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Modulation of Horizontally Acquired Genes by the Hha-YdgT Proteins in *Salmonella enterica* Serovar Typhimurium▽†

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We describe a transcriptomic study of the effect of *hha* and *ydgT* mutations in *Salmonella enterica* serovar Typhimurium. A large number of genes showing altered expression are located in AT-rich horizontally acquired DNA sequences. Many of these genes have also been reported to be targets for H-NS. As Hha and YdgT interact with H-NS, our findings strongly suggest that Hha and/or YdgT must form complexes with H-NS when they silence these DNA regions.

The genomes of all members of genera belonging to the family *Enterobacteriaceae* contain at least one copy of a gene that encodes a member of the Hha-YmoA family of proteins (23, 24). These low-molecular-mass proteins show high degrees of similarity, and all of them have been identified as modulators of the expression of virulence factors (7, 30). Hha regulates, among other genes, expression of *Escherichia coli* α-hemolysin and *esp* operons (23, 29, 31, 36), the *Salmonella enterica* serovar Typhimurium *hilA* modulator of *Salmonella* pathogenicity island 1 (SPI1) (14, 32), and virulence genes in SPI2 (37). The Hha paralogue YdgT contributes to modulation of virulence genes in *S. enterica* serovar Typhimurium SPI2 (6, 37). The YmoA protein regulates the expression of several *Yersinia* virulence factors, as well as the RovA transcriptional activator (7, 13, 27). The Hha-YmoA proteins are encoded exclusively in the genomes of members of the family *Enterobacteriaceae* and in conjugative plasmids isolated from these organisms (24). Cells in which Hha has been depleted exhibit phenotypic properties similar to those of cells lacking the nucleoid-associated protein (NAP) H-NS, and it has been proposed that Hha-like proteins represent a new class of NAPs (27).

Several lines of evidence have shown that proteins belonging to the Hha family interact with members of the H-NS family to modulate gene expression (13, 16, 17, 31, 32, 33). Hha-like proteins mimic the H-NS oligomerization domain (23, 30), and interaction of Hha with H-NS increases the repressive ability of H-NS (25, 31). H-NS is the most extensively studied example of a NAP that has a role as an environmentally dependent modulator of gene expression (11, 35). H-NS is considered a transcriptional repressor, playing a relevant role in silencing xenogeneic DNA (12, 22, 28, 34).

It remains to be determined whether the set of H-NS-regulated genes coincides with the set of Hha-regulated genes or whether the latter is simply a subset of the former. A genome-wide analysis of the modulatory role of Hha-like proteins has not been performed. Here we describe a transcriptomic study of the effect of depletion of Hha-like proteins in *S. enterica* serovar Typhimurium and provide evidence that one of the main targets for Hha and/or its paralogue YdgT are the genes in horizontally acquired DNA sequences that are silenced by H-NS.

The genome of *Salmonella* contains, in addition to *hha*, a copy of the *ydgT* gene, which codes for an Hha paralogue, YdgT. When cells grow under nonstress conditions, YdgT does not significantly contribute to modulation. Nevertheless, this protein is overexpressed in *hha* mutants, which attenuates the *hha* phenotype (33). To prevent attenuation, we used a double *hha ydgT* mutant (strain SV5015HY). This deletion mutant, obtained by gene replacement, was constructed as described by Datsenko and Wanner (9). To obtain the *ydgT* mutant, the antibiotic resistance of plasmid pKD4 (kanamycin) was amplified using primers YDGTKAM1 and YDGTKAM2. To obtain the *hha* mutant, the antibiotic resistance of plasmid pKD3 (chloramphenicol) was amplified using primers HHAP1 and HHAP2. Both constructions were verified by using primers YDGTSA and YDGTSA2, primers HHA3 and HHA5, and primers corresponding to pKD3 (c1/c2) and pKD4 sequences (k1/k2) (primer sequences are described in Table S1 in the supplemental material). Strain SV5015HY (*hha ydgT*) was obtained by P22 HT transduction of the *hha* deletion into the *ydgT* strain (Table 1). Transcriptomic analyses were performed by using a *Salmonella* microarray that contained 6,119 probes corresponding to 5,116 open reading frames, 21 rRNAs, 86 tRNAs, 51 small RNAs, and 845 intergenic regions. The methods used for RNA extraction, retrotranscription, labeling, hybridization, microarray scanning, and data analysis will be described elsewhere (J. F. Mariscotti and F. García del Portillo, submitted for publication). Compared to the wild-type (wt) strain, strain SV5015HY showed altered expression of about 1,000 genes. The mRNA levels of 471 genes were >2-fold higher in the *hha ydgT* mutant, indicating that Hha and YdgT repress gene expression in the wt strain. The mRNA levels of 504 genes were >2-fold lower in the mutant strain, indicating

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid[a]	Genotype	Reference or source
Strains		
SV5015	SL1344 His^+	J. Casadesús
SV3081	LT2/pSLT$^-$	39
SV5015HY	Δ*hha*::Cm Δ*ydgt*::Km	This study
SV5015HY-2	Δ*hha* Δ*ydgt*	This study
SV4478	LT2 Δ*finO*::Km	3
SV5015-finO	SV5015/pSLT Δ*finO*::Km	This study
SV5015HY-2-finO	SV5015HY-2/pSLT Δ*finO*::Km	This study
Plasmids		
pLG338-30	ori_{pSC101}; Ap^r	8
pUBM22	pBR322 *hha* Ap^r	29
pKD46	Red helper plasmid, Ap^r	9
pKD3	Template plasmid, Cm^r	9
pKD4	Template plasmid, Km^r	9
pCP20	FLP helper plasmid, Ap^r Cm^r	5

[a] The *S. enterica* serovar Typhimurium strains were derived from SV5015, a His^+ derivative of strain SL1344 (19).

that Hha and YdgT activate gene expression in the wt strain (see Tables S1 and S2 in the supplemental material). Up-regulation predominated in genes belonging to several functional categories, including genomic islands (SPI1 to SPI5 and other genes related to horizontally acquired DNA regions), and the pSLT plasmid. In contrast, down-regulation predominated in genes belonging to the surface structure, cell motility-secretion, and translation functional categories (Fig. 1).

Most of the genes in SPI1 to SPI5 (93.22% of the genes) were predominantly overexpressed in strain SV5015HY (Table 2). To confirm the transcriptomic results obtained, we used reverse transcription (RT)-PCR to analyze the mRNA levels of some of the genes that were differentially expressed. Ready-to-Go RT-PCR beads (Amersham Biosciences) and primer pairs shown in Table S1 in the supplemental material were used. The genes selected were *prgH* (which encodes a component of the needle complex of the type III secretion apparatus of SPI1), *hilC* and *invF* (which encode invasion regulatory proteins of SPI1), *sseB* (which encodes a component of the translocation machinery of SPI2), and *ssrA*/*ssrB* (which encodes a two-component regulatory system of SPI2). RT-PCR analysis confirmed overexpression of all these genes in strain SV5015HY (Fig. 2).

To examine the effect of deregulation of virulence genes in the SPIs, we used the competitive index (2). Equivalent numbers of cells from strains SV5015 and SV5015HY were combined and used to inoculate an animal host (BALB/c mice) (input). Bacteria were recovered after 48 h from the spleen and liver (output), and the competitive index was determined. The competitive indexes were 0.08 ± 0.05 and 0.05 ± 0.03 in spleen and liver homogenates, respectively. As previously reported for *S. enterica* serovar Typhimurium strain SL1344 (6, 37), the *hha* and *ydgT* alleles are responsible for an attenuated virulence phenotype.

Several (42.52%) of the putative open reading frames in the virulence plasmid pSLT exhibited altered expression in strain SV5015HY. Most of them (97%), including those in the *tra* operon, were overexpressed (Table 2). To further examine the

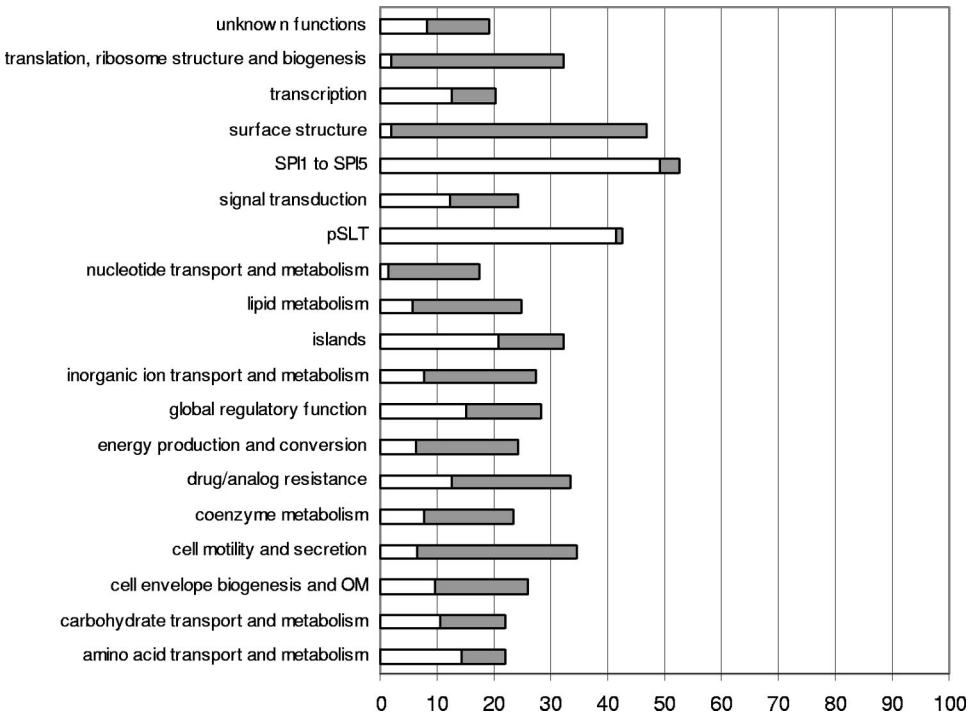

FIG. 1. Changes in expression of genes belonging to functional groups and in pathogenicity islands (26, 38). The bars indicate the percentages of genes belonging to each group that show altered expression in SV5015HY cells. The gray bars indicate the proportions of genes that are down-regulated ($M < 0$) and the open bars indicate the proportions of genes that are up-regulated ($M > 0$) for each group (where M is the fold change $\log_2$ ratio). OM, outer membrane.

TABLE 2. Hha-YdgT-dependent expression of SPI, flagellar, and pSLT genes identified by transcriptomic analysis

Gene[a]	*hha ydgT*/wt expression ratio	Protein function
SPI5		
pipB	4.18	Secreted effector protein
pipC	3.08	Pathogenicity island-encoded protein C
sopB	14.37	Secreted effector protein
SPI2		
STM1381	0.30	Putative cytoplasmic protein
STM1382	3.46	Putative regulatory protein
STM1390	3.28	Putative regulatory protein
ssrB	5.26	Transcriptional activator
ssrA	8.85	Sensor kinase
ssaE	4.91	Secretion system effector
sseB	12.04	Translocation machinery component
sscA	5.24	Secretion system chaperone
sseC	9.88	Translocation machinery component
sseD	7.16	Translocation machinery component
sseE	3.19	Secreted effector protein
sscB	4.44	Secretion system chaperone
ssaH	3.82	Type III secretion system apparatus protein
ssaI	3.72	Type III secretion system apparatus protein
ssaJ	3.38	Needle complex inner membrane lipoprotein
ssaK	2.89	Type III secretion system apparatus protein
ssaV	2.49	Type III secretion system apparatus protein
ssaO	3.72	Type III secretion system apparatus protein
ssaT	0.34	Type III secretion system apparatus protein
SPI1		
avrA	7.73	Secreted effector protein
sprB	13.36	Transcriptional regulator
hilC	2.55	Invasion regulatory protein
orgC	4.69	Putative cytoplasmic protein
orgB	16.80	Needle complex export protein
prgK	6.34	Needle complex inner membrane lipoprotein
prgJ	8.17	Needle complex minor subunit
prgI	2.45	Needle complex major subunit
prgH	12.77	Needle complex inner membrane protein
hilA	2.78	Invasion protein transcriptional activator
sptP	3.52	Protein tyrosine phosphatase/GTPase activating protein
sicP	13.74	Secretion chaperone
iacP	3.48	Acyl carrier protein
sipA	3.59	Secreted effector protein
sipD	28.15	Translocation machinery component
sipC	19.90	Translocation machinery component
sipB	14.17	Translocation machinery component
spaS	5.22	Type III secretion protein
spaR	2.45	Needle complex export protein
invJ	13.18	Needle length control protein
invI	13.18	Needle complex assembly protein
invB	6.04	Secretion chaperone
invA	3.47	Needle complex export protein
invF	3.39	Invasion regulatory protein
invH	4.03	Needle complex outer membrane lipoprotein precursor
STM2913	0.37	Putative permease
SPI3		
slsA	4.41	Putative inner membrane protein
cigR	3.71	Putative inner membrane protein
mgtC	4.18	Mg^{2+} transport protein
STM3767	0.28	Putative cytoplasmic protein
STM3770	4.21	Putative phosphotransferase system enzyme IIC
STM3774	2.52	Putative inner membrane protein
STM3782	3.81	Putative phosphotransferase system galactitol-specific enzyme IIC
Flagellar genes		
flgN	0.16	Putative FlgK/FlgL export chaperone
flgM	0.22	Anti-FliA factor
flgB	0.16	Flagellar basal body rod protein
flgC	0.21	Flagellar basal body rod protein
flgD	0.13	Flagellar basal body rod modification protein
flgE	0.24	Flagellar hook protein
flgF	0.16	Cell-proximal portion of basal body rod
flgH	0.17	Flagellar L-ring protein precursor
flgI	0.37	Flagellar P-ring protein precursor
flgJ	0.29	Flagellar biosynthesis protein
flgK	0.22	Flagellar hook-associated protein
cheB	0.16	Chemotaxis-specific methylesterase
cheW	0.12	Chemotaxis docking protein
fliD	0.41	Flagellar hook-associated protein
fliS	0.41	Flagellar protein FliS
fliT	0.33	Possible FliD export chaperone
fliF	0.20	Flagellar M-ring protein
fliH	0.16	Flagellar assembly protein
fliN	0.30	Flagellar motor switch protein
fliO	0.22	Flagellar biosynthetic protein
fljB	0.14	Flagellar biosynthesis protein
pSLT genes		
rcK	11.04	Resistance to complement killing
srgA	5.58	Putative thiol-disulfide isomerase or thioredoxin
orf7	8.08	Putative bacterial regulatory protein
pefI	6.94	Putative bacterial regulatory protein
orf6	9.58	Putative outer membrane protein
orf5	8.08	Putative outer membrane protein
pefA	4.29	Major fimbrial subunit
repA2	4.08	DNA replication protein
PSLT025	0.46	Putative cytoplasmic protein
PSLT045	3.81	Putative resolvase
PSLT046	11.00	Putative carbonic anhydrase
tlpA	2.89	Alpha-helical coiled-coil protein
psiB	4.98	Plasmid SOS inhibition
psiA	4.24	Plasmid SOS inhibition
traA	4.84	Pilus subunit
traL	3.62	Pilus assembly protein
traK	8.34	Pilus assembly protein
traP	6.96	Conjugative transfer protein
traC	3.28	ATP-binding protein
trbI	2.55	Pilus assembly protein
traU	5.30	Pilus assembly protein
trbC	3.82	Pilus assembly protein
traF	5.05	Pilus assembly protein
trbB	3.57	Conjugative transfer protein
traG	10.89	Mating pair stabilization and pilus assembly protein
traS	4.41	Entry exclusion protein
traT	3.90	Surface exclusion protein
PSLT107	2.75	Putative cytoplasmic protein
finO	2.61	FinP binding protein
traJ	2.55	Conjugative transfer regulation
traM	3.30	Conjugative transfer mating signal
traN	3.01	Conjugative transfer aggregate stability
traQ	2.29	Conjugative transfer fimbrial synthesis

[a] Bold type indicates the genes containing binding sites for H-NS as described by Navarre et al. (28).

effect of the *hha* and *ydgT* mutations on conjugation of plasmid pSLT, mating experiments were performed using a conjugation-derepressed derivative of plasmid pSLT, *finO*::Km (4). The antibiotic resistance cassettes associated with *hha* (chloramphenicol) and *ydgT* (kanamycin) mutations in strain SV5015HY were first deleted. To do this, we used FLP recombinase of plasmid pCP20 (9) and obtained strain SV5015HY-2. P22 HT transduction was used to transfer the *finO*::Km allele from plasmid pSLT of SV4478 into SV5015 and SV5015HY-2, generating strains SV5015-finO and SV5015HY-2-finO, respectively. The recipient used in the mating experiments was strain SV3081, a pSLT-cured strain, which was transformed with plasmid pLG338-30 to confer ampicillin resistance for selection of transconjugants. The frequency of plasmid transfer was calculated per donor bacterium. The frequency of pSLT $FinO^-$ plasmid transfer was 10-fold higher when strain SV5015HY-2 was used as the donor than when the wt strain was used as the donor (1.75×10^{-4} and 1.84×10^{-5}, respectively). These results show that the Hha-YdgT proteins participate in the regulation of plasmid pSLT conjugation. The par-

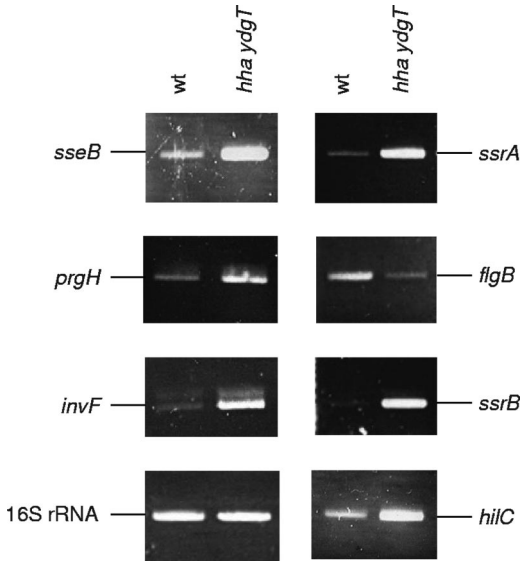

FIG. 2. RT-PCR analysis of transcription of the *prgH*, *hilC*, *invF*, *sseB*, *ssrA*, and *ssrB* genes from strains SV5015 (wt) and SV5015HY (*hha ydgT*). 16S rRNA was used as a control to confirm that equivalent quantities of templates were loaded.

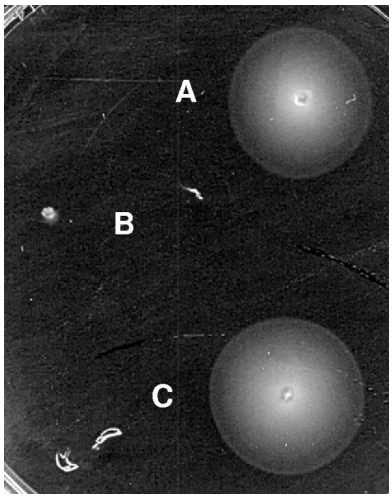

FIG. 3. Growth of strains SV5015 (wt) (colony A), SV5015HY (*hha ydgT*) (colony B), and SV5015HY(pUBM22) (colony C) on a motility agar plate.

ticipation of Hha-like proteins in the modulation of conjugative plasmid transfer has also been reported previously for the IncH1 plasmid R27 (15).

These results show that Hha-like proteins negatively modulate the expression of horizontally acquired genes in enteric bacteria, either directly or indirectly. This observation coincides with data for target genes previously described for Hha-like proteins (23). Hha-YdgT may negatively modulate gene expression either directly or indirectly. A good example of this is the virulence genes of SPI1; Hha modulates the master regulator, the *hilA* gene (14, 20).

Genes showing reduced expression in strain SV5015HY belong to several functional categories. The decreased expression of many of these genes may be due to an indirect effect of the double mutation on cell physiology (most likely the genes in the translation, ribosome structure, and biogenesis functional categories) or on specific transcriptional repressors. Remarkably, one significant set of genes showing reduced expression includes many genes involved in flagellar biogenesis (Table 2). We also used RT-PCR to confirm deregulation of *flgB*, which encodes a flagellar basal body rod protein (Fig. 2). We also compared strains SV5015 and SV5015HY in motility agar plates (18). Strain SV5015HY formed a very small halo (Fig. 3). This phenotype was complemented by expressing a plasmid that contains *hha* (pUBM22). Neither *hha* nor *ydgT* single mutants exhibited the drastic effect on motility shown by the *hha ydgT* double mutant (data not shown). Hence, the lower expression of several flagellar genes in strain SV5015HY resulted in reduced motility. The doubling time in LB medium of SV5015HY was 40% lower than that of the wt strain. The decrease in the growth rate may be explained by the down-regulation of the genes involved in translation, ribosomes, and biogenesis, as shown in the transcriptomic analysis (Fig. 1). Nevertheless, the difference in the doubling times of the strains analyzed is not sufficient to explain the difference in the reduced halos formed in motility plates.

Hha-like proteins form nucleoprotein complexes with H-NS (13, 15, 23, 30, 33). Recent results presented independently by different groups have shown that this NAP silences the expression of horizontally acquired DNA under nonpermissive conditions (22, 28, 34). Hence, a significant set of genes that are silenced by H-NS should also be silenced by Hha-YdgT. To confirm this, we compared the reported H-NS binding sites in the *Salmonella* genome (28) and our transcriptomic data. The results (Table 2) clearly support the hypothesis that Hha and YdgT interact with H-NS to favor silencing of xenogeneic DNA. Although these results were obtained with two different *Salmonella* strains (SV5015 and 14028) and the experimental approaches differed, 87% of SPI genes were repressed by Hha-YdgT and are reported to contain binding sites for H-NS. The coincidence is also high for other SV5015HY up-regulated genes on other genomic islands (34%) and for the pSLT plasmid (46%). It has been suggested that H-NS is not an effective silencer at all binding sites for some horizontally transferred sequences (28). This suggestion is supported by our finding that Hha-like proteins also participate in silencing xenogeneic DNA. Coregulation of genes in SPIs by H-NS and Hha-YdgT can be also inferred from two recent independent reports. The Hha and YdgT proteins repress expression of SPI2 virulence genes (37). Furthermore, H-NS down-regulates SPI2 expression, in competition with the two-component activator system SsrA-SsrB (40). The results reported here show that the Hha and YdgT proteins also repress *ssrA* and *ssrB* expression, thereby supporting the hypothesis that the Hha/H-NS complex modulates SPI2 gene expression.

For many other functional categories, the coincidence between Hha-regulated genes and H-NS-regulated genes was low. This finding can probably be attributed to H-NS modulating housekeeping functions in the absence of Hha-YdgT or to indirect effects of the double *hha ydgT* mutation on cell physiology. Some well-characterized examples of H-NS-modulated operons are *bgl* and *proU* (10, 21). We have been unable

to show that Hha-YdgT modulates these operons (unpublished results). We therefore suggest that the regulatory regions of the H-NS target genes include two categories: genes for which H-NS requires the formation of heteromeric complexes with Hha-like proteins in order to achieve efficient repression and genes repressed by H-NS homooligomers. Many of the H-NS-modulated genes that are located in horizontally acquired DNA, among others, belong to the first category.

The mechanism by which H-NS activates gene expression is not well characterized. This is also the case for Hha-YdgT. Nevertheless, we also found coincidences between genes down-regulated in strain SV5015HY and genes down-regulated in *hns* mutants. Genes related to flagellar biogenesis are positively regulated by H-NS in *E. coli* or *Salmonella* (1, 28). Our transcriptomic analysis showed that the Hha and YdgT proteins also positively regulate flagellar genes. Again, these findings point to participation of Hha-like proteins in some of the cellular processes that require H-NS.

Microarray data accession number. The complete data set has been deposited under accession number E-MEXP-1303 at htpp:/www.ebi.ac.uk/arrayexpress.

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