The Effect of Cell Growth Phase on the Regulatory Cross-Talk between Flagellar and Spi1 Virulence Gene Expression

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Abstract

The flagellar regulon controls Salmonella biofilm formation, virulence gene expression and the production of the major surface antigen present on the cell surface: flagelin. At the top of a flagellar regulatory hierarchy is the master operon, flhDC, which encodes the FlhD4C2 transcriptional complex required for the expression of flagellar, chemotaxis and Salmonella pathogenicity island 1 (Spi1) genes. Of six potential transcriptional start-sites within the flhDC promoter region, only two, P1<sub>flhDC</sub> and P5<sub>flhDC</sub>, were functional in a wild-type background, while P6<sub>flhDC</sub> was functional in the absence of CRP. These promoters are transcribed differentially to control either flagellar or Spi1 virulent gene expression at different stages of cell growth. Transcription from P1<sub>flhDC</sub> initiates flagellar assembly and a negative autoregulatory loop through FlhD4C2-dependent transcription of the rfiM gene, which encodes a repressor of flhDC transcription. Transcription from P1<sub>flhDC</sub> also initiates transcription of the Spi1 regulatory gene, hiiD, whose product, in addition to activating Spi1 genes, also activates transcription of the flhDC P5 promoter later in the cell growth phase. The regulators of flhDC transcription (RcsB, LrhA, RifM, HiiD, SlyA and RtsB) also exert their control at different stages of the cell growth phase and are also subjected to cell growth phase control. This dynamic of flhDC transcription separates the roles of FlhD4C2 transcriptional activation into an early cell growth phase role for flagellar production and a late cell growth phase role in virulence gene expression.

Introduction

Tens of millions of human cases of Salmonellosis, a foodborne gastroenteritis caused by Salmonella enterica, occur worldwide every year killing more than a hundred thousand people annually (World Health Organization Fact sheet N°139, August 2013). Typhoid fever caused by Salmonella Typhi kills an equivalent number of people each year. A prominent player in Salmonella pathogenesis is the bacterial flagellum. The bacterial flagellum is an ion-powered, complex motor organelle that endows bacterial cells, such as Escherichia coli and Salmonella enterica, with the ability to propel themselves through liquid medium and across hydrated surfaces [1]. Motility also plays an important role in biofilm formation and in the ability of many pathogens to reach their sites of infection and establish disease [2,3].

Early work on the discovery of Salmonella virulence genes identified a transposon insertion in the flagellar filament cap gene, flaD, as defective for survival of cells in macrophages [4]. However, flaD is in an operon with the flaT gene whose product is a regulator of the flagellar and Spi1 virulence genes master regulatory complex FlhD<sub>C2</sub>C<sub>2</sub> [5,6]. The transposon insertion in flaD was polar on flaT gene expression and thus identified regulation of FlhD<sub>C2</sub>C<sub>2</sub> activity as critical for Salmonella virulence. The two proteins that make up the FlhD<sub>C2</sub>C<sub>2</sub> transcriptional regulatory complex are co-expressed with the flaDC operon, class I promoter, which is at the top of a complex transcriptional hierarchy for both flagellar and Spi1 virulence genes expression. The decision whether or not to produce flagella is regulated at the levels of flhDC transcription, translation, FlhD<sub>C2</sub>C<sub>2</sub> assembly and stability [7]. Positive regulators of flhDC operon transcription include cAMP-CRP, Fis, Fur, H-NS and QseB [9–14]. A large number of regulatory factors are also reported to inhibit flhDC transcription. These factors include, LrhA, ResB, RtsB, SlyA, DskA, PelI-SrgD, FimZ, HldR, OmpR and RifM [15–20]. The FlhD<sub>C2</sub>C<sub>2</sub> activity generates an auto-regulatory loop by activating transcription of the rfiM gene encoding a LysR-type DNA binding protein RifM, which in turn inhibits the transcription of flhDC [21]. The post-transcriptional factors regulating flhDC include, CsrA [22,23], Hsp70 chaperone DnaK [24] and ClpXP protease [25]. Recently an FlhD<sub>C2</sub>C<sub>2</sub> repressed gene, ydiV [26], was shown to code for a protein (YdiV) that will bind to FlhD<sub>C2</sub>C<sub>2</sub>, in its free or DNA-bound form, remove FlhD<sub>C2</sub>C<sub>2</sub> from DNA and serves as an adapter that targets FlhD<sub>C2</sub>C<sub>2</sub> for ClpXP-dependent degradation [27,28].

In Salmonella, an initial characterization of the flhDC promoter region identified six transcriptional start sites (TSSs) [13]. In a recent study, only four of the original six TSSs were detected [29]. The presence of six TSSs in the Salmonella flhDC regulatory region combined with the presence of DNA binding sites of CRP, LrhA, RtsB, HilD, ResB, HNS and others indicated a complex level of transcriptional regulation.
Author Summary

Flagellar-mediated motility is fundamental to Salmonella pathogenesis, which takes the lives of hundreds of thousands of people each year. The genes of the Salmonella pathogenicity island 1 and those of the flagellar regulon are part of the same transcriptional hierarchy. We report the novel finding where the key control of this network takes place at the flhDC promoter region. We followed the transcription from the two “live” flhDC promoters as a function of the cell growth phase. P1 comes on early in the cell cycle, while P5 comes on late. Transcription of P5 is HiID dependent, which represents a totally new finding and Salmonella specific; there is no HiID in E. coli flhDC control, no P5 transcription. P1 & P5 can express flhDC to equivalent levels, yet only P1-dependent expression produces motility UNLESS we artificially induce P5 EARLY in the cell cycle. This work is the foundation for the cell cycle stages a Salmonella bacterium experiences during host infection. This is a significant conceptual advance in Salmonella pathogenesis: one can no longer consider gene regulation at 37°C and OD 0.6 as a reflection of the Salmonella infection cycle; the whole cell growth cycle must be considered in understanding this complex biological processes.

Salmonella enterica is an intracellular facultative pathogen causing a range of diseases in a variety of hosts [30]. Important virulence factors required for Salmonella invasion of epithelial cells and development of Salmonellosis are encoded within the Salmonella pathogenicity island 1 (Spi1) genes. Spi1 encodes a virulence-associated type III secretion system (T3SS) as part of an injectosome structure required for the secretion and injection of multiple effector proteins into the cytoplasm of host cells [31–36]. Expression of Spi1 genes is controlled in response to specific environmental signals in a complex hierarchical process with multiple transcriptional regulators. These include, HilA, a member of the OmpR/ToxR family of transcriptional regulators, which promotes transcription of genes encoding the necessary components for a functional Spi1 injectosome system [32,35,37,38]. Also included are the hilC and hilD genes whose products are members of the Ara/XylS family of transcriptional regulators that control hilA gene transcription. HilD is at the top of the regulatory network controlling Spi1 expression because most regulators controlling hilA transcription appears to be HilD-dependent [39,40].

It is noteworthy to mention that many protein components of the Spi1 and flagella T3SS exhibit a significant degree of amino acid identity, leading to the production of remarkably similar T3SS structures [16,33,34,41,42]. Furthermore, many of the transcriptional and posttranslational regulatory factors of flhDC also target the main transcriptional regulators of Spi1, such as HilA and HilD [11,43–52]. In addition, the ATP-dependent Lon protease was shown to degrade both FlhDC and HilD [24,25]. Coordinated expression of Spi1 and flagellar genes has been recently demonstrated [53]. In Salmonella, expression of Spi1 genes is activated by FlI (54–57), which is encoded within the flagellar flaC2 operon. FlIZ activates the hilD gene expression at the posttranslational level and HilD in turn promotes transcription of the rtsB operon, which encodes a pathogenesis-related DNA-binding regulatory proteins. RtsA and RtsB reciprocally regulate both the Spi1 and flagellar genes [17]. The direct binding of RtsB to the flhDC promoter region inhibits flhDC transcription and motility [17].

We decided to investigate how input from different regulatory factors might integrate multiple environmental or cell cycle signals into the control of flhDC expression in Salmonella enterica. We explored how and when positive and negative regulators affect flhDC expression throughout the cell growth cycle. We measured the effect of RcsB, LhaA, RfM, SlyA, RtsB and HilD regulatory factors on flhDC operon transcription at different cell growth phases. We characterized the specific TSSs within the flhDC promoter region and their involvement in the positive and negative control of flhDC cell-cycle dependent transcription. Finally, we examined how the individual TSSs and protein regulatory factors controlled the interconnection between the flagellar and Spi1 regulons.

Results

Dynamics of flhDC operon transcription in liquid culture after induction from stationary phase

To investigate flhDC operon transcription at different phases of the cell growth, we constructed a transcriptional fusion of the flhDC promoter region to the luciferase operon of Photobacterium luminescens (luxCDBAE operon). Because the flhDC operon is autoregulated negatively by RfM and positively by HilD, we designed strains harboring an intact copy of the flhDC operon under the control of its native promoter (PflhDC) and an in-frame fusion of a second copy of the promoter region of flhDC (through the first 272 nucleotides of flhD coding sequence) to the luciferase operon: DUP[PflhDC-luxCDBAE*Km*PflhDC-flhD*C] (Figure 1A). Thus, individual PflhDC promoter regions transcribe both the luminescence operon reporter and the flhDC operon. This results in a strain with luminescence readout for the level of transcriptional activation of flhDC under conditions that also preserves the wild-type expression of the flagellar regulon including flhDC autoregulation through FlhDC-dependent expression of rtsM and hilD genes. For simplicity, we will refer to the construct as PflhDC-DUP.

Following batch inoculation of an overnight culture of the PflhDC-DUP strain into fresh media with shaking at 30°C, transcription of the flhDC genes declined 4-fold during the initial lag phase transition to log phase growth to a minimal value (Figure 1B). This observation is consistent with that reported in an earlier study [11]. After the transition to log phase growth, transcription of flhDC increased more than 10-fold between OD 0.3 and 1.2, followed by a decline in flhDC transcription as cells enter late log and stationary phase growth (Figure 1B).

Dynamics of flhDC operon transcription during cell cycle growth in liquid culture in the absence of transcriptional regulators

In Salmonella enterica, flagellar regulon transcription is highest during the exponential phase of growth and decays in late stationary phase [58]. Transcription of the flagellar master regulatory operon, flhDC, is under both negative and positive control by multiple regulatory factors. Null mutations in any one of the rcsB, rtsM, lhaA, slyA, and rtsB genes result in increased transcription of the flhDC operon, which is consistent with an inhibitory activity on flhDC expression. HilD is an activator of flhDC transcription such that over-expression of the hilD gene increases flhDC expression (Singer et al. submitted). The diversity of transcription factors controlling expression of flhDC reflects the complexity of flhDC transcriptional regulation and suggests that flhDC transcription is controlled when Salmonella cells are experiencing different metabolic or environmental states, or
Figure 1. Growth phase dependent transcription of the flhDC operon promoter in Salmonella enterica serovar Typhimurium is controlled by LrhA, RcsB, HilD, SlyA and RtsB. (A) Diagram depicting a duplicated chromosomal region that includes fusion of the flhDC promoter region (P_{flhDC-C} (728 bp upstream of the start codon of flhD and the first 272 nucleotides of flhD coding region)) to the luciferase operon of Photorhabdus luminescens in addition to a wild-type flhdc promoter-operon region. (B) A time course plot showing P_{flhDC-C} expression at increasing cell density of strain Pwt_{flhDC-C-luxCDBAE-P_{flhDC-C}flhD}^C (TH18684) grown in LB media at 30°C with shaking. Luciferase activity was measured along with the OD595. Plots represent the recorded luciferase activity divided by the OD595. (C & D) Time course plots showing P_{flhDC-luxCDBAE} expression at increasing cell density in the absence of flhDC regulators. Individual regulators of flhDC promoter (P_{flhDC-luxCDBAE}) transcription were removed by deletion in the P_{flhDC-luxCDBAE-P_{flhDC-luxCDBAE}}^C background. Plots for specific individual strains are identified at the right of their corresponding plots. (wt = wild-type (TH18684), rcsB = ΔrcsB::tetRA (TH19230), lrhA = ΔlrhA::tetRA (TH18722), rtsB = ΔrtsB::T-POP (TH18724), slyA = ΔslyA::T-POP (TH18720) and hilD = ΔhilD::tetRA (TH19654)). (C) Loss of RcsB, LrhA or RtsB resulted in increased transcription of the flhDC operon at early growth phase. (D) Effect of removal of virulence-related genes slyA, rtsB or hilD differentially affected flhDC operon transcription. Deletion of either the rtsB or slyA gene resulted in increased flhDC operon transcription once cells reach stationary phase contrary to a deletion in the hilD gene, which resulted in increased flhDC transcription once bacterial cells enter mid exponential phase. The OD595 values are shown at the bottom of the chart. Values are the average of three independent experiments done in duplicate. Error bars represent standard deviation.

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different growth conditions under which these transcriptional factors are active. We examined both the timing and magnitude of individual regulatory proteins on flhDC transcriptional control throughout the cell’s growth phase. We tested flhDC transcriptional levels as a function of the cell’s growth phase in strains missing the individual negative regulators RcsB, LrhA, RflM, RtsB, SlyA and the positive regulator HilD (Figures 1C & D). As was presented above for the wild-type strain, this was done by growing Pwt_{flhDC} cells in liquid culture at 30°C using luciferase as the reporter for flhDC transcription levels. Luciferase levels were determined at specific optical densities shown in Figure 1. As expected, removal of individual inhibitors resulted in an increase in flhDC transcription levels while removal of HilD decreased flhDC transcription. Importantly, our assay revealed a growth phase-dependent hierarchy of the effect of these regulators. At OD 0.3, basal flhDC transcription was elevated in the absence of LrhA and RcsB, while removal of RflM, RtsB, SlyA or HilD exhibited the same basal level of transcription as wild type (Figures 1C & 1D). This suggests that RcsB and LrhA act earlier, during lag phase, to inhibit flhDC transcription. This effect could also represent a carry-over of repression from stationary phase that keep flhDC transcription low during the transition to log growth. In the absence of RflM we observed an earlier transition to flhDC activation than in the other mutant strains. Since FlhD4C2 transcribes the rflM gene and RflM protein inhibits flhDC transcription (flhDC auto-inhibition), this result suggests that flhDC auto-inhibition through RflM occurs during early exponential phase to control when full FlhD4C2-dependent gene expression occurs at log phase. The negative effect of RtsB and SlyA on flhDC transcription was detected as cells enter early stationary phase. We also observed that the maximum flhDC transcription level peaked earlier for both the hilD and rflM mutants at OD 1, while the wild type and mutants in rcsB, lrhA, slyA and rtsB peaked around OD 1.2.

The data presented in Figure 1C demonstrate that initial flhDC transcription is kept low by a combination of repressors including at least RcsB and LrhA. Initial FlhD4C2 expression during the stationary to log phase transition produces enough RflM to maintain a low level of flhDC transcription until an OD of ~0.3 is reached. After OD 0.3, flhDC expression increased significantly, but RflM, RcsB and LrhA reduce the overall level. Interestingly,
the wild-type level is balanced by the presence of the HilD activator of flhDC transcription, the hilD-activated inhibitor of flhDC transcription RtsB and by the virulence associated factor SlyA (Figure 1D).

The effect of growth conditions on flhDC transcription as a function of cell growth

In order to obtain more detailed information relating the effect of specific regulatory proteins on flhDC transcription as a function of the cell's growth phase, we determined luciferase levels for the P\textsubscript{w\textsubscript{flhDC}}-luc operon grown in liquid culture at 30°C in 96 well plates with a microplate reader. Using this assay system, we could measure the activity of flhDC transcription at shorter times intervals (6 min) with continuous shaking at 150 rpm. We observed the same trend of regulation of the flhDC operon as seen in batch cultures for bhcA, rcsB (Figure 2A), rflM (Figure 2B), slyA and rtsB (Figure 2C), and hilD mutants (Figure 2D). However, the pattern observed in 96 well plates was somewhat different compared to the batch growth. We observed that activation of flhDC transcription took place earlier at OD\textsubscript{0.2} rather than OD\textsubscript{0.3}. Consistent with this observation, the differences between the activity of flhDC in wild-type versus mutant strains also occurred at an earlier OD measurement in microtiter plate growth compared to growth in batch culture. The cells in 96 well plates reached maximum expression at OD\textsubscript{0.6} compared to OD\textsubscript{1.2} in the batch culture. We attribute these differences to the mode of growth in 96 well plates (150 rpm) where bacterial cells are grown in much lower volumes and likely to be subjected to different oxygen levels in the medium compared to batch cultures. It has been shown that activation of flgA, a gene under the control of flhDC, under static conditions (no shaking of 96 well plates) occurred immediately after dilution of an overnight culture into LB-1% Saut [53]. When we tested the activation of flhDC operon in standing batch culture in LB, we observed that flhDC transcription increased at OD\textsubscript{0.12} (Figure S1), which is earlier compared to what we observed either in batch shaking (OD\textsubscript{0.3}) or 96 well grown cultures (OD\textsubscript{0.2}). Moreover, the shutdown of flhDC transcription observed in standing cultures took place after cells reach an OD\textsubscript{0.6} compared to shaking batch culture where the shutdown started at an OD\textsubscript{1.2}.

Growth phase transcriptional dynamics of flhDC transcriptional regulators

Because flhDC transcription is differentially regulated by different transcription factors in a growth phase dependent manner, we hypothesized that the effect of each of these regulators is observed at the time when they are produced during the cell growth cycle. To investigate this possibility we placed the luxCDBAE operon reporter under control of the promoters of the six regulatory genes bhcA, rcsB, rflM, slyA, rtsB and hilD, whose products have been demonstrated to bind directly to the flhDC promoter region and monitored their expression profile at different optical densities (binding of RflM or SlyA to the flhDC promoter region has not been reported). We monitored the activities of these constructs in 96 well plates over time. We observed that the transcription of the autoregulated gene bhcA is immediately activated following dilution from an overnight culture, and before the activation of flhDC (Figure S2A). Transcription of rcsD (which is the first gene of the rcsD operon transcribed from the rcsD promoter) also initiated before flhDC (Figure S2B), whereas transcription of rflM overlapped with that of flhDC (Figure S2B). Since rflM transcription is dependent on FlhD1C2, these results suggest that low basal levels of FlhD1C2 are sufficient to promote rflM gene transcription. In addition, transcription of rtsB reached a maximum at OD\textsubscript{0.35} and decayed very quickly (Figure S2B) compared to the rest of the regulators tested in this study. The transcription of hilD gene is under positive autoregulatory control by HilD itself [59] and by HilD-activated RtaA [17]. In addition, the product of an flhDC activated gene, FlIZ, controls HilD at a posttranslational level [54,57]. We observed that transcription of hilD increased at OD\textsubscript{0.4} (Figure S2C), at the same time when HilD promoted transcription of flhDC (Figure 2D). The expression of the HilD-activated rtsA gene (the first gene of the rtsA operon) appeared to be activated at the same time as hilD (Figure S2C). Transcription of the slyA gene was activated just after flhDC transcription started and before initiation of hilD and rtsB transcription, with a peak of expression at entry into stationary phase (Figure S2D). These results suggest that there is a hierarchy of transcription of the factors regulating flhDC transcription that mirrors their effect on the transcriptional regulation of the flhDC operon.

We next asked if the protein levels of the regulatory factors controlling flhDC transcription were also growth phase dependent. We performed Western blot analysis of whole cell lysates of Salmonella strains (LrhA-HA, RcsB-3×Flag, RflM-HA, SlyA-HA, RtsB-HA and HilD-Flag) at different optical densities (Figure 3A). We established that LrhA is present at an early time point during cell growth (OD\textsubscript{0.2}) with maximum expression at OD\textsubscript{0.6} followed by a decay at late stationary phase (note that both the N-terminal and C-terminal HA-tag fusion to LrhA are made but not functional and therefore there is no positive feedback regulation of bhcA transcription by LrhA protein [18]). The level of RcsB protein, the transcriptional regulator of the phosphorelay system RcsDBC, also appeared to be growth phase dependent because RcsB protein was detected early in the growth phase (OD\textsubscript{0.2}) and increased at the stationary phase of cell growth. The FlhD1C2 activated RflM, was produced early in the growth phase (OD\textsubscript{0.2}), and increased at OD\textsubscript{0.4} followed by a quick decay during the rest of the cell's growth phase. HilD protein, the positive activator of flhDC transcription, was detected at OD\textsubscript{0.4} and increased at stationary phase (Figure 3A), RtsB, whose gene is under the transcriptional control by HilD, was not detected early in the growth phase and was present at OD\textsubscript{1.3}. The absence of RtsB at an early time point in the blot might be due to the detection limits for low protein levels in our experiment (See CHIP, Figure 3B, where RtsB was already associated with the promoter of flhDC at OD\textsubscript{1}). In contrast, the negative regulator SlyA was produced during all the phases of cell growth, with a sharp increase at OD\textsubscript{1}. These results demonstrate a hierarchy at the level of expression of flhDC regulators that specifically mimics the differential dynamics of flhDC operon transcription.

In vivo binding by regulators of flhDC transcription to the flhDC promoter region

We examined the in vivo binding dynamics within the flhDC promoter region by these regulatory factors. At different optical densities (0.4 to 1.4), chromatin immunoprecipitations (ChIP) were conducted using strains with individually tagged transcriptional factors, RcsB, RflM, HilD, RtsB, LrhA and SlyA (Figure 3B). Expression of RcsB and binding of RscB to its target DNA at the flhDC promoter was detected throughout the entire growth phase. However, RcsB bound levels increased as cells progressed to exponential phase (OD 0.4 to 0.6) followed by decreased binding at latter stages of growth. The transcriptional regulator RflM binding to DNA was detected at OD\textsubscript{0.4} with maximal binding at OD\textsubscript{0.6}, but was no longer bound the flhDC promoter region beyond OD\textsubscript{0.6}. HilD, a transcriptional activator of flhDC, was...
bound to the flhDC promoter region at OD~0.4 increasing to a maximum bound level at OD~0.8 and followed by absence of bound HilD at OD~1. SlyA was not physically associated with the flhDC promoter at OD~0.4 and ~0.6, but was bound to the flhDC promoter region at OD~0.8. There was no binding of RtsB to the flhDC promoter at an early time point of cell growth OD~0.4 to 0.6. Binding by RtsB had initiated by OD 0.8 and increased through OD 1.4. We were unable to immunoprecipitate LrhA tagged protein because C-terminal or N-terminal tagged LrhA behaved like bclA null mutant (Figure S5i). These results highlight the binding dynamics of different regulators to the flhDC promoter region resulting in a dynamic of flhDC operon transcription.

Molecular analysis of the individual flhDC transcriptional start-sites

Six transcriptional start sites, designated P1\textsubscript{flhDC}, P2\textsubscript{flhDC}, P3\textsubscript{flhDC}, P4\textsubscript{flhDC}, P5\textsubscript{flhDC} and P6\textsubscript{flhDC}, within Salmonella flhDC promoter region were obtained by primer extension [13]. However only P1\textsubscript{flhDC}, P3\textsubscript{flhDC}, P4\textsubscript{flhDC} and P5\textsubscript{flhDC} were detected by RNA-Seq based approach [29]. Each of these TSSs was preceded by a hexamer motif (−10 box) with the consensus invariant residues adenine at position 2 (A2) and thymine at position 6 (T6), except for P4 (Figure 4A). To investigate the authenticity of these TSSs, we made alterations of the −10 sequences targeting the conserved residues A2 and T6 by changing them to a cytosine residue (C) and also by totally changing the −10 box to a GTTGGT sequence (Figure 4B). As controls, additional mutations were made in each −10 box, in a nucleotide other than A2 or T6 (Figures 4B & S3A) that supposedly should not alter significantly the effect of RNAP on transcription [60]. Because flhDC is subjected to negative and positive transcriptional feedback, mutations of the promoters responsible for transcription of flhDC operon in the wild-type strain might affect the positive and negative transcriptional regulation of flhDC operon in the wild-type strain (described above). Mutations of wild-type sequence P1\textsubscript{flhDC} (TATAGT) to GTTGGT (P1\textsuperscript{−1}flhDC), TCTAGC (P1\textsuperscript{−2}flhDC) or TCTAGT (P1\textsuperscript{−3}flhDC) but not TACAGT (P1\textsuperscript{−4}flhDC) were associated with a significant reduction of flhDC transcription (Figure 4C). Mutations of the wild-type P5\textsubscript{flhDC} (TATGCT) to TCTGCC (P5\textsuperscript{−1}flhDC) or TCTGCC (P5\textsuperscript{−3}flhDC) but not to TAGCCT (P5\textsuperscript{−4}flhDC) reduced significantly the

Figure 2. Precise transcriptional regulation of the flhDC operon is growth phase dependent. Transcription kinetics for the flhDC operon in various mutant backgrounds with the P\textsubscript{wt}flhDC-luxCDBAE-P\textsubscript{wt}flhDC-luxCDflhDC operon reporter construct measured in a 96 well plate growth format. The luciferase activity was investigated in seven genetic backgrounds: (A) wild-type (TH18684) empty circles, ΔrcsB::tetRA (TH19230) filled circles, ΔlrhA::tetRA (TH18722) filled diamonds, (B) ΔrtfM::CF (TH18716) filled triangle, ΔrfsB::CAN (TH18724) filled circles, ΔslyA::T-POP (TH18720) filled squares and (D) Δhild::tetRA (TH19654) filled diamonds. The genotypes of the strains are indicated in the left of their plots at the level of their maximum A.U’s. Cells from overnight cultures were diluted 1 to 500 in LB and 200 μl was inoculated into 96 well dark plates that were sealed with a breath easy membrane and incubated at 30 °C in a plate reader with 5 min orbital shaking at 150 rpm. After a pause of 5 second following shaking, luminescence and OD595 of the inoculated wells were read during 95 second. The luminescence was recorded with a 0.1 s integration time for normalization. Arbitrary units (A.U.) were calculated as luminescence reading divided by OD595. The average at each time point was normalized to the maximum A.U. of the wild-type strain. Each data point represents six experiments performed in triplicate in different days. Error-bars indicate standard deviations. A representative growth curve is shown in the second axis of the plots. doi:10.1371/journal.ppat.1003987.g002
transcription of \(flhDC\) to the same extent as the mutation of \(-10\) to GTTGGT (P5\(^{\text{r}}\) \(flhDC\)) (Figure 4D). These results indicated that P1\(_{\text{flhDC}}\) and P3\(_{\text{flhDC}}\) are bona-fide promoters.

Analysis of mutations of \(-10\) sequences of the P2\(_{\text{flhDC}}\) and P0\(_{\text{flhDC}}\) (overlapping with the CRP binding site which is required for the transcription of \(flhDC\) from P1\(_{\text{flhDC}}\) promoter) and P3\(_{\text{flhDC}}\) (overlapping with the LrhA binding site) and P4\(_{\text{flhDC}}\) were not conclusive (Supplementary Text S1 & Figure S3).

We further investigated the authenticity of the six putative TSSs of the \(flhDC\) operon, by engineering strains with combined mutations in the promoter region of \(flhDC\) leaving only one wild-type \(-10\) sequence from the six described promoters. Thus, P1\(^*\) designates a strain that has only a functional P1 promoter, etc. We also constructed a control strain with combined mutations in all the six promoters, AP\(_{\text{flhDC}}\) (All Promoters mutated). We established that P1\(^{\text{r}}_{\text{flhDC}}\) and P5\(^{\text{r}}_{\text{flhDC}}\) were able to promote \(flhDC\) operon transcription but to a lesser extent to what is observed in the wild-type strain (Figure 4E). The transcription of \(flhDC\) was totally abolished in strains harboring P2\(^{\text{r}}_{\text{flhDC}}\), P3\(^{\text{r}}_{\text{flhDC}}\), P6\(^{\text{r}}_{\text{flhDC}}\) and AP\(_{\text{flhDC}}\) mutants, while P4\(^{\text{r}}_{\text{flhDC}}\) mutants showed very low level of \(flhDC\) transcription (1.8% relative to the wild-type strain) (Figure 4E). These results suggested that in the wild-type background P1\(_{\text{flhDC}}\) and P5\(_{\text{flhDC}}\) are the main promoters driving \(flhDC\) operon transcription with a marginal activity from the P4\(_{\text{flhDC}}\) promoter. Yanagihara et al., 1999; have demonstrated that P0\(_{\text{flhDC}}\) is only active in the absence of CRP, we confirmed that P0\(_{\text{flhDC}}\) (only P6 is functional) is inhibited by CRP, because in a crp null mutant there was an increase of transcription of P6\(_{\text{flhDC}}\) compared to wild-type (Figure S3H).

Since only mutations in P1\(_{\text{flhDC}}\) and P5\(_{\text{flhDC}}\) promoters significantly affected the expression of \(flhDC\), we would expect the level of transcription of \(flhDC\) operon in the absence of both P1 and P5 promoters to be similar to the level of transcription of \(flhDC\) operon in the absence of all \(flhDC\) promoters (P1 through P6). To investigate this hypothesis, we measured the luciferase activity in a strain with combined mutations in P1\(_{\text{flhDC}}\) and P5\(_{\text{flhDC}}\) promoters (P1\(^{\text{r}}\) P5\(^{\text{r}}\) \(flhDC\)) and compared it to the luciferase activity of a wild-type strain and to a strain with all six promoters mutated (strain AP\(_S\)). We observed that transcription of \(flhDC\) operon in strain P1\(^{\text{r}}\) P5\(^{\text{r}}\) \(flhDC\) was totally abolished to the same levels observed in a strain with all \(flhDC\) promoters mutated (Figure 4F). These results demonstrated that in a wild-type background P1\(_{\text{flhDC}}\) and P5\(_{\text{flhDC}}\) are the major promoters driving transcription of \(flhDC\) operon. We concluded that transcription of the \(flhDC\) operon in strain P1\(_{\text{flhDC}}\) (harboring mutations of the \(-10\) box of P1\(_{\text{flhDC}}\)) is driven from the P3\(_{\text{flhDC}}\) promoter and that transcription of the \(flhDC\) operon in strain P5\(_{\text{flhDC}}\) (harboring mutations of \(-10\) box of P5\(_{\text{flhDC}}\)) is driven from P1\(_{\text{flhDC}}\).

**Dynamics of \(flhDC\) transcription from P1\(_{\text{flhDC}}\) and P5\(_{\text{flhDC}}\) promoters**

Once we established that P1\(_{\text{flhDC}}\) and P5\(_{\text{flhDC}}\) are the main promoters driving transcription of the \(flhDC\) operon, we monitored the expression of the P1\(_{\text{flhDC}}\) and P5\(_{\text{flhDC}}\) promoters at different optical densities using Pwt\(_{\text{flhDC}}\), P1\(_{\text{flhDC}}\) and P5\(_{\text{flhDC}}\) constructs (Figure 5A). The transcription profile of \(flhDC\) operon in strains P1\(_{\text{flhDC}}\) (P5-expressed) and P5\(_{\text{flhDC}}\) (P1-expressed) demonstrated that both promoters are required for transcription of \(flhDC\) because the expression of \(flhDC\) operon in constructs P1\(_{\text{flhDC}}\) (P5-expressed) and P5\(_{\text{flhDC}}\) (P1-expressed) did not reach the expression levels of the wild-type strain, Pwt\(_{\text{flhDC}}\) (both P1 and P5 are expressed) (Figure 5A). Moreover, transcription of \(flhDC\) operon from P1\(_{\text{flhDC}}\) is activated earlier than P5\(_{\text{flhDC}}\) because (i) the transcription profile of the \(flhDC\) operon in construct P5\(_{\text{flhDC}}\)
(P1-expressed) overlapped with that of the wild-type strain from OD 0.1 to OD 0.4, (Figure 5A) and (ii) there was a delay in the transcription of $flhDC$ operon in construct P1

$flhDC$

(P1-expressed) started declining at OD $0.4-0.5$, meanwhile, transcription of $flhDC$

operon in strain P1\textsuperscript{−}$_{flhDC}$ (P5-expressed) was more pronounced at a later growth stage accounting for $\sim60\%$ relative to the wild-type at OD $0.6$ (Figure 5A). It is apparent from the dynamic profile of $flhDC$ operon transcription, that P5

$flhDC$

promoter transcription occurs concomitantly with a cessation or decline in the transcription from P1

$flhDC$

(Figure 5A). These results indicate that P1

$flhDC$

is an early promoter, whose activation drives the transcription of $flhDC$ operon synthesis at early growth phase followed by a cessation or decline once P5

$flhDC$

promoter is activated.
HilD specifically activates transcription from the P5flhDC promoter

We have demonstrated that HilD is a positive regulator of flhDC transcription (Figure 1D & 2D). As shown (Figure 2D), when cells are grown in the 96 well plate format, the effect of HilD on the transcription of flhDC takes place starting at OD~0.4. In order to determine which of the two promoters, P1flhDC or P5flhDC, is the target of the positive regulation by HilD, we compared the dynamic profile of flhDC transcription in P1flhDC, P1hilD and P5hilD constructs in a wild-type and its isogenic strain hilD null mutant (Figures 5B & C). We established that, relative to the wild-type strain background, a deletion of hilD (i) reduced P1flhDC promoter transcription; (ii) abolished the transcription of flhDC operon in a P1hilD (P5-expressed) (Figure 5B) and (iii) 

Figure 5. Transcription levels of the P1flhDC and the P5flhDC promoters during the cell growth phase and their regulation by HilD and RflM. (A) Luciferase activity was measured in three genetic backgrounds: PwtflhDC-luxCDBAE-PwtflhDCflhD+C (TH18684) filled square, P1hilD-luxCDBAE-PwtflhDCflhD+C (TH18889) filled circle and P5hilD-luxCDBAE-PwtflhDCflhD+C (TH18895) filled triangle. Luciferase activity relative to the wild-type strain is shown (first axis) along with the OD 595 (Second axis). In the absence of the P5 promoter (P5flhDC) the flhDC operon (transcribed from P1) was activated earlier than the isogenic strain that transcribed flhDC from the P5 promoter (P5hilD). Transcription of the flhDC operon from the P1 promoter (P5hilD) was activated at the same time as with the wild-type promoter (PwtflhDC) at OD~0.2. When cells reach an OD of 0.4, P1hilD promoter activity (P5flhDC) ceased and declined afterwards. Transcription from the P5flhDC start site (P1hilD) took place at an OD of ~0.35. (B & C) HilD promotes transcription of the flhDC operon from the P5flhDC promoter. Luciferase activity relative to the wild-type strain is shown (first axis) along with the OD 595 (Second axis). Luciferase activity was investigated in five strains: PwtflhDC-luxCDBAE-PwtflhDCflhD+C (TH18684), P1hilD-luxCDBAE-PwtflhDCflhD+C (TH18889), P5hilD-luxCDBAE-PwtflhDCflhD+C ΔhiID::TetRA (TH19965), P5hilD-luxCDBAE-PwtflhDCflhD+C (TH18895) and P5flhDC-luxCDBAE-PwtflhDCflhD+C ΔhiID::TetRA (TH19966). Transcription of flhDC operon from the P5flhDC promoter (P1hilD) was totally abolished in the absence of HilD. The absence of HilD had no effect on the transcription of flhDC operon from the P1hilD promoter (P5hilD). Each data point of the plots represents the average of five independent replicates performed in different days of six measurements for wild-type flhDC promoter and three measurements for the rest of strains. (D & E) RflM feedback inhibits transcription of the flhDC operon. Luciferase activity of P1hilD and P5hilD flhDC promoters expressing the luxCDBAE reporter is presented as a function of the cell growth phase in isogenic strains in the presence (WT) and absence (ΔRflM) of RflM. Luciferase levels at different points during the cell growth phase were measured for the (D) P1hilD-luxCDBAE-PwtflhDCflhD+C, and (E) P5hilD-luxCDBAE-PwtflhDCflhD+C duplication constructs. Growth conditions and luciferase activity were analyzed as described in Figure 2. A representative growth curve is shown in each plot. Plots represent the average of five independent replicates performed in different days of six measurements for each data point. Error bars represent standard deviation. (F) Overproduction of RflM inhibits transcription from the P1hilD and P5hilD promoters. Luciferase activity for PwtflhDC-luxCDBAE-PwtflhDCflhD+C, P1hilD-luxCDBAE-PwtflhDCflhD+C and P5hilD-luxCDBAE-PwtflhDCflhD+C was investigated, when cells reached an OD 1, in two genetic backgrounds: ParaBADF, and ParaBAD(ΔRflM). Growth conditions and luciferase activities were analyzed as described in Figure 4. To induce expression of rFlM from the arabinose promoter (ParaBAD(ΔRflM)), arabinose was added at 0.2% (indicated by an arrow). (i) indicates wild-type level of rFlM. Chart represents the average of three independent experiments.

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Figure 6. Effects of RcsB, LrhA, RtsB and SlyA on transcription of P1_{flhDC} and P5_{flhDC}. For these assays, we compared the transcription from the P1_{flhDC} (defective in the P1 start-site) and the P5_{flhDC} (defective in the P5 start-site) promoter constructs. Plots represent luciferase activity divided by the OD595 values shown at the bottom of the chart. (A) RcsB, LrhA and RtsB but not SlyA repressed transcription from the P1_{flhDC} promoter. Luciferase activity of P5_{flhDC}-luxCDBAE-P{flhD}+C transcriptional fusion (P1-expressed) was investigated in five genetic backgrounds: wild-type (TH18895), ΔrcsB::tetRA (TH20237), rtsB::T-POP (TH19976), ΔlrhA::tetRA (TH19974), slyA::T-POP (TH19975). (B) RcsB, LrhA and SlyA but not RtsB are negative regulators of P5_{flhDC} promoter. Luciferase activity of P1_{flhDC}-luxCDBAE-P{flhD}+C transcriptional fusion (P5-expressed) was measured in wild-type (TH18889), rcsB::tetRA (TH20236), rtsB::T-POP (TH19972), ΔlrhA::tetRA (TH19970) and slyA::T-POP (TH19971). (C) RcsB inhibits hilD transcription in an flhDC independent manner. Luciferase activities of the P_{hilD}-luxCDBAE transcriptional fusion in wild-type (rcsC+) (TH19425), ΔrcsC::T-POP (TH19687) and ΔhilD::FCF (TH19690) backgrounds were recorded as described in Figure 1. FliZ, a post-translational activator of HilD, promotes transcription of the auto-regulated hilD gene. Tetracycline (Tc) was used at 3 μg/ml to induce rcsC transcription in the ΔrcsC::T-POP background resulting in activation of RcsB. Upon RscB activation (rcsC+T-POP +Tc), transcription of hilD was abolished. The inhibitory effect of RcsB on hilD transcription (40-fold) is more dramatic than the four-fold decrease in the absence of FliZ. Results are the average of two independent experiments performed in duplicate. Error bars represent standard deviation. (D) RflM inhibits hilD transcription in an flhDC independent manner. Luciferase activity of strains harboring a hilD transcriptional fusion, P_{hilD}-luxCDBAE, was measured in four genetic backgrounds, araBAD::FCF (TH20541) (Column 1), araBAD::rflM+ (TH20542) (Column 2) and araBAD::FCF P_{flhDC}::T-POP (TH20543) (Column 3, 5 and 7) and araBAD::rflM+ P_{flhDC}::T-POP (TH20544) (Columns 4, 6 and 8). P_{araBAD::rflM+} strains, in the presence of arabinose (Ara and +) leads to the overexpression of rflM and P_{araBAD::FCF} serves as a control. Addition of tetracycline (Tet and +) to P_{flhDC}::T-POP strains allows the overexpression of flhDC and in the absence of tetracycline the cells are flhDC–. Cells were diluted 1 to 500 from an overnight culture into LB in the presence arabinose, tetracycline or arabinose and tetracycline. 0.2% arabinose (Ara) was added to induce transcription of rflM and 3 μg/ml tetracycline (Tet) to induce transcription of flhDC. At an OD595=1, the luciferase activity was recorded as described in Figure 4.

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did not affect the transcription of flhDC operon in construct P5-flhDC (P1-expressed) (Figure 5C). These results indicate that HilD promotes transcription from P3-flhDC and has no apparent effect on P1-flhDC promoter transcription.

The negative autoregulation of flhDC transcription via RflM is at the P1-flhDC promoter

Transcription of the flhDC operon is subjected to negative feedback by RflM, which is activated at the transcriptional level by FlhD4C2 [21]. To further study the effect of the negative autoregulation on flhDC operon transcription kinetics, we monitored the transcription profile, over time, in the three strains Pwt-flhDC, P1-flhDC and P5-flhDC in the absence and presence of RflM. We established that there was an increase in the transcription from Pwt-flhDC in the absence of RflM (Figure 2B). We demonstrated that the P1-flhDC promoter is under negative autoregulation by RflM because the expression of flhDC operon in strain P1-flhDC (P5-expressed) was similar between the wild-type and its isogenic rflM null mutant (Figure 5D). Additionally, we found that RflM did not appear to regulate P5-flhDC because flhDC transcription in strain P5-flhDC (P1-expressed) increased in the absence of RflM (Figure 5E). These results demonstrated that in the wild-type background the P1-flhDC promoter is subjected to negative autoregulation through RflM, while transcription from P5-flhDC appeared to be RflM independent.

We employed an alternative approach to confirm which of the flhDC promoters is specifically inhibited by the transcription factor RflM. We monitored the transcription of flhDC in a strain that overproduces RflM under control of the arabinose promoter, P\textsubscript{araBAD}\textsubscript{TPOP}gf\textsubscript{RflM}. In the presence of arabinose, used to induce overexpression of flhDC transcription, we observed an inhibition of transcription of flhDC operon in the three strains tested, Pwt-flhDC, P1-flhDC and P5-flhDC (Figure 5F). These results suggest that RflM is able to inhibit transcription of flhDC operon from both promoters, P1 and P5, which is in contradiction to the specific inhibition of the P1-flhDC but not the P5-flhDC promoter by RflM observed in Figures 5D & E. RflM protein production or stability appears to decline in function of cell growth cycle (Figure 3A), suggested that continuous production of RflM might affect indirectly the expression of P3-flhDC. Because HilD is an activator of the P5-flhDC promoter, we hypothesized that overexpression of RflM inhibits transcription of hilD gene. In order to test this hypothesis, we monitored the activity of a luciferase transcriptional fusion of the hilD promoter, P\textsubscript{hilD}\textsubscript{TPOP}, in two genetic backgrounds: (i) P\textsubscript{hilD}\textsubscript{TPOP}:FCF and (ii) P\textsubscript{hilD}\textsubscript{TPOP}:gf\textsubscript{RflM}. We observed that under conditions that overproduce RflM, presence of arabinose, there was an inhibition of transcription of the autoregulated gene hilD (Figure 6, compare columns 1 to column 2). Note that the strains used to determine luciferase activity are all flhDC\textsuperscript{+}, and overexpression of RflM inhibits flhDC transcription required for production of the posttranslational regulator of HilD. Thus, the effect of RflM, on hilD transcription could be indirect through inhibiting flhDC. To test if the effect of RflM on hilD, is direct or indirect we used two additional strains (i) P\textsubscript{hilD}\textsubscript{TPOP}:FCF P\textsubscript{flhDC}\textsubscript{TPOP} and (ii) P\textsubscript{hilD}\textsubscript{TPOP}:gf\textsubscript{RflM} P\textsubscript{flhDC}\textsubscript{TPOP}. For the P\textsubscript{flhDC}\textsubscript{TPOP} backgrounds the flhDC operon is transcribed from the tetracycline (Te)-inducible tetC promoter, and as such are flhDC\textsuperscript{+} in the absence of tetracycline and flhDC\textsuperscript{+} in the presence of tetracycline. First, we observed that flhDC controlled transcription of the hilD gene, because in the absence of Te, there was a 2-fold decrease in the P\textsubscript{hilD}\textsubscript{TPOP} strain background (Figure 6D, compare column 3 to column 5). Moreover, we demonstrated that under condition of RflM overexpression, there was a higher level of inhibition of hilD transcription compared to the reduction observed in the P\textsubscript{flhDC}\textsubscript{TPOP} background (Figure 6D, compare column 5 to column 6). The overproduction effect of RflM was not rescued by addition of Te to induce flhDC transcription, an activator of hilD transcription (Figure 6D, compare column 6 to column 8). These results demonstrated that RflM could inhibit transcription of the hilD gene in an flhDC independent manner. Thus flhDC and rflM have opposite effects on the transcription of hilD, where flhDC is an indirect positive regulator of HilD, yet high levels of RflM inhibit hilD transcription. Since HilD is an activator of P5-flhDC transcription, we conclude that the negative effect of RflM overproduction on transcription of P3-flhDC is indirect and through inhibition of hilD gene transcription.

Targeting of the flhDC promoter region by RcsB, LrhA, SlyA and RtsB

The presence of two principal TSSs within the flhDC operon promoter region combined with the hierarchical regulation by different transcriptional factors, suggests that there is differential regulation at the promoter by different transcriptional regulators at different cell growth phases. We investigated which of the specific regulators: RcsB, LrhA, SlyA and RtsB control transcription of flhDC through the P1-flhDC and P5-flhDC promoters start-sites.

1. P1-flhDC is negatively regulated by RcsB, RsB and LrhA but not by SlyA. To determine if RcsB, RsB, LrhA and SlyA regulate P1-flhDC, we monitored the transcription of flhDC operon of the construct P5-flhDC (P1-expressed) in strains defective in either the rcsB, lhaA, slyA or rtB genes. We observed increased transcription in the P5-flhDC background, in either rcsB, lhaB or lhaB null mutants compared to the wild-type strain (Figure 6A). The transcription of P1-flhDC increased 5-fold, 2-fold and 1.6-fold in rcsB, lhaB and rtB mutant strains, respectively. These results demonstrated that RcsB, LrhA and RtsB repress transcription from P1-flhDC. However, a null mutation in the slyA gene did not affect transcription from P1-flhDC because there were no differences in the transcription levels for the P5-flhDC mutant promoter at any point of time during all the growth phases between the wild-type and the slyA mutant. The same effect of RcsB, LrhA and SlyA was also observed in strain P1-flhDC (this strain is P5P4P3P6P2\textsuperscript{−}) (Figure S4A). However, there was no effect of rtB mutation on the expression of P1-flhDC, which could be attributed to either the low level of expression flhDC in construct P1-flhDC or to the additional mutations (P5P4P3P6P2\textsuperscript{−}) present in the P1-flhDC construct (See supplementary Text S1).

2. P5-flhDC is negatively regulated by RcsB, LrhA, SlyA but not by RtsB. We monitored the transcription of the flhDC operon in construct P1-flhDC (P5-expressed) in strains lacking either the rcsB, lhaA, slyA or rtb genes. We demonstrate that the transcription from P3-flhDC promoter is regulated by RcsB, LrhA and SlyA proteins, because transcription of flhDC in construct P1-flhDC (P5-expressed) increased in rcsB (2-fold), lhaA (2-fold) and slyA (1.8-fold) mutant strains (Figure 6B). We also demonstrated that transcription of the P5-flhDC promoter is not regulated by RtsB protein because transcription of flhDC in construct P1-flhDC (P5-expressed) was independent of RtsB (Figure 6B). In strain P5-flhDC (this strain is P4P3P6P2P1\textsuperscript{−}), we observed the same regulation as with P1-flhDC (Figure S4B).

The transcription kinetics of the P1-flhDC (P5-expressed) in the ΔrcsB mutant was different than that of the Pwt-flhDC or the P5-flhDC (P1-expressed) in the absence of RcsB. While, there was a relief in the inhibition of transcription for the Pwt-flhDC or the P5-flhDC (P1-expressed) constructs at earlier time points of cell growth, the rcsB mutation resulted in increased transcription in the construct P1-flhDC (P5-expressed) only at later stage of growth (Figure 6B). It has been demonstrated that RcsB regulates flhDC transcription by...
direct binding to an RcsB binding sequence located 11 bp downstream of P1_{flhDC}. Inspection of the DNA upstream region of flhDC operon did not reveal the presence of any additional consensus RcsB-binding site, suggesting that the inhibitory effect of RcsB on transcription from the P5_{flhDC} promoter might be indirect and through the repression of an activator or activation of a repressor. It has been shown that RscB inhibits hilD transcription [61], whose activation is under the control of hilD. Because hilD is an activator of transcription from the P5_{flhDC} promoter, we hypothesized that the effect of RscB on P5_{flhDC} transcription was due to derepression of hilD transcription in an rcsB mutant background. To test this hypothesis, we monitored the expression of a transcriptional fusion of hilD to luciferase, P_{fliZ)::lux}, in wild-type, ΔfliC::FCF and ΔrcsC::T-POP strains (Figure 6C). We used an rcsC::T-POP allele that results in tetracycline(Tc)-dependent transcription of the rcsC gene [62] and thus activation of the transcription factor RscB, to monitor the effect of RscB on hilD transcription. We used a fliZ null mutant to detect if the effect of RscB on hilD is through the flhDC regulated gene fliZ which is the post-translational activator of the autoregulated hilD gene. We observed that FliZ regulated transcription of hilD, because there was a 2-fold reduction of P_{fliZ} dependent transcription in the strain lacking fliZ compared to the fliZ+ background (Figure 6C). However, under conditions that over-express RscC (addition of Tc in the presence of the ΔrcsC::T-POP allele to induce transcription of rcsC), the transcription of hilD was abolished (Figure 6C). Compared to the effect of deleting fliC overexpression of RscC exerted a more pronounced inhibitory effect on hilD transcription. These results demonstrate that RscB inhibits transcription of hilD in both flhDC dependent and independent manners and suggested that the P5_{flhDC} promoter is indirectly regulated by the RcsB transcriptional factor.

The timing of transcription of flhDC as a prerequisite for motility

There appears to be five stages of flhDC transcription that are controlled by three clusters of response regulators. Deletion of either, rcsB, lhaA or rpsL resulted in increased motility compared to the wild-type strain [18,19,62]. We observed that null mutations in any of the late regulators: hilD, rtsB or slyA, did not affect motility (Figure 7A). Based on the expression profiles of the flhDC operon in these mutant strains, these results establish that Salmonella wild-type motility will only need to reach a threshold of flhDC expression for motility, while increased flhDC expression later in the growth phase has no further effect on motility. It is noteworthy to mention that factors that affected the early transcription of the P1_{flhDC} promoter: LrhA, RscB (Figure 6A) and Rjm (Figure 5E) affected motility while transcriptional factors, HilD and SlyA, that regulate P5_{flhDC} promoter late in the growth phase (Figure 5B & 6B) did not affect motility (Figure 7A). Moreover, RtsB, by inhibiting transcription from P5_{flhDC} at later stages of growth (Figure 6B), did not inhibit motility suggesting that the growth phase combined with activation of flhDC promoters is important for motility (Figure 7A). It is noteworthy to mention that the factors that affected transcription of P5 flhDC but not motility are bona fide virulence factors.

We decided to study the effect of the flhDC promoter mutations on the motility of Salmonella. We constructed strains harboring single mutation in each of the promoters separately. Thus P1 refers to a strain that has a mutation in P1 promoter, etc. Note that these strains in contrast to strains harboring the luciferase constructs do not harbor a duplication of the flhDC operon. We demonstrated that strains defective in P1_{flhDC} start-site transcription (only P1 is mutated) were non-motile while P5_{flhDC} defective strains (only P5 is mutated) exhibited no apparent reduction of motility (Figure 7B). There was a motility defect of the strains P2− and P6− that is related to the effect of CRP (as discussed earlier and in Supplementary material). The motility of P3− and P4− were not significantly different from the wild-type strain. These results confirmed that in the wild-type background transcription from P1_{flhDC} is a prerequisite for motility while P5_{flhDC} is not required for motility. These results also suggested that the right timing of expression of flhDC is essential for motility. If this hypothesis is correct, we could expect that if flhDC is expressed from P5_{flhDC} promoter at an early time point it should confer a motility phenotype. To test this hypothesis we used the non-motile strain P5+ (only P5 is functional and the other promoters are mutated) (Figure 7C) to isolate suppressors of motility inhibition. This strain was used in order to limit isolating mutations in the other promoters of flhDC that would otherwise suppress motility [16]. We isolated a spontaneous suppressor that restores motility to the P5− strain (Figure 7C) and mapped the mutation to the promoter region of hilD gene (addition of a thymine residue at position −51 from the start codon of HilD and resulting in higher expression of hilD (labeled hilDΔp)). The isolation of this mutation confirmed that HilD regulates the P5_{flhDC} promoter. If the hypothesis that the timing of expression of flhDC as a prerequisite for motility is correct, then a hilD-up mutation should promote transcription of flhDC operon from P5 promoter at early growth phase. To test this hypothesis we used a transcriptional lac fusion to flhB, a class 2 promoter that is positively regulated by FlhDC-C2, as readout to determine the expression of the P5 promoter transcription. The transcription of flhB indicates the presence of FlhD1-C1-dependent transcription. Transcription of flhB in the P5+ strain was very low during early growth phases and increased when cells reached an OD of 1.4 (Figure 7D). These results suggest that P5+ cells are able to express flagellar genes at later stage of cell’s growth phase yet they are not motile. Interestingly, overexpression of hilD, hilDΔp mutant resulted in a premature activation of P5_{flhDC}, leading to the transcription of flhB at early growth phase and similar to the timing and levels of the wild-type strain (Figure 7D). These results suggested that the timing of FlhD1-C2 production during an early growth phase is critical for motility.

Discussion

The complex networks and the number of factors necessary for the production of functional flagella and the resulting motility, though beneficial for the bacteria, represent a significant requirement on the cell’s resources [63,64]. At the top of this cascade sits the flhDC operon [7]. We established now that Salmonella flhDC operon is primarily transcribed from two promoters, P1_{flhDC} and P5_{flhDC}. The activities of these two promoters are coupled to five stages controlling flhDC transcription and each stage is differentially controlled by a set of transcriptional factors: (1) repression of transcription of flhDC during the initial growth phase by LrhA and RscB (2) repression by Rjm at early exponential phase (3) activation through the action of HilD at mid exponential phase (4) repression by SlyA and RtsB at the onset of stationary phase, and finally (5) shut down at late stationary phase.

Dynamics of flhDC operon transcription in a growth phase dependent manner

The pre-log steady state transcription of flhDC regulation is controlled by two transcription factors, RscB and Lrha. Null mutation in any of these transcriptional regulators, promoted flhDC transcription early in the growth phase and this inhibition
was maintained throughout the rest of the growth phase (exponential and stationary). We found that the effect of LrhA and RcsB was coincident with activation of transcription of their respective genes. As cell densities reached an OD of 0.2–0.3, transcription of flhDC increased. The increased flhDC transcription resulted in transcription of fliM, which in turn resulted in the feedback inhibition of flhDC transcription. This effect was consistent with the concurrent transcriptional activation of flhDC.

Figure 7. Time-dependent transcription of flhDC operon controls motility of Salmonella. (A) A representative image of motility of the wild-type strain compared to (A) slyA, rtsB, spi1 and hilD null mutants. Null mutations in slyA, rtsB, spi1 or hilD does not affect motility compared to the wild-type strain (B) A representative image of motility of the wild-type strain compared to constructs harboring single promoters mutations in the flhDC regulatory region. (C & D) Early transcription of P5*flhDC promotes motility (C) the motility defect of P5* construct (only P5 is active and the promoters P1, P2, P3, P4 and P6 are mutated) was rescued by a mutation that overexpresses hilD (P5* hilDup) and (D) transcription of class 2 gene, fliL, of the P5* construct in a wild-type strain compared to its isogenic strain hilD up (mutation that overexpress HilD). β-galactosidase activity (Miller Units) of a lac fusion to fliL gene was investigated in three genetic backgrounds: wild-type, P5*flhDC and P5*flhDC hilDup strains. Values are average of two experiments done in duplicate at different ODs.

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and flIM, where a surge of transcription of flIM mimicked that of flhDC and decayed quickly compared to the rest of the regulators controlling flhDC transcription. At the protein level, RflM appeared to follow the same early production and a quick decay as observed at the transcriptional level. We conclude that RflM limits flhDC transcription perhaps to efficiently control the kinetic expression of the middle and late flagellar class genes to facilitate flagellum assembly. Class 2 promoters respond differently to FlhD4C2 levels allowing the cell to control the timing of an individual class 2 operon transcription with respect to the other class 2 operons. Auto-repression at the transcriptional level has been shown to reduce relative variance and duration of fluctuations, and consequently limits noise in downstream expression [65,66]. Expression of flIC, encoding the filament component of the flagellum, has been demonstrated to be bistable [67,68]. We suggest that RflM would fulfill the noise reduction of flagellar class 2 and class 3 promoters during exponential growth phase, by controlling class 1 flhDC operon transcription. In support of this hypothesis, a null mutation of flIM gene has been shown to increase heterogeneity of flIC expression in a cell population when compared to wild-type [21].

Once bacteria reach mid-exponential phase growth, there is a second layer of control on flhDC operon transcription. This control is positive, and is brought on by the effect of a virulence-associated transcription factor, HilD. There was a delay in the positive effect of HilD compared to the negative effect exerted by RtsB, LrhA and RflM. This delayed HilD effect on flhDC operon transcription was due to the time required to activate HilD expression through FliD-C2-dependent FliZ production. FliD-C2 activates fliZ gene transcription from a flagellar class 2 promoter and FliZ, in turn, activates hilD expression at the post-translational level [57]. Finally, a third layer of flhDC transcription takes place and, unexpectedly, is also controlled by HilD. HilD activates the transcription of two regulatory factor genes, rtsB [17] and slyA (data not shown). RtsB and SlyA are two DNA binding regulators, which then act to inhibit flhDC transcription.

There is no doubt that flagellar motility provides a significant survival advantage over non-motile bacteria in many environmental situations. Furthermore, the link between production of flagella and other regulatory networks [69–72] would be affected if an unchecked production of flagella occurs. The overexpression of the flagellar regulon also attenuates Salmonella virulence [73]. These observations could explain the array of negative regulators controlling transcription of flhDC operon and keeping a check on the flagellar synthesis as well as FlhD-C2 production.

P1flhDC and P5flhDC are the main promoters driving flhDC transcription

While the literature reports the presence of either four or six transcription start-sites in the flhDC promoter region [13,29], our work suggests that only the P1flhDC and P5flhDC promoters are functional in a wild-type strain under laboratory growth conditions. First, we demonstrated that there was a reduction in flhDC operon transcription in the absence of P1flhDC or P5flhDC compared to the wild-type strain (Figure 4C & D). Second, we showed that flhDC operon transcription was totally abolished in P1flhDC-P5flhDC double mutant (Figure 4F). We confirmed that the P6flhDC promoter is active only in the absence of CRP [13]. Moreover, there was no apparent effect of P4flhDC, P3flhDC and P2flhDC promoters on flhDC transcription. In E. coli, CsrA, a carbon storage global regulator, activates flhDC expression in an RNaseE-dependent manner through protection of 5′end cleavage [23]. The 5′-UTR of the P5flhDC start-site transcript is 534 bases in length. We suspect that the presumed P3flhDC and P2flhDC start-sites resulted from RNaseE-dependent RNA-processing and/or degradation of the P5flhDC transcript. The P4flhDC start-site might also result from RNA processing; however, the isolation of mutants in the −10 region of P4flhDC that result in increased flhDC transcription suggests there might be unknown conditions where transcription from P4flhDC occurs [16].

Activation of flhDC operon transcription from the P1flhDC promoter establish two disparate regulatory loops

Genes with multiple transcription start-sites combined with auto-regulatory networks have been described in other systems. These include, Salmonella phoP, Bordetella pertussis bvgA, E.coli rrrA, and Salmonella flhDC operon [27,74–78]. These four cases bear similarity with flhDC operon transcription from P1flhDC and P5flhDC promoters. However, the case of flhDC is more elaborate, where two disparate pathways are used as feedback control. First, we demonstrated a sequential activation of P1flhDC and P5flhDC transcripts that are growth phase dependent (Figure 5A). The P1flhDC promoter activating two regulatory pathways resulting in both a negative and a positive regulatory loop and each of these loops has a specific effect on the flhDC operon promoters. The negative loop starts with P1flhDC leading to the production of FliD-C2 that activates flIM, which in turn feedback inhibits the P1flhDC promoter (Figure 5E). The positive feedback loop is also generated from P1flhDC, where transcription of flhDC operon from P1flhDC leads to fliZ gene transcription followed by FlhD4C2 production of flhDC, leading to the production of HilD then activates the second flhDC transcriptional cycle from P5flhDC (Figure 5B). Paradoxically, HilD controls transcription of rtsB and slyA genes, whose products binds to the flhDC promoter region (Figure 3B) and inhibit transcription, from P1flhDC and P5flhDC, respectively (Figures 6A & B).

Importance of timing of flhDC transcription activation on motility and virulence

The three promoter classes of the flagellar regulon, class 1, class 2 and class 3; are expressed in a temporal cascade that coincides with flagellum assembly [79]. The control of flagella production is ultimately determined through the production of FliD-C2. However, when flhDC is highly over-expressed the cells are not motile for reasons that are not understood. Thus, an intricate temporal control of gene expression and specific quantities of a functional FliD-C2 master regulator are essential for motility. For example, the activator of type I fimbriae gene expression, FimZ, represses flhDC transcription suggesting that adherence is impeded in the presence of functional flagella. Neither deletion of flhDC nor over-expression of flhDC affect type I fimbriae gene expression suggesting that the presence of fimbriae (at wild-type levels) does not impede swimming. FliD-C2 activity is also required in other cell processes such as SpeI gene expression and other genes less characterized such as the rfbABC operon [80], which is implicated in surfactin production and the modABC operon [81], which is involved in anaerobic respiration. This leads us to speculate that P1flhDC is required for flagella production and P5flhDC is required for growth in various environmental conditions such as in biofilms or in host cells. One possibility is that the activation of flhDC transcription from P5flhDC might represent a mechanism of protein amplification by a surge of transcription, when it is necessary to turn on the SpeI regulatory network, even under conditions where flagella synthesis is inhibited at the level of fliA and flIC. This scenario can be very useful after infection when the bacteria requires expression of virulence factors to survive the physical and immune clearance of the eukaryotic host.
Flagella appear to be required for reaching and selecting point of entry of bacteria into host cells [81]. The low pH of the stomach will cause flagella already present to depolymerize [82]. In the intestine, the early transcription of flhDC operon from the P1 promoter provides the transcription factor, FlhD4C2, for expression of functional flagellar machinery to reassemble filaments and allow bacterial cells to swim to selected points of entry into epithelia cells. At the time of invasion, expression of both T3SS1 and flagella has been shown to be required. Thus, in the second step, the already expressed flhDC from P1-flhDC promoter activates transcription of flhDC, the posttranslational regulator of HilD. In turn, HilD promotes transcription of SPI1 genes, leading to invasion. Thus P1-expressed flhDC fulfills two functions: driving the cells near the point of entry and also boosting the expression of SpI1, necessary for invasion, through its effect on HilD. It is noteworthy to mention that invasion of epithelial cells is a rapid process occurring within 10 to 15 minutes after introduction of S. typhimurium into the intestinal lumen [83]. Translocation of bacteria across the epithelial barrier and into the underlying tissue is observed within 2 hours after infection of ligated ileal loops [83,84]. Interestingly Salmonella can replicate within two distinct intracellular environments: intravacuolar and cytosolic [85]. Once inside the host, the expression of both flagella and SpI1 appear to be downregulated but not abolished with most of the cytosolic population expressing both flagella and SpI1 at latter stage of invasion. Thus P1-expressed flhDC acts as a bistable regulator of HilD: it is expressed constitutively and is activated at the posttranslational level by FliZ [50], ultimately leading to activation of transcription from the P5-flhDC promoter. This positive autoregulation also generates a subsequent inhibition of flhDC operon transcription, of both P1-flhDC and P5-flhDC promoters, by two HilD-induced regulatory factors SlyA and RsbV, themselves regulated by different environmental cues. The activation of transcription from P5-flhDC would lead to higher expression of FlhD4C2. Though not necessary for motility, it could affect expression of HilD. Because, HilD is required for Salmonella survival inside host cells, this positive circle of activation might be well suited for virulence.

Materials and Methods

Bacterial strains, primers and standard genetic manipulations

Bacterial strains and primers used in this study are listed in Table S1 and Table S2, respectively (Supplementary Information). Bacterial cells were routinely grown in Luria-Bertani (LB) broth and, when necessary, supplemented with appropriate antibiotics at the following concentrations: Kanamycin (5–10 μg/ml), tetracycline (15 μg/ml) in agar plates and for induction of T-Pop 3.5 μg/ml. L-arabinose was used at 0.2% (w/v) when needed. Motility agar plates were prepared as described earlier [62]. The generalized transducing phage of S. typhimurium P22HT105/1 int-201 was used in all transductional crosses [88]

Construction of transcriptional fusions to a luciferase reporter

For the construction of strain TH18684 DUP[flhDC8093-luxCDBAE-luxKm-FlhD4C2(?)], primers 5104 and 5103 [designed to delete the replication origin and tetracycline resistance (TcR) cassette of the plasmid pRG38 [89]] were used to amplify the kanamycin cassette of pKD3. The PCR product was electroporated into TH18710 (LT2/pKD46/pRG38) followed by selection for kanamycin resistance (KmR). KmR colonies were pooled and infected with P22 to produce a transducing lysate. This lysate was used to transduce LT2 selecting KmR. The KmR transductants were replica-plated in LB+, Km and LB+ Tc-sensitve (TcR) and KmR colonies should have resulted from integration of PflhDC-luxCDBAE into the chromosome generating a duplication of the promoter region of the flhDC operon. To check the integration of a single copy of PflhDC-luxCDBAE-Km and to screen for the presence of any duplication of the luxCDBAE upon integration, a set of primers [1401 (revert for luxC)-3091 (forward upstream of FlhDC promoter region not present in the plasmid pRG38)] demonstrated the correct integration of the plasmid at the flhDC promoter region. A second PCR reaction using [Primers 267 (Km) and 1403 (luxE)] demonstrated the correct placement of Km cassette after the luciferase operon. Amplification with primers, 1403 and 1401, indicated a single copy integration of the plasmid without its origin of replication. Five candidates were obtained having a single integration of FlhDC-luxCDBAE-into the chromosome. One of the five candidates was sequenced and used in this study (TH18684). The Duplication of PflhDC was maintained in the presence of 5–10 μg/ml Km.

Mutations in the promoter region of PflhDC-lux were constructed using the λ-Red recombinase system, as reported previously [90].

Conclusions

Our finding can be rationalized in terms of a model (Figure 8). Two regulatory factors, LrhA and RcsB regulate flhDC by inhibiting transcription from P1-flhDC and P5-flhDC. The effect of RcsB is more dominant on P1-flhDC then on P5-flhDC whereas LrhA represses more strongly P5-flhDC than P1-flhDC. Transcription activation of P1-flhDC by CRP leads to a rapid transcription of flhD, which in turn reduces transcription of P1-flhDC and limits a rapid class 2 and class 3 genes expression. The FlhD4C2 complex, already produced, allows motility to proceed and also promotes activation of HilD at the posttranslational level through FliZ, ultimately leading to activation of transcription from the P5-flhDC promoter. This positive autoregulation also generates a subsequent inhibition of flhDC operon transcription, of both P1-flhDC and P5-flhDC promoters, by two HilD-induced regulatory factors SlyA and RsbV, themselves regulated by different environmental cues.
using the primers listed in Table S2. All transcriptional fusion constructs using the luciferase operon reporter used the strain TH18727: (DUP\[(P\text{flhDC8093)::tetRA-luxCDBAE*Km*P}_{\text{flhDCflhD}}+\text{flhC}]+\text{flhC})/pKD46) as the electroporation recipient. Individual fusion constructs with specific promoter regions were designed as follows: the \text{rcsB} promoter region included 400 bp upstream of the start codon through 230 bp of coding region, the \text{rcsD} promoter region included 466 bp upstream of the start codon through 260 bp of coding region, the \text{slyA} promoter region included 258 bp upstream of the start codon and 290 bp of the coding region, the \text{hilD} promoter region included 300 bp upstream of the start codon through 240 bp of coding region, the \text{rtsA} promoter region included 264 bp upstream of the start codon through 290 bp of coding region, the \text{rtsB} promoter region included 880 bp upstream of the start codon through 200 bp of coding region and the \text{rflM} promoter region included 460 bp upstream of the start codon through 284 bp of coding region. The promoter regions defined above were amplified by PCR using the respective primers listed in Table S2, and electroporated into strain TH18727, using the Lambda-Red recombinase system selecting for replacement of tetRA element with a PCR-amplified DNA fragment [90].

Construction of tagged proteins
Chromosomal FLAG-tagged \text{HilD}, \text{RcsB} and chromosomal HA-tagged \text{RtsB}, \text{SlyA}, \text{RflM}, \text{HilD} and \text{LrhA} were generated by the Lambda-Red recombinase system, as described previously [91] using gene-specific primer pairs, as shown in Table S2. All strains were verified by PCR amplification and DNA sequence analysis.
Chromatin-Immunoprecipitation (CHIP)

CHIP was performed as in [93] with modifications. Bacterial batch cultures were grown at 30°C to different ODs, at which point formaldehyde (final concentration of 1%) was added to cells. After 20 min at room temperature in an orbital shaker, cross-linking was quenched by the addition of glycine (500 mM) for 10 minutes. Samples were then placed on ice for an additional 10 minutes to complete quenching. Cells were collected by centrifugation, and washed twice with cold phosphate-buffer saline (pH 7.5). Cells pellets were resuspended in 1 ml of lysis buffer (10 mM Tris, pH 8.0, 20% sucrose, 50 mM NaCl, 10 mM EDTA, 10 µg/ml of lysozyme) and incubated at 37°C for 30 min. Following lysis, 1 ml of immunoprecipitation buffer (50 mM HEPES-KOH, pH 7.5, 150 mM NaCl, 10 mM EDTA, 1% Triton X-100, 0.1% sodium deoxycholate, 0.1% sodium dodecyl sulfate) and phenylmethylsulfonyl fluoride (final concentration of 1 mM) were added. To shear cellular DNA to an average size of 500 to 1,000 bp, the cell extracts were sonicated on ice using Misonix Sonicator 3000 with a microtip at power 2 for three 10 s pulses, with 30 s rests on ice between pulses. The lysates were clarified by centrifugation and the supernatant were treated with 5 µl RNaseA (10 µg/ml) at 37°C for 30 minutes. The treated supernatant was retained for use as the input sample in the immunoprecipitation experiments. Aliquots of sheared samples were un-cross-linked by incubation for 2 h at 42°C and 6 h at 65°C in 0.5× elution buffer containing freshly added 0.8 mg/ml of Proteinase K. DNA was purified using a PCR purification Kit (Biolane). An aliquot of purified DNA was run in a 1.25% agarose gel to confirm the shearing of DNA to 500–1,000 bp and DNA was quantified using Nanodrop spectrophotometer. An Aliquot of the input sample (2 µg) was used for each immunoprecipitation experiment. The sample was incubated with 50 µl of proteinPlus A/G beads (Santa Cruz) and 4 µl of HA monoclonal antibody (Covance) or Flag M2 antibody (Sigma) for 90 min at room temperature on a rotating wheel. An immunoprecipitation experiment without antibody was also set up as a negative control. The beads were collected by centrifugation and subsequently washed three time with immunoprecipitation buffer and once with immunoprecipitation buffer plus 300 mM NaCl, once with wash buffer (10 mM Tris-HCl, pH 8.0, 250 mM LiCl, 1 mM EDTA, 0.5% Nonidet-P40, 0.5% sodium deoxycholate) and finally with PBS buffer (pH 7.5). Immunoprecipitated complexes were then removed from the beads by treatment with elution buffer (50 mM Tris-HCl [pH 7.5], 10 mM EDTA, 1% SDS). Cross-linking of immunoprecipitated samples was reversed by incubation for 2 h at 42°C and 6 h at 65°C in 0.5× elution buffer with 0.8 mg/ml of Pronase (Roche). Prior to analysis, DNA was purified from the immunoprecipitate by using a PCR purification kit (Biolane) and resuspended in 30 µl of TE and quantified using a Nanodrop spectrophotometer. Two micrograms of the fragmented DNA, isolated from DNA-protein complexes, was used as the input in all ChIP assays. Following purification, Real-time PCRs were run on a C1000 thermal cycler (BioRad) to analyze immunoprecipitated DNA. DNA samples were used in a 20 µl reaction mix containing a 1 µM concentration of each oligonucleotide and 10 µl of 2x SYBR-Green Reaction mix. Two pairs of primers, 3569-3477 and 3753-3090 covering the promoter region of fbdC were used (Table S2).

PCR conditions were as follows: Initial denaturation at 95°C for 3 min, and 40 cycles of 95°C for 15 s and 60°C for 1 min, followed by the default melting curve program of the PCR machine. Fold-enrichments were determined by the 2ΔΔCt method described in SA Biosciences User manual. To account for chromatin sample preparation differences, CHIP DNA fractions Ct values (Mean threshold cycles) were normalized ΔΔCt(normalized ChIP) to the Input DNA fraction Ct values by substituting the Ct-values of

Growth conditions and luciferase assays

LB+Km medium containing 1% tryptone, 0.5% yeast extract, and 0.5% NaCl was used for growth of all bacterial cultures to determine the transcription activities of luciferase. Overnight cultures in LB+Km cultures were adjusted to the same OD 595 nm, then, 8-ml glass tubes containing 2 ml of LB+Km were inoculated with a 500-fold dilution of the bacterial suspensions and incubated at 30°C in a water bath with shaking at 250 rpm. For determination of luciferase activity in batch cultures, samples (200 µl) were taken at different time point and the light production along with the OD595 were measured in 96 well plates in a microplate reader (PolarStar Optima). For the determination of luciferase activity in batch cultures, samples (200 µl) were washed three times with PBS-T (PBS) for 45 minutes at room temperature. Following three washes in PBS-T and one wash in PBS. Labeled proteins bands were detected using the Odyssey Infrared Imaging System (LI-COR Biosciences, Lincoln, NE, USA).
sample from the corresponding no antibody control. The percentage input of each ChIP fraction was calculated using 2ΔCt(normalized ChIP)/(ΔCt(normalized) + ΔCt(normalized NoAb)). The IP fold enrichment was then calculated using 2ΔCt(normalized ChIP)/ΔCt(normalized NoAb) to evaluate the fold amount of starting material of the sample applied in the real-time PCR.

Supporting Information

Figure S1 Effect of static culture growth on the transcription of the flhDC operon in Salmonella enterica serovar Typhimurium. This plot represents the luminescence divided by the corresponding OD595 (A.U.) of a static culture. An overnight culture of strain Pwt_flhDC (TH18684) at 37°C was diluted 1 to 500 into fresh LB media. Cells were then incubated statically at 30°C and luminescence was recorded along with the OD595. OD values are shown at the bottom of the chart. Values are the average of two experiments done in duplicate. (TIF)

Figure S2 Transcription dynamics of factors that regulate flhDC transcription mimic the time in the cell growth phase where their effect on flhDC operon transcription is exerted. Luciferase activity was investigated in wild-type strain harboring Pwt_flhDC-luxCDABE-Pwt_flhDC/flhDC’ C (TH18684), P4::luxCDABE (TH19254), P5::luxCDABE (TH20087), P6::luxCDABE (TH19426). Luciferase activity was recorded and plotted as described in Figure 2. (A) Transcription of the auto-regulated flhA gene promoter was activated immediately after dilution of an overnight culture into LB media and earlier than the transcription of the flhDC operon. (B) The transcriptional profiles of rrlM and rcsD promoters are shown in the second axis along with the Pwt_flhDC. The activation of the rrlM promoter transcription, expressed from an FliD(C)_C-dependent promoter, was concomitant with that of flhDC operon transcription (Pwt_flhDC), happening at earlier time point of the cell growth phase. The transcription from the rrlM promoter (P_rrlM) diminished before cells enter stationary phase compared to promoter transcription for other regulator factors shown in this figure. Transcription from the rcsD promoter (P_rcsD) shown in the second axis) was activated before that of Pwt_flhDC. (C & D) Activation of promoters of the virulence related genes, implicated in the regulation of flhDC transcription, took place after initiation of flhDC transcription. A representative growth curve is shown in (A, C & D). For (B), the OD595 is shown at the bottom of the chart. (TIF)

Figure S3 Analysis of mutations of the putative promoters P2, P3, P4 and P6. (A) The wild-type sequence of ~100 bp of the putative TSSs and their mutant alleles are shown. (B, C, D & E) Charts represent the luciferase activities of the Pwt_flhDC-luxCDABE-Pwt_flhDC/flhDC’ C reporter construct in wild-type and isogenic strains carrying mutations in individual start-site ~10 boxes. Cells were grown overnight in LB and diluted 1 to 500 in fresh media, and grown at 30°C with shaking and luciferase activities were recorded at two optical densities (0.5, black bars and 1, grey bars). Charts of luciferase activity in strains with mutations in the P2 (B), P3 (C), P4 (D), P6 (E) promoters of flhDC operon compared to the wild-type flhDC promoter activity that was set at 100%. Each specific mutation is indicated under their corresponding bars. (F) Luciferase activity of strains P5::P6::P5+ (harboring mutations in P1, P3 and P4) and P5::P6::P1+ (harboring mutations in P5, P4 and P3). Results are the average of three independent experiments done in duplicate. Error bars represent standard deviation. (G & H) Mutations in the flhDC P2::flhDC and P6::flhDC promoter start-sites inhibit CRP-mediated transcriptional activation of P1::flhDC start-site. (G) CRP does no longer affect transcription of flhDC in strains deficient in P1, P2 and P6 promoters. Luciferase activity of Pwt::flhDC, P1::flhDC, P2::flhDC and P6::flhDC was measured in two genetic backgrounds: wild-type (wt) and its isogenic null mutant (crp (TH19180). Plots represent the ratio of the luciferase activity measured in wild-type strain relative to crp null mutant. (H) CRP represses transcription of P6::flhDC promoter. Luciferase activity of P1::flhDC (only P1 is active the rest of the promoters are mutated) and P6::flhDC (only P6 is active the rest of the promoters are mutated) was measured in two genetic backgrounds: wild-type and its isogenic null mutant crp. Plots represent the ratio of the luciferase activity measured in crp null mutant relative to the wild-type. (TIF)

Figure S4 Effects of RcsB, LrhA, RtsB and SlyA regulators on flhDC P1::flhDC and P3::flhDC transcription. For these assays, we compared the transcription of flhDC promoter region constructs (A) The P3::flhDC (defective in P1, P2, P3, P4, and P6 start-sites) promoter construct transcribed flhDC primarily from the P5 start-site. (B) The P1::flhDC (defective in P2, P3, P4, P5 and P6 start-sites) promoter construct transcribed flhDC primarily from the P1 start-site. (C) ResB and LrhA but not RtsB or SlyA repressed transcription of flhDC in P1::flhDC construct. Luciferase activity of P1::flhDC-luxCDABE::Pwt::flhDC’ C transcriptional fusion was investigated in five genetic backgrounds: wild-type (TH18890), ΔcrsB::T-POP (TH19221), Δrbc::T-POP (TH19180), bcrA::T-POP (TH19603), sylB::T-POP (TH19618) (D) ResB, LrhA and SlyA but not RtsB repressed transcription of flhDC in P5::flhDC construct. Luciferase activity of P5::flhDC-luxCDABE-Pwt::flhDC’ C transcriptional fusion was measured in wild-type (TH18890), ΔcrsB::T-POP (TH19221), Δrbc::T-POP (TH19180), bcrA::T-POP (TH19603) and sylB::T-POP (TH19618). Plots represent luciferase activity divided by the OD595 and plotted against the OD595 values shown at the bottom of the chart. (TIF)

Figure S5 Effects of HA or Flag-tagged regulators, LrhA, ResB, SlyA, HiiD, RtsB and RM on flhDC operon transcription. Luciferase activity of Pwt::flhDC-luxCDABE-Pwt::flhDC’ C transcriptional fusion was measured in wild-type, lrlA-NA, flhA-Flag, slyA-Flag, hiiD-Flag, rtsB-Flag and gEMHA. Cells were diluted 1 to 500 from an overnight culture into LB and grown at 30°C. Plots represent luciferase activity measured at OD 1 compared to the wild-type expression set at 100%. (TIF)

Table S1 List of strains used in this study. (DOCX)

Table S2 List of primers used in this study. (DOCX)

Text S1 Analysis of the promoters of the flhDC operon. (DOCX)

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Author Contributions

Conceived and designed the experiments: CM KTH. Performed the experiments: CM. Analyzed the data: CM KTH. Contributed reagents/materials/analysis tools: CM KTH. Wrote the paper: CM KTH.
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