H-NS Silencing of the *Salmonella* Pathogenicity Island 6-Encoded Type VI Secretion System Limits *Salmonella enterica* Serovar Typhimurium Interbacterial Killing

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The secretion of bacterial toxin proteins is achieved by dedicated machineries called secretion systems. The type VI secretion system (T6SS) is a widespread versatile machine used for the delivery of protein toxins to both prokaryotic and eukaryotic cells. In *Salmonella enterica* serovar Typhimurium, the expression of the T6SS genes is activated during macrophage or mouse infection. Here, we show that the T6SS gene cluster is silenced by the histone-like nucleoid structuring H-NS protein using a combination of reporter fusions, electrophoretic mobility shift assays, DNase footprinting, and fluorescence microscopy. We further demonstrate that derepression of the *S. Typhimurium* T6SS genes induces T6SS-dependent intoxication of competing bacteria. Our results suggest that relieving T6SS H-NS silencing may be used as a sense-and-kill mechanism that will help *S. Typhimurium* to homogenize and synchronize the microbial population to gain efficiency during infection.

During the course of infection, bacteria produce and secrete bacterial toxins. Secretion of these toxin proteins is achieved by dedicated, specialized machineries called secretion systems. The type VI secretion system (T6SS) is required for the virulence of several Gram-negative pathogens. In *Vibrio cholerae*, the T6SS translocates VgrG1, a toxin that carries a domain responsible for actin cross-linking in eukaryotic cells (1–4). However, the role of the T6SS is not limited to virulence toward eukaryotes; an increasing number of reports demonstrate that the T6SS is also involved in interbacterial intoxication (5–10). With bacteriocins and contact-dependent inhibition (CDI), the T6SS is therefore involved in shaping bacterial communities (11–14) by delivering antibacterial toxins, including murein hydrolases, DNases, and phospholipases, directly into the target recipient bacterial cell (5, 6, 15; for recent reviews see references 14, 16 and 17). The attacker cell is protected from these toxins by the coproduction of cognate proteins that confer immunity (15, 18). The T6SS therefore confers a growth advantage to bacteria in mixed cultures and has been suggested to be important in environmental niches where bacterial competition for nutrients is critical for survival or for the uptake of DNA for transformation and acquisition of new traits (14, 19). However, while the role of the T6SS in interbacterial competition has been evidenced and characterized under laboratory conditions, its role in shaping bacterial communities of the microbiota is not yet clearly elucidated.

At the molecular level, the T6SS is assembled from 13 proteins, called core components, that form a transenvelope apparatus anchoring a cytoplasmic tubular structure to the membrane (20–24). Based on structural homologies with bacteriophage tail components, this tubular structure has been proposed to be constituted of an inner tube assembled by stacked hexameric rings of the Hcp protein, resembling the tail tube of bacteriophages, tipped by the VgrG protein (21, 25–27). The current model describes the internal tube as a conduit for the secretion of the effector toxins (13, 28–30). This model has been recently supported by data demonstrating direct contacts between the Hcp protein and

effectors (31). However, additional mechanisms have recently been reported, such as the tip protein, VgrG, or an adaptor protein, PAAR (proline, alanine, alanine, arginine), serving as a carrier for effectors (17, 32, 33). The Hcp internal tube is wrapped into a coating cylinder resembling the sheath of contractile phages (21, 26, 34). Cryo-electron and fluorescence microscopies showed that this sheath-like structure is dynamic and undergoes cycles of elongation and contraction (21). Similarly to the bacteriophage infection mechanism, it has been proposed that upon contact with a bacterial neighbor, contraction of the T6SS sheath propels the Hcp tube toward the target cell (2, 9, 35, 36). Because of the T6SS function in bacterial virulence and interbacterial competition, the expression of T6SS genes needs to be tightly controlled. A broad variety of transcriptional, translational, and posttranslational regulatory mechanisms have been identified and characterized (13, 37, 38).

In *Salmonella enterica* serotypes, T6SS gene clusters are encoded within different pathogenicity islands (SPIs), the most

Received 14 February 2015 Returned for modification 10 March 2015

Accepted 16 April 2015

Accepted manuscript posted online 27 April 2015

Citation Brunet YR, Khodr A, Logger L, Aussel L, Mignot T, Rimsky S, Cascales E. 2015. H-NS silencing of the *Salmonella* pathogenicity island 6-encoded type VI secretion system limits *Salmonella enterica* serovar Typhimurium interbacterial killing. *Infect Immun* 83:2738–2750. doi:10.1128/IAI.00198-15.

Editor: A. J. Bäuml

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Supplemental material for this article may be found at <http://dx.doi.org/10.1128/IAI.00198-15>.

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FIG 1 Summary of genome-wide studies on H-NS in *Salmonella* Typhimurium focusing on the T6SS gene cluster. (A) Schematic representation of the *Salmonella* Typhimurium SPI-6-encoded T6SS gene cluster. The names of the different genes are indicated (the *tss* nomenclature has been used for the T6SS core components except for the generic names for *hcp*, *vgrG*, and *clpV*). The two promoters identified in this study are indicated by arrows. The noncore clusters and the potential promoters identified by Mulder et al. (41) are indicated by horizontal brackets and asterisks, respectively. The positions and orientations of the *lacZ* transcriptional reporter fusions used in this study are indicated by the dashed arrows (beginning of the arrow represents the fusion joint). (B and C) Summary of the ChIP-on-chip experiments showing the enrichment of H-NS and of the RNA polymerase (RNAP) (56) (B) or the enrichment on H-NS for each gene (57) (C). In panel B, the locations of the six DNA fragments used for the electrophoretic mobility shift assays (Fig. 4) are indicated above the graph (black underlining, fragment with H-NS binding sites predicted with high scores; gray underlining, fragment with no predicted H-NS binding site, used as a negative control). (D) Summary of the microarray data (from reference 57). Bars represent the fold change for each T6SS gene in WT cells compared to the level in *hns* mutant cells (–10 means that the transcript is 10-fold more abundant in *hns* cells than in wild-type cells [57]). The STM gene numbers are indicated between the bars of the corresponding genes in panels C and D (e.g., STM02xx).

commonly distributed being the T6SS associated with SPI-6 (39). In *S. enterica* serovar Typhimurium (*S. Typhimurium*), the causative agent of gastroenteritis, only a single T6SS gene cluster is found on the genome, within SPI-6. This T6SS gene cluster is composed of two operons organized in divergent orientations (Fig. 1A) (39, 40). A recent bioinformatics study demonstrated that the genes encoding the core components are separated by

noncore clusters of genes, probably acquired more recently (Fig. 1A) (41). The gene cluster encodes all the genes required to assemble a functional secretion apparatus, as well as three effectors (STM0277, *tae4*; STM0291 and STM0292, *rhs*) and their cognate immunity proteins (STM0278, *tai4*; STM0291b and STM0293, *rhsI*) (42–45). *tae4* encodes a toxin with peptidoglycan hydrolase activity, whereas the Rhs C-terminal domains carry putative nu-

clease activities (42–45). The upstream operon (*STM0266-STM0271*) consists of a number of genes encoding machine subunits and two genes of unknown function. The downstream operon (*STM0272-STM0289*) consists of the remaining machine core component genes and the toxin/antitoxin pairs. The expression of T6SS genes encoded within the SPI-6 pathogenicity island is not detected under laboratory *in vitro* conditions (41, 46, 47); however, promoter-reporter and transcriptional profiling studies showed that the expression of these genes is activated in the late stages of macrophage and epithelial cell infection (41, 48). Indeed, the SPI-6 T6SS was first identified as a modulator of intracellular proliferation in macrophages (46, 49). Recent studies further revealed that the SPI-6 T6SS is an important actor for virulence toward mice and for chicken gastrointestinal colonization (50–52). The undetectable level of T6SS expression under *in vitro* conditions suggests that these genes are silenced and that this silencing might be counteracted *in vivo*. The major bacterial silencing protein is the histone-like nucleoid structuring protein (H-NS). H-NS usually binds to and represses the expression of A/T-rich genes, such as those acquired by horizontal gene transfer, and therefore acts as a general xenogeneic silencer (53–55). Upon examination of published *S. Typhimurium* genome-wide H-NS transcriptional profiling reports and of data from combined chromatin immunoprecipitation and microarray (ChIP-on-chip) analysis (56–57) (Fig. 1B to D), we noted a significant enrichment in H-NS binding to the SPI-6 T6SS gene cluster (Fig. 1B and C) and upregulated expression of these genes in *hns* mutant cells (Fig. 1D). In this study, we confirm that H-NS acts as a silencer for SPI-6 T6SS genes in *S. Typhimurium* using chromosomal reporter fusions. Electrophoretic mobility shift assays further demonstrate that H-NS binds to different regions of the gene cluster. DNase I footprinting of the noncoding region between the two divergent operons revealed that H-NS binds to discrete sites and then spreads from them to upstream and downstream regions. Finally, we show that derepression of the T6SS leads to the assembly of dynamic sheath-like structures and provides a growth advantage to *S. Typhimurium* in bacterial competition experiments.

MATERIALS AND METHODS

Bacterial strains, growth conditions, and chemicals. *Escherichia coli* K-12 DH5 α and W3110 strains were used for cloning procedures and competition assays, respectively. The *S. enterica* serovar Typhimurium strain LT2 (kindly provided by Josep Casadesus, University of Sevilla, Spain) was used for this study. The *S. Typhimurium* LT2 strain is leaky for *rpoS*, a mutation that counteracts the lethality of the *hns* null mutation (57). Strains were routinely grown in LB broth at 37°C with aeration. Plasmids were maintained by the addition of ampicillin (100 $\mu\text{g} \cdot \text{ml}^{-1}$), kanamycin (50 $\mu\text{g} \cdot \text{ml}^{-1}$), or chloramphenicol (40 $\mu\text{g} \cdot \text{ml}^{-1}$).

Strain construction. Strains and oligonucleotides used for strain constructions are listed in Table S1 in the supplemental material.

(i) Construction of *E. coli* mutant strains. The *E. coli* W3110 *hns* mutant strain was constructed by P1 transduction of the BW25112 *hns::kan* cassette obtained from the Keio collection (58) into W3110. Deletion of the kanamycin cassette was obtained by using the F₁ recognition target (FRT)-specific flippase-encoded pCP20 plasmid (59).

(ii) Construction of *S. Typhimurium* mutant strains. Isogenic mutant strains were constructed by λ -Red recombination engineering using the one-step inactivation procedure developed by Datsenko and Wanner (59) using pKD4-amplified PCR products. λ -Red functions were expressed from plasmid pKD46 (59). Strains were verified by colony PCR before transfer of the mutations into new LT2 cells by P22 transduction.

Cassettes were excised with the pCP20 plasmid as described previously (59).

(iii) Construction of chromosomal transcriptional fusions to *lacZ*. Chromosomal transcriptional fusions were inserted at the original locus as described previously (60). Briefly a kanamycin resistance cassette (from pKD4) (59) flanked by two FRT sites was inserted downstream of both *STM0271* and *STM0272* promoter regions or in place of the *STM0277* (*tae4*) or *STM0285* (*tssM*) gene in *S. Typhimurium* LT2 using λ -Red recombination and then excised using pCP20, generating strains carrying a single FRT site. Chromosomal *lacZ* fusions were constructed by integrating the plasmid pCE36 (60) and selected on LB agar plates supplemented with kanamycin at 37°C. Strains were verified by PCR before P22 transduction of the chromosomal fusions into new LT2 cells.

(iv) Construction of the chromosomal *tssB-sfgfp* fusion. To insert the gene encoding the superfolder green fluorescent protein (sfGFP) on the chromosome of *S. Typhimurium*, we first engineered a pKD4 derivative plasmid carrying the *sfgfp* gene upstream of the FRT site. This plasmid was constructed by restriction-free cloning (61). Then the sfGFP-FRT-Kan-FRT fragment was PCR amplified and inserted in frame at the 3' end of *tssB* using λ -Red recombination. The strain was verified by colony PCR before transfer of the mutations into new LT2 cells by P22 transduction and cassette excision using pCP20.

(v) Construction of *S. Typhimurium hns* mutant strains. The *hns* mutation was transferred by P22 transduction from a P22 lysate of *Salmonella* ATCC 14028 Δhns (62) kindly provided by Françoise Norel (Institut Pasteur, Paris, France).

β -Galactosidase activity assay. β -Galactosidase activities were measured as described previously (63) from mid-exponential-growth-phase cells (optical density at 600 nm [OD₆₀₀] of 0.8) as *S. Typhimurium hns* cells present a growth defect. The values reported are the averages of β -galactosidase activities of 27 measurements, representing experimental triplicates of three independent clones for each transduction (three independent *hns* P22 transductions).

Flow cytometry. *Salmonella* cells were grown overnight in LB broth, diluted 100-fold in LB broth, and grown to an OD₆₀₀ of ~ 1 . Cells were harvested, diluted in LB broth to $\sim 10^6$ cells $\cdot \text{ml}^{-1}$, and fixed by addition of paraformaldehyde (3.2% final concentration) for 10 min. Fixed cells were sorted at a debit rate of 7.5 $\mu\text{l} \cdot \text{min}^{-1}$ and a sheath pressure of 2×10^4 Pa using an A50 Micro flow cytometer instrument (Apogee) equipped with a 50-mW argon ion laser (488 nm) for excitation. Prior to sorting, flow cytometer settings were calibrated with Apogee Flow Systems 1- μm beads. Acquisition was triggered with small (224/65,535) and large (980/65,535) scatters to discriminate bacterial populations from the noise. Data were acquired with the PC Control, version 3.40, and Histogram, version 110.0, software programs (Apogee) and analyzed using FlowJo, version 10 (TreeStar, Inc.). Experiments were carried out in duplicate with a technical duplication. Each experiment gave similar results. The data are presented as the number of total cells (from the four replicates) as a function of the fluorescence level (in arbitrary units).

5' RACE assay. Total RNA was isolated from 8×10^9 LT2 or LT2 *hns* exponentially growing (OD₆₀₀ of ~ 0.8) cells using a PureYield RNA Midiprep system (Promega). RNAs were eluted with 1 ml of water, cleaned with DNase (Ambion), and precipitated overnight at -80°C by ammonium sulfate-ethanol procedures. After a washing step, pellet RNA was resuspended into 45 μl of nuclease-free water. RNA quality and integrity were tested on agarose gel and by the absorbance ratio at 260/280 nm. Total RNAs (80 $\mu\text{g} \cdot \text{ml}^{-1}$) were then subjected to transcriptional +1 mapping using a 5' rapid amplification of cDNA ends (RACE) system (Invitrogen). The oligonucleotides designed for amplification and cloning of cDNA are listed in Table S1 in the supplemental material.

H-NS purification. *E. coli* K-12 H-NS was purified according to published protocols (64). The *S. enterica* Typhimurium LT2 and *E. coli* H-NS proteins share 95% identity and 98% similarity at the protein level.

Electrophoretic mobility shift assays (EMSAs). PCR products were generated with Phusion Taq polymerase (New England BioLabs) using *S.*

Typhimurium genomic DNA (purified using a DNeasy blood and tissue kit; Qiagen), a mix of deoxynucleoside triphosphates (dNTPs) supplemented with [α - 32 P]dGTP (2.5 μ Ci per PCR in a total volume of 50 μ l; Perkin-Elmer), and oligonucleotide pairs (see Tables S1 and S2 in the supplemental material) and then column purified (Wizard Gel and PCR Cleanup kit; Promega). PCR products were incubated for 30 min at 20°C with the indicated concentration of H-NS (see Fig. 4) in a final volume of 10 μ l in H-NS binding buffer (20 mM HEPES, pH 8.0, 8 mM magnesium aspartate, 60 mM potassium glutamate, 5 mM dithiothreitol [DTT], 0.05% [vol/vol] Nonidet P-40 [Sigma], and 0.3 mg $\cdot$ ml $^{-1}$ bovine serum albumin [BSA; New England BioLabs]). After incubation, the mixture was loaded on a prerun 8% nondenaturing polyacrylamide (Tris-borate) gel, and DNA and DNA complexes were separated at 100 V in Tris-borate buffer (45 mM Tris base, 45 mM boric acid, 100 mM MnCl $_2$ buffer). Gels were fixed in 10% trichloroacetic acid for 10 min and exposed to Kodak BioMax MR films.

DNase I footprints. A fragment was obtained by PCR using Phusion Taq polymerase and a couple of primers in which one was labeled prior to the PCR. The primer was end labeled with [γ - 32 P]ATP (3,000 Ci $\cdot$ mmol $^{-1}$) using phage T4 polynucleotide kinase (New England BioLabs) and purified on a Sephadex G-25 column (Amersham Biotech). The 5'-end-radiolabeled fragment (2.5 nM) was incubated for 30 min at 20°C with the indicated concentration of H-NS (see Fig. 5A) in H-NS binding buffer (see the paragraph above on EMSAs). The DNA was then incubated for 30 s with 0.25 μ g $\cdot$ ml $^{-1}$ of DNase I (Worthington Biochemicals), and the reaction was quenched by the addition of 180 μ l of phenol-chloroform (5:1; pH 8.0) and 180 μ l of DNase Stop buffer (0.2 M sodium acetate, pH 5.0, 100 μ g $\cdot$ ml $^{-1}$ calf thymus DNA, 200 mM EDTA, 100 μ g $\cdot$ ml $^{-1}$ glycogen) successively. After centrifugation (15,000 $\times$ g for 3 min), the aqueous phase was submitted to ethanol precipitation. Samples were washed, dried, and resuspended in 5 μ l of formamide blue buffer (Tris-borate-EDTA [TBE] buffer supplemented with 90% [wt/vol] formamide, 0.01% [wt/vol] xylene cyanol, 0.025% [wt/vol] bromophenol blue). Samples were heated at 90°C for 4 min and loaded on 7% (wt/vol) polyacrylamide-8 M urea denaturing gels. Gels were fixed in 20% ethanol-10% acetic acid for 10 min, dried, and exposed to a phosphor storage screen (GE Healthcare).

H-NS binding quantification/quantitative gel analysis. Quantitative analyses of the DNase footprint were performed as previously described (65). Briefly, the intensity of each band in digital images of the gels was quantified using ImageQuant, version 5.0, software (GE Healthcare) and normalized with the intensity of the corresponding band in the absence of protein (66). Nonlinear least-square curve fitting of the data was carried out using the Origin software (OriginLab) and the following binding equation: $Y = L + (U - L) \times k^n x^n / (1 + k^n x^n)$, where L is the lower y value, U is the upper y value, K is the affinity constant, and n is the Hill coefficient. The values obtained for U and L were used to normalize the data from 1 to 0 to obtain the value for the normalized band intensity. We determined the fractional saturation of sites and fitted these data by the nonlinear least-squares method. Apparent dissociation equilibrium constants (K_d s) for H-NS binding were calculated from the curves.

Fluorescence microscopy. Overnight cultures of *S. Typhimurium* cells bearing the chromosomal *tssB-sfgfp* fusion were diluted 1:100 into LB medium and cultivated at 37°C to an OD $_{600}$ of $\sim$ 1.0. Cells were washed in phosphate-buffered saline (PBS), resuspended to an OD $_{600}$ of 50 in PBS, and spotted on a thin pad of 1.5% agarose in PBS covered with a coverslip. Fluorescence and phase-contrast micrographs were recorded using an automated and inverted epifluorescence microscope (TE2000-E-PFS; Nikon, France), equipped with a CoolSNAP HQ 2 camera (Roper Scientific SARL, France) and a 100 $\times$ /1.4 DLL objective and the Perfect Focus System (PFS), as previously described (9, 67). Images were collected every 15 s, using an exposure time of 100 ms for sfgFP fluorescence and 5 ms for phase contrast using MetaMorph software (Molecular Devices). sfgFP and phase-contrast channels were adjusted and merged using ImageJ.

Bacterial growth competition assay. Growth competition assays were performed using *S. Typhimurium* and its *hns* derivative as an attacker and *E. coli* K-12 W3110 and *S. Typhimurium* strains bearing the pUA66-*rrnB* plasmid (68) as prey, as described previously (9, 10). Briefly, cells were grown in LB medium at 37°C to an OD $_{600}$ of 1, adjusted to an OD $_{600}$ of 0.5, and mixed at a 4:1 ratio (attacker/prey). Then, 25 μ l of the mixture was spotted in triplicate onto prewarmed dry agar plates and incubated overnight at 30°C. Bacterial spots were cut out, and cells were resuspended in 1 ml of LB medium. Triplicates (150 μ l each) were transferred into wells of a black 96-well plate (Greiner), and the optical density at 600 nm and fluorescence (excitation, 485 nm; emission, 530 nm) were measured with a Tecan Infinite M200 microplate reader (nine measurements per mixture per tested combination). The relative fluorescence was expressed as the intensity of fluorescence divided by the absorbance at 600 nm after the value of a blank, nonfluorescent sample (the attacker alone) was subtracted. The experiments were done in triplicate with identical results, and the results of a representative experiment are reported. For enumeration of viable prey cells, the bacterial suspensions recovered from the spots were serially diluted and spotted on selective kanamycin plates.

Protein separation, transfer, and immunodetection. SDS-polyacrylamide gel electrophoresis (SDS-PAGE), Western blotting, and immunodetection were performed using Bio-Rad material and standard procedures. Proteins were immunodetected using anti-GFP monoclonal antibody (Roche) and anti-Pal antibodies from our laboratory collection (69) and secondary antibodies coupled to alkaline phosphatase and revealed using 5-bromo-4-chloro-3-indolylphosphate and nitroblue tetrazolium chloride.

RESULTS

H-NS silences the expression of the two divergent T6SS gene operons. cDNA microarray comparisons of *S. enterica* Typhimurium wild-type (WT) and *hns* strains identified regions of the genome silenced by H-NS (57). Among these regions, the expression of the T6SS genes encoded within *Salmonella* pathogenicity island 6 (SPI-6) increased 1.9- to 10.1-fold in *hns* mutant cells (Fig. 1D). Silencing by H-NS is generally caused by direct binding of this protein on target A/T-rich sequences. Using genome-wide ChIP-on-chip analysis for systematically testing H-NS binding on the *S. Typhimurium* genome, Lucchini et al. (56) and Navarre et al. (57) reported a 1.5- to 6-fold increase of H-NS binding to the T6SS locus (Fig. 1B and C).

To confirm these data, we first tested whether an *hns* mutation impacted the expression of the two divergent operons encoding the T6SS in strain *S. Typhimurium* LT2. Strain LT2 was used in this study as it carries an *rpoS* gene starting with a rare UUG start codon that results in decreased RpoS activity and avirulence (70, 71) and counteracts the lethal effect of the *hns* mutation (57). Hence, LT2 tolerates *hns* mutations, but such mutants exhibit a reduced growth rate (57). Expression of the two operons was tested using chromosomal transcriptional fusions in which the β -galactosidase gene was inserted on the chromosome at the T6SS locus in place of the *STM0271* or *STM0272* (*clpV*) gene. Additional *lacZ* fusions were created within the downstream gene operon (*STM0272-STM0289*) in place of the *STM0277* (*tae4*) and *STM0285* (*tssM*) genes. β -Galactosidase activity measurements showed that the expression of the two operons, as well as that of the two internal reporter fusions, was detected at very low levels (4 to 7 Miller units) in Luria broth (Fig. 2, open bars) independently of the growth phase (data not shown). Similar measurements in minimal Eagle's or M63 medium also demonstrated that the *S. Typhimurium* SPI-6 T6SS loci are expressed at similar levels (data

FIG 2 H-NS represses the expression of the type VI secretion gene operons. The activities of chromosomal reporter fusions of *STM0271*, *STM0272* (*clpV*), *STM0277* (*tae4*), and *STM0285* (*tssM*) to the *lacZ* reporter gene were measured in the WT *Salmonella enterica* Typhimurium LT2 (open bars) and its *hns* isogenic mutant (dotted bars). The β -galactosidase activities are the average of 27 measurements.

not shown). When the *hns* mutation was transduced into these reporter strains, the activity of the four reporter fusions increased to reach 200 to 300 Miller units (Fig. 2, dotted bars). These results demonstrate that H-NS silences the expression of both T6SS gene operons in *S. Typhimurium*.

Identification of transcriptional +1 sites. In a recent study, Kröger and colleagues used a genome-wide approach to systematically sequence mRNAs with the goal of identifying +1 transcriptional sites on the *S. Typhimurium* chromosome (47). The observation that no +1 transcriptional sites were identified in the

SPI-6 T6SS locus suggested that T6SS mRNAs are not produced *in vitro* (47). We used 5' RACE assays to define the +1 transcriptional sites of *STM0271* and *clpV*, the first genes of the two divergent T6SS operons, using mRNAs purified from *S. Typhimurium* WT and *hns* cells. In agreement with the results published by Kröger et al. (47) and with the reporter fusions, we did not obtain PCR products from WT total mRNA extracts, but PCR products were amplified from *hns* mutant mRNA extracts. Sequencing of these PCR products indicated +1 sites (Fig. 3). Potential -10 and -35 boxes can be readily identified at appropriate positions with respect to these +1 sites (Fig. 3A and B).

Binding of H-NS on the T6SS locus. *In silico* analyses using Virtual Footprint software (72) predict the existence of a large number of putative H-NS binding sites within the *S. Typhimurium* T6SS locus, distributed in the promoter region or within coding sequences. To test whether H-NS binds to the T6SS locus *in vitro*, we selected five DNA fragments that bear putative H-NS binding sites (Fig. 1B, fragments 1 to 5), including a fragment encompassing the divergent promoter (fragment 2) and a control fragment within the *clpV* gene for which no H-NS binding site is predicted (Fig. 1B, fragment 6). The six fragments were radiolabeled and subjected to electrophoretic mobility shift assays using purified *E. coli* H-NS (which shares 98% similarity and identical DNA-binding sites with the *S. Typhimurium* H-NS protein). Figure 4 shows that fragments 1 to 5 were retarded in the presence of increasing amounts of H-NS. Fragment 6 was not retarded in the presence of up to 300 nM H-NS. Image analyses of the shifts estimated that H-NS binds to fragments 1 to 5 with apparent K_d s of 50 to 75 nM. We conclude that H-NS specifically binds on different regions of the *S. Typhimurium* SPI-6 T6SS locus.

FIG 3 Identification of the promoters of the two divergent operons. (A) Sequence of the *STM0271-STM0272* (*clpV*) intergenic region. The +1 transcriptional start sites identified by 5' RACE and the corresponding -10 and -35 boxes are indicated in bold letters (roman letters, promoter for *STM0272* [*clpV*]; italics, promoter for *STM0271*). The translational start sites are indicated for the two genes. The regions protected by H-NS in the DNase footprint (Fig. 5) are indicated by solid lines while extensions of these protected regions are indicated by dotted lines. DNase hyperactivity sites are indicated by asterisks. The sequence is numbered identically to the DNase footprint (relative to the *clpV* +1 transcriptional start site). (B) Sequence alignment of the *clpV* and *STM0271* promoters with the *E. coli* σ^{70} -RNA polymerase binding consensus. Bases identical to the consensus are shaded in gray.

FIG 4 H-NS binds to several regions on the type VI secretion gene cluster. Electrophoretic mobility shift assays using radiolabeled DNA fragments corresponding to regions of the T6SS gene cluster (numbered 1 to 6) (Fig. 1B; see also Table S2 in the supplemental material). Fragment 6 corresponds to a negative control (no H-NS binding site predicted). Radiolabeled fragments were mixed with increasing concentrations of purified *Escherichia coli* H-NS (0, 5, 7.5, 10, 20, 50, 75, 100, and 300 nM).

H-NS binds to and spreads on the T6SS divergent promoter.

To gain further insights into H-NS binding on the divergent promoter region, we performed DNase I protection assays. The analysis of the DNase I footprint shown in Fig. 5A demonstrates that H-NS binds to four regions within the fragment: -32 to -50 , -100 to -110 , -126 to -136 , and -156 to -164 (positions relative to the *clpV* +1 transcriptional site) (Fig. 3A and 5A, solid lines). We also noticed a number of DNase hyperactive sites (Fig. 3A and 5A, asterisks). DNase hyperactive sites reflect local distortion of the DNA structure, induced by H-NS binding in adjacent regions, that might affect binding of transcriptional regulators or of the RNA polymerase (Pol) (54). Interestingly, these sites are gathered within the -32 to $+18$ region, suggesting that distortion of the extended RNA polymerase binding site might contribute to H-NS silencing of the SPI-6 T6SS promoter. H-NS binding is also clearly observable when the densities of the bands from the DNase I footprint are plotted as a function of H-NS concentration (Fig. 5B). DNase footprints also suggest H-NS cooperativity as higher H-NS concentrations led to the extension of the protected region to adjacent zones downstream and upstream (Fig. 3A and 5A, dotted lines). The degree of protection by H-NS at positions -107 (high affinity) and -147 (low affinity) was quantified (Fig. 5C). H-NS binds with an estimated apparent dissociation constant (K_d) of 30 nM at position -107 while the K_d at position -147 is estimated at 350 nM. This observation suggests that H-NS spreads from an initial, high-affinity binding site used as a nucleation center to occupy an extensive DNA region. These characteristics of H-NS have already been studied in detail in previous work and have been proposed to be responsible for gene silencing (54, 65, 73).

Derepression of *S. Typhimurium* T6SS gene expression leads to assembly of dynamic T6SS sheath structures. Once expressed, the T6SS genes encode proteins that form a membrane complex anchoring a dynamic cytoplasmic structure (21, 24). This tubular structure is structurally and functionally related to the sheaths of contractile bacteriophages and undergoes cycles of extension and contraction (21, 26, 34). This dynamic can be followed by time-lapse fluorescence microscopy by fusing the superfolder green fluorescent protein (sfGFP) to the C terminus of one of the T6SS's sheath components, TssB (9, 21, 35, 36, 74). The *sfgfp* gene was

introduced on the *S. Typhimurium* chromosome, in frame with the *tssB* coding sequence. In agreement with the results of the reporter fusion assays, the TssB-sfGFP fusion protein was not detectable by Western blot analyses (Fig. 6A), and the total cell fluorescence was very low (Fig. 6B and C). When the *hns* mutation was introduced, a significant level of TssB-sfGFP was detected by immunoblotting with anti-GFP antibody (Fig. 6A). The homogeneity of the population was assessed by flow cytometry. Figure 6B shows that although the LT2 *tssB-sfgfp* cells present fluorescence signal similar to that of WT LT2 cells, nearly 90% of the *hns tssB-sfgfp* population displays a high level of fluorescence. Expression of *tssB-sfgfp* in *hns* cells was accompanied by the observation of fluorescent intracellular tubular structures in ~60% of the cells (Fig. 6D). Time-lapse fluorescence microscopy recordings showed that these structures were dynamic, oscillating between extended and contracted conformations, with dynamic behaviors similar to those observed in *V. cholerae*, *Pseudomonas aeruginosa*, and enteroaggregative *E. coli* (Fig. 6E) (9, 21, 35, 36, 74). It is worth noting that *S. Typhimurium* cells presented a behavior similar to that observed in *P. aeruginosa* cells: an attacked sibling cell responded by triggering the assembly of a T6SS at the vicinity of the attack (Fig. 6E, blue arrowheads indicate response to contraction of the T6SS in the attacker cells indicated by red arrowheads) (35, 74, 75).

Derepression of the *S. Typhimurium* T6SS genes induces interbacterial intoxication. The T6SS has been shown to provide a fitness advantage to *P. aeruginosa*, *Burkholderia thailandensis*, *V. cholerae*, *Serratia marcescens*, *Citrobacter rodentium*, and enteroaggregative *E. coli* in mixed cultures due to its antibacterial activity (5–10, 15). The effectors delivered by these machineries target the peptidoglycan layer of the target prey cell (18, 42) or have phospholipase or DNase activity (45, 76–79). To prevent self-intoxication, these toxin proteins are produced in combination with a specific cognate immunity protein that protects the donor cell. In *S. Typhimurium*, the *STM0277* gene, named *tae4*, encodes a muramidase and is located upstream of *tai4* (*STM0278*), which encodes the cognate immunity protein (Fig. 1A) (42–44). This observation suggests that the *S. Typhimurium* T6SS might target bacteria. We therefore asked whether the T6SS provides a growth advantage to *S. Typhimurium* in mixed cultures. We first tested

FIG 5 H-NS DNase footprint of the divergent T6SS promoter. (A) DNase footprint. DNase accessibility to a radiolabeled DNA fragment corresponding to the divergent promoter of the T6SS gene cluster (from -173 to $+100$ relative to the $+1$ transcriptional site of *STM0272*) in the presence of increasing concentrations of purified *Escherichia coli* H-NS protein (5, 10, 25, 50, 100, 125, 250, 500, and 1,000 nM). The Maxam-Gilbert (G+A) chemical degradation is shown on the left. The protected regions are indicated on the right by solid bars. Extensions of the primary protected region due to H-NS cooperativity are indicated with dotted lines (the orientation of the arrows indicates the orientation of H-NS polymerization). Regions of DNase I hyperactivity are indicated by asterisks. (B) Line representation of the protection pattern of H-NS (gray, free DNA; orange, H-NS at 25 nM; black, H-NS at 125 nM). The densities of the DNase footprint bands (indicated in arbitrary units) are plotted as a migration (from the top to bottom of the DNase footprint). (C) Quantification of H-NS binding at positions -107 and -147 . The intensities (band intensity at the indicated H-NS concentration relative to the band intensity in the free DNA fragment) are plotted as a function of the H-NS concentration (in nM). The dissociation constants (K_d s) were calculated as indicated in Materials and Methods.

the interspecies competition using an *E. coli* K-12 strain (W3110, a strain devoid of T6SS genes) as prey. Since WT *E. coli* and *S. Typhimurium* *hns* cells have different growth behaviors, an *E. coli* *hns* mutant strain was used in these experiments in order to have comparable generation times. As shown in Fig. 7A, the *S. Typhimurium* WT strain had no impact on *E. coli* growth. However, derepression of the SPI-6 T6SS by introduction of the *hns* mutation in *S. Typhimurium* caused *E. coli* cells to die. The intoxication of *E. coli* by *S. Typhimurium* *hns* cells is due to the SPI-6 T6SS as *S. Typhimurium* *hns* cells carrying a deletion of the upstream ($\Delta STM0271$ -*STM0266*) or downstream ($\Delta STM0272$ -*STM0289*) T6SS gene operon had no impact on *E. coli* growth (Fig. 7A). We further tested intraspecies activity. Here, again, although the WT *S. Typhimurium* strain had no impact on its own growth, the *S. Typhimurium* *hns* strain presented antibacterial activity against its parental, wild-type strain (Fig. 7B). This activity was dependent on the SPI-6 T6SS as an *hns* strain with a deletion of the downstream or upstream operon had no intoxication activity. It is worth noting that the WT prey cells are not protected, suggesting that the T6SS gene operon in these cells is not expressed at all, even during the attack. Interestingly, T6SS-disabled *S. Typhimurium*

hns prey cells have different behaviors: while cells deleted of the upstream operon were resistant to *hns* attacker cells, cells deleted of the downstream operon were intoxicated (Fig. 7B). The latter result suggests that immunity genes to T6SS-delivered effectors are encoded within the downstream operon and is consistent with the presence of *tai4* and *rhl* (the genes encoding the immunities to Tae4 and Rhs, respectively) in this operon.

DISCUSSION

In this study, we provide evidence that expression of the *S. Typhimurium* SPI-6 T6SS gene cluster is silenced by the nucleoid-structuring protein H-NS. H-NS binds to different regions of the cluster on high-affinity sites and then spreads onto adjacent regions to silence the expression of these genes. Once derepressed, the T6SS genes are produced and assemble a functionally active machine that is used to intoxicate competing bacteria.

ChIP-on-chip genome-wide studies on the *S. Typhimurium* chromosome showed that H-NS binds to several regions, including the horizontally acquired SPI-6 pathogenicity island (56, 57). This island comprises a gene cluster encoding a type VI secretion system consisting of two divergent operons (39, 40). Using elec-

FIG 6 Derepression of the *S. Typhimurium* SPI-6 T6SS leads to assembly of the secretion apparatus. (A) Western blot analyses of *S. Typhimurium* WT and Δhns cells engineered to produce the TssB-sfGFP from the original locus. Total cell extracts from 2×10^8 cells were analyzed by SDS-PAGE. The TssB-sfGFP and the control Pal protein were immunodetected using anti-GFP (upper panel) and anti-Pal (lower panel) antibodies. The two proteins are indicated, as well as the molecular mass markers (at left; kDa). (B) Flow cytometry analyses of WT cells, WT cells carrying the chromosomal *tssB-sfGFP* fusion, and Δhns cells carrying the chromosomal *tssB-sfGFP* fusion. The number of cells (n) is plotted against the fluorescence level (AU, arbitrary units). Peaks reflect populations with different fluorescence levels. The number of individual cells in each population is proportional to the surface area of the corresponding peak and is indicated on the right of the peak (ratio to the total population). (C and D) Fluorescence microscopy analyses of *S. Typhimurium* WT and Δhns cells producing the TssB-sfGFP fusion protein (GFP channel). Scale bar, 10 μm . (E) Fluorescence microscopy time-lapse recordings of *S. Typhimurium* Δhns cells producing TssB-sfGFP (one image taken every 15 s, merged differential interference contrast and GFP channels). The arrowheads point the extension (blue) and contraction (red) of the T6SS sheath-like structures. For better visualization, the bacterial limits are outlined in black. Scale bar, 2 μm .

trophoretic mobility shift assays, we confirmed that the purified H-NS protein directly binds to the T6SS genes, including the interoperon region carrying the two divergent promoters. H-NS binding is accompanied by silencing of T6SS gene expression, as shown with chromosomal reporter fusions positioned on different locations within the gene cluster. H-NS-dependent silencing of the T6SS genes was suggested from the microarrays data (56) and more recently by the observation that no +1 transcriptional site was identified in the SPI-6 T6SS region using sequencing of total mRNA of the wild-type strain (47). Indeed, using 5' RACE on total RNA isolated from the WT strain, we were unable to amplify a PCR product corresponding to the SPI-6 T6SS region; however, when 5' RACE was performed on total RNA isolated from LT2 Δhns cells, we identified two transcriptional sites, upstream of the *clpV* and *STM0271* genes, the two first genes of the

divergent operons. The corresponding -10 and -35 boxes can be readily identified. DNase footprinting on the *clpV* promoter further showed that H-NS binds to different regions, including the -35 box, and then spreads on downstream and upstream fragments. This observation is consistent with the two-step mechanism of H-NS silencing in which H-NS first binds to high-affinity sites that nucleate cooperative binding, leading to H-NS polymerization onto adjacent DNA regions (54, 55, 65, 73). H-NS-dependent silencing of the *S. Typhimurium* T6SS gene cluster is not an isolated case as previous studies showed that the *Acinetobacter baumannii*, *Pseudomonas aeruginosa* HSI-2 and HSI-3, *Pseudomonas putida*, and *Vibrio parahaemolyticus* T6SS gene clusters as well as the *Edwardsiella tarda* T6SS *evpP-evpO* genes are repressed by H-NS-like proteins (80–84).

Recently, Mulder et al. noted that the SPI-6 T6SS gene region

FIG 7 Derepression of the *S. Typhimurium* T6SS confers a growth advantage in inter- and intraspecific competitions. Data are from interspecific (A) and intraspecific (B) growth competition assays. The indicated attacker and *gfp*⁺ prey strains were mixed, spotted onto LB agar plates, and incubated for 14 h. Recovered mixtures were analyzed for total relative fluorescence (expressed in arbitrary units [A.U.]). The recovered prey cells were numbered on selective plates, and values are reported in the lower graphs (each symbol represents values from three independent assays, and the average is indicated by a horizontal bar). WT, wild type; hns, Δ *hns* strains; dw, deletion of the SPI-6 T6SS downstream operon, Δ *STM0272-STM0289*; up, deletion of the SPI-6 T6SS upstream operon, Δ *STM0271-STM0266*).

comprises genes encoding the T6SS core components that share synteny with the genes of the *Burkholderia mallei* T6SS-3 gene cluster (41); however, in *S. Typhimurium*, these core genes are separated by genes acquired more recently, named noncore clusters 1 to 4 (cluster 1, *STM0275-STM0278*; cluster 2, *STM0283-STM0284*; cluster 3, *STM0286-STM0288*; cluster 4, *STM0290-STM0298*) (Fig. 1A) (41). These regions correspond to DNA fragments that do not encode T6SS core components but, rather, accessory components and/or effector/immunity pairs. Indeed, the ChIP-on-chip data (Fig. 1B) showed that H-NS preferentially binds to these regions, and our EMSAs (Fig. 4) demonstrated that H-NS binds to these regions with high affinity.

Interestingly, although the SPI-6 T6SS gene cluster is silenced *in vitro*, transcriptional profiling, reporter fusion, real-time PCR, and Western blot studies showed that its expression increased in the late stages of macrophage infection (41, 46, 48). Indeed, SPI-6 T6SS mutant strains present defects in intracellular replication and systemic dissemination in mice (41, 46). All of these studies point out that a functional SPI-6 T6SS is expressed during *in vivo* infection and further suggest that H-NS silencing should be counteracted under these conditions. Derepression of H-NS-silenced genes, known as countersilencing, can occur by competition with sequence-specific DNA binding regulators or by disruption of H-NS nucleoprotein filaments by DNA bending (53, 85). A number of transcriptional activators, including PhoP, SlyA, and LeuO, have been shown to antagonize H-NS silencing in *E. coli* and *S. Typhimurium* (86–91). However, deletion of *phoP* or *slyA* had only a minor effect on the expression of the SPI-6 T6SS genes (41, 92). The roles of LeuO and SinR, a transcriptional regulator encoded within the SPI-6 pathogenicity island, in countersilencing the expression of the SPI-6 T6SS gene cluster need to be tested.

We observed that derepression of the SPI-6 T6SS gene cluster in *hns* mutant cells induces assembly and function of the secretion machine and causes intoxication of *E. coli* prey cells. As previously shown for *P. aeruginosa*, *V. cholerae*, and enteroaggregative *E. coli* (9, 35, 74, 75), T6SS activity in *S. Typhimurium* cells induces higher T6SS activities in neighboring cells, a phenomenon referred to as dueling (Fig. 6E). This dueling appears only if the attacked cell is a sibling with the *hns* mutation as intraspecific competition showed that WT prey cells are efficiently killed. This further suggests that the attack is not sufficient to induce T6SS assembly but, rather, that a functional T6SS requires both proper transcriptional expression and sensing of the attack coupled to posttranslational activation. A mechanism of posttranslational activation, named the threonine phosphorylation pathway (TPP), has been described in *P. aeruginosa* and *Agrobacterium tumefaciens*. It requires sensing and signaling of the attack by the TagQRST proteins, leading to the PpkA-dependent phosphorylation of a forkhead-associated protein (FHA) or of the TssL T6SS core component and, ultimately, to the activation of the late stages of T6SS assembly (93–97). However, no homologue subunits to the TagQRST-PpkA-PppA-FHA threonine phosphorylation pathway are encoded within the *S. Typhimurium* SPI-6 T6SS gene cluster, suggesting that the mechanism of sensing and posttranslational activation is different from that described in *P. aeruginosa* or *A. tumefaciens*. As shown in Fig. 7, we observed that *S. Typhimurium hns* cells have antibacterial activity on *S. Typhimurium* WT prey cells but had no effect on the isogenic *hns* mutant cells. These data are consistent with the H-NS-mediated silencing of *tai4* (*STM0278*), a gene encoding an immunity protein to the peptidoglycan hydrolase *tae4* (*STM0277*) in the WT background. In the absence of activation of the T6SS in prey cells, the action of

Tae4 delivered by attackers cannot be counteracted. The activation of the gene cluster (and particularly of Tae4) during the late stages of infection might also explain the previous observation that *tai4* mutant cells display significant replication defects (41). Here, again, the absence of the immunity protein upon activation of the T6SS gene cluster may cause self-intoxication and decreased replication behavior. Although we cannot rule out that the *S. Typhimurium* SPI-6 T6SS has a role in pathogenesis, these data clearly show that this gene cluster confers antibacterial properties to *S. Typhimurium*. However, our antibacterial competition experiments also showed that the expression of the *S. Typhimurium* T6SS genes is not activated when WT cells are in contact with competing bacteria (Fig. 7). This and the data demonstrating activation of these genes during infection (41, 46, 48) strongly suggest that the antibacterial activity of the *S. Typhimurium* T6SS is important when *Salmonella* is in contact with eukaryotic cells. A similar situation may exist for the *P. aeruginosa* HSI-1, *V. cholerae*, and *A. tumefaciens* T6SSs, which are activated during infection and have antibacterial activity (78, 98, 99). Indeed, a very recent study suggested that the *V. cholerae* T6SS-dependent antibacterial activity influences the indigenous microbiota in the host (99). What can be the role of specifically activating the SPI-6 T6SS-dependent antibacterial activity during infection? One may hypothesize that it may help *S. Typhimurium* to clear its niche, either in the early stage of infection by competing with the gut flora or after phagocytosis by clearing the *Salmonella*-containing vacuole (SCV); however, the SCV usually contains a clonal population of bacteria, issued from a single bacterium (100). Additionally, activating the T6SS might help to synchronize or homogenize the *S. Typhimurium* kin population at a specific stage of infection as cells for which the SPI-6 T6SS has not been activated would be eliminated. H-NS derepression of the T6SS genes might therefore fine-tune the infection process by discarding disabled cells of the progeny. It would be interesting to perform *in vivo* experiments in mice or macrophages in which WT *S. Typhimurium* cells are challenged with T6SS- or *Tai4*-deficient bacteria. This will help us better understand the role of the T6SS in shaping bacterial communities during infection.

ACKNOWLEDGMENTS

We thank Manon Vinay, Nathalie Franche, and Mireille Ansaldi (LCB, Marseille, France) for help with the flow cytometry analyses, Javier López-Garrido and Josep Casadesús (Seville, Spain) for sharing ideas, protocols, and strains and for fruitful discussions, Françoise Norel (Institut Pasteur, Paris, France) for P22 phage *hns* lysates and for sharing protocols, Laure Journet and Laetitia Houot for critical reading of the manuscript, Julie Viala for discussions regarding *Salmonella*, as well as the members of the Cascales, Llobès, Mignot, Cambillau, Bouveret, and Sturgis research groups for discussions. We are indebted to Annick Brun, Isabelle Bringer, and Olivier Uderso for technical assistance and to Adhémair Patackess for encouragement.

Work in E.C.'s laboratory is supported by the Centre National de la Recherche Scientifique, the Aix-Marseille Université, and grants from the Agence Nationale de la Recherche (ANR-10-JCJC-1303-03 and ANR-14-CE14-0006-02). Work in S.R.'s laboratory was supported by an ANR grant (ANR-09-BLAN-0367-02). Y.R.B., A.K. and L.L. were supported by fellowships from the French Ministry of Research.

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