

## Repression of Intracellular Virulence Factors in *Salmonella* by the Hha and YdgT Nucleoid-Associated Proteins<sup>∇</sup>

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**The Hha/YmoA family of nucleoid-associated proteins is involved in gene regulation in enterobacteria. In *Salmonella enterica* serovar Typhimurium, virulence genes required for intracellular growth are induced following host cell invasion but the proteins responsible for repressing these genes prior to host cell entry have not been fully identified. We demonstrate here that Hha is the major repressor responsible for silencing virulence genes carried in *Salmonella* pathogenicity island 2 prior to bacteria sensing an intracellular environmental cue.**

Virulence gene regulation in bacterial pathogens is a highly coordinated process involving extracellular sensors and transcription factors that are activated in response to specific environmental cues (4). In *Salmonella enterica* serovar Typhimurium, the virulence factors required for intracellular growth are encoded on a large pathogenicity island called *Salmonella* pathogenicity island 2 (SPI-2), which encodes a type III secretion system and a two-component regulatory system called SsrA-SsrB that activates this type III system in the intracellular environment (2, 5). SsrA is a sensor kinase that phosphorylates the cognate response regulator, SsrB, leading to the induction of SPI-2 gene expression. Because standard LB broth is non-inducing for SPI-2 gene expression, we (6) and others (10) previously established an in vitro-acidified culture medium (LPM) with micromolar concentrations of phosphate and magnesium that induces robust SsrB-dependent gene expression, the assembly of the type III secretion system, and the secretion of virulence effectors at pH 5.8. This growth medium is suitable for the production of SPI-2-secreted proteins from cultures ranging in scale from a few milliliters (6) to 40-liter fermentors (our unpublished data).

The specific environmental context required for SPI-2 activation implied the existence of a repressing system to silence intracellular virulence genes in SPI-2 in the absence of an activating environmental signal. Because SPI-2 lacks an obvious negative regulator by sequence similarity to other repressors, we hypothesized that SPI-2 integrates with ancestral negative regulatory proteins to achieve appropriate genetic control. We found one repressor, YdgT, which affected the expression of SPI-2 genes and contributed to the contextual activation of virulence in animals (7). However, other unidentified repressors were likely involved because *ydgT* null mutants still repressed SPI-2 genes in LB medium. YdgT is a

member of the Hha/YmoA family of small nucleoid-like proteins involved in gene regulation (8, 13, 16, 20). YmoA modulates the expression of *Yersinia enterocolitica* virulence factors, including Yop proteins, the YadA adhesin, and invasins (8, 11). Hha was originally shown to increase the cytoplasmic expression of hemolysin in *Escherichia coli* (18) and has also been shown to negatively regulate invasion genes in *Salmonella* (12) and virulence genes in the locus of enterocyte effacement in enterohemorrhagic *E. coli* (22). Heretofore, there has been no report on the role of Hha in SPI-2 gene expression. Here, we identify Hha as the major repressor that silences SPI-2 virulence genes prior to bacteria sensing an activating environmental cue consisting of low Mg<sup>2+</sup>, low PO<sub>4</sub><sup>3-</sup>, and acidification. These data add important insight into the understanding of intracellular virulence gene regulation in *Salmonella* and make accessible new culture conditions and a genetic probe for examining this essential virulence determinant in *Salmonella* serovar Typhimurium.

Because the *ydgT* mutants created in our earlier work (7) still repressed intracellular virulence genes in SPI-2 when grown in LB medium, we hypothesized the existence of another repressor protein that contributed to SPI-2 gene regulation. We focused our attention on the small nucleoid-like protein Hha because of its amino acid similarity to YdgT and YmoA (7). To begin, we deleted *hha* from wild-type *Salmonella* serovar Typhimurium strain SL1344 and from a  $\Delta ydgT$  strain to create single and double mutants, respectively (Table 1). Chromosomal deletions of *hha* were created using the  $\lambda$  Red recombination method as described by Datsenko and Wanner (9). The primers for PCRs were designed to contain 5' end complementarity to the *hha* gene and 3' end complementarity to the FRT (Flp recognition target)-flanked antibiotic resistance cassettes of plasmids pKD3 and pKD4. The primer sequences used were as follows: *hhaF*, 5'-GCG TGT TCT CTA AAA AGT AAT GTA GCG TGA TTA ACG GTG TAG GCT GGA GCT GCT TCG-3'; and *hhaR*, 5'-CTT GTT AAA AAT TAT TAC AAT CAT AGG TAG AAT TTA TGT CTG ATA AAC CAT ATG AAT ATC CTC CTT A-3'. The resulting PCR products were purified, electroporated into *Sal-*

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TABLE 1. Strains used in this study

Strain	Genotype or description	Source or reference(s)
<i>E. coli</i>		
BKC1-14	DH5 $\alpha$ <i>supE44</i> $\Delta$ <i>lacU169</i> ( $\phi$ 80 <i>lacZ</i> $\Delta$ <i>M15</i> ) <i>hsdR17</i> <i>recA1</i> <i>endA1</i>	Our collection
BKC3-59	SM10 $\lambda$ <i>pir</i> <i>thi</i> <i>thr</i> <i>leu</i> <i>tonA</i> <i>lacY</i> <i>supE</i> <i>recA</i> ::RP4-2-Tc::Mu Km $\lambda$ <i>pir</i> (pP <sub><i>sseA</i></sub> :: <i>tnpR</i> :: <i>lacZ</i> ), Ap <sup>r</sup>	6
<i>Salmonella enterica</i> serovar Typhimurium		
BKC1-1	SL1344, wild type, Sm <sup>r</sup>	23
BKC3-25	SL1344, P <sub><i>sseA</i></sub> ::pP <sub><i>sseA</i></sub> :: <i>tnpR</i> :: <i>lacZ</i>	6
BKC5-49	SL1344 $\Delta$ <i>ydgT</i>	7
BKC9-49	SL1344 <i>hha</i> ::Kan	This study
BKC9-60	SL1344 $\Delta$ <i>ydgT</i> <i>hha</i> ::Kan	This study
BKC1-55	SL1344 <i>ssrB</i> ::Kan	14
BKC5-56	SL1344 <i>ssrB</i> ::Kan $\Delta$ <i>ydgT</i>	7
BKC9-50	SL1344 <i>ssrB</i> ::Kan <i>hha</i> :: <i>cat</i>	This study
BKC5-57	SL1344 $\Delta$ <i>ydgT</i> P <sub><i>sseA</i></sub> ::pP <sub><i>sseA</i></sub> :: <i>tnpR</i> :: <i>lacZ</i>	7
BKC9-73	SL1344 <i>hha</i> ::Kan P <sub><i>sseA</i></sub> ::pP <sub><i>sseA</i></sub> :: <i>tnpR</i> :: <i>lacZ</i>	This study
BKC9-75	SL1344 $\Delta$ <i>ydgT</i> <i>hha</i> ::Kan P <sub><i>sseA</i></sub> ::pP <sub><i>sseA</i></sub> :: <i>tnpR</i> :: <i>lacZ</i>	This study
BKC6-10	SL1344 <i>ushA</i> :: <i>cat</i> , Cm <sup>r</sup>	3, 7
BKC10-50	SL1344( <i>phha</i> ), Ap <sup>r</sup>	This study
BKC10-51	SL1344 $\Delta$ <i>hha</i> ( <i>phha</i> )	This study

*monella* strains, and subjected to selection as described previously (9).

Using established culture conditions for the induction of SPI-2 gene expression (6, 7), we examined the expression of a prototypical SPI-2 gene, *sseB*, which is controlled by the SsrB-dependent effector/secretory operon promoter in SPI-2 (5). The growth of wild-type *Salmonella* in LPM (pH 5.8) induced robust expression of SseB as shown previously (6), with a slight increase in SseB levels in a *ydgT* mutant background as expected. Under these inducing conditions, strains that were deleted for *hha* or double mutants lacking both *ydgT* and *hha* showed greater accumulation of SseB in bacterial lysates than wild-type *Salmonella* (Fig. 1A).

The above data suggested that Hha might be involved in the repression of genes carried in SPI-2 under certain environmental conditions. As a more stringent test of this hypothesis, we cultured wild-type *Salmonella* and the repressor mutants in LB broth, a rich medium that is highly repressing for SPI-2 gene expression, and examined bacterial lysates for SseB. As reported in our previous work (7), the loss of *ydgT* did not lead to increases in SPI-2-encoded proteins compared to the wild type under noninducing conditions, such as growth in LB broth (Fig. 1B). However, deletion of *hha* or both *hha* and *ydgT* led to a 4.3-fold or 5.6-fold increase, respectively, in SseB protein levels as determined by Western blot and densitometry analyses (Fig. 1B), suggesting a derepression of the promoter activity of *sseB* in the mutants under study. To verify that the specific deletion of *hha* in our strains was responsible for the derepression of *sseB*, we cloned the full-length *hha* gene into pFLAG-CTC (Sigma) as an NdeI/BglII DNA fragment but eliminated the carboxyl-terminal FLAG tag in our cloning

strategy by incorporation of the native stop codon from *hha*. This generated a complementation plasmid with IPTG (isopropyl- $\beta$ -D-thiogalactopyranoside)-inducible expression of the wild-type *hha* gene (designated *phha*). This complementation plasmid was transformed into wild-type and  $\Delta$ *hha* *Salmonella* serovar Typhimurium strains, and the strains were grown in LB as described above. The expression of the wild-type *hha* strain from the complementation plasmid prevented SseB accumulation from the  $\Delta$ *hha* strain and also eliminated the low basal expression of SseB from wild-type *Salmonella* serovar Typhimurium (Fig. 1C), indicating that the deletion of *hha* was responsible for this overexpression phenotype.

In order to examine the transcriptional activity of the promoter controlling *sseB*, we next integrated into the chromosome a single-copy transcriptional fusion of the *sseAB* promoter to *lacZ* (P<sub>*sseA*</sub>::*lacZ*) by homologous recombination as

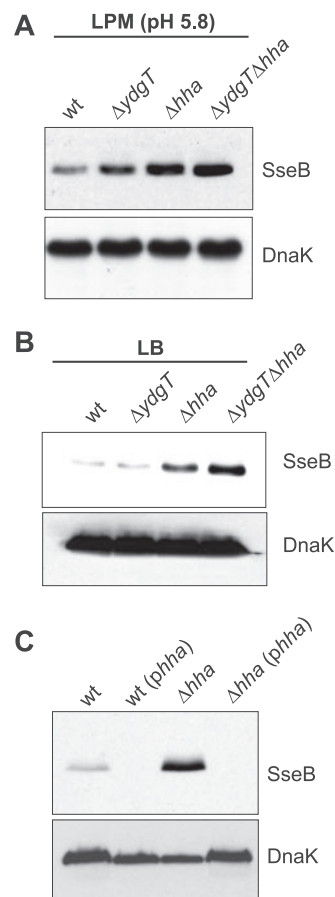


FIG. 1. Deletion of *hha* increases SseB protein levels. Bacterial cultures (wild type [wt] and  $\Delta$ *ydgT*,  $\Delta$ *hha*, and  $\Delta$ *ydgT*  $\Delta$ *hha* mutants) were grown under SPI-2-inducing conditions in LPM (pH 5.8) (A) and under SPI-2-repressing conditions in LB broth (B) as described in the text. Bacterial cell lysates were probed by Western blot analysis for the SPI-2-encoded protein, SseB (top panels), and a control protein (intracellular DnaK) (bottom panels). (C) Complementation of *hha* deletion. Wild-type *Salmonella* serovar Typhimurium and the  $\Delta$ *hha* mutant were transformed with *phha*, carrying the full-length wild-type *hha* strain from an IPTG-inducible promoter. Strains were grown in LB and probed by Western blot analysis for SseB as described in the text. The data shown are representative of experiments performed three times.

described previously (6). Reporter strains were constructed in wild-type bacteria and *ydgT*, *hha*, and *ydgT hha* mutants, creating strains designated with  $P_{sseA}::pP_{sseA}-lacZ$  nomenclature. Reporter strains were inoculated into either LB or LPM (pH 5.8) to give an  $A_{600}$  of  $\sim 0.05$  and then incubated with shaking at 37°C for various times. Samples were removed at regular intervals for the determination of numbers of CFU,  $A_{600}$ , and  $\beta$ -galactosidase activity by using a chemiluminescence assay described previously (7). Relative light units of  $\beta$ -galactosidase activity were measured using a top-reading plate luminometer (Molecular Devices, Sunnyvale, CA) after a 60-minute incubation of bacterial lysate and substrate. To facilitate direct comparisons between strains,  $\beta$ -galactosidase activity was normalized to the optical density of the paired culture. Under SPI-2-inducing conditions (LPM at pH 5.8), the transcriptional activity of  $P_{sseA}$  was detected from wild-type bacteria and  $\beta$ -galactosidase activity from *ydgT* mutant bacteria was slightly increased compared to that from wild-type bacteria during the exponential phase of the growth cycle (Fig. 2A). When the experiment was conducted with *hha* mutants and with *ydgT hha* double mutants,  $\beta$ -galactosidase activity was significantly higher in both mutant strains at all time points examined (Fig. 2A). Under these conditions, normalized  $\beta$ -galactosidase activity reached significantly greater maximal levels in strains deleted for *hha*. To further examine the transcriptional activity of SPI-2 under conditions that normally repress SPI-2 gene expression, we repeated the  $\beta$ -galactosidase activity assays using strains grown in LB under SPI-2-repressing conditions. As expected, *sseA* promoter activity was low in wild-type *Salmonella* and in *ydgT* mutants during the exponential phase of growth (Fig. 2B). As cultures plateau into stationary phase, the transcriptional activity of SPI-2 promoters increases, but this expression is not dependent on the acidification of the culture medium and thus represents a different mode of activation, unlike classical SPI-2 gene expression (6). In  $\Delta hha$  and  $\Delta ydgT \Delta hha$  strains,  $\beta$ -galactosidase activity was 16-fold and 15-fold higher than in the wild-type strain, respectively, at 2 h after subculture (Fig. 2B) and remained significantly higher throughout log phase. Remarkably, the level of  $\beta$ -galactosidase activity in log-phase *hha* and *hha ydgT* mutant strains in non-inducing growth medium was greater than the level of activity observed for wild-type *Salmonella* grown in SPI-2-inducing medium. Next, to examine whether the increase in *sseAB* promoter activity from  $\Delta hha$  strains required the response regulator, SsrB, we deleted *hha* from a strain lacking *ssrB* to generate an *ssrB hha* double mutant. We integrated the chromosomal  $P_{sseA}::pP_{sseA}-lacZ$  reporter into this strain and examined the  $\beta$ -galactosidase activity of the SPI-2 promoter as described above. The deletion of *ssrB* eliminated the increase in  $\beta$ -galactosidase activity from the *hha* strain (Fig. 2C), indicating that SsrB was required for this transcriptional activity. As expected, the  $\beta$ -galactosidase activities of both wild-type and *ssrB* mutant *Salmonella* serovar Typhimurium strains were low (Fig. 2C). Together, these experiments demonstrated that the deletion of *hha* results in the overproduction of SPI-2-encoded SseB protein and the transcriptional activation of the *sseAB* promoter under classically noninducing conditions where wild-type *Salmonella* represses this expression. This increase in promoter activity requires SsrB since an *ssrB hha* double mutant shows transcriptional activity similar to those of wild-type bac-

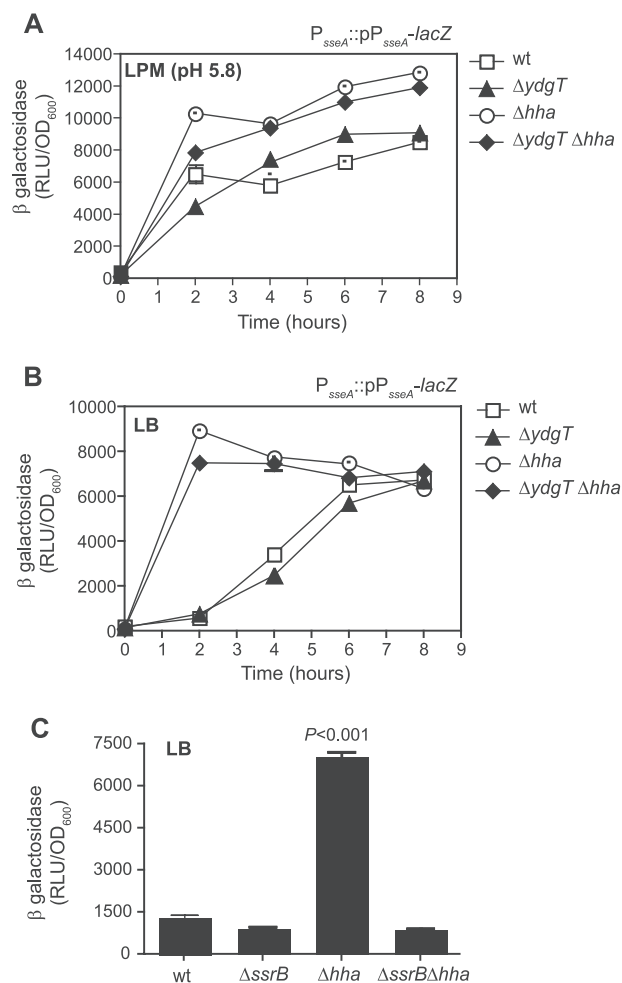


FIG. 2. Transcriptional activity of the SPI-2 effector operon promoter ( $P_{sseA}$ ) is derepressed upon deletion of *hha* and requires SsrB for activity. The  $\beta$ -galactosidase activity of a chromosomal  $P_{sseA}::lacZ$  reporter was examined in the indicated strains during growth under (A) SPI-2-inducing conditions (LPM at pH 5.8) and (B) noninducing conditions (LB). The data are means and standard deviations (SD) from triplicate determinations from two independent experiments. (C) The  $\beta$ -galactosidase activity of a chromosomal  $P_{sseA}::lacZ$  reporter was examined in wild-type *Salmonella* (wt), in mutants lacking the response regulator, *ssrB*, or the repressor, *hha*, and in a double mutant lacking both *ssrB* and *hha*. The data are the means and SD from triplicate determinations from two experiments.  $P$  was  $<0.001$  for the  $\Delta hha$  strain compared to the wild type. RLU, relative light units; OD<sub>600</sub>, optical density at 600 nm.

teria and an *ssrB* mutant under these conditions. Importantly, the identification of Hha as the major repressor of SPI-2 gene expression makes available facile culture conditions under which to examine the SPI-2 regulon and identify potentially new genes that coregulate with this virulence system.

Previously, we reported that *Salmonella* serovar Typhimurium YdgT was involved in the contextual control of virulence factor expression during murine typhoid (7). To examine the in vivo phenotype of our *hha* and *hha ydgT* mutant strains, we performed competitive infections of mice (1) by using a wild-type strain (SL1344 *ushA::cat*) (7) and each of the mutants generated in this work. Groups of C57BL/6 mice ( $n = 5$

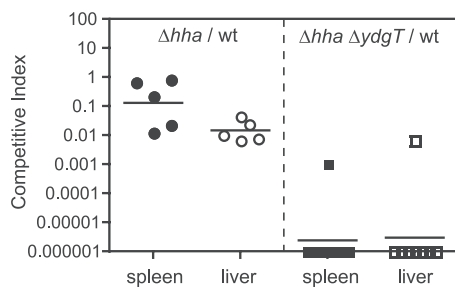


FIG. 3. Competitive infections in mice. Mice were infected with a mixed inoculum of wild-type *Salmonella* serovar Typhimurium (wt) and either an *hha* mutant (left) or an *hha ydgT* double mutant (right). The CI for the *hha* strain and *ydgT hha* strain were determined for the spleen and liver at 72 h postinfection. Each data point represents one animal, and horizontal bars indicate geometric means. CI for *hha*/wt,  $P = 0.021$  (spleen) and  $P = 0.001$  (liver); CI for *ydgT hha*/wt,  $P < 0.0001$  for spleen and liver.

to 10) were infected by the oral route with  $\sim 10^7$  CFU of a mixed inoculum of wild-type *Salmonella* and the mutants indicated. All animal procedures were approved by the McMaster University Animal Care Committee and were in accordance with guidelines set by the Canadian Council on Animal Care. Competitive indices (CI) for the spleen and liver of infected mice were calculated at 72 h postinfection by using the following equation:  $CI = (\text{mutant/wild type})_{\text{output}}/(\text{mutant/wild type})_{\text{input}}$ . The mean CI for the spleen and liver for the *hha* mutant were 0.125 ( $P = 0.02$ ) and 0.0151 ( $P < 0.0001$ ), respectively, indicating that the *hha* mutant was significantly attenuated compared to wild-type bacteria (Fig. 3). This level of attenuation was similar to that observed previously for single *ydgT* mutants at the same time point (7). However, the deletion of both *ydgT* and *hha* reduced the virulence of the double mutant by 6 orders of magnitude, producing mean CI values for the spleen and liver of  $2.63 \times 10^{-6}$  ( $P < 0.0001$ ) and  $3.16 \times 10^{-6}$  ( $P < 0.0001$ ), respectively. These data support our previously articulated notion (7) that the negative regulation of virulence genes is a key evolutionary strategy that is essential for pathogen virulence. However, further research of the extent to which SPI-2 deregulation contributes to this attenuation is required.

A previous report describing the role of Hha in the transcriptional regulation of *hilA*, a positive activator of virulence genes in SPI-1 (12), examined bacterial invasion into the ileal mucosae of anesthetized mice. Our in vivo data extend this previous observation by demonstrating a prominent role for *hha* in the virulence of systemic murine typhoid and by confirming the importance of *ydgT* for systemic colonization (7), as demonstrated by the severe virulence defect of the *ydgT hha* double mutant. The major virulence defect of the *hha ydgT* double mutant strain, significantly more attenuated than either the *hha* or *ydgT* single mutant, suggests a potential negative synergistic interaction between YdgT and Hha that warrants additional work to determine the exact nature of this genetic interaction. We are also currently engaged in studies to determine the relationship between the SsrB transcriptional activator and the repressors identified in our work because the transcriptional reporter data indicate that SsrB is required for the overexpression phenotype of *hha* mutants. It was recently re-

ported that H-NS, another nucleoid-associated protein in gram-negative bacteria, could silence genes acquired by horizontal gene transfer (15, 17). Given that H-NS can form heterodimeric complexes with both Hha and YdgT (19, 21), it is possible that H-NS is also involved in the repression phenotype we observe. However, since the deletion of *hns* is lethal in *Salmonella* serovar Typhimurium unless additional mutations in the alternative sigma factor gene, *rpoS*, or the virulence regulator, *phoP*, are also introduced (17), uncovering the potential role of H-NS in this repressive activity will require further study.

In addition to identifying a major regulatory node controlling intracellular virulence of *Salmonella*, this study draws attention to highly accessible culture conditions and genetic mutants in which to examine SPI-2 regulation and the expanded regulon coexpressed with the SPI-2 type III secretion system. In addition, the *hha* mutant and *hha ydgT* double mutant we describe are novel genetic probes of an essential virulence pathway in *Salmonella*, facilitating a detailed examination of genetic interactions between virulence gene repressors and transcriptional activators that collectively control the virulence behavior of this pathogen. We believe that the seemingly diametric actions of activators and repressors sculpt the virulence gene program during the evolution of bacterial pathogenesis, which is then fine-tuned by using multiple regulatory inputs for the optimal colonization of host niches.

These data support the notion that both positive regulators and repressors of virulence genes are crucial for the control of bacterial pathogenesis in animals. In the case of *Salmonella* serovar Typhimurium, the integration of a two-component regulatory system acquired by horizontal gene transfer (SsrA-SsrB) with ancestral repressors such as YdgT and Hha is a salient feature controlling the virulent behavior of the pathogen during the infection of host animals. Thus, such regulators would seem to be befitting targets for new anti-infectives that upset the highly coordinated expression of virulence traits in *Salmonella* and likely other pathogens. Realizing the therapeutic potential of targeting virulence gene regulators stands to bridge a widening innovation gap in anti-infective targets and to revolutionize our approach to fighting pathogens in human and animal medicine.

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