The Hcp-like protein HilE inhibits homodimerization and DNA binding of the virulence-associated transcriptional regulator HilD in Salmonella

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Running title: HilE controls dimerization and DNA binding of HilD

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Abstract
HilD is an AraC-like transcriptional regulator that plays a central role in Salmonella virulence. HilD controls the expression of the genes within the Salmonella pathogenicity island 1 (SPI-1) and of several genes located outside SPI-1, which are mainly required for Salmonella invasion of host cells. The expression, amount and activity of HilD are tightly controlled by the activities of several factors. The HilE protein represses the expression of the SPI-1 genes through its interaction with HilD; however, the mechanism by which HilE affects HilD is unknown. In this study, we used genetic and biochemical assays revealing how HilE controls the transcriptional activity of HilD. We found that HilD needs to assemble in homodimers to induce expression of its target genes. Our results further indicated that HilE individually interacts with each the central and the C-terminal HilD regions, mediating dimerization and DNA binding, respectively. We also observed that these interactions consistently inhibit HilD dimerization and DNA binding. Interestingly, a computational analysis revealed that HilE shares sequence and structural similarities with Hcp proteins, which act as structural components of type 6 secretion systems in Gram-negative bacteria. In conclusion, our results uncover the molecular mechanism by which the Hcp-like protein HilE controls dimerization and DNA binding of the virulence-promoting transcriptional regulator HilD. Our findings may indicate that HilE’s activity represents a functional adaptation during the evolution of Salmonella pathogenicity.

Introduction
The genus Salmonella groups pathogenic bacteria for human and many animals; it comprises only two species, S. enterica and S. bongori, the former is further divided into six subspecies and more than 2500 serovars. Depending on the host, S. enterica serovar Typhimurium (S. Typhimurium) can cause diseases ranging from gastroenteritis to life-threatening systemic infection (1,2). For instance, in humans, calves and chicken S. Typhimurium causes self-limiting gastroenteritis, whereas in laboratory mice it causes a systemic infection resembling that produced by S. Typhi in humans; thus, S. Typhimurium is frequently used as a model to study the molecular mechanisms mediating the Salmonella virulence (1,3,4). Horizontal gene transfer events have greatly contributed to the evolution of the Salmonella pathogenicity (5,6). Most of the genes gained by Salmonella are clustered in chromosomal regions called Salmonella pathogenicity islands (SPIs) (4,5). SPI-1 is a 40 kb region conserved in the two Salmonella species, which includes 39 genes
encoding a type 3 secretion system (T3SS-1), their chaperones and effector proteins, as well as some transcriptional regulators that control the expression of many virulence genes located within and outside SPI-1 (4,7). The T3SSs are molecular syringes that extend from the membranes of several bacteria, composed of a basal body and a needle-like complex, through which effector proteins are injected from the bacterial cytoplasm into the cytoplasm of eukaryotic cells (8). *Salmonella* injects the SPI-1 effector proteins into the intestinal epithelial cells through the T3SS-1, which induces cytoskeletal rearrangements promoting the *Salmonella* invasion of these eukaryotic cells leading to enteritis (1,4,8).

The expression of the SPI-1 genes is controlled by several environmental clues, such as osmolarity, oxygen tension, pH, short- and long-fatty acids concentration and bile (4,9-11). In vitro, expression of the SPI-1 genes is induced at early stationary phase when *Salmonella* is grown in the nutrient-rich lysogenic broth (LB) (12,13). Several regulators control the expression of the SPI-1 genes, including HilD, HilA and InvF, all encoded in SPI-1, which act in a cascade fashion: HilD, a member of the AraC/XylS transcriptional regulators family, directly induces the expression of HilA, which in turn activates the expression of InvF; HilA and InvF activate the expression of all components of the T3SS-1, their chaperones and effector proteins (4,14). HilD induces the expression of HilA directly, or through a positive feed-forward loop that it forms with HilC and RtsA (15,16). HilC and RtsA are AraC-like transcriptional regulators that bind the same DNA sequence recognized by HilD; HilC is encoded within SPI-1, whereas RtsA is encoded in another island. HilD also controls the expression of many other virulence genes located outside SPI-1, including acquired and ancestral genes, directly, or indirectly through HilA, InvF or other regulators (12,17-25). In agreement with its role as a master transcriptional regulator for a high number of genes, the expression, concentration and activity of HilD is tightly controlled. Transcription of *hilD* is positively auto-regulated (15,26) and is repressed by H-NS (27), whereas its translation is repressed by CsrA (28). In addition, Fur and FliZ control HilD at post-translational level (29,30); the Lon protease degrades HilD, thus mediating its concentration (31,32); the CpxR/A two-component system decreases the stability of HilD through Lon-dependent and independent mechanisms (33). Furthermore, several compounds affect HilD: propionate and bile salts decrease its stability (9,10); butyrate and oleate negatively affect its regulatory activity, whereas acetate and formate enhance this (11,34-36); and L-arabinose affects HilD expression at post-transcriptional level (37).

The activity of HilD is also negatively controlled by HilE through protein-protein interaction (38). However, the specific effect of this interaction on HilD has remained unknown. The *hilE* gene resides in a region of the *Salmonella* chromosome similar to a pathogenicity island (38), supporting that it was acquired by horizontal transfer. The expression of *hilE* is positively regulated by the PhoPQ and PhoBR two-component systems, as well as by FimZ and LuxO (39,40), whereas this is negatively regulated by Mlc and the small RNA IsmR (41,42), which provides additional inputs controlling the activity of HilD.

Here we show that HilE negatively affects dimerization and DNA binding of HilD, by interacting with both the central and C-terminal regions of HilD, which mediate dimerization and DNA binding, respectively. Therefore, our results demonstrate how HilE regulates HilD activity. Additionally, our results revealed that HilE shares sequence and structural similarities with proteins called Hep (Haemolysin-coregulated protein), which are structural components of type 6 secretion systems in Gram-negative bacteria, supporting the hypothesis that HilE was adapted to act as an important regulatory protein during the *Salmonella* pathogenicity evolution.

**Results**

**HilE interacts with the central region and with the C-terminal region of HilD.**

To investigate how HilE negatively affects the activity of HilD, we analyzed the interaction of HilE with different regions of HilD by using the LexA-based genetic system for heterodimerization, which is similar to a two-hybrid system to analyze protein-protein...
interactions (43,44). It is important to remark that the interaction between HilE and HilD was previously demonstrated with this system (38). In the LexA-based genetic system for heterodimerization, the WT LexA DNA binding domain (LexA<sub>DBDwt</sub>) and a derivative mutant of this (LexA<sub>DBDmut</sub>) are expressed from two different plasmids in the *Escherichia coli* SU202 reporter strain, which carries a chromosomal sulA-lacZ transcriptional fusion containing a LexA hybrid operator (43,44). LexA<sub>DBDwt</sub> and LexA<sub>DBDmut</sub> cannot affect the expression of sulA-lacZ; however, when they are fused to proteins that interact between them, an active dimer of LexA<sub>DBDwt</sub> and LexA<sub>DBDmut</sub> is formed, which is able to bind the hybrid LexA operator on sulA-lacZ and thus to repress the expression of this fusion. Fusion proteins of LexA<sub>DBDwt</sub> with full-length or distinct regions of HilD, and of LexA<sub>DBDmut</sub> with full-length HilE, were generated and assessed. The LexA<sub>DBDwt</sub>-HilD fusions were named according to the amino acid position for the ends of the HilD fragment carried (Fig. 1A). As expected, the combination LexA<sub>DBDwt</sub>-HilD<sub>1-309</sub> (full-length HilD) + LexA<sub>DBDmut</sub>-HilE (full-length HilE), but not LexA<sub>DBDwt</sub> + LexA<sub>DBDmut</sub> or LexA<sub>DBDmut</sub>-HilD + LexA<sub>DBDmut</sub> mediated repression of sulA-lacZ (Fig. 1B), further confirming the interaction between HilD and HilE. Interestingly, the fusion proteins LexA<sub>DBDwt</sub>-HilD<sub>1-220</sub> or LexA<sub>DBDmut</sub>-HilD<sub>221-309</sub>, in combination with LexA<sub>DBDmut</sub>-HilE, also repressed the expression of sulA-lacZ (Fig. 1B), indicating that HilE interacts with the regions of HilD spanning the amino acids 1 to 220 and 221 to 309. In contrast, the combination LexA<sub>DBDwt</sub>-HilD<sub>1-130</sub> + LexA<sub>DBDmut</sub>-HilE did not repress the expression of sulA-lacZ, supporting that HilE does not interact with the N-terminal region of HilD from amino acid 1 to 130. However, the lack of interaction between LexA<sub>DBDwt</sub>-HilD<sub>1-130</sub> and LexA<sub>DBDmut</sub>-HilE could be due to a not correctly folding of LexA<sub>DBDmut</sub>-HilD<sub>1-130</sub>. As expected, the combinations LexA<sub>DBDwt</sub>-HilD<sub>1-130</sub> + LexA<sub>DBDmut</sub>- LexA<sub>DBDmut</sub>-HilD<sub>1-220</sub> + LexA<sub>DBDmut</sub> or LexA<sub>DBDmut</sub>-HilD<sub>221-309</sub> + LexA<sub>DBDmut</sub> used as negative controls, did not affect the expression of sulA-lacZ (Fig. 1B). A Western blot analysis showed expression signals for all the LexA<sub>DBDmut</sub>-HilD fusion proteins tested (Fig. 1C). To note, the LexA<sub>DBDmut</sub>-HilD<sub>221-309</sub> fusion repressed the sulA-lacZ fusion, in combination with LexA<sub>DBDmut</sub>-HilE (Fig. 1B), even when its expression level was lower than that of the LexA<sub>DBDwt</sub>-HilD<sub>1-309</sub> and LexA<sub>DBDmut</sub>-HilD<sub>1-220</sub> fusions (Fig. 1C), indicating that differences in the amount of the LexA<sub>DBDwt</sub>-HilD fusion proteins assessed did not affect the results of these assays. In agreement with a previous report (45), expression of LexA<sub>DBD</sub> was not detected in our Western blot analysis (Fig. 1C). These results support that HilE interacts with regions spanning amino acids 130 to 220 and 221 to 309 of HilD.

Pull-down assays were performed to confirm the HilE interactions with HilD. For this, we purified HilE fused to the Trx protein and a 6X-His tag (Trx-His-HilE) as described in Experimental procedures. The Trx-His-HilE fusion protein repressed the SPI-1-mediated protein secretion profile of the WT *S. Typhimurium* strain (Fig. 2A), which supports that it is able to negatively affect HilD. Trx-His-HilE was used as the bait protein in the pull-down assays; firstly it was immobilized on Ni-NTA resin and then whole-cell extracts containing either LexA<sub>DBD</sub> or LexA<sub>DBDmut</sub>-HilD<sub>1-309</sub>, LexA<sub>DBDmut</sub>-HilD<sub>1-130</sub>, LexA<sub>DBDmut</sub>-HilD<sub>1-220</sub>, LexA<sub>DBDmut</sub>-HilD<sub>221-309</sub> prey proteins were loaded to the Ni-NTA resin carrying Trx-His-HilE. As expected, Trx-His-HilE captured the LexA<sub>DBDmut</sub>-HilD<sub>1-309</sub> and LexA<sub>DBDmut</sub>-HilD<sub>1-220</sub> prey proteins, but not LexA<sub>DBDwt</sub>-HilD<sub>1-130</sub> or LexA<sub>DBDwt</sub> (Fig. 2B), confirming the interaction of HilE with full-length HilD and the central region of HilD. LexA<sub>DBDmut</sub>-HilD<sub>221-309</sub> showed interaction with HilE in the LexA-based genetic system (Fig. 1B); however in the pull-down assays this interaction was not evident (Fig. 2B), which could be explained by the low level of expression showed by LexA<sub>DBDmut</sub>-HilD<sub>221-309</sub> (Fig. 1C). As a control, parallel pull-down assays were performed using Trx-His as the bait protein, which was unable to capture any of the LexA<sub>DBDmut</sub>-HilD prey proteins tested (Fig. 2C).

Taken together, these results indicate that HilE binds to both the central region and the C-terminal region of HilD.

**HilE does not affect the stability of HilD**

The interaction with HilE could affect the stability of HilD, to investigate this, we determined the in...
**HilD acts as a dimer and its central region mediates the interaction itself**

Several AraC-like transcriptional regulators act as dimers (46-49). Therefore, we thought that the interaction of HilE with the central region of HilD could affect dimerization of HilD. To investigate this possibility, we first analyzed whether HilD indeed dimerizes by using the LexA-based genetic system for homodimerization (43,44). In this case, LexA_{DBDwt} is expressed from a plasmid in the E. coli SU101 reporter strain, which carries a chromosomal sulA-lacZ transcriptional fusion containing the LexA WT operator (43,44). LexA_{DBDwt} does not affect the expression of sulA-lacZ; however, when it is fused to a protein that interacts itself, an active dimer of LexA_{DBDwt} is formed, which is able to bind the WT LexA operator on sulA-lacZ and thus represses the expression of this fusion. The same LexA_{DBDwt} fusion proteins used before in the heterodimerization assay, were now tested in the homodimerization assay. LexA_{DBDwt} alone and the fusion protein LexA_{DBDwt-H-NS}, whose dimerization capacity has been tested before (50), were used as negative and positive controls, respectively. Both, LexA_{DBDwt-H-NS} and LexA_{DBDwt-HilD1-309}, but not LexA_{DBDwt}, repressed the expression of sulA-lacZ (Fig. 4A), indicating that HilD dimerizes. A Western blot analysis showed expression signals for the LexA_{DBDwt-HilD1-309} and LexA_{DBDwt-H-NS} fusion proteins (Fig. 4B). To confirm the dimerization of HilD, the size of the maltose binding protein (MBP)-HilD fusion, purified in native conditions, was analyzed by gel-filtration chromatography. The MBP-HilD fusion protein eluted from the gel-filtration chromatography as a ~178 kDa product (supplemental Fig. S1), which corresponds to a size similar to that expected for a dimer of this protein.

Next, we sought to determine if dimerization is required for the regulatory activity of HilD. For this, the ability to induce the expression of a hilA-cat transcriptional fusion of LexA_{DBDwt-HilD1-309}, LexA_{DBDwt-HilD21-309} and LexA_{DBDwt-HilD1-309} which carry the DNA binding domain of HilD, was tested in an E. coli K-12 strain. Since hilA is a gene directly and positively controlled by HilD (4,51) and HilD is not present in E. coli K-12. As a negative control, LexA_{DBDwt} was also assessed; additionally, as a reference for the expression of hilA-cat in the presence or absence of HilD, the activity of this fusion was also determined in the WT S. Typhimurium strain and its isogenic ΔhilD mutant. The expression of hilA-cat was induced in the presence of LexA_{DBDwt-HilD1-309} or LexA_{DBDwt-HilD1-309}, which show dimerization, but it was not induced by LexA_{DBDwt-HilD21-309} that does not dimerize (Fig. 5). As expected, the expression of hilA-cat was also induced in the WT S.
Typhimurium strain, but not in the ΔhilD mutant, or in the presence of LexA<sub>DBDwt</sub> (Fig. 5). These results support that HilD needs to form dimers to induce the expression of target genes. To confirm this, we generated and analyzed the chimeric protein LexA<sub>DBDwt</sub>-LZ-HilD<sub>221-309</sub>, which is a fusion of LexA<sub>DBDwt</sub>, the leucine zipper (LZ) motif of the GCN4 transcriptional factor of <i>Saccharomyces cerevisiae</i>, and the DNA binding domain of HilD (amino acids 221 to 309) (Fig. 6A). The LZ motif has been used before as a dimerization module (52). The ability of the LexA<sub>DBDwt</sub>-LZ-HilD<sub>221-309</sub> fusion protein to undergo dimerization and to induce the expression of hilA was assayed and compared with that of the LexA<sub>DBDwt</sub>-HilD<sub>221-309</sub> and LexA<sub>DBDwt</sub>-HilD<sub>1-309</sub> fusion proteins. Both LexA<sub>DBDwt</sub>-LZ-HilD<sub>221-309</sub> and LexA<sub>DBDwt</sub>-HilD<sub>1-309</sub>, but not LexA<sub>DBDwt</sub>-HilD<sub>221-309</sub>, repressed the expression of sulA-lacZ in the <i>E. coli</i> SU101 reporter strain (Fig. 6B), indicating that LexA<sub>DBDwt</sub>-LZ-HilD<sub>221-309</sub> dimerizes through the heterologous LZ motif. Notably, LexA<sub>DBDwt</sub>-LZ-HilD<sub>221-309</sub> and LexA<sub>DBDwt</sub>-HilD<sub>1-309</sub>, but not LexA<sub>DBDwt</sub>-HilD<sub>221-309</sub>, induced the expression of the hilA-cat fusion in <i>E. coli</i> K-12 (Fig. 5), showing that the LZ motif generates dimers of the DNA binding domain of HilD which are able to induce expression of target genes.

In all, these results demonstrate that HilD dimerizes through its central region spanning amino acids 130 to 220, which is essential for the regulatory activity of HilD.

### HilE negatively affects the dimerization of HilD

Based on the results described above, we now analyzed whether HilE affects the dimerization of HilD. For this, the LexA<sub>DBDwt</sub>-HilD<sub>1-309</sub> fusion protein, which carries the dimerization domain of HilD, was tested for homodimerization in the presence of the pA6-HilE1 plasmid expressing HilE, or in the presence of the pMPM-A6Ω vector. Expression of HilE from pA6-HilE1 drastically reduced the SPI-1-mediated protein secretion profile of the WT <i>S. Typhimurium</i> strain (Fig. 7A), supporting that the amount of HilE reached from this plasmid can negatively affect HilD. The effect of HilE on LexA<sub>DBDwt</sub>-LZ-HilD<sub>221-309</sub> and LexA<sub>DBDwt</sub>-H-NS, which dimerize through the LZ motif and the H-NS dimerization domain, respectively, was also assessed as negative controls. Interestingly, HilE affected the repression of sulA-lacZ mediated by the dimerization of LexA<sub>DBDwt</sub>-HilD<sub>1-309</sub>; in contrast, it did not affect the repression of sulA-lacZ mediated by the dimerization of LexA<sub>DBDwt</sub>-LZ-HilD<sub>221-309</sub> or LexA<sub>DBDwt</sub>-H-NS (Fig. 7B). These results indicate that HilE negatively affects the dimerization of HilD, but not that of H-NS or the LZ motif tested.

### HilE directly affects the DNA binding domain of HilD

The results described above indicate that HilE affects the dimerization of HilD by interacting with the central region of this regulator, which in turn would indirectly inhibit its regulatory activity. However, our results revealed that HilE also interacts with the C-terminal region of this regulator, which in turn would indirectly inhibit its regulatory activity. However, our results revealed that HilE also interacts with the C-terminal region of this regulator, which in turn would indirectly inhibit its regulatory activity. Therefore, the higher negative effect of HilE on the regulatory activity of LexA<sub>DBDwt</sub>-HilD<sub>1-309</sub> than that of LexA<sub>DBDwt</sub>-LZ-HilD<sub>221-309</sub> (Fig. 8), could be the result of a double effect of HilE on LexA<sub>DBDwt</sub>-HilD<sub>1-309</sub> on the dimerization and the DNA binding domain, and just one effect of HilE on LexA<sub>DBDwt</sub>-LZ-HilD<sub>221-309</sub> on the DNA binding domain.

### HilE inhibits the DNA binding of HilD

Our results strongly support that HilE inhibits the DNA binding activity of HilD. To confirm this, we...
performed competitive electrophoretic mobility shift assays (EMSAs) with purified Trx-His-HilE and MBP-HilD proteins and a 50-bp fragment of the regulatory region of hilC carrying a HilD-binding site. As shown below, purified Trx-His-HilE interacts with HilD (Fig. 2B) and, on the other hand, in a previous study we demonstrated that purified MBP-HilD binds to target genes in EMSAs (12). The hilC fragment was incubated with a constant concentration of MBP-HilD (0.5 μM) without or with increasing concentrations of Trx-His-HilE (0.3, 0.5, 1.0, 1.5 and 2 μM). Parallel binding reactions were performed with purified Trx-His instead Trx-His-HilE, as negative controls. From the concentration of 1.0 μM, Trx-His-HilE, but not Trx-His, drastically reduced the formation of the DNA/MBP-HilD complex (Fig. 9A and B), indicating that Trx-His-HilE interferes with the DNA binding of MBP-HilD. Additional binding reactions ruled out a possible DNA binding activity of Trx-His-HilE, even at the highest concentration tested (2.0 μM) (Fig. 9C). Thus, these results further confirm the interaction of HilE with HilD and demonstrate that this interaction inhibits the HilD binding to DNA.

**HilE shares sequence and structure similarities with Hcp proteins from T6SSs**

HilE was reported several years ago as a protein that does not present homology with any other protein in the databases (38). However, our recent BLASTp analysis revealed that the HilE sequence presents an identity of around 30% with Hcp proteins from type 6 secretion systems (T6SSs) of different Gram-negative bacteria (Fig. 10A and data not shown). To determine whether HilE also presents structural analogy with the Hcp proteins, it was modeled by I-TASSER server (53,54). HilE was successfully modeled yielding a structure with a C-score of 1.07 and a TM-score of 0.86. The predicted structure is composed by a tight β-barrel domain with two β-sheets, with 4 and 5 β-strands each, flanked in one side by an α-helix (Fig. 10B). The overall modeled structure of HilE highly resembles that described for the monomeric subunits of Hcp proteins: Hcp1 of *Pseudomonas aeruginosa* (55), Hcp1 of *Acinetobacter baumannii* (56), EvpC of *Edwardsiella tarda* (57), a T6SS effector (T6SSe) of *Yersinia pestis* (Filippova, E.V. *et al*. unpublished) and Hcp2 of *Salmonella Typhimurium* (58) (Fig. 10C), showing TM-values of 0.95, 0.89, 0.88, 0.86 and 0.78, respectively, with these proteins. TM-values >0.5 indicates structures sharing the same topology. These results are consistent with structural similarity shared between HilE and Hcp proteins.

**Discussion**

Negative regulation of HilE on the SPI-1 virulence genes is important for *Salmonella* fitness (59). Previously, it was shown that HilE negatively controls the expression of the SPI-1 genes by interacting with HilD, a central positive regulator for SPI-1 (38). In this study, we show that HilE specifically regulates the DNA binding activity of HilD, by inhibiting the dimerization and by directly acting on the DNA binding domain of this regulator. In agreement with this, a recent study also found that HilE blocks the DNA binding of HilD (60). Our results indicate that HilD requires dimerization to induce the expression of target genes. Consistently, the HilD-binding sites on different genes have two direct repeat sequences (21,27,51). Several other AraC-like transcriptional regulators act as dimers, such as ToxT, ExsA, UreR and AggR, which control expression of virulence genes in *Vibrio cholerae, Pseudomonas aeruginosa, Proteus mirabilis* and *E. coli* (45,48,49,61,62). HilE interacts with the central region of HilD and thus inhibits its dimerization, which indirectly would affect the DNA binding activity of this regulator. Additionally, HilE interacts with the C-terminal region of HilD containing the DNA binding domain, which negatively affects the transcriptional activity of HilD independently of its dimerization. This double interaction and effect supports a tight control of the HilD activity by HilE. In enterogaegregative *E. coli* (EAEC), the Aar protein interacts with the central region of AggR, containing the dimerization domain, inhibiting the dimerization and the DNA binding of this AraC-like regulator (62). Similarly, in *P. aeruginosa*, the ExsD protein interacts with the N-terminal region of ExsA, containing the dimerization domain, thus preventing the dimerization and DNA binding of this regulator (45,61,63). Neither Aar nor ExsD seem to directly compromise the DNA binding domain of their target regulators. One example of a protein that directly acts on the DNA binding
domain of a transcriptional regulator is the control exerted by CarS on the CarA repressor in Myxococcus xanthus. CarS binds to the DNA binding domain of CarA and thus inhibits its interaction with target genes; interestingly, the structure of CarS mimics the operator DNA recognized by CarA (64). Further studies, such as three-dimensional analysis, are required to determine the precise stoichiometry and the amino acids mediating the interaction between HilE and HilD, which is a matter of our current investigation.

Protein-protein interaction has been shown to regulate the stability of transcriptional regulators. For instance, FliT interacts with the FlhDC complex, the central positive regulator of the flagellar genes, which enhances the degradation of FlhC subunit by the ClpXP protease (65). Our results discarded a negative effect of HilE on the stability of HilD in the growth conditions tested, further supporting that HilE only controls the regulatory activity of HilD. However, since HilD positively auto-regulates (15,26), HilE indirectly controls the expression and thus the amount of HilD.

Interestingly, we found that HilE shares sequence identity and structure analogy with Hcp proteins from different Gram-negative bacteria; thus, HilE could be postulated as a Hcp-like protein. Important to note, the Hcp-like proteins, including HilE, conserve high structure similarity but low sequence identity among them (55) (Fig. 10A). The Hcp proteins are structural components of the T6SSs, which are translocation machines that resemble an inverted phage tail, involved in different functions such as antibacterial activity and virulence (66,67). Interestingly, the T6SSs components, including the Hcp proteins, are closely related to those of the phages tail, which work as channels for DNA delivery into bacteria (66,67). Specifically, the Hcp proteins form a hexameric ring structure that stacks in a head-to-tail fashion, forming the tube through which T6SS effector proteins are either injected from the bacterial cytoplasm into the prey cell, or released to the extracellular milieu (66-68). Furthermore, the Hcp proteins can also act as chaperones that help to the translocation of the T6SS effector proteins (69). S. Typhimurium possesses a functional T6SS, which is encoded in SPI-6 (70). If HilE has a structural/chaperone role in this T6SS remains to be determined.

The hilE gene is located in a genomic region that shows characteristics to be acquired by Salmonella (supplemental Fig. S2) (38); BLASTp analysis indicated that the other proteins also encoded in this Salmonella genomic island do not show homology with any protein related to the T6SSs. It is tempting to speculate that HilE diverged from an ancestral T6SS or phage Hcp protein. Thus, our results could illustrate the adaptation of a structural protein, during the evolution of Salmonella, to act as a regulator of virulence genes expression.

**Experimental procedures**

**Bacterial strains and growth conditions**

Bacterial strains used in this study are listed in supplemental Table S1. Bacterial cultures were grown in Lysogeny broth (LB) containing 1% tryptone, 0.5% yeast extract and 1% NaCl at pH 7.5. When necessary, media were supplemented with the following antibiotics: ampicillin (200 µg/ml), streptomycin (100 µg/ml), tetracycline (12 µg/ml) or kanamycin (20 µg/ml). Cultures for the determination of chloramphenicol acetyltransferase activity (CAT) and for the LexA-based genetic system assays were performed as we described previously (12,28,50).

**Construction of plasmids**

Plasmids and primers used in this study are listed in supplemental Table S1 and Table S2, respectively. To construct the plasmids pSR658-HilD1, pSR658-HilD2, pSR658-HilD3, pSR658-HilD4 and pSR658-HilD5, fragments of the hilD gene were amplified by PCR using the primers pairs HilD-SacI/HilDexR-PstI, HilD-SacI/HilD-130, HilD130-2/HilDexR-PstI, HilD-SacI/HilD-220 and HilD-221/HilDexR-PstI, respectively. The resulting PCR products were digested with SacI and PstI and cloned into the vector pSR658 digested with the same restriction enzymes. To construct the pSR659-HilE1 plasmid, the hilE gene was amplified by PCR using the primers HilE-SacI and HilE-HindIII-3’. The resulting PCR product was digested with SacI and HindIII and
cloned into the vector pSR659 digested with the same restriction enzymes. To construct the plasmid pSR658-HilD6, the DNA fragment encoding 35 amino acids of the leucine zipper (LZ) motif from GCN4 of Saccharomyces cerevisiae was amplified by PCR using the primers LZ-F and HilD221LZR and DNA of the pUT18C-zip plasmid as template. The fragment encoding the region of HilD from codons 221 to 309 (HilD221-309) was also amplified by PCR using the LZ-HilD221F and HilDexR-PstI primers. Then, the LZ and HilD221-309 fragments were fused by overlap extension PCR using the LZ-F and HilDexR-PstI primers. The resulting PCR product was digested with XhoI and PstI and cloned into the pSR658 vector digested with the same restriction enzymes. To construct the pA6-HilE1 and pK6-HilE1 plasmids, the hilE gene was amplified by PCR using the HilE-Ncol-2 and HilE-His6 primers. The resulting PCR product was digested with Ncol and HindIII and cloned into the pMPM-A6Ω or pMPM-K6Ω vectors digested with the same restriction enzymes. To construct the plasmid pET32-HilE expressing the Trx-His-HilE fusion protein, hilE gene was amplified by PCR using the HilE-Ncol-2 and HilE-PUT-BamHI primers. The resulting PCR product was digested with Ncol and BamHI and cloned into the pET32b(+) vector digested with the same restriction enzymes.

HilD dimerization assays

To test dimerization of HilD, the plasmids pSR658, pSR658-HilD1 or pSR658-HNS were transformed into the E. coli SU101 reporter strain for homodimerization assays, which carries the chromosomal sulA-lacZ transcriptional fusion (43,44). Likewise, to determine the region of HilD containing the dimerization domain, the plasmids pSR658, pSR658-HilD1, pSR658-HilD2, pSR658-HilD3, pSR658-HilD4 or pSR658-HilD5, were transformed into the same reporter strain. Transformants were grown in LB with tetracycline and 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside) to induce expression of LexA<sub>DBD</sub> and LexA<sub>DBD</sub>-fusion proteins. Samples were collected at an optical density at 600 nm (OD<sub>600</sub>) of 1.0 and used for the determination of β-galactosidase activity.

To test if HilE affects the dimerization of HilD, the E. coli SU101 reporter strain was first transformed with the plasmids pSR658, pSR658-HilD1, pSR658-HNS or pSR658-HilD6; and then transformed with the pMPM-A6Ω vector or the pA6-HilE1 plasmid expressing HilE from the arabinose inducible promoter. Transformants were grown in LB with tetracycline and ampicillin, as well as with 1 mM IPTG to induce the expression of LexA<sub>DBD</sub> and LexA<sub>DBD</sub>-fusion proteins, and with 0.1% L-Arabinose to induce the expression of HilE. Samples were collected at an OD<sub>600</sub> of 1.0 and used for the determination of β-galactosidase activity.

HilD-HilE heterodimerization assays

To test the interaction between HilD and HilE, the E. coli SU202 reporter strain for heterodimerization assays, which carries the sulA-lacZ transcriptional fusion with a hybrid LexA operator (43,44) was first transformed with the plasmids pSR658, pSR658-HilD1, pSR658-HilD2, pSR658-HilD4 or pSR658-HilD5, and then transformed with the pSR659 vector or the pSR659-HilE1 plasmid. Transformants were grown in LB with tetracycline and ampicillin, and with 1 mM IPTG to induce expression of LexA<sub>DBD</sub> and LexA<sub>DBD</sub>-fusion proteins. Samples were collected at an optical density at 600 nm (OD<sub>600</sub>) of 1.0 and used for the determination of β-galactosidase activity.

β-galactosidase assays

The β-galactosidase assay and protein quantification to calculate specific activities were performed as previously described (71).

CAT assays

The CAT assays and protein quantification to calculate CAT specific activities were performed as previously described (72).

Western blotting

Whole-cell extracts were prepared from bacterial samples collected at the indicated time points from LB cultures. Ten micrograms of each extract were subjected to electrophoresis in SDS-12% polyacrylamide gels, and then transferred to 0.45
µm pore size nitrocellulose membranes (Merck), using a semidy transfer apparatus (Bio-Rad). The membranes containing the transferred proteins were blocked in 5% nonfat milk overnight and then incubated with anti-c-Myc (Sigma, Cat No. M4439) or anti-His (Roche, Cat No. 11922416001) monoclonal antibodies, at 1:5000 and 1:1000 dilutions respectively, or with anti-LexA (Abcam, Cat No. ab14553) or anti-GroEL (Sigma, Cat No. G6532) polyclonal antibodies, at 1:10.000 and 1:100.000 dilutions, respectively. Horseradish peroxidase-conjugated anti-mouse (Sigma, Cat No. A9044) or anti-rabbit (Rockland, Cat No. 611-1302), at a dilution of 1:10.000, were used as the secondary antibodies. Bands on the blotted membranes were developed by incubation with the Western Lightning Chemiluminescence Reagent Plus (Perkin-Elmer) and exposed to Kodak X-Omat films or Armosham Imager 600 (GE Healthcare).

Expression and purification of MBP-HilD

Expression and purification of MBP-HilD were performed as we previously described (12).

Expression and purification of Trx-His and Trx-His-HilE

The Trx-His and Trx-His-HilE proteins were expressed in E. coli BL21/DE3 carrying the pET32b(+) or pET32-HilE plasmids, respectively, and purified from soluble bacterial extracts by using Ni-NTA agarose columns (Qiagen). Bacterial cultures were grown in 250 ml of LB with ampicillin up to an OD$_{600}$ of 0.6, at 37°C. Then, the expression of Trx-His or Trx-His-HilE was induced by adding 1 mM IPTG and the cultures were incubated at 30°C for 4 h. Bacteria were collected by centrifugation at 4°C and the pellets were washed once with ice-cold binding buffer (5 mM Imidazole, 500 mM NaCl, 20 mM Tris-HCl, pH 8.0) and resuspended in 30 ml of the same buffer. Cells were lysed with French press and bacterial debris were removed by centrifugation at 4°C. The soluble bacterial extracts were loaded into Ni-NTA agarose columns previously equilibrated with 50 ml of binding buffer. The columns were washed with 100 ml of binding buffer and then with 100 ml of wash buffer (20 mM Imidazole, 500 mM NaCl, 20 mM Tris-HCl, pH 8.0). The proteins were eluted with elution buffer (250 mM Imidazole, 500 mM NaCl, 20 mM Tris-HCl, pH 8.0). The collected fractions were analyzed in a 12% SDS-PAGE. Those fractions containing the purified Trx-His and Trx-His-HilE proteins were loaded into a Slide-A-Lyzer 7K cassette (Thermo) and dialyzed at 4°C in a buffer containing 20 mM Tris HCl pH 8.0, 40 mM KCl, 1mM EDTA and 20% (v/v) glycerol. Protein concentration was determined by the Bradford procedure. Aliquots of the purified Trx-His and Trx-His-HilE proteins were stored at -70°C.

Electrophoretic mobility assays (EMSAs)

EMSAs were performed using the purified MBP-HilD, Trx-His-HilE or Trx-His proteins and a 50-bp DNA fragment of hilC containing a binding site of HilD. The 50-bp hilC fragment was generated by annealing the complementary primers HilCRR-F and HilCRR-R. For this, the primers, each at a final concentration of 15 µM, were boiled together at 95°C for 10 min and then slowly cooling to room temperature. Competitive binding reactions were performed by mixing ~100 ng of the hilC fragment with MBP-HilD (0.5 µM) and increasing amounts of Trx-His-HilE or Trx-His (0.3, 0.5, 1.0, 1.5 and 2.0 µM), in a total volume of 20 µl of binding buffer containing 100 µg/ml bovine serum albumin (BSA), 30 mM HEPES pH 7.5, 5 mM EDTA, 3 mM dithiothreitol (DTT), 200 mM KCl, 25 mM MgCl$_2$ and 5% glycerol. For non-competitive EMSAs, only increasing amounts of Trx-His-HilE purified protein (0.3, 0.5, 1.0, 1.5 and 2.0 µM) were used. Protein-DNA binding reactions were incubated at 37°C for 20 min and then electrophoretically separated in 6% non-denaturing polyacrylamide gels in 0.5x Tris-borate-EDTA buffer, at room temperature. The DNA fragments were stained with ethidium bromide and visualized with an Alpha-Imager UV transilluminator (Alpha Innotech Corp.).

HilD stability assays

HilD stability assays were performed as we described previously (33). Values for HilD-Myc bands were normalized with respect to those for GroEL bands and then the relative percentage of HilD-Myc at each indicated time, with respect to
time 0, was calculated. The half-life time \((t_{1/2})\) of HilD was calculated by one phase decay equation.

**Protein Secretion Analysis**

Protein secretion assays were performed as we described previously (28). Samples were analyzed in a 12% SDS-PAGE and stained with Coomassie Brilliant Blue R-250.

**Gel-filtration assay**

The purified protein MBP-HilD was subjected to gel filtration chromatography analysis by using AKTA-FPLC system (Superdex 200 HiLoadTM 26/60 column, GE Healthcare Life Sciences, USA), with a flow rate of 0.5 ml/min and a pressure limit of 0.3 MPa, in a buffer containing 200 mM Tris–HCl pH 8.0, 150 mM NaCl, at 20°C. The column was pre-calibrated by using Gel Filtration Molecular Weight Markers Kit (Sigma-Aldrich) including Cytochrome C from horse heart (12.4 kDa), Carbonic Anhydrase from bovine erythrocytes (29 kDa), Albumin bovine serum (66 kDa), Alcohol Dehydrogenase from yeast (150 kDa) and β-Amylase from sweet potato (200 kDa). The relative molecular mass of MBP-HilD was determined by comparison to the five-point molecular weight calibration curve.

**Pull-down assays**

Pull-down assays were performed with purified and Trx-His-HilE or Trx-His fusion proteins and whole-cell extracts from E. coli SU101 expressing LexA<sub>DBDwt</sub>, LexA<sub>DBDwt</sub>-HilD<sub>1-220</sub>, LexA<sub>DBDwt</sub>-HilD<sub>1-220</sub>, LexA<sub>DBDwt</sub>-HilD<sub>1-309</sub> or LexA<sub>DBDwt</sub>-HilD<sub>213-309</sub>. To immobilize the bait protein, 15 µg of purified Trx-His-HilE or Trx-His were incubated for 1 h at 10°C with 80 µL of Ni-NTA resin (Qiagen) previously equilibrated with lysis buffer (50 mM NaH<sub>2</sub>PO<sub>4</sub>, 300 mM NaCl, 5 mM Imidazole, pH 8.0), in a 1.5 ml microcentrifuge tube. The resin containing the immobilized bait protein was washed with 1 ml of wash buffer (70 mM NaH<sub>2</sub>PO<sub>4</sub>, 300 mM NaCl, 35 mM Imidazole, pH 8.0) by centrifugation at 4000 g for 1 min. The supernatant was carefully removed and then the resin was incubated for 1 h at 10°C with 80 µL of the whole-cell extract containing the respective prey protein. To remove the unbound proteins, the resin was washed five times with 1 ml of wash buffer by centrifugation at 4000 g for 1 min. Finally, the resin was resuspended with 20 µL of SDS loading buffer containing 1.5% of β-mercaptoethanol and boiled for 5 min at 99°C. After this, the samples were analyzed by Western-blotting.

**Sequence alignment and structure prediction of HilE**

The sequence of HilE and that of some Hcp proteins were aligned using the Clustal Omega server (73). The sequence of HilE was submitted to I-TASSER server for structural modeling (53,54), a final model with C-score of 1.07, TM-score of 0.86 and RMSD of 2.7 Å, was selected. All molecular graphics were done in PyMOL version 1.8 (The PyMOL Molecular Graphics System, Version 1.8. Schrödinger, LLC).

**Statistical analysis**

Data from CAT and β-galactosidase assays were analyzed using one-way analysis of variance (ANOVA) with the Tukey’s multiple comparison test. A \(P\) value of \(< 0.05\) was considered significant. This statistical analysis was performed using Prism 6 program version 6.01 (GraphPad Software, San Diego, CA).
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Conflicts of interest

The authors declared that they have no conflicts of interest with the content of this article.
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HilE controls dimerization and DNA binding of HilD

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**FOOTNOTES**

Abbreviations used are: SPI-1, *Salmonella* pathogenicity island 1; T3SS, type 3 secretion system; Hcp, haemolysin-coregulated protein; T6SS, type 6 secretion system; LexA<sub>DBDwt</sub>, LexA DNA binding domain wild type; LexA<sub>DBDmut</sub>, LexA DNA binding domain mutated; MBP, maltose binding protein; Trx, thioredoxin; LZ, leucine zipper; CAT, chloramphenicol acetyltransferase.
Figure 1. HilE interacts with two different regions of HilD. (A) Schematic representation of the LexA<sub>DBD</sub>-HilD and LexA<sub>DBDmut</sub>-HilE fusion proteins tested. Numbers indicate the residues of LexA<sub>DBD</sub>, HilD or HilE carried in the respective fusion protein. (B) Expression of the sulA-lacZ fusion was determined in the E. coli SU202 reporter strain containing the pair of plasmids pSR658 and pSR659 (LexA<sub>DBD</sub>-HilD<sub>1-309</sub> + LexA<sub>DBDmut</sub>-HilE), pSR658-HilD1 and pSR659-HilE1 (LexA<sub>DBD</sub>-HilD<sub>1-130</sub> + LexA<sub>DBDmut</sub>-HilE), pSR658-HilD1 and pSR659 (LexA<sub>DBD</sub>-HilD<sub>1-220</sub> + LexA<sub>DBDmut</sub>-HilE), pSR658-HilD2 and pSR659-HilE1 (LexA<sub>DBD</sub>-HilD<sub>221-309</sub> + LexA<sub>DBDmut</sub>-HilE), pSR658-HilD2 and pSR659 (LexA<sub>DBD</sub>-HilD<sub>221-309</sub> + LexA<sub>DBDmut</sub>-HilE). The β-galactosidase activity was determined from samples collected of bacterial cultures grown in LB at 37°C up to an OD<sub>600</sub> of 1.0. Expression of the LexA<sub>DBD</sub> fusion proteins was induced by adding 1mM IPTG to the medium. The data are the averages of three independent experiments performed in duplicate. Bars represent the standard deviations. *** Expression statistically different compared to that reached in the absence of HilE (p<0.001); n.s., no significant difference. (C) Expression of the LexA<sub>DBD</sub>, LexA<sub>DBDmut</sub>-HilD, LexA<sub>DBD</sub>-HilD<sub>1-130</sub>, LexA<sub>DBDmut</sub>-HilD<sub>1-220</sub> and LexA<sub>DBD</sub>-HilD<sub>221-309</sub> proteins was analyzed by Western blotting using polyclonal anti-LexA antibodies. Whole cell lysates were prepared from samples of bacterial cultures grown in LB at 37°C up to an OD<sub>600</sub> of 1.0. As a loading control, the expression of GroEL was also determined using polyclonal anti-GroEL antibodies. MW, protein molecular weight standards (Precision Plus Protein™ BIO-RAD). Arrowheads indicate the expected bands.
Figure 2. Pull-down assays showing the interaction between HilE and HilD. (A) Secretion of the SPI-1-encoded proteins SipA, SipB, SipC and SipD was tested in the WT S. Typhimurium strain and its isogenic ΔhilD mutant, as well as in the WT S. Typhimurium strain carrying the pET32-HilE plasmid expressing Trx-His-HilE or the pET32b(+) vector expressing Trx-His. The bacterial cultures were grown for 9 h in LB at 37°C, in the presence (+) or not (-) of 1 mM IPTG to induce or not the expression of Trx-His-HilE or Trx-His. Supernatants of the cultures were analyzed in SDS-PAGE at 12%. FliC is a flagellar protein whose secretion is SPI-1 independent. Bait proteins Trx-His-HilE (B) or Trx-His (C) immobilized on Ni-NTA resin were incubated with whole-cell extracts containing the LexA<sub>DBD<sub>wt</sub></sub>, LexA<sub>DBD<sub>wt</sub>-HilD<sub>1-309</sub></sub>, LexA<sub>DBD<sub>wt</sub>-HilD<sub>1-130</sub></sub>, LexA<sub>DBD<sub>wt</sub>-HilD<sub>1-220</sub></sub> or LexA<sub>DBD<sub>wt</sub>-HilD<sub>221-309</sub></sub> prey proteins. After washing, the proteins captured by the Trx-His-HilE or Trx-His bait proteins were analyzed by Western blotting using polyclonal anti-LexA antibodies. The Trx-His-HilE or Trx-His bait proteins were also detected with monoclonal anti-His<sub>6</sub> antibodies. MW, protein molecular weight standards (Precision Plus Protein™ BIO-RAD). Arrowheads indicate the expected bands. An asterisk indicates bands showing cross-reaction with the polyclonal anti-LexA antibodies.
Figure 3. HilE does not affect the stability of HilD. (A) Stability of HilD-Myc was determined in the ΔhilD, ΔhilD ΔhilE, and ΔhilD Δlon mutants of S. Typhimurium carrying the pBAD-HilD1 plasmid, grown in LB at 37°C. Expression of HilD-Myc, from the arabinose-inducible promoter of pBAD-HilD1, was induced with 0.05% L-arabinose for 45 min; then, transcription and translation were halted by the addition of a cocktail of antibiotics and glucose, and samples of bacterial cultures were taken at indicated times. HilD-Myc was detected from whole-cell lysates of the samples by Western blotting using monoclonal anti-Myc antibodies. As a loading control, the expression of GroEL was also determined using polyclonal anti-GroEL antibodies. A representative Western blot of three independent experiments is shown. Figure is composed by four different blots. (B) Densitometric analysis of the HilD-Myc bands from the Western blots is indicated as the relative percentage of HilD-Myc at each time with respect to time 0. Intensity values of HilD-Myc bands were normalized with those respective of GroEL bands. The data are the averages of three independent experiments. Bars represent the standard deviation and $t_{1/2}$ indicates the half-life of HilD. MW, protein molecular weight standards (Precision Plus Protein™ BIO-RAD).
HilE controls dimerization and DNA binding of HilD

Figure 4. HilD forms homodimers through its central region. (A) Expression of the sulA-lacZ fusion was determined in the E. coli SU101 reporter strain containing the plasmids pSR658 (LexA_{DBDwt}), pSR658-HilD1 (LexA_{DBDwt-HilD1-309}) or pSR658-HNS (LexA_{DBDwt-H-NS}). (B) Expression of LexA_{DBDwt}, LexA_{DBDwt-HilD1-309} and LexA_{DBDwt-H-NS} was analyzed by Western blotting using polyclonal anti-LexA antibodies. As a loading control, the expression of GroEL was also determined using polyclonal anti-GroEL antibodies. MW, protein molecular weight standards (Precision Plus Protein™ BIO-RAD). Arrowheads indicate the expected bands. (C) Expression of the sulA-lacZ fusion was determined in the E. coli SU101 reporter strain containing the plasmids pSR658 (LexA_{DBDwt}), pSR658-HilD1 (LexA_{DBDwt-HilD1-309}), pSR658-HilD2 (LexA_{DBDwt-HilD1-130}), pSR658-HilD4 (LexA_{DBDwt-HilD1-220}), pSR658-HilD5 (LexA_{DBDwt-HilD221-309}) or pSR658-HilD3 (LexA_{DBDwt-HilD130-309}). The β-galactosidase activity was determined from samples collected of bacterial cultures grown in LB at 37°C up to an OD\textsubscript{600} of 1.0. Expression of LexA_{DBDwt} and the LexA_{DBDwt} fusion proteins was induced by adding 1mM IPTG to the medium. The data are the averages of three independent experiments performed in duplicate. Bars represent the standard deviations. *** Expression statistically different compared to that reached in the presence of LexA_{DBDwt} (p<0.001).
Figure 5. Dimerization is required for the HilD regulatory activity. Expression of the hilA-cat fusion contained in the philA-cat1 plasmid was tested in the WT S. Typhimurium SL1344 and its isogenic ΔhilD mutant, as well as in the E. coli MC4100 strain carrying the plasmids pSR658 (LexA_{DBDwt}), pSR658-HilD1 (LexA_{DBDwt}-HilD1_{309}), pSR658-HilD5 (LexA_{DBDwt}-HilD221_{309}), pSR658-HilD3 (LexA_{DBDwt}-HilD130_{309}) or pSR658-HilD6 (LexA_{DBDwt}-LZ-HilD221_{309}). The CAT-specific activity was determined from samples collected of bacterial cultures grown in LB at 37°C up to an OD₆₀₀ of 1.2. Expression of LexA_{DBDwt}, LexA_{DBDwt}HilD1_{309}, LexA_{DBDwt}HilD221_{309}, LexA_{DBDwt}HilD130_{309} and LexA_{DBDwt}-LZ-HilD221_{309} was induced by adding 1mM IPTG to the medium. The data are the averages of three independent experiments performed in duplicate. Bars represent the standard deviations. *** Expression statistically different compared to that reached in the presence of LexA_{DBDwt} (p<0.001).
**Figure 6.** LexA<sub>DBD</sub><sub>wt</sub>-LZ-HilD<sub>221-309</sub> dimerizes. (A) Schematic representation of LexA<sub>DBD</sub><sub>wt</sub>-LZ-HilD<sub>221-309</sub>. Numbers indicate the residues of LexA<sub>DBD</sub> or HilD carried in this fusion protein. (B) Expression of the sulA-lacZ fusion was determined in the E. coli SU101 reporter strain containing the plasmids pSR658 (LexA<sub>DBD</sub><sub>wt</sub>), pSR658-HilD1 (LexA<sub>DBD</sub><sub>wt</sub>-HilD<sub>1-309</sub>), pSR658-HilD5 (LexA<sub>DBD</sub><sub>wt</sub>-HilD<sub>221-309</sub>) or pSR658-HilD6 (LexA<sub>DBD</sub><sub>wt</sub>-LZ-HilD<sub>221-309</sub>). The β-galactosidase activity was determined from samples collected of bacterial cultures grown in LB at 37°C up to an OD<sub>600</sub> of 1.0. Expression of LexA<sub>DBD</sub><sub>wt</sub>, LexA<sub>DBD</sub><sub>wt</sub>-HilD<sub>1-309</sub>, LexA<sub>DBD</sub><sub>wt</sub>-HilD<sub>221-309</sub> and LexA<sub>DBD</sub><sub>wt</sub>-LZ-HilD<sub>221-309</sub> was induced by adding 1mM IPTG to the medium. The data are the averages of three independent experiments performed in duplicate. Bars represent the standard deviations. *** Expression statistically different compared to that reached in the presence of LexA<sub>DBD</sub><sub>wt</sub> (p<0.001); n.s., no significant difference.
HilE controls dimerization and DNA binding of HilD

Figure 7. HilE inhibits the dimerization of HilD. (A) Secretion of the SPI-1-encoded proteins SipA, SipB, SipC and SipD was tested in the WT S. Typhimurium strain and its isogenic ΔhilD mutant, as well as in the WT S. Typhimurium strain carrying the pA6-HilE1 plasmid expressing HilE from an arabinose-inducible promoter, or carrying the pMPM-A6Ω vector, in the presence (+) or not (-) of increasing concentrations of L-Arabinose. Bacterial cultures were grown for 9 h in LB at 37°C and supernatants were analyzed in SDS-PAGE at 12%. FliC is a flagellar protein whose secretion is SPI-1 independent. MW, protein molecular weight standards (Precision Plus Protein™ BIO-RAD). (B) Expression of the sulA-lacZ fusion was determined in the E. coli SU101 reporter strain containing the pair of plasmids pSR658 and pA6-HilE1 (LexA_{DBDwt} + HilE), pSR658 and pMPM-A6Ω (LexA_{DBDwt} + vector), pSR658-HilD1 and pA6-HilE1 (LexA_{DBDwt}-HilD1-309 + HilE), pSR658-HilD1 and pMPM-A6Ω (LexA_{DBDwt}-HilD1-309 + vector), pSR658-HNS and pA6-HilE1 (LexA_{DBDwt}-H-NS + HilE), pSR658-HNS and pMPM-A6Ω (LexA_{DBDwt}-H-NS + vector), pSR658-HilD6 and pA6-HilE1 (LexA_{DBDwt}-LZ-HilD221-309 + HilE) or pSR658-HilE6 and pMPM-A6Ω (LexA_{DBDwt}-LZ-HilD221-309 + vector). The β-galactosidase activity was determined from samples collected of bacterial cultures grown in LB at 37°C up to an OD_{600} of 1.0. Expression of LexA_{DBDwt}, LexA_{DBDwt}-HilD1-309, LexA_{DBDwt}-HNS and LexA_{DBDwt}-LZ-HilD221-309 was induced by adding 1mM IPTG to the medium. 0.1% L-Arabinose was also added to induce the expression of HilE from pA6-HilE1. The data are the averages of three independent experiments performed in duplicate. Bars represent the standard deviations. *** Expression statistically different compared to that reached in the absence of HilE (p<0.001); n.s., no significant difference.
Figure 8. HilE directly affects the DNA binding domain of HilD. Expression of the *hilA-cat* fusion contained in the philA-cat1 plasmid was tested in the *E. coli* MC4100 strain carrying the pair of plasmids pSR658-HilD1 and pK6-HilE1 (LexA<sub>DBD<sub>wt</sub>-HilD<sub>1-309</sub> + HilE), pSR658-HilD1 and pMPM-K6Ω (LexA<sub>DBD<sub>wt</sub>-HilD<sub>1-309</sub> + vector), pSR658–HilD6 and pK6-HilE1 (LexA<sub>DBD<sub>wt</sub>-LZ-HilD<sub>221-309</sub> + HilE), pSR658–HilD6 and pMPM-K6Ω (LexA<sub>DBD<sub>wt</sub>-LZ-HilD<sub>221-309</sub> + vector), pSR658 and pK6-HilE1 (LexA<sub>DBD<sub>wt</sub> + HilE) or pSR658 and pMPM-K6Ω (LexA<sub>DBD<sub>wt</sub> + vector). The CAT-specific activity was determined from samples collected of bacterial cultures grown in LB at 37°C up to an OD<sub>600</sub> of 1.2. Expression of LexA<sub>DBD<sub>wt</sub>, LexA<sub>DBD<sub>wt</sub>-HilD<sub>1-309</sub> and LexA<sub>DBD<sub>wt</sub>-LZ-HilD<sub>221-309</sub> was induced by adding 1mM IPTG to the medium. 0.1% L-Arabinose was also added to induce the expression of HilE from pK6-HilE1. The data are the averages of three independent experiments performed in duplicate. Bars represent the standard deviations. *** Expression statistically different compared to that reached in the absence of HilE (p<0.001).
Figure 9. HilE inhibits the DNA binding activity of HilD. Competitive non-radioactive EMSAs were performed to analyze the effect of HilE on the DNA binding activity of HilD. A 50-pb DNA fragment of *hilC*, containing a HilD binding site, was incubated with purified MBP-HilD (0.5 µM) and increasing concentrations (0, 0.3, 0.5, 1.0, 1.5, 2.0 µM) of purified (A) Trx-His-HilE or (B) Trx-His proteins. (C) EMSA was performed to evaluate DNA binding activity of HilE. The 50-pb DNA fragment of *hilC* was incubated with increasing concentrations of purified Trx-His-HilE (0, 0.3, 0.5, 1.0, 1.5, 2.0 µM). The DNA-protein complexes are indicated with asterisk and were resolved in a nondenaturing 6% polyacrylamide gel and stained with ethidium bromide.
Figure 10: Sequence and structure comparison of HilE with Hcp proteins. (A) Sequence alignment of HilE and five Hcp proteins: Hcp1 of *P. aeruginosa* (Hcp1-Pa), Hcp1 of *A. baumannii* (Hcp1-Ab), EvpC of *E. tarda* (EvpC-Et), T6SSe of *Y. pestis* (T6SSe-Yp) and Hcp2 of *S. Typhimurium* (Hcp2-Stm). (B) Ribbon representation of the HilE predicted structure with I-TASSER server, colored by secondary structure: β-strands (blue), α-helix (orange) and loops (grey). (C) Overlapping of the HilE predicted structure (blue) with the crystallographic structure of the proteins Hcp1-Pa (PDB 4KSR) (red), Hcp1-Ab (PDB 4W64) (green), EvpC-Et (PDB 3EAA) (yellow), T6SSe-Yp (PDB 3V4H) (cyan) and Hcp2-Stm (PDB 5XEU) (magenta).
The Hcp-like protein HilE inhibits homodimerization and DNA binding of the virulence-associated transcriptional regulator HilD in *Salmonella*

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