HilD induces expression of a novel *Salmonella* Typhimurium invasion factor, YobH, through a regulatory cascade involving SprB

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HilD is an AraC-like transcriptional regulator encoded in the *Salmonella* pathogenicity island 1 (SPI-1), which activates transcription of many genes within and outside SPI-1 that are mainly required for invasion of *Salmonella* into host cells. HilD controls expression of target genes directly or by acting through distinct regulators; three different regulatory cascades headed by HilD have been described to date. Here, by analyzing the effect of HilD on the yobH gene in *Salmonella enterica* serovar Typhimurium (*S*. Typhimurium), we further define an additional regulatory cascade mediated by HilD, which was revealed by previous genome-wide analyses. In this regulatory cascade, HilD acts through SprB, a LuxR-like regulator encoded in SPI-1, to induce expression of virulence genes. Our data show that HilD induces expression of sprB by directly counteracting H-nS-mediated repression on the promoter region upstream of this gene. Then, SprB directly activates expression of several genes including *yobH*, *slrP* and *ugtL*. Interestingly, we found that YobH, a protein of only 79 amino acids, is required for invasion of *S*. Typhimurium into HeLa cells and mouse macrophages. Thus, our results reveal a novel *S*. Typhimurium invasion factor and provide more evidence supporting the HilD-SprB regulatory cascade.

The genus *Salmonella* groups facultative anaerobic Gram-negative bacteria and is divided into two species, *S*. *enterica* and *S*. *bongori*. The former is responsible for diseases ranging from gastroenteritis to severe systemic infections in a wide range of hosts, and it comprises 6 subspecies that are further divided into serovars. The broad-host-range *S*. *enterica* serovar Typhimurium (*S*. Typhimurium) is a common cause of gastroenteritis in humans and many animals worldwide; furthermore, it can also cause systemic infection in humans and some animals, including laboratory mice. For this reason, *S*. Typhimurium is frequently used as a model for studying the host-pathogen interactions during infection with *Salmonella*.

During its evolution, *Salmonella* has acquired numerous DNA fragments through different horizontal transfer events; those encoding virulence factors are denominated *Salmonella* pathogenicity islands (SPIs). Up to 22 SPIs have been described among *Salmonella* serovars, being SPI-1 and SPI-2 the most importantly involved in gastroenteritis and systemic disease, respectively. SPI-1 is present in the two *Salmonella* species, in contrast, SPI-2 is present only in the *S*. *enterica* species, which supports that SPI-1 was acquired earlier than SPI-2 during the *Salmonella* pathogenicity evolution.

SPI-1 is a ∼40 kb chromosomal region of *Salmonella* that contains 39 genes, which encode a type III secretion system (T3SS-1; a syringe-like molecular apparatus that extend from the membranes of bacteria), several effector proteins and their respective chaperones, as well as some transcriptional regulators. Translocation of effector proteins into the cytoplasm of eukaryotic cells, through the T3SS-1, favors invasion of *Salmonella* into these cells by a trigger mechanism, which involves cytoskeletal rearrangements known as “membrane ruffles”. Invasion of *Salmonella* into the intestinal epithelium induces strong intestinal inflammatory response leading to gastroenteritis; in turn, this generates different antimicrobial activities that displace most of the intestinal microbiota, which heighten the intestinal colonization by *Salmonella*. Consistently with their role in intestinal disease, the SPI-1 genes are expressed in vivo when *Salmonella* resides in the intestinal lumen and in the cytosol of epithelial cells. Moreover, expression of the SPI-1 genes is regulated by distinct molecules or conditions present in the host environment.
the intestine of humans and animals, such as short- and long-fatty acids, high osmolarity, bile, low level of aeration and neutral pH. In laboratory conditions, the SPI-1 genes are expressed in nutrient-rich media like the lysogeny broth (LB), where they are expressed in the late exponential (early stationary) phase of growth, which somehow mimics the intestinal environment.

Expression of the SPI-1 genes is controlled by a myriad of global and Salmonella-specific regulators. HilD, an AraC-like transcription regulator encoded within SPI-1, is the apex of different regulatory cascades controlling expression of tens of Salmonella virulence genes. HilD positively regulates expression of the SPI-1 genes and many other related genes encoded outside SPI-1, by inducing expression of HilA, an OmpR-ToxR-like transcriptional regulator, which in turn induces expression of InvF, also an AraC-like transcriptional regulator; HilA and InvF, both encoded in SPI-1, directly induce expression of the rest of SPI-1 genes. HilD controls expression of hilA directly or through a feed-forward loop that it forms with HilC and RtsA, encoded within and outside SPI-1, respectively; although these AraC-like regulators recognize the same DNA motif, HilD is dominant over HilC and RtsA. On another hand, HilD positively controls expression of the FlhDC complex, the master positive transcriptional regulator of the flagellar/chemotaxis genes, which are also required for the invasion phenotype of Salmonella.

In addition to HilD, HilC, HilA and InvF, SPI-1 also codes for SprB, which belongs to the LuxR/Uhp family of transcriptional factors. SprB is not required for expression of the SPI-1 genes, but it is expressed under the same conditions that favor expression of these genes; furthermore, a previous study indicates that expression of SprB is positively regulated by HilA. Firstly, SprB was shown to directly regulate expression of the siaABCDEF operon located in SPI-4; however, recent transcriptomic analyses support that SprB controls expression of several virulence and hypothetical genes, but not that of the siaABCDEF operon.

Data from this study, together with previous results from genome-wide analyses, define an additional regulatory cascade headed by HilD. In this regulatory cascade, HilD induces expression of SprB, which in turn activates expression of several target genes including yobH, SirP, and YobL; SirP and YobL have been involved in Salmonella virulence. Our results show that HilD directly induces expression of sprB by antagonizing repression mediated by H-NS. Interestingly, we found that the yobH gene is required for invasion of HeLa cells and mouse macrophages by S. Typhimurium, which reveals a novel invasion factor of Salmonella. Thus, the HilD-SprB regulatory cascade represents a novel pathway that controls expression of virulence genes in Salmonella.

Results

HilD positively controls the expression of yobH (SL1344_1770). Previous RNA-sequencing (RNA-seq) analysis indicates that the HilD transcriptional regulator positively controls the expression of the S. Typhimurium SL1344_1770 gene, which is located outside SPI-1 and codes for a hypothetical protein of 79 amino acids. Additional RNA-seq and co-expression analyses also support that HilD is involved in the expression of SL1344_1770. Orthology of SL1344_1770, which show high sequence identity and a conserved genomic context, are denominated yobH in Escherichia coli and several other bacteria; thus, we kept the name of yobH for SL1344_1770.

To confirm the regulation of yobH by HilD, a transcriptional fusion of the intergenic region upstream of yobH to the cat (chloramphenicol acetyltransferase) reporter gene was constructed in the pKK232-8 plasmid. Specific activity from this fusion was quantified in the wild type (WT) S. Typhimurium strain SL1344 and its derivative ΔhilD mutant, grown in nutrient-rich lysogeny broth (LB) at 37°C, conditions that induce the expression of genes regulated by HilD. The activity of the yobH-cat fusion showed a 3-fold reduction in the ΔhilD mutant, compared with its expression in the WT strain; in addition, the expression of HilD from the pK6-HilD plasmid increased around 5-fold the activity of this fusion in the ΔhilD mutant (Fig. 1A). To investigate if yobH indeed codes for a protein and to further confirm the positive regulation of yobH by HilD, we tested the expression of the YobH-FLAG putative protein (YobH tagged with a 3XFLAG epitope) in the WT S. Typhimurium strain and its derivative ΔhilD mutant. A specific signal for YobH-FLAG was detected in the WT strain, with the expected size for this protein (Fig. 1B). The amount of YobH-FLAG was almost abolished in the ΔhilD mutant; as expected, it was restored at WT levels by the expression of HilD from the pK6-HilD plasmid (Fig. 1B). Taken together, these results show that HilD induces the expression of yobH.

YobH is involved in the S. Typhimurium invasion of host cells. HilD positively regulates expression of numerous genes mainly required for the invasion of Salmonella into host cells. For instance, recently we found that HilD controls expression of the gdhD invasion gene, which is located outside SPI-1. To determine whether YobH is required for this Salmonella virulence phenotype, we evaluated the invasion ability of the WT S. Typhimurium strain and its isogenic yobH mutant in HeLa cells and RAW264.7 mouse macrophages. Additionally, we constructed a complemented ΔyobH mutant (ΔyobH + yobH-FLAG-kan), by inserting yobH into the chromosome of the ΔyobH mutant as described in Fig. S1, which was also assessed in the invasion assays. Furthermore, the ΔhilD and ΔssrB mutants were also tested in these assays as positive and negative controls, respectively; SsrB is a transcriptional regulator that is required for Salmonella intracellular replication but not for invasion of host cells. The ΔyobH mutant showed a ~3-fold decrease in the invasion of both HeLa cells and RAW264.7 macrophages with respect to the WT and the complemented ΔyobH + yobH-FLAG-kan strains (Fig. 2A,B). As expected, the ΔhilD mutant was unable to invade the HeLa cells and RAW264.7 macrophages, whereas the ΔssrB mutant invaded these cells at similar levels to those showed by the WT and the complemented ΔyobH mutant.
∆yobH + yobH-FLAG-kan strains (Fig. 2A,B). Important to note, the number of bacteria present in the starting inoculums used in the invasion assays showed a variation of only 18% between the different strains tested (Fig. S2). Together, these results indicate that YobH is a novel invasion factor of S. Typhimurium.

HilD controls expression of yobH through SprB. We sought to determine if HilD regulates the yobH virulence gene directly or indirectly. For this, we performed electrophoretic mobility shift assays (EMSAs) by using affinity-purified maltose-binding protein (MBP)-HilD and a DNA fragment spanning the intergenic region upstream of yobH. DNA fragments carrying the regulatory region of hilA or sopB were also assessed in these assays as positive and negative controls, respectively; HilD binds to hilA but not to sopB23. As show in Fig. S3A, MBP-HilD did not shift the yobH fragment or that of sopB, even at the highest protein concentration tested (1 μM). In contrast, MBP-HilD shifted the positive control, hilA, at concentrations from 0.1 to 1 μM (Fig. S3B). These assays indicate that HilD does not interact with the regulatory region of yobH; alternatively, HilD could require an additional factor to bind to yobH.

To investigate whether HilD requires another S. Typhimurium regulator to induce the expression of yobH, we monitored the activity of the yobH-cat fusion in the WT E. coli MCA100 strain, which lacks HilD and the other
Salmonella-specific regulators, in the presence of the pK6-HilD plasmid expressing HilD or in the presence of the pMPM-K6Ω vector. As a positive control, activity of the hilA-cat fusion was also tested; hilA is directly regulated by HilD.47,48 As expected, the yobH-cat and hilA-cat fusions showed low or undetectable expression levels in E. coli (Fig. S3C,D). The activity of hilA-cat, but not that of yobH-cat, was induced by HilD in E. coli (Fig. S3C,D), indicating that an additional factor, present in S. Typhimurium SL1344 but not in E. coli MC4100, is required for the HilD-mediated expression of yobH.

Several studies have shown that HilD induces expression of a high number of virulence genes through distinct regulatory cascades involving the HilA, InvF, HilC, RtsA, SsrA/SsrB and FlhDC transcriptional regulators.25,28,29,32,33 To determine if any of these regulators are required for the HilD-mediated expression of yobH, activity of the yobH-cat fusion was measured in the WT S. Typhimurium strain and its isogenic ΔhilD, ΔinvF, ΔhilC, ΔrtsA, ΔssrB and ΔflhDC mutants. As positive controls, the ΔSPI-1 and ΔhilD mutants were also tested; SPI-1 encodes HilID, HilIA, InvF and HilIC. Activity of the yobH-cat fusion was affected in the ΔSPI-1 and ΔhilD mutants, but not in the ΔhilA, ΔinvF, ΔhilC, ΔrtsA, ΔssrB and ΔflhDC mutants, with respect to the WT strain (Fig. 3), suggesting that HilD induces expression of yobH through a regulatory cascade different to those well characterized before this study.

Previous RNA-seq analyses indicate that HilD induces expression of two additional Salmonella-specific transcriptional regulators, SprB and SinR.28,45, encoded in SPI-1 and SPI-6, respectively. Moreover, we previously confirmed that HilD directly induces expression of sinR.28 SprB has been involved in the expression of several S. Typhimurium virulence genes28,46 whereas SinR remains uncharacterized. To investigate whether SprB and/or SinR are involved in the HilD-mediated expression of yobH, we monitored the activity of the yobH-cat fusion in the ΔsprB and ΔsinR mutants. Surprisingly, the activity of this fusion was reduced in the ΔsprB mutant, as in the ΔSPI-1 and ΔhilD mutants, whereas SinR remains uncharacterized. To investigate whether SprB and/or SinR are involved in the HilD-mediated expression of yobH, we monitored the activity of the yobH-cat fusion in the ΔsprB and ΔsinR mutants. Surprisingly, the activity of this fusion was reduced in the ΔsprB mutant, as in the ΔSPI-1 and ΔhilD mutants, whereas it was not affected in the ΔsinR mutant (Fig. 3), suggesting that SprB is required for the expression of yobH. Expression of SprB from the pK6-SprB plasmid, under an arabinose inducible promoter, restored the activity of yobH-cat in both the ΔsprB and the ΔhilD mutants (Fig. 4A). In contrast, expression of HilD from the pK6-HilD plasmid induced the activity of yobH-cat in the ΔhilD mutant (Fig. 1A), but not in the ΔsprB mutant (Fig. 4A). Similarly, SprB restored the expression of YobH-FLAG in the ΔhilD mutant, whereas HilD was unable to induce the expression of YobH-FLAG in the ΔsprB mutant (Fig. 4B). Altogether, these results support that SprB acts downstream of HilD for the expression of yobH.

Results from a chromatin immunoprecipitation sequencing (ChIP-seq) analysis revealed that SprB binds to the regulatory region of yobH in vivo.28 We sought to confirm the SprB binding on yobH by EMSAs; however, we were unable to purify the 6XHis-tagged SprB protein, probably due to its high insolubility. Alternatively, we investigated if SprB requires any other Salmonella-specific regulator to induce the expression of yobH. For this, the activity of the yobH-cat fusion was tested in the WT S. Typhimurium strain and in the WT E. coli MC4100 strain carrying the pMPM-K6Ω vector or the pK6-SprB plasmid. Activity of a cat transcriptional fusion of sirA, a gene expected to be not controlled by SprB, was also tested as negative control; an ortholog of sirA (uvrY) is present in E. coli K-12.28,30 The presence of SprB induced the activity of yobH-cat in E. coli to levels similar to those reached by this fusion in the WT S. Typhimurium strain (Fig. 5A); in contrast, SprB did not affect the activity of the sirA-cat fusion (Fig. 5B). These results are in line with the notion that SprB directly activates expression of yobH.

The results described above strongly suggest that HilD positively regulates the expression of sprB. To confirm this, we quantified the expression of sprB in the WT S. Typhimurium strain and its isogenic ΔhilD mutant. Expression of sprB seems to be controlled by both the regulatory region upstream of hilIC, which generates a hilIC-sprB transcript, and that located upstream of sprB.28 HilD regulation on the promoter upstream of hilIC has been extensively shown in previous studies.28,30,38,46,51 Thus, we evaluated the effect of HilD on the regulatory region upstream of sprB by constructing and analyzing a sprB-cat transcriptional fusion carrying this region. As shown in Fig. 6A, activity of the sprB-cat fusion was 2-fold reduced in the ΔhilD mutant, compared with its activation in the WT strain; furthermore, expression of HilD from the pK6-HilD plasmid increased 3-fold the activity of this fusion in the ΔhilD mutant, indicating that HilD induces expression of sprB by also acting on the regulatory region upstream of this gene.
To investigate whether HilD regulates expression of sprB (by acting on the regulatory region upstream of this gene) directly or through an additional Salmonella-specific factor, we determined the activity of the sprB-cat fusion in the WT E. coli MC4100 strain carrying the pK6-HilD plasmid or the pMPM-K6Ω vector. As expected, activity of the sprB-cat fusion was 3-fold lower in the E. coli strain than in the WT S. Typhimurium strain.

**Figure 4.** HilD induces the expression of yobH through SprB. (A) Activity of the yobH-cat transcriptional fusion from the pyobH-cat plasmid, was determined in the WT S. Typhimurium SL1344 strain and its isogenic ΔsprB mutant containing or not the pMPM-K6Ω vector, or the pK6-SprB or pK6-HilD plasmids, as well as in the ΔhilD mutant containing the pMPM-K6Ω vector or the pK6-SprB plasmid. Means and standard deviations from three independent experiments performed in duplicate are shown. Statistically different values are indicated (**p < 0.001). (B) Expression of YobH-FLAG in WT S. Typhimurium SL1344 strain and its isogenic ΔsprB mutant containing the pMPM-K6Ω vector or the pK6-HilD plasmid, as well as in the ΔhilD mutant containing the pMPM-K6Ω vector or the pK6-SprB plasmid, was analyzed by Western blotting by using an anti-FLAG monoclonal antibody. GroEL was detected as a loading control with an anti-GroEL polyclonal antibody. Blots were cropped from different parts of the same gel. CAT specific activity and YobH-FLAG expression were quantified from samples of bacterial cultures grown for 9 h in LB at 37°C.

**Figure 5.** SprB induces expression of yobH in the absence of Salmonella-specific regulators. Activity of the yobH-cat (A) and sirA-cat (B) transcriptional fusions from the pyobH-cat and psirA-cat plasmids, respectively, was determined in the WT S. Typhimurium SL1344 strain and in the WT E. coli MC4100 strain containing or not the pMPM-K6Ω vector or the pK6-SprB plasmid expressing SprB under an arabinose inducible promoter. CAT specific activity was quantified from samples of bacterial cultures grown for 9 h in LB containing 0.001% L-arabinose, at 37°C. Means and standard deviations from three independent experiments performed in duplicate are shown. Statistically different values are indicated (**p < 0.001).
HilD counteracts H-NS-mediated repression on sprB. HilD induces expression of target genes mainly by counteracting H-NS-mediated repression on the respective promoters. To know whether HilD induces expression of sprB by a similar way, we analyzed if inactivation of H-NS leads to HilD-independent expression of this gene. Since a Salmonella Δhns mutant exhibits a severe growth defect, we inactivated H-NS activity by the overexpression of the H-NSG113D dominant negative mutant, which is affected in DNA binding activity but still forms heterodimers with the WT H-NS monomers. For this purpose, activity of the sprB-cat fusion was quantified in the WT S. Typhimurium strain and its isogenic Δhild mutant containing the pT6-HNS-G113D or pT6-HNS-WT plasmids, which express H-NSG113D and WT H-NS, respectively, or containing the empty vector pMPM-T6Ω. Expression of H-NSG113D, but not WT H-NS, increased the activity of sprB-cat in the ΔhilD mutant, at similar levels to those reached by this fusion in the WT strain (Fig. 7A). Consistently, activity of sprB-cat was also induced in an E. coli Δhns mutant, compared with the WT E. coli strain (Fig. S4). Furthermore, EMSAs revealed that purified H-NS-FLAG-His (H-NS-FH) protein binds to the regulatory region upstream of sprB, from a concentration of 0.45 µM, but it does not bind to the regulatory region upstream of ppK, used as a negative control in these assays (Fig. 7B). Previous genome-wide binding studies indicate that H-NS interacts with the region upstream of sprB in vivo. In contrast to the observed for sprB, the activity of the yokH-cat fusion was not increased in the E. coli Δhns mutant and H-NS-FH.
did not bind to the regulatory region upstream of yobH (Fig. S5). These results show that H-NS directly represses expression of sprB, but not of yobH, and that when the activity of H-NS is inactivated, or when H-NS is absent, expression of sprB becomes independent of HilD, which supports that HilD acts on this gene as an anti-H-NS factor.

To determine whether HilD indeed displaces H-NS from sprB, we performed competitive EMSAs. A DNA fragment carrying the regulatory region of sprB was first incubated with a constant concentration of H-NS-FH (0.6 µM) and then increasing amounts of MBP-HilD (0.2, 0.4, 0.6, 0.8 and 1 µM) were added. Binding reactions containing only H-NS-FH or MBP-HilD were also tested. The DNA-protein complexes were detected by staining the DNA fragments with ethidium bromide; additionally, the presence of H-NS-FH on these complexes was detected by Western blot with anti-FLAG antibodies. As shown in Fig. 7C, the DNA-H-NS complex was shifted by the presence of MBP-HilD to a slower-migrating complex similar to that formed only by MBP-HilD (upper panel); furthermore, the immunoblots showed that the presence of MBP-HilD decreased the amount of H-NS-FH bound to the tested DNA fragment (lower panel), which indicates that HilD is able to remove H-NS from sprB.

Altogether, these results demonstrate that HilD induces expression of sprB by antagonizing H-NS-mediated repression on this gene.

The HilD-SprB regulatory cascade controls expression of the slrP and ugtL virulence genes. Previous RNA-seq analyses indicate that HilD and SprB positively controls expression of several other genes in common, in addition to yobH, including slrP and ugtL. To further define if HilD and SprB also act in a cascade fashion on slrP and ugtL, we constructed and analyzed cat transcriptional fusions carrying the regulatory region of the slrP or ugtL genes. Activity of the slrP-cat and ugtL-cat fusions was quantified in the WT S. Typhimurium strain and its derivative ∆hilD mutant containing the pK6-SprB plasmid or the pMPM-K6Ω vector. As a negative control, an invF-cat transcriptional fusion was quantified in the WT S. Typhimurium SL1344 strain and its isogenic ∆hilD mutant containing or not the pMPM-T6Ω vector, or the pT6-HNS-WT or pT6-HNS-G113D plasmids, with (+) and without (−) induction (0.1% L-arabinose). CAT specific activity was quantified from samples of bacterial cultures grown for 9 h in LB at 37 °C. Means and standard deviations from three independent experiments performed in duplicate are shown. Statistically different values are indicated (***p < 0.001). (B) EMSAs were performed with purified H-NS-FH (0, 0.2, 0.45 and 0.7 µM) and a DNA fragment containing the regulatory region of sprB. A DNA fragment containing the regulatory region of ppK was used as a negative internal control. The DNA-protein complexes, indicated by an asterisk, were resolved in a nondenaturing 6% polyacrylamide gel and stained with ethidium bromide. (C) Competitive nonradioactive EMSAs between H-NS and HilD on the regulatory region of sprB. Purified H-NS-FH protein was added at 0.6 µM (lanes 3 to 8) and purified MBP-HilD protein was added at 0.2, 0.4, 0.6, 0.8 and 1 µM (lanes 4 to 8, respectively). No proteins were added in lane 1 and MBP-HilD was added at 1 µM in lane 2. The DNA-protein complexes were resolved in a nondenaturing 6% polyacrylamide gel. The upper panel shows the protein-DNA complexes stained with ethidium bromide and the lower panel shows the immunoblot detection of H-NS-FH from the DNA-protein complexes. Blots for DNA or protein detection were cropped from different gels.
The systemic infection in the mouse model was studied. The results are in agreement with previous analyses by transposon-directed insertion-site sequencing (TraDIS) and demonstrate that the HilD-SprB regulatory cascade induces expression of the \(\text{slrP}\) and \(\text{ugtL}\) genes. Activity of the \(\text{slrP-cat}\), \(\text{ugtL-cat}\) and \(\text{invF-cat}\) transcriptional fusions from the \(\text{pslrP-cat}\), \(\text{psugL}\)-cat and \(\text{pinvF}\)-cat plasmids, was determined in the WT S. Typhimurium SL1344 strain and its isogenic \(\Delta\text{hilD}\) mutant containing the pMPM-K6Ω vector or the pK6-SprB plasmid. CAT specific activity was quantified from samples of bacterial cultures grown for 9h in LB at 37°C. Means and standard deviations from three independent experiments performed in duplicate are shown. Statistically different values are indicated (**p < 0.001; ***p < 0.0001).

**Figure 8.** The HilD-SprB regulatory cascade induces expression of the \(\text{slrP}\) and \(\text{ugtL}\) genes. Activity of the \(\text{slrP-cat}\), \(\text{ugtL-cat}\) and \(\text{invF-cat}\) transcriptional fusions from the \(\text{pslrP-cat}\), \(\text{psugL}\)-cat and \(\text{pinvF}\)-cat plasmids, was determined in the WT S. Typhimurium SL1344 strain and its isogenic \(\Delta\text{hilD}\) mutant containing the pMPM-K6Ω vector or the pK6-SprB plasmid. CAT specific activity was quantified from samples of bacterial cultures grown for 9h in LB at 37°C. Means and standard deviations from three independent experiments performed in duplicate are shown. Statistically different values are indicated (**p < 0.001; ***p < 0.0001).

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**Discussion**

Acquisition of SPI-1 was a pivotal event for the evolution of *Salmonella* pathogenicity, not only by the virulence factors encoded in this island, which provide ability to invade host cells, but also by the additional factors for invasion encoded outside SPI-1 that have been recruited through the control of their expression by the SPI-1 regulator HilD. In this study, we identify a novel invasion factor, YobH, whose expression is controlled by HilD. Our results demonstrate that YobH is required for the S. Typhimurium invasion of HeLa cells and mouse macrophages. In agreement with these results, a previous analysis by transposon-directed insertion-site sequencing (TraDIS) suggests that YobH plays a role in the intestinal colonization of *S. Typhimurium* in chicks and cows, but not in the systemic infection in the mouse model. The *yobH* gene is located outside SPI-1, in a chromosomal region conserved in many bacteria, including *S. bongori* and *E. coli* K-12; YobH shares 79% sequence identity with its ortholog from *E. coli* K-12. A previous study indicates that HilD directly regulates expression of the \(\text{fbhDC}\) operon, encoding the master regulator of the flagellar genes, which is also conserved in *E. coli* K-12 and many other bacteria. YobH and its orthologs from different bacteria are on average 80 amino acids long and have no assigned function; they are annotated as putative membrane, exported or uncharacterized proteins. Our preliminary results support that YobH is secreted in *S. Typhimurium* (data not shown). How is YobH secreted and what is the specific function of YobH for invasion are topics of our current investigation.

HilD induces expression of a high number of target genes by acting directly or through distinct regulators, in growth conditions that somehow resemble the intestinal environment (SPI-1-inducing conditions), such as those that we assessed in this study. At present, three different regulatory cascades headed by HilD have been well characterized: the HilD-Hila-InvF, HilD-SsrA/SsrB and HilD-FlhDC cascades. Additionally, HilD forms a feed-forward positive loop with HilC and RtsA, which amplifies the activation of the HilD-Hila-InvF cascade, and probably also the activation of the other regulatory cascades and genes controlled directly by HilD. Our data, together with previous results obtained from genome-wide expression and binding analyses, define an additional cascade formed by HilD to induce expression of virulence genes. In this regulatory cascade, HilD induces expression of the \(\text{yobH}\), \(\text{slrP}\) and \(\text{ugtL}\) virulence genes through SprB, a *Salmonella*-specific LuxR-like regulator encoded in SPI-1. Previous studies revealed that HilD and RtsA induce expression of \(\text{yobH}\) by an undefined way. \(\text{SlrP}\) (*Salmonella* leucine-rich repeat protein) is an effector protein with ubiquitin ligase activity that is translocated into mammalian cells through both T3SS-1 and T3SS-2. \(\text{UgtL}\) is an inner membrane protein that mediates resistance to antimicrobial peptides by modifying lipid A in the lipopolysaccharide; furthermore, it is involved in the activation of the PhoP/PhoQ two-component regulatory system in response to mildly acidic pH. \(\text{UgtL}\) is required by *S. Typhimurium* for killing and for the intestinal colonization of mice; moreover, TraDIS analysis supports that \(\text{UgtL}\) is important for the intestinal colonization of *S. Typhimurium* in pigs. Importantly to note, expression of both \(\text{slrP}\) and \(\text{ugtL}\) is also controlled directly by the PhoP/PhoQ two-component system, in growth...
conditions that somehow mimic the intracellular environment of host cells (SPI-2-inducing conditions)\(^\text{53,65}\), where the HilD-mediated regulation on target genes is not evident\(^\text{25,30,38,48,51}\); in contrast, expression of yobH seems to be not regulated by PhoP/PhoQ\(^\text{46}\). PhoP forms with the SlyA regulator a feed-forward loop that controls expression of ugtL in SPI-2-inducing conditions\(^\text{60,65}\). Thus, expression of both ugtL and strP is controlled by at least two distinct regulatory mechanisms that act in response to different environmental conditions. HilD-SprB and PhoP-SlyA would induce expression of ugtL and strP in different niches where the activity of these genes is required for the Salmonella infection of hosts. For instance, activity of UgtL is needed for the intestinal colonization and for the systemic infection of mice\(^\text{58,59}\). On another hand, it is tempting to speculate that HilD-SprB helps to reach the levels of UgtL required for the subsequent UgtL-mediated activation of the PhoP/PhoQ system in response to acidic pH\(^\text{58}\), a cue present in the intracellular environment. Following this idea, it has been shown that activated PhoP represses expression of hilD, hilA and rtsA, and thus the SP-1 invasion genes\(^\text{47}\); therefore, the HilD-SprB cascade represents an additional branch that further expands the HilD virulence regulon, connecting the activity of several genes located outside SPI-1 with the capability for invasion of host cells encoded within SPI-1.

Our results demonstrate that HilD positively controls expression of sprB by acting on the regulatory region upstream of this gene. Previous studies indicate that HilD can also control expression of hilC by acting on the hilC gene, located upstream of sprB; a hilC-sprB transcript was detected in a previous study\(^\text{42}\) and direct regulation of hilC by HilD is well documented\(^\text{60,50,38,48,51}\). We show that HilD induces expression of sprB by directly displacing the repressor H-NS from the regulatory region upstream of this gene; a mechanism that HilD follows to induce expression of other target genes\(^\text{30,37,41-43}\). H-NS represses expression of hilC\(^\text{43,53,69,70}\), which suggest that HilD induces expression of the hilC-sprB transcript also by antagonizing H-NS mediated repression. In contrast to HilD, which is required for the expression of target genes only in the presence of H-NS, we found that SprB is required for the expression of yobH even in the absence of H-NS, which supports that it does not act as an anti-H-NS factor. There is growing evidence to suggest that other LuxR-like regulators mainly act as classical activators, which induce expression of target genes by favoring binding of the RNA polymerase on promoters\(^\text{71-74}\). Whether SprB antagonizes a repressor different to H-NS or whether it acts as a classical activator remains to be elucidated.

Our data reveal a novel Salmonella invasion factor and further define an additional regulatory cascade mediated by HilD for the expression of Salmonella virulence genes. A model that summaries the results from this study is depicted in Fig. 9.

**Methods**

**Bacterial strains and growth conditions.**  Bacterial strains used in this study are listed in Table S1. Bacterial cultures for the determination of chloramphenicol acetyl transferase (CAT) activity and for Western blot assays were grown in LB as described previously\(^\text{25,30,36}\). When necessary, the medium was supplemented with the following antibiotics: ampicillin (200 μg/ml), streptomycin (100 μg/ml), kanamycin (20 μg/ml) or tetracycline, (12 μg/ml).

**Construction of plasmids.**  Plasmids and primers used in this study are listed in Tables S1 and S2, respectively. To generate the yobH-cat, sprB-cat, strP-cat and ugtL-cat transcriptional fusions, the regulatory regions of yobH, sprB, strP and ugtL were amplified by PCR using the primer pairs SL1770-FW22/SL1770-RV11, sprB-catF/sprB-catR, strPB2-Fw22/sprPB3-Rv11 and ugtL-Fw/ugtL-Rv, respectively, and chromosomal DNA from the WT S. Typhimurium strain as template. The resulting PCR products were purified with the Zymoclean Gel DNA Recovery Kit (Zymo Research), digested with BamHI and HindIII enzymes and then cloned into the pKK232-8 vector\(^\text{86}\) digested with the same restriction enzymes. To construct the p2795-YobH-FLAG plasmid, the yobH::3XFLAG-kan) strain. P22 transduction was used to transfer the yobH::3XFLAG-kan allele from the strain DTM127 into the strains JPTM25 and DTM122, generating the strains DTM126 and DTM131, respectively. The kanamycin resistance cassette was excised from the strains DTM121, DTM124, DTM127, DTM129 and DTM131, by using the pCP20 plasmid expressing the FLP recombinase, as described previously\(^\text{49}\), generating the strains DTM122, DTM125, DTM128, DTM130 and DTM132, respectively. The complemented DTM126 strain was generated by inserting the yobH::3XFLAG-kan into the chromosome.
of the DTM125 strain, using a previously reported method based on the λRed recombinase system and the p2795-YobH-FLAG plasmid. All modified strains were verified by PCR amplification and sequencing.

**Chloramphenicol acetyltransferase (CAT) assays.** The CAT activity and protein quantification to calculate CAT specific activities were determined as previously described.

**Statistical analysis.** Data were analyzed with GraphPad Prism 5.0 software (GraphPad Inc., San Diego, CA) using One-Way analysis of variance (ANOVA) with the Tukey's multiple comparison test. A P-value of <0.05 was considered significant.

**Electrophoretic mobility shift assays (EMSAs).** Fragments spanning the regulatory regions of yobH, hilA, sopB, sprB and ppK were obtained by PCR amplification using the primer pairs SL1770-FW22/SL1770-RV11, hilA1FBamHI/hilA2RHindIII, SigDBH1F/SigDH3R, sprB-catF/sprB-catR and PPK-Fw1/PPK-Rv1, respectively, and chromosomal DNA from the WT S. Typhimurium strain as template. Binding reactions were performed as described previously. For competitive EMSAs, the sprB fragment was first incubated with 0.6 µM H-NS-FH for 15 min and then incubated with increasing concentrations MBP-HilD for an additional 20 min. Binding mixtures were electrophoretically separated in 6% nondenaturing acrylamide in 0.5X Tris-borate-EDTA buffer, at room temperature. DNA bands were visualized by staining with ethidium bromide, in an Alpha-Imager UV transilluminator (Alpha Innotech Corp.).

**Expression and purification of proteins.** Expression and purification of MBP-HilD and H-NS-FH were performed as described previously.

**Western blotting.** Western blot assays were performed as described previously. Anti-FLAG M2 monoclonal antibodies (Sigma) were used at 1:2,000 or 1:3,000 dilutions, for detection of YobH-FLAG and H-NS-FH, respectively. Anti-GroEL polyclonal antibodies were used at a dilution of 1:100,000. Horseradish peroxidase-conjugated secondary antibodies (Pierce), anti-mouse or anti-rabbit, were used at a dilution of 1:10,000. Blots were developed by incubation with the Western Lightning Chemiluminescence Reagent Plus (Perkin-Elmer) and then exposition to KodaK X-Omat films.

**Invasion assays.** Invasion of HeLa cells or RAW264.7 macrophages was determined by gentamicin protection assays as described previously. Briefly, HeLa cells or RAW264.7 macrophages were grown in high-glucose Dulbecco's Modified Eagle Medium (GIBCO 12100-046) supplemented with 10 mM sodium pyruvate solution,
20 mM L-glutamine and 10% (v/v) heat-inactivated fetal bovine serum, at 37 °C and a 5% CO2 atmosphere, in 24-well tissue culture plates. Monolayers of HeLa cells or RAW264.7 macrophages, from each well, were infected during 10 min with the respective bacterial suspension obtained from LB cultures, using a multiplicity of infection (MOI) of 10:1 (bacterial to eukaryotic cells). After the time of infection, monolayers were washed and then incubated during 1 h in DMEM containing 50 μg/ml gentamicin to eliminate extracellular bacteria. DMEM was removed and the HeLa cells and RAW264.7 macrophages from each well were lysed in 1 ml and 200 μl of 0.2% (w/v) sodium deoxycholate in 1X PBS, respectively. To obtain the intracellular CFUs per well, serial dilutions of each cell lysate were plated onto LB agar supplemented with 100 μg/ml streptomycin. CFUs from the starting inoculums were also quantified.

Data Availability
All data generated or analyzed during the current study are available from the corresponding author on reasonable request.

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Author Contributions
V.H.B. designed and supervised the research; M.M.B. and R.M. performed experimental research; M.M.B., R.M. and V.H.B. analyzed data; and M.M.B. and V.H.B. wrote the paper. All authors read and approved the final manuscript.

Additional Information
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