1 environmental regulation converges in the modulation of the *hilA* gene (Ellermeier & Slauch, 2007). *hilA* is located on SPI1 and encodes the HilA protein, a member of the OmpR/ToxR family of regulators (Bajaj et al., 1995, 1996; Lee et al., 1992). Induction of HilA switches on the expression of SPI1 genes, including the T3SS. Different studies have shown that low oxygen, high osmolarity, the exponential phase of growth and slightly alkaline pH induce *hilA* expression and consequently the invasion genes (Bajaj et al., 1996; Jones & Falkow, 1994; Chubiz et al., 2010) Other recent studies have shown that, when *Salmonella* cells are grown in aerated Luria–Bertani (LB) medium at 37 °C, *hilA* induction occurs at the onset of the
stationary phase (Song et al., 2004; Mangan et al., 2006; Bustamante et al., 2008). These culture conditions have been used to further analyse the effects of modulators such as ppGpp (Song et al., 2004) and integration host factor (IHF) (Mangan et al., 2006), and the regulatory cross-talk between SPI1 and SPI2 (Bustamante et al., 2008). Most likely, low oxygen/exponential growth phase and aeration/early stationary phase mimic different environmental inputs that Salmonella cells detect when growing in natural environments, and lead to hilA induction.

HilA expression is subject to both positive and negative modulation. Three AraC-like transcriptional activators have been reported to bind to the hilA promoter: HilC, HilD and RtsA (Ellermeier & Slauch, 2004; Ellermeier et al., 2005). hilC and hilD are also located on SPI1, and rtsA maps outside it. Each of these activators can independently bind to the hilA promoter and activate hilA expression. HilD is dominant, and there is no hilA expression in its absence. In response to different stimuli, HilD activates HilC and RtsA transcription. Enhanced HilC/RtsA levels, in turn, activate hilA transcription (Ellermeier & Slauch, 2007). Interestingly, hilD mRNA levels have been found to be dependent upon DNA adenine methylation (Dam) activity, thus suggesting posttranscriptional control of HilD expression (López-Garrido & Casadesús, 2010). Several other global modulators also activate hilA expression, among them the two-component systems EnvZ/OmpR and BarA/SirA. Their effect seems to be indirect, as they sense osmolarity and bile/short-chain fatty acids, respectively (Altier, 2005; Ellermeier et al., 2005; Lucas & Lee, 2001). Other global regulators that positively modulate hilA expression are Fur (Ellermeier & Slauch, 2008) and the nucleoid-associated proteins Fis, HU (Schechter et al., 2003) and the bacterial IHF (Mangan et al., 2006). IHF binds to many target DNA sequences and plays a role as a transcription factor (Browning et al., 2010). IHF bends target DNA sequences, and usually modulates gene expression together with other nucleoid-associated proteins and/or other transcriptional factors.

hilA expression is also negatively regulated by several factors. A crucial repressor of SPI1 genes, including hilA, is HilE. It is likely that the effect of HilE is exerted by direct interaction with HilD, thus blocking its activity (Baxter et al., 2003). In turn, HilE expression is sensitive to several inputs, including the two-component systems PhoP/PhoQ and PhoR/PhoB, which either directly or indirectly activate HilE expression, thus leading to SPI1 repression. Negative modulation of hilA is also accomplished by global modulators such as Hha (Fahlen et al., 2001) and H-NS (Olekhnovich & Kadner, 2006; Schechter et al., 2003). Both belong to the superfamily of the nucleoid-associated proteins (Dorman, 2004; Madrid et al., 2007) and have been shown to interact to modulate gene expression (Ellison & Miller, 2006; Madrid et al., 2002; Nieto et al., 1991, 2002). The nucleoid-associated protein H-NS is a well-characterized example of a modulator that influences gene expression in response to environmental stimuli such as osmolarity, pH and temperature (Hulton et al., 1990; Hommans et al., 2001; Ono et al., 2005). Hha mimics the H-NS oligomerization domain (Madrid et al., 2007), copurifies with H-NS (Nieto et al., 2002) and targets a subset of the H-NS-modulated genes, several of which have been acquired by horizontal gene transfer, such as several virulence determinants (Vivero et al., 2008; Baños et al., 2009).

The SPI1 can be considered as a paradigm of a bacterial virulence determinant that responds to a large number of environmental and physiological stimuli through a complex array of interacting regulatory networks. Despite the fact that a large number of stimuli and modulators have been hitherto identified as influencing SPI1 expression, in only a few instances has a potential environmental stimulus been associated with a specific regulatory system. Relevant questions that remain to be answered relate to the precise identification of the stimuli to which the different regulators respond and the integration of the different regulatory circuits that govern hilA expression. In the present study we extend earlier work on the role of Hha and H-NS in the silencing of hilA expression (Olekhnovich & Kadner, 2006) and link it to environmental factors and the physiological state of the bacterial cells. Under non-permissive conditions (i.e. low temperature, low osmolarity and exponential growth phase), H-NS plays a crucial role in silencing hilA expression and Hha potentiates H-NS-mediated silencing. Moreover, we provide evidence to indicate that H-NS silencing of hilA is antagonized by IHF.

**METHODS**

**Bacterial strains, plasmids and culture conditions.** Bacterial strains and plasmids used in this study are listed in Table 1.

*S. Typhimurium* SV5015 is a His⁺ derivative of strain SL1344 (Baños et al., 2009). To construct the transcriptional lac fusion in hilA, the gene was first disrupted in the SV5015 strain using the procedure of Datsenko & Wanner (2000). The oligonucleotides used for this construction were HILAP1/HILAP2 (Supplementary Table S1), which ampliﬁed the antibiotic resistance of plasmid pKD3 with extensions corresponding to sequences of hilA. After verification of the predicted deletion using the combination of oligonucleotides HILAP1UP/KT and K2/HILAP2DOWN, antibiotic-resistance determinants were eliminated using an FLP/FRT-mediated site-specific recombination method, as described by Cherepanov & Wackernagel (1995). The FRT-generated site was used to integrate plasmid pKG136 (Ellermeier et al., 2002), thereby generating the transcriptional *hilA::lacZY* fusion strain SV5015UB2. The SV5015UB2 strain was used as a donor strain to transfer the *hilA::lacZY* fusion to strains SV5015H1, SV5015AV1 and SV5015HAV1 using phage P22 HT (Sternberg & Maurer, 1991), generating strains SV5015UB2H, SV5015UB2AV1 and SV5015UB2HAV1, respectively.

Chromosomal deletions of the genes *hilC, hilD,lhfa* and *lhfb* were done in strain SV5015UB2 by the λ Red recombiant method, as described by Datsenko & Wanner (2000). The antibiotic-resistance determinant of plasmid pKD3 was amplified using primers HILCP1/HILCP2, HILDP1/HILDP2, IHFAP1/IFHAP2 and IHBFP1/IHBFP2 (Supplementary Table S1). The resulting PCR products were purified and electroporated into strain SV5015UB2. The mutants were
confirmed by PCR using the primers CAT-C1 and CAT-C2 (chloramphenical resistance; Cm') in combination with specific primers located in the remaining gene sequence nearby (Supplementary Table S1, P1UP/P2DOWN series primers).

Plasmid pBADHNSt was constructed by cloning the hns\_TEPEC gene from plasmid pHSGHNSTE into the pBAD18 vector. The resulting PCR product was inserted into the pBAD18 vector previously digested with Eco\_RI. The liberated Eco\_RI fragment was excised from the agarose and purified using a QIAquick Gel Extraction kit (Qiagen). The resulting Eco\_RI fragment was inserted into the pBAD18 vector previously digested with Eco\_RI, giving plasmid pBADHNSt. DNA sequencing was performed to confirm that the insertion was correctly in-frame with the pBAD18 vector.

Cultures were grown either in LB medium (10 g NaCl l\(^{-1}\)) at 25 or 37 °C, or in salt-free LB medium at 37 °C. The cultures were diluted 1:100 from an overnight culture in 20 ml fresh medium in 100 ml flasks, and incubated with vigorous shaking at 200 r.p.m. (Innova 3100 water bath shaker, New Brunswick Scientific) until the desired OD\(_{600}\) was achieved (0.4 and 2.0 for exponential and early stationary phase cultures, respectively). For HNST\(_{TEPEC}\) induction from the pHSGHNSTE plasmid, the cultures were diluted 1:200 from an overnight culture in 20 ml fresh LB containing l-arabinose (Sigma) at a final concentration of 0.02%. The cultures were incubated at 37 °C (200 r.p.m.) in 100 ml flasks and cells were collected at the early stationary phase (OD\(_{600}\) 2.0). Medium SOC (Miller, 1992) was used for the recovery of transformants. Antibiotics, where appropriate, were added to the following final concentrations (µg ml\(^{-1}\)): carbenicillin, 50; kanamycin, 50; chloramphenicol, 25.

**Genetic and molecular procedures.** Basic molecular genetic manipulations were performed as described by Sambrook & Russell (2001). Enzymes were used according to the manufacturer’s recommendations. Introduction of plasmids into *E. coli* and *Salmonella* Typhimurium strains was performed by electroporation of 10 % glycerol-washed cells using an Eppendorf gene pulser (Electroporator 2510). Plasmids isolated in *E. coli* were first passed through restriction-deficient *S. Typhimurium* strain LB5000 before transformation of competent cells.

**β-Galactosidase assays.** β-Galactosidase activity measurements were performed as described by Miller (1992).

**Electrophoresis and Western blotting analysis of proteins.** Protein samples were analysed by SDS-PAGE at 15 %. From the gels, proteins were transferred to nitrocellulose membranes. Western blot analysis was performed with polyclonal antibodies raised against

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**Table 1. Strains and plasmids used in this study**

Abbreviations: Cb, carbenicillin; Cm, chloramphenicol; Km, kanamycin; Tc, tetracycline.

<table>
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<tr>
<th>Strain or plasmid</th>
<th>Description</th>
<th>Source or reference</th>
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<td>J. Casadesús*</td>
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**Plasmids**

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<td>Elliott &amp; Geiduschek (1984)</td>
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<td>h1dA promoter region (~505 to +372) on pTE103</td>
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Identification of the IHF-binding site on the hilA promoter. The putative IHF-binding site was determined using Virtual Footprint software (Münch et al., 2005) (http://prodoric.tu-bs.de/vfp/). The hilA promoter spanning from positions −505 to +372 (relative to the transcriptional start site) was subjected to analysis using the position weight matrix IHF from E. coli K-12. The best match is shown in Fig. 4(a), where the hilA promoter is presented from position −308 to −76 in order to minimize the figure width.

Electrophoretic mobility shift assays (EMSA). For competitive EMSA, an 877 nt fragment corresponding to the hilA promoter (nucleotides −505 to +372) was generated by PCR using primers HILAECORI5/HIATABAMHI23 (Supplementary Table S1). Ten picomoles of the PCR product was radiolabelled with [γ-32P]ATP (PerkinElmer) using T4 Polynucleotide Kinase (Fermentas) according to the manufacturer’s instructions. For each reaction, 35 fmol DNA template was mixed with H-NS–His or IHF proteins in binding buffer (250 mM HEPES, pH 7.4, 350 mM KCl, 5 mM EDTA, 5 mM DTT, 500 μg BSA ml⁻¹, 25 %, v/v, glycerol) and incubated at 37 °C for 15 min. Increasing concentrations of H-NS–His or IHF protein were added when indicated, and the reaction was continued at 37 °C for 15 min. The samples (20 μl) were separated on a 5 % polyacrylamide/0.5 x TBE gel. The bands were visualized using Quantity One software (Bio-Rad). Purified H-NS–His protein was obtained as described by Nieto et al. (1991). IHF protein was kindly supplied by Professor Fernando de la Cruz, Universidad de Cantabria and Instituto de Biomedicina y Biotecnología de Cantabria, Santander, Spain.

Transcription assays and primer extension. The hilA promoter corresponding to nucleotides −505 to +372 was PCR-amplified using the oligonucleotides HILAECORI5 and HILABAMHI23 (Supplementary Table S1). The resulting PCR product together with plasmid pTEhilA was used as template in the in vitro transcription assays.

To determine whether H-NS and/or IHF have an effect on the initiation of transcription, single-round transcription was carried out as follows. In vitro transcription reactions were performed in a 25 μl mixture containing 150 ng of the supercoiled plasmid pTEhilA, 200 μM each ribonucleotide triphosphate (rNTP), 5 U RNA polymerase (RNAP) (USB), and H-NS and IHF in the amounts indicated in Fig. 6. When indicated, IHF was incubated with the DNA in transcription buffer (20 mM Tris/HCl, pH 8.0, 25 mM KCl, 5 mM MgCl₂, 2.5 mM DTT, 25 μg BSA ml⁻¹) for 10 min at 37 °C prior to the transcription assay. Then, H-NS was added and the reaction was incubated for 10 min at 37 °C. At this point, the rNTPs rATP, rCTP and rUTP were added to the mixture to stall the RNAP at position 17 in the absence of rGTP, and after 2 min 100 ng heparin μl⁻¹ (final concentration) and GTP were added. The reaction was continued for 20 min at 37 °C and then stopped by inactivation of the RNAP at 75 °C for 10 min.

The in vitro-synthesized RNA was used as template for primer extension reactions. Primer extension products were detected by primer extension using 300 fmol [γ-32P]ATP-labelled HILAECORI5/HIATABAMHI23 primer (Supplementary Table S1). RevertAid H Minus M-MuLV reverse transcriptase (Fermentas) was used according to the manufacturer’s instructions. After incubation for 1 h at 42 °C, 10 μl loading buffer (95 % formamide, and 0.025 % each of bromophenol blue and xylene cyanol) was added; the sample was boiled for 2 min at 95 °C and chilled on ice, and 20 μl was loaded onto a 10 % polyacrylamide gel containing 7 M urea. The bands were visualized using Quantity One software (Bio-Rad).

RESULTS

Effect of temperature, osmolarity and growth phase on hilA expression in strain SV5015

Some reports have established that low-osmolarity and low-temperature conditions result in reduced hilA expression (Ono et al., 2005; Schechter et al., 2003), and others have shown that hilA expression is growth phase-dependent, being hilA-induced at the onset of the stationary phase when cells are grown in aerated LB medium (Song et al., 2004).

We first assessed the possibility that the effect of growth phase on hilA expression is temperature- and/or osmolarity-dependent. To do this, hilA transcription was evaluated in SV5015UB2 wild-type (wt) cells growing aerobically under different temperature and osmolarity conditions (25 and 37 °C, and low and high osmolarity, i.e. salt-free and conventional LB medium, respectively). Both exponential and early stationary phase samples were collected for each growth condition. The results obtained (Fig. 1) clearly showed that, as expected, hilA expression was repressed both at low osmolarity and at low temperature. Under these conditions, no growth phase effect was apparent. In contrast, growth at high osmolarity and temperature (LB medium, 37 °C) resulted in a growth phase-dependent induction of hilA. Altogether, these results show that, when growing in an aerated rich medium such as LB, high levels of hilA expression require a combined set of external stimuli.
(high temperature and osmolarity) and a specific physiological state of the cell (stasis).

Temperature, osmolarity and growth phase-dependent silencing of hilA by H-NS and Hha

In the Enterobacteriaceae, H-NS and Hha are involved in thermo- and osmoregulation of several genes. We next assessed the role of the two proteins on low temperature- and low osmolarity-dependent hilA silencing at the exponential as well as at the early stationary growth phase. Strains SV5015UB2, SV5015UB2 Δhha, SV5015UB2 Δhns and SV5015UB2 Δhns Δhha were grown either in salt-free LB medium at 37 °C or in LB medium at 25 °C. β-Galactosidase expression was monitored in cells collected at the exponential phase of growth, as well as at the early stationary phase (Fig. 2). With respect to low-temperature growth conditions (Fig. 2a), the effect of hns, hha and hns hha alleles on hilA expression indicated that H-NS plays the predominant role in growth phase-independent hilA thermosilencing. The effect of the hha allele alone at 25 °C was almost undetectable. With respect to low-osmolarity conditions, hilA expression was derepressed in both hha and hns genetic backgrounds (Fig. 2b). The hns mutant showed a significantly higher derepression than the hha mutant. The double hns hha mutant showed the highest β-galactosidase activities at both low osmolarity and low temperature, indicating that Hha enhances H-NS-mediated hilA silencing. hilA transcription in the double hha hns mutant either at low osmolarity or at low temperature was still higher than in the wt strain under growth conditions that led to stationary phase hilA induction (growth in LB medium at 37 °C, stationary phase) (Fig. 1). This highlights the role of both proteins in hilA silencing at low osmolarity and low temperature in a growth phase-independent manner.

We next tested the role of H-NS and Hha proteins on hilA expression in cells growing in LB medium at high temperature (37 °C). Again, samples from strain SV5015UB2 and the corresponding hns, hha and hha hns derivatives were collected at both the exponential and early stationary growth phases, and hilA transcriptional expression was determined by monitoring β-galactosidase activity (Fig. 2c). In exponential phase cells, the results obtained provided evidence that H-NS and Hha repress hilA expression. Moreover, both proteins had an additive effect on hilA expression. In early stationary phase cells, H-NS did not further silence hilA expression. A modest effect repressing hilA was seen for Hha (1.5-fold induction in hha mutants when compared with wt cells). Earlier results have also indicated this differential role of Hha and H-NS on hilA expression (Olekhnovich & Kadner, 2006). Considering that in hns mutants Hha levels are increased (Hommais et al., 2001), one might speculate that the excess of Hha in an hns mutant may moderately repress hilA transcription. To support this hypothesis, hilA transcription was monitored in strain SV5015UB2, which ectopically expresses Hha from plasmid pUBM22 (Nieto et al., 1991). Overexpression of Hha resulted in reduced hilA transcription (Fig. 3).

It is well established that both high and low temperature influence H-NS silencing (Falconi et al., 1998; Madrid et al., 2007; Ono et al., 2005). In this work we were able to
observe that both temperature and osmolarity modified the ability of H-NS to silence hilA. Remarkably, a combination of high osmolarity and high temperature reduced the capacity of H-NS to repress hilA. These results can be interpreted as temperature and osmolarity influencing the physicochemical properties of the DNA or the H-NS protein itself and partially alleviating H-NS silencing of hilA. Nevertheless, complete alleviation of H-NS-mediated hilA silencing also required that cells enter the stationary phase. This suggests that factors other than temperature or osmolarity, such as proteins that exhibit anti-H-NS activity, might play a role in alleviating hilA silencing.

**Stationary phase induction of hilA in strain SV5015 requires IHF**

We next tested the hypothesis that cellular factors that antagonize H-NS hilA silencing account for hilA derepression in cells growing in LB medium at 37 °C and entering the stationary phase. It has been shown that IHF plays a critical role in the expression of genes required by S. Typhimurium to undergo the physiological changes associated with the transition from the exponential to the stationary phase of growth, including virulence gene expression (Mangan et al., 2006). Among others, hilA and several other SPI1 genes are downregulated in both ihfA and ihfB mutant derivatives of strain SL1344. Moreover, it is well known that HilD, HilC and RtsA are required for proper hilA induction in cells growing aerobically in LB medium and entering the stationary phase. Therefore, we hypothesized that either the HilD-HilC-RtsA regulatory cascade or IHF could play a role in antagonizing H-NS silencing of hilA to promote its expression under permissive conditions. To further address this question, we first tried to construct double hilC hns, hilD hns and hns ihf null mutants. Whereas hilA induction at the onset of the stationary phase should be abolished in mutants lacking the hypothetical H-NS antagonist, the combination of the hns allele with a mutant allele in the gene encoding the H-NS antagonist would restore hilA induction. Double hilC hns and hilD hns mutants could be easily constructed; however, a double hns ihf mutant appeared not to be viable in strain SV5015. As an alternative, we decided to partially interfere with H-NS activity by expressing the H-NSTEPEC protein. This protein corresponds to a naturally occurring truncated form of the H-NS protein that acts as an H-NS antagonist (Williamson & Free, 2005). Hence, hilA transcription in hilC hilD or ihf mutants would correspondingly increase when H-NS activity was reduced by expressing H-NSTEPEC only if HilC, HilD or IHF acted as an H-NS

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**Fig. 3.** Overexpression of Hha represses hilA transcription in cells growing in LB at 37 °C. (a) Expression of β-galactosidase (in Miller units; MU) from the chromosomal hilA–lacZYA transcriptional fusion in cells harbouring no plasmid, plasmid pBR322 or plasmid pUBM22. (b) Immunodetection of Hha protein in the corresponding cell extracts.

**Fig. 4.** Effect of ihfA and ihfB alleles on growth phase-dependent hilA induction. (a) Schematic illustration of a partial sequence of the hilA promoter. Numbers indicate the distance from the transcriptional start site. The boxes indicate the H-NS-binding sites (Olekhnovich & Kadner, 2006) and the nucleotides shown in grey type indicate the putative IHF-binding site found using Virtual Footprint software (http://prodoric.tu-bs.de/vfp/). The consensus sequence in grey type is shown above the putative IHF-binding site (WATCAANNNTTR, where W is an A or T, N is any of four nucleotides and R is an A or G). (b) Cells from LB cultures grown at 37 °C were harvested at the exponential and early stationary phases of growth and β-galactosidase activity (in Miller units; MU) was monitored. The data shown are the means and SDs of three independent experiments.
antagonist. We first assessed hilA transcription in hilD and double hilC hilD mutants, either in an H-NS+ or in an H-NS reduced activity background (H-NST\textsubscript{EPEC} expressed). To achieve this, the mutants were transformed with plasmid pBADHNSt, which encodes the arabinose-inducible truncated form of the H-NS protein that exhibits anti-H-NS activity (Williamson & Free, 2005). The effect of the expression of H-NST\textsubscript{EPEC} protein on hilA expression was monitored in cells growing in LB medium at 37 °C (Table 2). Antagonizing with H-NS either in the hilD or in the double hilC hilD mutant did not result in hilA induction in cells entering the stationary phase. These results coincide with those reported for S. Typhimurium strain LT2 (Olekhnovich & Kadner, 2006).

We next tested the role of IHF in stationary phase-dependent hilA induction. A putative IHF-binding site was detected upstream of hilA (Fig. 4a). ihfA and ihfB derivatives of strain SV5015 were constructed and hilA expression was studied (Fig. 4b). When cells were grown in LB medium at 37 °C, at stationary phase, hilA induction did not take place in cells lacking either of the two IHF subunits. Under other growth conditions (low temperature, low osmolarity), neither of the two ihf mutant alleles modified hilA expression (data not shown). We next studied hilA expression in cells lacking IHF and exhibiting a reduced H-NS activity. Interestingly, a partial inhibition of H-NS activity in cells lacking IHF resulted in a significant induction of hilA expression, up to about 50 % of the expression obtained in the wt cells (Table 2). These in vivo results suggest that, at least under certain environmental conditions (high temperature and osmolarity, cells entering the stationary phase), the presence of IHF antagonizes H-NS-mediated hilA silencing.

**In vitro binding of H-NS and IHF to the hilA regulatory region**

To further test the hypothesis that IHF interferes with H-NS-mediated silencing of hilA, we performed competitive EMSAs between H-NS and IHF on the hilA promoter region. A 877 bp fragment spanning from −505 to +372, which contains the putative IHF-binding site, was used (see Fig. 4a). The DNA was incubated with the lowest concentrations of either H-NS or IHF that gave a complete shift of free DNA (1 or 0.16 µM, respectively). After 15 min at 37 °C, increasing amounts of the other protein were added, and incubation was continued for an additional 15 min. When H-NS was prebound to DNA, addition of increasing amounts of IHF generated lower-migrating protein–DNA complexes until a fragment was obtained with a mobility similar to that of the IHF and DNA complex. In contrast, when IHF was prebound to DNA, addition of increasing amounts of H-NS did not alter the mobility of protein–DNA complexes (Fig. 5).

**IHF alleviates H-NS-mediated repression of hilA transcription**

The effect of ihfA or ihfB mutant alleles on hilA expression in cells growing in LB medium and entering the stationary phase, the effect on hilA transcription of the H-NS antagonist in double ihf hns mutants, as well as the gel-shift data presented above suggest that the IHF protein functions as a transcriptional regulator of the hilA gene. To determine the effect of IHF on hilA transcription, *in vitro* transcription assays, using purified recombinant H-NS and IHF proteins, were performed on the hilA promoter (Fig. 6). First of all, increasing concentrations of H-NS repressed hilA expression. Addition of 1 µM H-NS resulted in a 4.7-fold repression of transcription. To test the effect of increasing concentrations of IHF on the yield of transcripts from the hilA promoter, the concentration of H-NS was fixed at 1 µM. Quantification of the transcripts showed that the presence of 0.5 µM IHF in the transcription assay did not have a stimulatory effect in the absence of H-NS. Nevertheless, when IHF was present at 0.5 µM in the reaction mix, it stimulated transcription twofold in the presence of 1 µM H-NS. The increase in the degree of

<table>
<thead>
<tr>
<th>Strain genotype</th>
<th>β-Galactosidase activity*</th>
<th>Ratio†</th>
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<tbody>
<tr>
<td></td>
<td>LB</td>
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<tr>
<td>hilA–lacZYA</td>
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<td>1059</td>
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<tr>
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</tr>
<tr>
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<td>611.7</td>
</tr>
<tr>
<td>hilA–lacZYA ΔihfB::Cm pBADHNSt</td>
<td>301.1</td>
<td>508.8</td>
</tr>
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</table>

*β-Galactosidase activities (Miller units; MU) were measured after growth to early stationary phase (OD\textsubscript{600} 2.0) in LB medium or LB+0.02 % l-arabinose at 37 °C.
†The ratio was calculated by dividing the β-galactosidase value of the strain with a plasmid (pBAD18 or pBADHNSt) by that of its cognate wt (hilA–lacZYA) parent.
stimulation in the presence of H-NS indicates that in vitro, IHF can alleviate H-NS-mediated repression of hilA transcription.

DISCUSSION

In this report we present data that help to clarify the role of H-NS and Hha proteins in environmental- and growth phase-dependent hilA modulation in S. Typhimurium strain SV5015. Regulation of hilA in S. Typhimurium represents an interesting example of the wide variety of stimuli that can affect virulence gene regulation and how complex some bacterial regulatory cascades turn out to be. In spite of the fact that several reports have shown that Hha, H-NS or an H-NS/Hha complex influences hilA expression (Fahlen et al., 2001; Olekhnovich & Kadner, 2006; Schechter et al., 2003), information integrating the role of H-NS/Hha-mediated hilA modulation in the bacterial response to different environmental inputs is not yet available. The pre-existing data have shown a role for H-NS/Hha in repressing hilA at low osmolarity (Olekhnovich & Kadner, 2006); however, H-NS and Hha differentially modulate hilA at high osmolarity. The reason for the latter effect remained to be clarified.

Both low temperature and low osmolarity represent non-permissive conditions for the expression of several virulence determinants in pathogens such as Salmonella and E. coli. Silencing of these determinants by H-NS or H-NS/Hha is a well-characterized process (Ellison & Miller, 2006; Goosen & van de Putte, 1995; Nieto et al., 2002; Ono et al., 2005). Our work shows that H-NS and Hha repress hilA under a set of well-defined environmental and physiological conditions and consequently account for SPI1 gene silencing. Low salt, low temperature or both environmental conditions together dictate H-NS/Hha-mediated hilA silencing. A recent report has shown that temperature, pH and ionic strength influence the ability of H-NS to repress gene expression, of which hilA is a good example.
well-characterized process that overcomes H-NS-mediated repression of several operons under specific physiological conditions (Stoebel et al., 2008). The RovA and Ler proteins have been shown to counteract H-NS-mediated silencing in *Yersinia enterocolitica* and *E. coli*, respectively (Bustamante et al., 2001; Ellison & Miller, 2006). In *Salmonella*, H-NS silencing of SPI-2 is antagonized by HilD or by SsrB proteins in response to different environmental conditions (Walters et al., 2007, 2011; Bustamante et al., 2008). It has been reported before that IHF antagonizes H-NS-dependent repression. A classical example is that of transcription from the Pe promoter of phage Mu, which is directly stimulated by IHF (Krause & Higgins, 1986). It has also been shown that IHF activates Pe transcription indirectly via the alleviation of H-NS-mediated repression (van Ulsen et al., 1996). Recent reports further reinforce the role of IHF in alleviating H-NS silencing in *Vibrio cholerae* (Stonehouse et al., 2008) and in the csgD operon of *E. coli* (Ogasawara et al., 2010).

In this work, we provide genetic and biochemical evidence indicating that IHF plays a key role in alleviating H-NS silencing in the *hilA* promoter. *In vivo* data show that, under specific environmental and physiological conditions (permissive conditions), IHF is required for *hilA* induction. Interference with H-NS activity compensates for the absence of IHF in *ihf* mutants. EMSA experiments suggest that binding of IHF to the *hilA* regulatory region might abolish H-NS binding and hence the silencing of such a promoter region. Finally, *in vitro* transcription data show that IHF interferes with H-NS repression of *hilA*. Our results link previous reports that show the roles of H-NS in modulating *hilA* (Olekhovich & Kadner, 2006) and of IHF in modulating stationary phase-dependent virulence expression in *Salmonella* (Mangan et al., 2006). By growing cells in aerated LB medium, we show that H-NS silencing of *hilA* ceases at the onset of the stationary phase, and in addition that IHF is required to alleviate H-NS silencing under these physiological conditions.

The results presented here provide evidence indicating that outside the host (i.e. low-temperature and/or low-osmolarity conditions), H-NS is a key element in silencing unwanted expression of the SPI1 genes. The Hha protein enhances the silencing effect of H-NS on *hilA*. Within the host, the combination of modified environmental factors (among others, increased temperature and osmolarity) and the activity of IHF abolish H-NS silencing of *hilA*. Thereafter, the regulatory cascade including HilD, HilC, RtsA, ppGpp and other regulatory factors, in response to several other signals, dictates *hilA* expression levels.

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