The \textit{Salmonella} Spi1 Virulence Regulatory Protein HilD Directly Activates Transcription of the Flagellar Master Operon \textit{flhDC}

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Infection of intestinal epithelial cells is dependent on the \textit{Salmonella enterica} serovar Typhimurium pathogenicity island 1 (Spi1)-encoded type III injectisome system and flagellar motility. Thus, the expression of virulence and flagellar genes is subject to tight regulatory control mechanisms in order to ensure the correct spatiotemporal production of the respective gene products. In this work, we reveal a new level of cross-regulation between the Spi1 and flagellar regulatory systems. Transposon mutagenesis identified a class of mutants that prevented \textit{flhDC} autorepression by overexpressing HilD. HilD, HilC, RtsA, and HilA comprise a positive regulatory circuit for the expression of the Spi1 genes. Here, we report a novel transcriptional cross talk between the Spi1 and flagellar regulons where HilD transcriptionally activates \textit{flhDC} gene expression by binding to nucleotides −68 to −24 upstream from the P5 transcriptional start site. We additionally show that, in contrast to the results of a previous report, HilA does not affect flagellar gene expression. Finally, we discuss a model of the cross-regulation network between Spi1 and the flagellar system and propose a regulatory mechanism via the Spi1 master regulator HilD that would prime flagellar genes for rapid reactivation during host infection.

The enteropathogenic Gram-negative bacteria of the genus \textit{Salmonella} are responsible for the food-borne illness gastroenteritis, localized infection of the small intestine, and systemic enteric (typhoid) fevers. Symptoms of \textit{Salmonella} infection include diarrhea, abdominal cramps, and fever (1). The effector-driven manipulations of the vertebrate host cells are dependent on two virulence-associated type III secretion systems (vT3SS; injectisome) (2–4) encoded in \textit{Salmonella enterica} serovar Typhimurium pathogenicity island 1 (Spi1) (5) and Spi2 (6).

The Spi1 and Spi2 virulence systems are responsible for different processes related to \textit{Salmonella} pathogenesis that occur at different time points during infection. Spi1 is needed for the invasion of the intestinal epithelium leading to gastroenteritis (7, 8), while Spi2 plays a role during trafficking to the basolateral side of epithelial cells (9) and during later \textit{Salmonella} replication and survival within macrophages (10, 11). Both systems are regulated in a spatial and temporal manner to ensure the production of gene products at the correct points during infection.

The Spi1 genes are highly regulated by a set of DNA-binding proteins, including the AraC-like regulators HilD, HilC, and RtsA. In a feed-forward loop, each of those regulators can activate the \textit{hilD}, \textit{hilC}, and \textit{rtsA} genes, as well as the gene encoding the transcriptional Spi1 activator HilA (12). HilD is a dominant regulator of \textit{hilA} transcription, while HilC and RtsA amplify \textit{hilA} gene expression (12, 13).

The needle-like injectisome system is evolutionarily related to the bacterial flagellum (14). Bioinformatic and structural analysis demonstrated that the two share many similar features (15); however, differences exist regarding the purpose of protein secretion in the two systems. In case of the flagellum, secreted substrate proteins are mainly needed for flagellum assembly. The flagellar type III secretion system (fT3SS) exports substrate subunits that assemble into a functional flagellum and regulatory factors that control the assembly process. Completed flagella are used by the bacterium to move in liquid environments and across hydrated surfaces by rotation of the rigid, helical flagellar filaments.

The virulence-associated vT3SS of Spi1 is essential for both the assembly of the injectisome needle-like structure (16, 17) and the secretion of effector proteins into host cells, where they can alter cellular processes to facilitate the infection process as described above.

Flagellar gene expression is under spatiotemporal control by a transcriptional hierarchy of three promoter classes. On top of the cascade is the flagellar master operon, \textit{flhDC}, which is under the control of a \(\sigma^{70}\)-dependent flagellar class 1 promoter. A functional FlhD\_C\_C\_ complex is required for subsequent flagellar class 2 promoter transcription. Flagellar class 2 gene products are required for the structure and assembly of a flagellar hook-basal body (HBB). One class 2 gene product, \(\sigma^{28}\) (encoded by the \textit{flaA} gene), is a sigma transcription factor that directs RNA polymerase to transcribe the flagellar class 3 promoters. The products of flagellar class 3 transcription are needed after HBB completion (18–20) to form the filament, motor force generators, and chemosensory components.

The flagellar master operon is under the control of a variety of different factors that either positively or negatively influence \textit{flhDC} expression. Both global positive regulators, such as the nucleoid proteins Fis and H-NS or the cyclic AMP (cAMP)-catabolite activator protein (CAP) complex (21–23), and negative regulators, such as RflM, the Spi1 regulator RtsB, or SlyA, bind within and act upon the \textit{flhDC} promoter (24, 25). The regulator RtsB is encoded in an operon with \textit{rtsA} and functions as a repressor of
flagellar class 1 gene expression. The rtsAB operon is transcriptionally activated by HilD, HilC, and RtsA. In addition, a regulatory feedback loop acting on flhDC transcription via RIM has been reported (26, 27). The inhibitory effect of RIM on flhDC expression has been shown to be dependent on the RcsCDB system (28), a positive regulator of Spi2 and other genes associated with bacterial cell growth in macrophages (29).

Significant amounts of biosynthetic resources and energy are required in order to synthesize, assemble, and rotate a functional flagellum (30, 31). For that reason, the expression of the flagellar system via the Spi1 master regulator HilA.

Thijs et al. showed direct binding of HilA to the flhDC region and downregulation of flhDC expression under invasive conditions (34, 48). Stewart et al. reported that the FlhD4C2 factor (34, 45–47). YdiV binds to FlhD and prevents autorepression of flhDC expression (41–44). In addition, FliZ positively regulates flagellar activity. Elevated levels of HilD protein activate flhD and 3 promoters and functions as a regulator of HilD protein gene transcription. FliZ is expressed from flagellar class 2 promoters and targets FlhD for ClpXP-dependent proteolytic degradation (34, 48). Stewart et al. reported that YdiV mutant strains were unable to fully repress flagellin production and thus caused increased caspase-1-dependent pyroptosis as a defense mechanism of Salmonella-infected macrophages (49). At the same time, an increased rate of macrophage killing was reported for ydiV-deficient Salmonella (50).

Controversial results have been reported regarding the regulation of the flagellar system via the Spi1 master regulator HilA. Thijs et al. showed direct binding of HilA to the flhDC promoter region and downregulation of flhDC expression under invasive conditions (51). However, earlier studies showed that HilA did not affect flhDC-lux transcriptional fusions when bacteria were grown in motility agar (52). An interconnecting cross talk can also be found between the Spi1 and Spi2 regulons at the level of HilD (53). During stationary growth in lysogenic broth (LB) in vitro, the onset of HilD-dependent Spi2 gene activation occurred at a later time point than the HilD-dependent activation of the Spi1 system. In experiments under conditions resembling the intracellular environment, HilD was not required for activation of the Spi2 regulon. These data of Bustamante et al. (53) suggest that the activation of the Spi2 system is purposefully regulated via two distinct pathways, which come into play depending on the environmental factors within a given niche. Cross talk between the Spi1, Spi2, and flagellar regulatory systems is therefore likely of great importance to Salmonella for spatiotemporal coordination of motility and virulence.

**MATERIALS AND METHODS**

**Bacterial strains, plasmids, and media.** Detailed information about bacterial strains and plasmids used in this study is listed in Table S1 in the supplemental material. Salmonella Typhimurium strain LT2 or ATCC 14028 was cultured in lysogenic broth (LB) (54) that was supplemented with kanamycin (50 μg ml−1), tetracycline (Tc 15 μg ml−1), or anhydrotratetrazacycline (1 μg ml−1) if needed. Gene expression from the arabinose promoter was induced by the addition of 0.2% l-arabinose. The Salmonella Typhimurium generalized transducing phage P22 HT105/1 int-201 was used in all crosses (55). Cultures of the virulent Salmonella Typhimurium strain ATCC 14028 were grown under Spi1-inducing conditions (high osmolarity and low oxygen) as described previously (26).

**Isolation of random T-Pop insertions.** Transposon T-Pop insertions in strain TH15941 [::Δrabort1007::flhDC::lacZ ΔflhC::MuJ fliA5886(R91C L207P)] (changes of R to C at position 91 and L to P at position 207 are encoded by fliA5886)] were isolated as described previously (26). Briefly, TH15941 expresses the flhDC C operon from the chromosomal araBAD promoter (ParaBAD) and carries a chromosomal flhC-lac transcriptional reporter fusion [::MuJ::flhC5213::MuJd], as well as a fliA null allele that is defective in DNA binding (56). TH15941 becomes Lac deficient in the presence of arabinose (Ara− Lac +) due to induction of flhDC C′ transcription from PparaBAD and autorepression of flhC-lac reporter transcription by FlhD4C2. T-Pop insertions were introduced into TH15941 carrying plasmid pN2881, and approximately 30,000 random T-Pop insertions were screened for the loss of FlhD4C2-mediated repression of flhDC in the presence of tetracycline.

**RNA isolation and quantitative real-time PCR.** RNA isolation was performed for three independent biological replicates using the RNeasy minikit (Qiagen). For removal of genomic DNA, RNA was treated with DNase I for 30 min at 37°C using the DNA-Free RNA Kit (Zymo Research). Subsequently, RNA samples were reverse transcribed according to the RevertAid first strand cDNA synthesis kit (Fermentas). Quantitative real-time PCR were performed using the EvaGreen quantitative PCR (qPCR) master mix (Bio-Rad) on a CFX96 real-time PCR instrument (Bio-Rad). Relative changes in mRNA levels were analyzed according to the method of Pfaffl (57) and normalized against the transcription levels of reference genes rpoB, rpoD, gyrB, and gmk.

**β-Galactosidase assays.** β-Galactosidase activity was measured as described previously using at least three independent biological replicates (26). Cultures were supplemented with 0.2% l-arabinose and 1 μg ml−1 anhydrotratetrazacycline if needed. ST14028 experiments were performed under Spi1-inducing (high-salt and low-oxygen) and regular LB conditions.

**Luminescence assay.** Luminescence measurements were made using a PerkinElmer 2030 microplate reader. Overnight cultures were diluted 1:100 in LB supplemented with 0.2% arabinose and grown in a microtiter plate for 3 h at 37°C. An amount of 25 μg ml−1 kanamycin was added to all cultures in order to retain the flhDC promoter duplication. Luminescence was measured for 3 s, and absorbance at 595 nm was measured before and after the luminescence readout for 0.1 s each time. The luminescence was normalized to the average optical density. Within one experiment, all samples were grown on the same plate. For each strain, at least four biological replicates were measured per plate. All samples were normalized against the wild-type control.

**Purification of HilD protein.** For HilD protein purification, HilD was fused to an Ulp1-cleavable His6-sumo tag (58). Protein expression was induced for 6 h by the addition of 0.2 mM isopropyl-β-D-thiogalactopyranoside (IPTG) during growth at 18°C with shaking at 120 rpm. Soluble
protein was purified using Ni-nitroliotricarboxylic acid (NTA) agarose (Protin; Macherey-Nagel) under native conditions. Purified His6-SUMO-HilD protein was dialyzed overnight and afterwards incubated with recombinantly produced His6-Ulp1 protease overnight at 4°C. His6-Ulp1 resulted in Tc-dependent expression of an sequence analysis revealed that this class of transposons had insertion but only in the presence of tetracycline (Tc). The T-POP T-POP transposon will transcribe genes adjacent to the site of insertion by incorporation. Sequencing reactions were carried out using the USB Thermo Sequenase (5'biotin-GATCATGCTGACACGTACGG-3'), fragment 2 (5'-GATCATGCTGACAGCTGAGGTATTTTAAGCTACACACACCAACAATTTTCGCATGCTGACACGTACGGGAGTATTTACAGAGAGAAAC-3') and fragment 3 (5'-GATCATGCTGACACGTGACACGTACGGGAGTTGATTAATCTTGAAATGAGCAAAAAGGTCAAAAGAGACTGCTCAAGATAAAGC3'). Gel shift assays were carried out using the LightShift chemiluminescent electrophoretic mobility shift assay (EMSA) kit (Thermo Scientific) according to the manufacturer’s protocol. Increasing amounts of purified HilD were incubated with 0.01 pmol of each of the biotinylated DNA fragments and 50 µg ml−1 unispecific competitor DNA [poly(dI-dC)] (Sigma) for 20 min at room temperature (RT). A 250-fold molar excess of the unlabeled DNA fragment was added to the highest protein concentration to demonstrate specific binding. DNA-binding-deficient (5'-GACACTGCTCAAGATAAAGC-3' and 5'-GCGACGCGCAAATGACC-3') gel shift assays were carried out using the LightShift chemiluminescent electrophoretic mobility shift assay (EMSA) kit (Thermo Scientific) according to the manufacturer’s protocol. Increasing amounts of purified HilD were incubated with 0.01 pmol of each of the biotinylated DNA fragments and 50 µg ml−1 unispecific competitor DNA [poly(dI-dC)] (Sigma) for 20 min at room temperature (RT). A 250-fold molar excess of the unlabeled DNA fragment was added to the highest protein concentration to demonstrate specific binding. Biotin-labeled DNA was detected after polyacrylamide gel electrophoresis, transfer to a nylon membrane, and UV cross-linking using chemiluminescence detection.

**RESULTS**

**HilD links Spi1 and flagellar gene regulation.** We have recently identified RfM (formerly EcnR) as a negative regulator of flhDC transcription that is activated by HilD in an flhDC-RfM feedback loop. Transposon T-POP insertions in the rifM gene resulted in loss of FlhDC autorepression (26). In this screen, a class of T-POP insertions were also isolated that lost FlhDC autorepression but only in the presence of tetracycline (Tc). The T-POP transposon will transcribe genes adjacent to the site of insertion by induction of a Tc-inducible promoter, P\_tet\_c, within the T-POP element. We hypothesized that transcription from the T-POP-encoded P\_tet\_c into genes adjacent to the site of T-POP insertion resulted in Tc-dependent expression of an flhDC activator. DNA sequence analysis revealed that this class of transposons had inserted upstream from the hilD coding region. The hilD-linked insertions required reduction of P\_tet\_c within the T-POP element for loss of FlhDC autorepression. We envisioned at least two mechanisms by which activation of HilD could bypass the autoregulatory effect of FlhDC overexpression. HilD could act to either repress rifM, the inhibitor of flhDC transcription, or activate flhDC expression and overcome the inhibition of flhDC via RfM. HilD is a known activator of hilA transcription; HilA then activates the transcription of genes encoding the Spil type III secretion apparatus and Spil effector proteins through InvF (24, 59, 60). HilD could also act directly on the rifM promoter region to repress rifM transcription or activate transcription of another protein that represses rifM. We first analyzed the potential inhibition of rifM gene transcription by HilD using quantitative real-time PCR. As shown in Fig. S1A in the supplemental material, HilD induction, surprisingly, resulted in about a 50% increase in rifM transcript levels. This suggested a direct activation of the rifM activator FlhDC by HilD. This was confirmed by analyzing the β-galactosidase activity of transcriptional rifM fusions, which showed an increase in rifM-lac transcription upon HilD overexpression (see Fig. S1B, lanes 2 and 3). Upon deletion of flhDC, the activating effect of HilD was neutralized and rifM transcription was abolished (see Fig. S1B, lane 4).

We next tested the possibility that HilD might act directly on flhDC transcription. As shown by the results in Fig. 1A, HilD overexpression had a strong positive effect on flhDC mRNA levels as analyzed by quantitative real-time PCR. This activation of flhDC expression was confirmed using an flhC-lac reporter fusion. As shown by the results in Fig. 1B, flhC-lac expression increased approximately 3-fold upon HilD overexpression in an attenuated Salmonella LT2 background under normal LB growth conditions (Fig. 1B, bar 2). In results comparable to those for the LT2 strain, flhC transcription upon HilD overexpression also increased in the virulent *Salmonella* background ST4028s grown under Spil-inducing conditions (Fig. 1B, bars 5 and 6). The induction of Spil gene transcription by HilD additionally resulted in a growth defect, as described previously (61). However, deletion of the Spil region of the chromosome [Δ(invH-sprB)] (Fig. 1B, Spil\_−) relieved the growth-inhibitory effect of HilD overproduction (not shown). The retarded growth under HilD-overproducing conditions had an effect on our growth-based β-galactosidase assays (Fig. 1B, compare bar 5, Spil\_+, to bar 6, Spil\_−), and the HilD-activating effect on flhDC operon transcription was therefore more pronounced in strains harboring the invH-sprB deletion (Spil\_−).

In order to further characterize the effect of HilD on flagellar gene expression, we analyzed transcriptional lac fusions to individual flagellar class 1, class 2, and class 3 promoters. Upon overexpression of HilD from the arabinose promoter (Fig. 1C, HilD\_+/−, HilD\_+/H9004\_C), we observed a 2-fold increase in flhC-lac (class 1) transcription and a 3-fold increase in both flIL-lac (class 2) and flfB-lac (class 3) expression (Fig. 1C). Mutant strains overexpressing a DNA-binding-deficient variant of HilD missing its helix-turn-helix binding motif (Fig. 1C, HilD\_ΔHilD\_+\_) were lacking the HilD effect and showed flagellar gene expression levels comparable to those of the wild-type control.

We next uncoupled the expression of the flagellar master regulator flhDC from any transcriptional regulation using a tetracycline-inducible flhDC promoter (62). Under P\_tet\_c-flhD\_C\_+ conditions, neither HilD nor HilD\_ΔHilD\_+ overexpression showed any effect on flagellar gene expression (Fig. 1D). These results sug-
Data were analyzed by the Student t test. Biological replicates are shown as individual data points (diamonds), and the relevant genotypes are indicated as follows: $\Delta$, the respective gene was deleted from the strain.

In a complementary experiment, we analyzed flagellar gene expression levels in a background overexpressing the SpI1 master regulator HilA. HilA was suggested to be a regulator of $flhDC$ transcription (51). As shown in Fig. S2 in the supplemental material, we could rule out an effect of HilA on flagellar gene transcription and thereby confirmed that the observed data were analyzed by the Student t test. Biological replicates are shown as individual data points (diamonds), and the relevant genotypes are indicated as follows: $\Delta$, the respective gene was deleted from the strain.

In a complementary experiment, we analyzed flagellar gene expression levels in a background overexpressing the SpI1 master regulator HilA. HilA was suggested to be a regulator of $flhDC$ transcription (51). As shown in Fig. S2 in the supplemental material, we could rule out an effect of HilA on flagellar gene transcription and thereby confirmed that the observed
HiILD effect on flhDC transcription was not an indirect effect via HilA.

The flhDC promoter region was reported to consist of six transcriptional start sites upstream from the flhDC gene and annotated P1 through P6 (63). To determine which flhDC transcriptional start site was activated by HiILD, we subsequently measured HiILD-mediated expression of lux fusions to individual flhDC promoters. Upon induction of HiILD, flhDC expression was detected for the construct with P1 through P6, as well as for the P5 promoter fusion (Fig. 1E). We therefore concluded that HiILD acts solely on the P5 promoter to activate flhDC transcription.

Many regulatory proteins that act on flhDC transcription bind to a region close to the P1 promoter, like RcsB (+5 to +19 nucleotides from the P1 transcription start site) or RtsB (−4 to +106 nucleotides from the P1 transcription start site) (24, 29). We tested possible dominant effects of simultaneous overproduction of both HiILD (acting as activator of P5 transcription) and RtsB (acting as repressor of P1 transcription) on flhDC operon transcription. As shown by the results in Fig. S3A in the supplemental material, RtsB overproduction decreased flhD-lac levels, whereas HiILD had an activating effect. We did not observe a change in flhD-lac expression compared to that in the wild type when we overproduced both RtsB and HiILD concurrently. This suggested that the repressor RtsB and the activator HiILD acted simultaneously on different promoters of flhDC. We further explored this possibility by analyzing the expression of lux fusions to individual flhDC promoters under conditions where either HiILD or RtsB was overexpressed (see Fig. S3B). As demonstrated by the results described above, the expression of HiILD activated flhDC expression from the P5 promoter, whereas the expression of RtsB had no effect on flhDC transcription from the P5 promoter. In contrast, RtsB overexpression showed a significant repression of flhDC expression from the P1 promoter, demonstrating that RtsB and HiILD can act independently as repressors and activators of different flhDC promoters.

To further test the idea that HiILD directly activates flhDC by binding to the flhDC promoter region, we performed electrophoretic mobility shift assays using purified HiILD protein and various promoter fragments of flhDC. DNA fragments comprising the P1 promoter (fragment 1; nucleotides −271 to −71 upstream from the flhD coding region), the P2, P3, P4, and P6 promoters (fragment 2; nucleotides −431 to −231 upstream from the flhD coding region), the P5 promoter (fragment 3; nucleotides −588 to −388 upstream from the flhD coding region and fragment 3′; nucleotides −668 to −462 upstream from the flhD coding region), and a control sequence outside the flhDC promoter region within the coding sequence of flhDC (designated flhDC coding; nucleotides + 403 to +672 downstream from the flhD start codon) were analyzed according to the schematic in Fig. 2A. As shown by the results in Fig. 2B, we observed binding of increasing concentrations of purified HiILD protein to DNA fragments comprising the P5 promoter (fragment 3 and 3′). Increasing amounts of HiILD decreased the amount of free DNA, while the amount of HiILD-bound DNA increased. Control DNAs (gyrA and flhDC coding) did not bind to purified HiILD, and neither did the flhDC promoter fragments 1 and 2.

We next performed DNase I footprinting to precisely map the HiILD binding site in the flhDC P5 promoter region. An flhDC promoter fragment comprising nucleotides −668 to −388 upstream from the flhD coding region was incubated with increasing concentrations of purified HiILD protein and, after partial digestion with DNase I, the resulting fragments were subjected to denaturing gel electrophoresis (Fig. 3A). We identified a region from −68 to −24 nucleotides upstream from the transcriptional start site of the P5 flhDC promoter that was protected from DNase I digestion in the presence of HiILD. In addition, the presence of HiILD resulted in an enhancement of DNase cleavage at nucleotides −51, −33, −28, and −27 upstream from the P5 transcriptional start, which indicates a potential DNA bend (Fig. 3A and B).

Comparison of known HiILD binding sites from the rtaA, hilC, hilD, and hilA promoters (64) with the identified P5 flhDC binding site revealed several nucleotides that were conserved between these sites (Fig. 3C; consensus; uppercase letters indicate predom-
HilD-Dependent Activation of *flhDC*

**FIG 3** DNase I footprinting demonstrates HilD binding to the *flhDC* P5 promoter region. (A) DNase I footprinting of an *flhDC* P5 promoter DNA fragment. A DNA fragment covering a region of nucleotides from position −668 to −388 upstream from the *flhDC* start codon was DIG labeled on the noncoding strand and incubated alone (lane P) and with increasing amounts of purified HilD protein (lane 1, 4.23 pmol; lane 2, 8.45 pmol; lane 3, 12.68 pmol; lane 4, 16.9 pmol; lane 5, 21.13 pmol; lane 6, 42.25 pmol) and digested with DNase I before being loaded on a sequencing gel. The vertical line indicates the region protected from DNase I digestion. Lanes C, T, A, and G show the specific nucleotides of the noncoding strand. Exposed nucleotides are highlighted by dots. (B) Partial nucleotide sequence of the P5 promoter of *Escherichia coli* digested with DNase I before being loaded on a sequencing gel. The vertical line indicates the region protected from DNase I digestion. Lanes C, T, A, and G show the specific nucleotides of the noncoding strand. Exposed nucleotides are highlighted by dots. (C) Comparison of HilD binding sites in the *flhDC*, *rtsA*, *hilC*, *hilD*, and *hilA* promoters. The alignment of HilD binding sites is as defined by DNase I footprinting in the present and previous studies (60, 64). Shading indicates the predominant (black) and conserved (gray) nucleotides. The consensus motif logo of the experimentally determined HilD binding sites is shown at the top and was generated using WebLogo (71). The proposed consensus is displayed at the bottom; uppercase letters indicate predominant nucleotides (>80% conserved), and lowercase letters indicate conserved nucleotides (>60% conserved).

**DISCUSSION**

In a previous study, we investigated the feedback regulation occurring at the level of the flagellar master regulator *flhDC*. Using a genetic screen, we identified RflM as a negative regulator of *flhDC* transcription and demonstrated that the FlhDC complex activated *rflM* expression in a regulatory feedback loop (26). Another class of transposon insertions that we obtained from our screen for regulators affecting *flhDC* autorepression resulted in overexpression of the *hilD* gene. This suggested a role of the Spi1 activator HilD in regulation of the flagellar master operon *flhDC*. In the present study, we show that HilD acts as a direct activator of *flhDC* expression via activation of the P5 transcriptional start site, thus revealing a novel transcriptional cross talk between the flagellar and virulence regulons in *Salmonella*. Importantly, a recent study by Kroeger et al. (65) analyzed the *Salmonella* transcriprome under 22 different infection-relevant environmental conditions. The RNA-sequencing data of this study reveal specific activation of the *flhDC* P5 promoter only under Spi1-inducing conditions. In addition, the P5 transcriptional start site and the upstream HilD binding site appear not to be present in *Escherichia coli*, indicating that HilD-dependent activation of *flhDC* evolved concurrently after horizontal gene transfer of Spi1 in *Salmonella*.

The transcriptional cross talk between the flagellar and Spi1 virulence regulons is complex, with several regulatory feedback mechanisms implemented on various levels (Fig. 4). The negative regulator of flagellar class 1 gene expression, RflM, is activated by the FlhDC protein complex, which additionally activates the expression of genes transcribed from flagellar class 2 promoters, including *fliZ*. FliZ protein is a posttranscriptional regulator of HilD protein. HilD acts in a positive-feedback loop together with HilC and RtsA to activate HilA and as an activator of flagellar class 1 gene expression through activation of the *flhDC* P5 promoter, as described in this work. RtsB is another Spi1-related regulator that is encoded in an operon together with *rtsA* and functions as a repressor of flagellar class 1 gene expression through repression of *flhDC* transcription at the level of the P1 promoter.

What might be the physiological relevance of this complex cross-regulation network? At the moment, this is unclear, but for the initiation of infection, motility plays a crucial role. Therefore, we speculate that the transcriptional link between the flagellar and virulence systems is of importance during a specific step in the *Salmonella* infection cycle, as outlined below.

The Spi1 and Spi2 virulence-associated genes are transcribed at different stages during *Salmonella* infection. In the insect patho-
gen *Xenorhabdus nematophila*, hemolysin production and a full virulence phenotype have been reported to be dependent on the flagellar protein Fliz, and mutants with mutations of fliAZ or flhDC had attenuated virulence (66). For the food-borne pathogen *Salmonella*, Fliz was previously described to posttranslationally modify the Spi1 regulator HilD, forwarding a positive effect to HilA, the transcriptional activator of Spi1 structural genes (41–44). However, recent data suggested that only a few of the known mechanisms involved in Spi1 regulation are in fact Fliz dependent (67). Transcriptional regulation between the Spi1 system, the related flagella, and the type I fimbria systems have been subject to intensive research within the last few years. However, the detailed mechanisms are not well understood. Saini et al. (68) recently described the cross-regulation between the three systems to constitute a gene expression hierarchy that has the expression of flagellar genes at the top of a complex cascade. Fimbrial genes are repressed during flagellar gene expression and vice versa. At the same time, flagellar gene expression increased the expression of Spi1 genes (68). However, it was shown that overproduction of the Spi1-related protein RtsB completely abolished flgA promoter activity, which can be explained by repression of the flagellar master regulatory operon flhDC. Saini et al. (68) argued that the loss of flagellar gene expression and motility, which is downregulated during intracellular growth, would correspond to the bacterium’s need to stay nonmotile after successful invasion. In this paper, however, we identified the Spi1 regulator HilD as a positive regulator of flagellar class 1 gene expression.

The activation of the flagellar system via HilD and simultaneous inactivation via RtsB argue for a dual or even multiprocess interplay between flagellar and Spi1 regulation. Thus, depending on the environmental niche and spatiotemporal stage of infection, the Spi1 regulator HilD could activate flhDC transcription through direct binding to the flhDC promoter or repress flhDC transcription via activation of RtsB. We postulate that during the early stages of epithelial cell infection, Spi1 gene expression is induced and motility is downregulated but flagellar genes are in a state ready to be immediately upregulated at some later time during infection by activation via HilD. The secretion of bacterial Spi1 effectors results in the internalization of *Salmonella* cells and formation of *Salmonella*-containing vacuoles. At this point, motility is probably no longer required or could hinder the infection process. In epithelial cells, flagellar proteins were downregulated during early infection (2 h p.i.), while the simultaneous expression of Spi1 and flagellar genes 4 to 6 h postinfection (resembling the late stages of infection) has been reported (39). Earlier results by Cummings et al. showed heterogeneous FliC expression by 60% of the bacterial population in Peyer’s patches 7 days p.i. (38). However, as soon as eukaryotic host cells burst or lyse, the bacteria released seek further host cells for infection. At this stage, it might be advantageous for the bacteria to have flagellar gene expression ready to allow for a fast switch back into a motile state that could be mediated by activation of the flagellar regulon via HilD. This argument is also supported by the results of Sano et al. (69), who observed the requirement for flagella in order for *Salmonella* to exit host macrophages. Accordingly, flagellum-negative cells were unable to escape from host cells. During the first 2 h of macrophage infection, intracellular *Salmonella* cells were nonflagellated, whereas flagellum reexpression was observed 4 h later, an effect also previously reported in *Legionella pneumophila* (70). Together, these results argue in favor of a HilD-mediated de novo synthesis of flagella during some later step in the infection process.

While many questions remain, analysis of the temporal course of gene expression will be crucial to understand the interplay between motility and virulence in detail. Further research will need to focus on the aspects of gene regulation...
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