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The non-motile phenotype of *Salmonella hha ydgT* mutants is mediated through PefI-SrgD

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Abstract

Background: Two ancestral nucleoid-associated proteins called Hha and YdgT contribute to the negative regulation of several virulence-associated genes in *Salmonella enterica* serovar Typhimurium. Our previous work showed that Hha and YdgT proteins are required for negative regulation of *Salmonella* Pathogenicity Island-2 and that *hha ydgT* double mutants are attenuated for murine infection. Interestingly, *hha ydgT* mutant bacteria exhibited a non-motile phenotype suggesting that Hha and YdgT have a role in flagellar regulation.

Results: In this study we show that the non-motile phenotype of *hha ydgT* mutants is due to decreased levels of the master transcriptional regulator $FlhD_4C_2$ resulting in down-regulation of class II/III and class III flagellar promoters and lack of surface flagella on these cells. The horizontally acquired *pefI-srgD* region was found to be partially responsible for this phenotype since deletion of *pefI-srgD* in a *hha ydgT* deletion background resulted in transient restoration of class II/III and III transcription, expression of surface flagella, and motility in the quadruple mutant.

Conclusion: These data extend our current understanding of the mechanisms through which Hha and YdgT regulate flagellar biosynthesis and further describe how *S.* Typhimurium has integrated horizontal gene acquisitions into ancestral regulatory networks.

Background

The pathogenic nature of *Salmonella enterica* has been shaped by the horizontal acquisition of virulence determinants [1,2]. In *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium), many virulence genes are organized in mobile elements such as pathogenicity islands, prophages, and the *Salmonella* virulence plasmid [3,4]. The increased pathogenic capacity conferred by such genes is dependent on their integration into ancestral regulatory networks of the cell, which can be accomplished by regulatory evolution following horizontal gene transfer [5].

The Hha/YmoA family of small nucleoid-associated proteins in *Enterobacteriaceae* [6] can participate in fine-tuning virulence gene expression in response to environmental cues [6,7]. For example, YmoA regulates expression of Yop proteins, YadA adhesin, Yst enterotoxin and invasin in *Yersinia enterocolitica* [7-9]. Hha negatively regulates the α-hemolysin genes *hlyCABD* in *Escherichia coli* [10], *hilA* encoded within *Salmonella* pathogenicity island 1 (SPI-1) in *S.* Typhimurium [11] and the locus of enterocyte effacement in enterohemorrhagic *E. coli* [12]. A third member, YdgT, similarly represses *hlyCABD* in *E. coli* [13]. We and others have shown that Hha and YdgT are repressors of the type III secretion system (T3SS) encoded in *Salmonella* Pathogenicity island 2 (SPI-2), where they provide an important negative regulatory input required for virulence [14-16]. Within their role as modulators of gene expression, Hha and YdgT repress other genes in horizontally acquired regions in *Salmonella* including the pathogenicity islands SPI-1 through SPI-5 and genes on the *Salmonella* virulence plasmid [16].

We and others have shown that *hha ydgT* mutants are non-motile [15,16], although the genetic basis linking the loss of Hha and YdgT to a non-motile phenotype was not known. Flagellar biosynthesis is an important virulence trait in enteric pathogens which can facilitate invasion of host intestinal epithelial cells [17]. Flagellar gene expression is governed by a three-tiered

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transcriptional hierarchy of early, middle, and late genes (Figure 1) [18]. The early genes *flhDC* encoding the master transcriptional regulator $FlhD_4C_2$, are at the top of the transcriptional hierarchy and are transcribed from the class I promoter [18]. $FlhD_4C_2$ in turn activates transcription of the middle genes encoding flagellar proteins comprising the hook-basal body, the alternative sigma factor FliA (σ^{28}) and its anti-sigma factor FlgM [19]. Upon assembly of the hook-basal body, FlgM is secreted, releasing FliA to activate transcription of the late genes from the class III promoter [20,21]. The late genes encode flagellin, and motor and chemotaxis proteins [18]. Within the flagellar transcriptional hierarchy, multiple regulators acting at either class I or class II have been identified [21]. Recently, new regulatory genes (*pefI-srgD*) in the *pef* fimbrial operon on the *Salmonella* virulence plasmid were found to encode synergistic negative regulators of flagellar gene expression [22]. Interestingly, the *pefI-srgD* locus was upregulated ~7-fold in *hha ydgT* mutants [16] suggesting that Hha and YdgT might impinge on *pefI-srgD* for control of flagellar gene expression. We show here that deletion of *pefI-srgD* in a non-motile *hha ydgT* deletion mutant leads to a transient restoration of class II/III and class III gene expression that is sufficient for assembly of surface flagella and motility.

Results

Flagellar-based motility and surface flagellar expression is abolished in *hha ydgT* mutants

During our characterization of Hha and YdgT-mediated repression of SPI-2 genes, we noted that *hha ydgT* mutant bacteria settled to the bottom of standing culture tubes whereas wild type cultures remained turbid. Previous work indicated that *hha ydgT* mutants failed to swim on motility plates but the contribution of the individual genes to this phenotype was not known and the ability of these strains to make surface flagella was not tested [16]. To test the contribution of individual genes to this non-motile phenotype, we used a standard soft agar motility assay and confirmed that *hha ydgT* mutants were non-motile in accordance with previous data (Figure 2A). This phenotype required deletion of both *hha* and *ydgT* as single Δ*hha* or Δ*ydgT* mutants remained motile (Figure 2B). To determine if the motility defect observed in Δ*hha* Δ*ydgT* was due to a defect in flagellar rotation or a lack of flagellar production we stained bacteria and examined them using transmission electron microscopy to visualize surface flagella. We found that while wild type bacteria were highly flagellated, Δ*hha* Δ*ydgT* bacteria did not assemble flagella on their surface (Figure 2C).

Transcriptional activity of class II/III and III promoters is decreased in a *hha ydgT* mutant

Flagellar biosynthesis is organized into a transcriptional hierarchy of three distinct classes. To understand the non-flagellated phenotype in greater detail, we measured the activity of transcriptional reporters corresponding to each of the three promoter classes driving the expression of green fluorescent protein (GFP). While the transcriptional activity in single *hha* or *ydgT* mutants was not significantly different when compared to wild type, transcriptional reporters for the hybrid class II/III promoter (*fliA*) [23,24] and class III promoter (*fliC*) were significantly reduced in the *hha ydgT* double mutant compared to wild type cells (Figure 3A). Since *flhDC* promoter activity did not differ between wild type and the *hha ydgT* mutant, we tested whether the inhibition of class II/III and class III gene expression in Δ*hha*

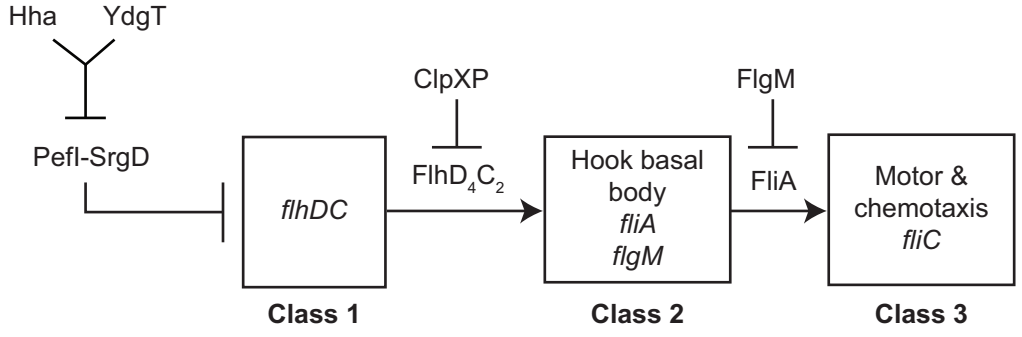

Figure 1 Organization of the flagellar biosynthesis transcriptional hierarchy. The early genes *flhDC* are transcribed from the class I promoter and encode the master transcriptional regulator $FlhD_4C_2$ which is able to bind within the class II promoter to activate transcription of the middle assembly genes in a σ^{70}-dependent manner. The middle assembly genes encode the hook-basal body structure which spans the inner and outer membrane, the sigma factor FliA (σ^{28}) and the anti-sigma factor FlgM. Once the hook-basal body is fully assembled, FlgM is exported through the hook-basal body allowing FliA to activate transcription of the late assembly genes from the class 3 promoter. Late assembly genes encode flagellin and proteins required for flagellar rotation and chemotaxis.

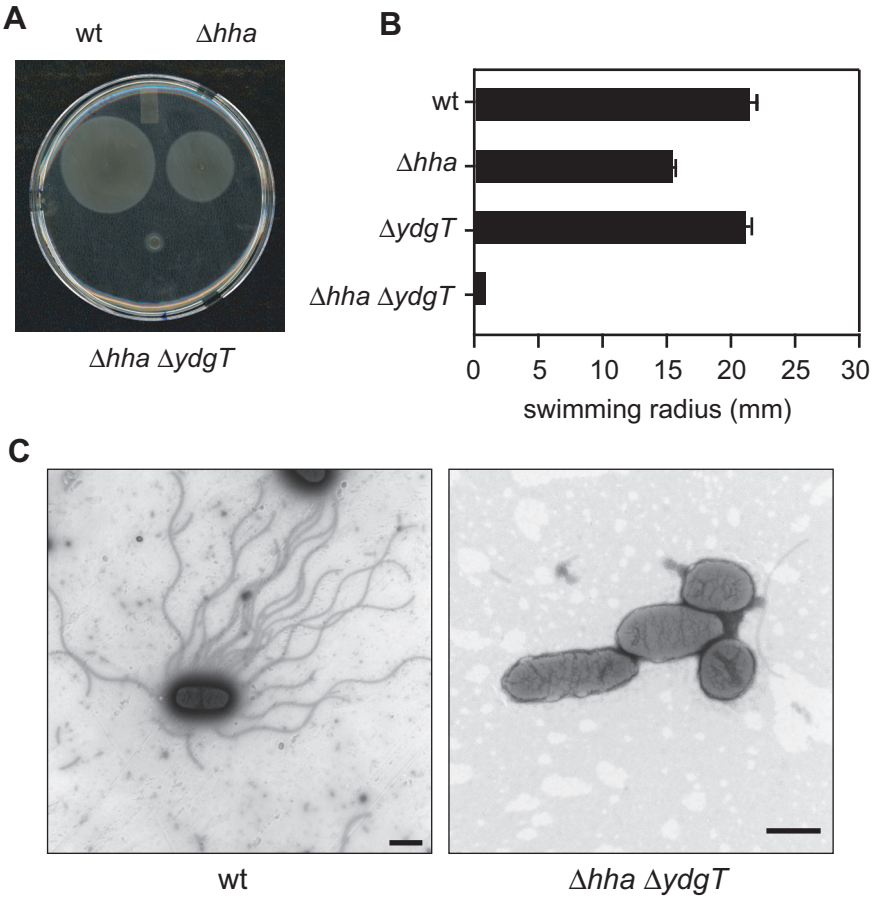

Figure 2 Repression of flagellar biosynthesis and motility is dependent on the loss of Hha and YdgT. (A) Wild type, Δ*hha*, Δ*ydgT* and Δ*hha* Δ*ydgT* were assessed for flagellar-based motility using a 0.25% soft agar motility assay in which 2 μL of overnight culture was inoculated into semi-solid agar and incubated at 37°C for 6 h. (B) The radius of the motility halo region was quantified after 6 h and is shown as means with standard errors. (C) Bacteria and surface flagella were negatively stained using a 0.1% uranyl acetate solution and visualized using scanning transmission electron microscopy. Data represents three independent experiments.

Δ*ydgT* involved an effect downstream of FlhD-FlhC protein production, since the $FlhD_4C_2$ complex is known to activate class II transcription. Using Western blot analysis with FlhC and FlhD-specific antisera, we observed a decrease in the levels of FlhC and FlhD in *hha ydgT* mutants compared to wild type (Figure 3B), which was consistent with the observed decrease in activity for $FlhD_4C_2$ target promoters. As a control we used a *clpXP* deletion mutant lacking the ClpXP protease that degrades the $FlhD_4C_2$ complex. As shown in Figure 3B, the levels of FlhC and FlhD were increased in Δ*clpXP* cells compared to wild type.

Loss of the fimbrial regulators PefI-SrgD restores motility in a *hha ydgT* background

We next wanted to identify potential negative regulators in Δ*hha* Δ*ydgT* that were acting to inhibit transcriptional regulation downstream of class I. Previous transcriptional profiling experiments showed that the *pefI-srgD* locus on the *Salmonella* virulence plasmid was upregulated ~7-fold following deletion of *hha ydgT* [16]. Subsequently, *pefI-srgD* genes were identified in a transposon mutagenesis screen as negative regulators of flagellar biosynthesis that worked in concert to inhibit motility [22]. Based on these data we hypothesized that the non-motile phenotype of *hha ydgT* mutants was mediated through its effect on *pefI-srgD*. If so, we reasoned that deletion of *pefI-srgD* in the *hha ydgT* mutant background would restore motility to this strain. We observed similar levels of motility (Figure 4A and Figure 4B) and surface flagella (Figure 4C and 4D) between wild type and Δ*pefI-srgD* bacteria, consistent with data from other groups [22]. However, as shown in Figure 4A, Figure 4B, and Figure 4C, deletion of *pefI-srgD* in the non-motile *hha ydgT* mutant restored

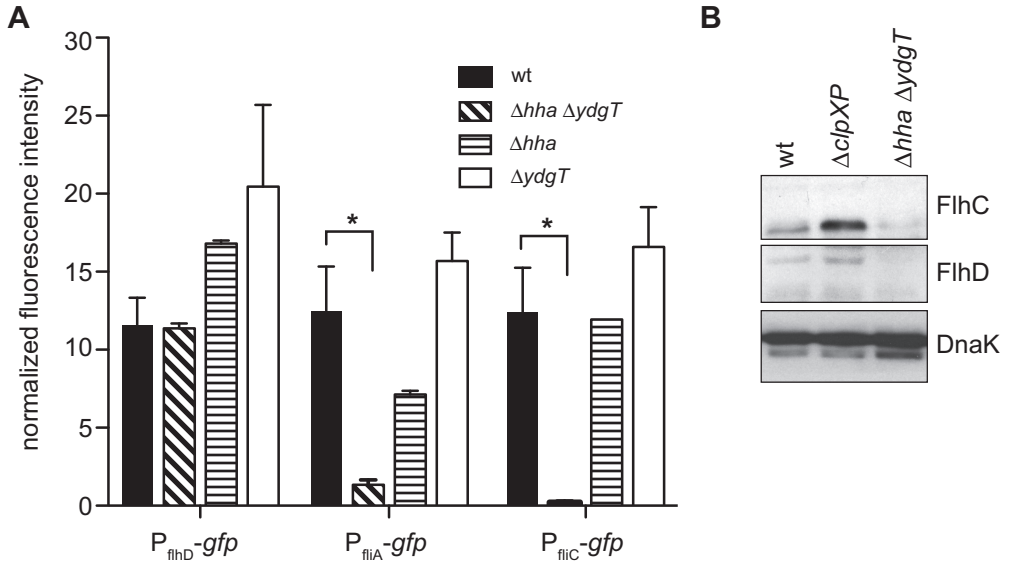

Figure 3 Loss of Hha and YdgT disrupts flagellar biosynthesis at the level of Class II/III activation. (A) Wild type and Δ*hha* Δ*ydgT* whole cell lysates were collected at OD_{600} ~ 0.4-0.6 and levels of FlhC and FlhD were determined by Western blot analysis. DnaK was used as a loading control. (B) Promoter activity at Class I (*flhD*), II/III (*fliA*) and III (*fliC*) was determined in wild type, Δ*hha*, Δ*ydgT* and Δ*hha* Δ*ydgT* using GFP reporter plasmid constructs. Fluorescence intensity (501/511 nm) was measured after 6 h and normalized to OD_{600} (RLU/OD_{600}). Data represents means and standard errors from three independent experiments.

surface flagella and motility to this strain. We noted that flagella distribution on the surface of Δ*hha* Δ*ydgT* Δ*pefI-srgD* quadruple mutants was less peritrichous and less abundant (Figure 4C and Figure 4D) than either wild type or Δ*pefI-srgD* suggesting that other regulators in addition to PefI-SrgD might be involved in regulating motility through the Hha and YdgT nucleoid-like proteins.

Class II/III and class III promoters are transiently activated upon loss of PefI-SrgD in Δ*hha* Δ*ydgT* bacteria

In transcriptional reporter experiments we were not able to detect class II/III or class III flagellar promoter activity in *hha ydgT* mutant bacteria despite similar class I gene expression levels relative to wild type. To determine if the restoration of motility in the Δ*hha* Δ*ydgT* Δ*pefI-srgD* mutant correlated with an increase in class II/III and class III promoter activity, we introduced the *gfp* transcriptional reporters into the *pefI-srgD* double mutant and the *hha ydgT pefI-srgD* quadruple mutant and measured promoter activity over time. Consistent with its role as a negative regulator of class I gene expression [22], P*flhD-gfp* activity was elevated in strains deleted for *pefI-srgD* compared to wild type, including the *hha ydgT pefI-srgD* mutant which showed the highest level of *flhD* promoter activity at ~3 h. In line with this, the quadruple mutant had a gain of transcriptional activity at class II/III and class III promoters that was apparent between 4-6 h (Figure 5). Although the level of reporter activity for the hybrid class II/III and class III reporters did not reach that of wild type cells, it was sufficient to restore the expression of surface flagella as shown by transmission electron microscopy, and to restore motility levels to ~80% of wild type.

Discussion

We have shown that Hha and YdgT positively regulate flagellar biosynthesis through their influence on the horizontally acquired flagellar regulators PefI-SrgD. The ability of Hha and YdgT to act as positive regulators is manifested only in the presence of both proteins, as single deletions of *hha* and *ydgT* had no apparent effect on flagellar biosynthesis. A similar phenomenon has been observed in the regulation of α-haemolysin production in *E. coli*. Loss of both Hha and YdgT was required to dramatically de-repress α-haemolysin production which correlated with the ability of YdgT to attenuate the *hha* mutant phenotype [13]. Similarly, Hha and YdgT may be able to compensate for any effect on flagellar biosynthesis in the single deletion mutants making it difficult to discern their individual roles in flagellar biosynthesis regulation.

PefI-SrgD were recently identified as negative regulators of flagellar gene expression as they inhibit class I activation at the top of the flagellar biosynthesis transcriptional hierarchy [22]. PefI-SrgD is located within the *pef* fimbrial operon on the *Salmonella* virulence plasmid and PefI acts to regulate *pef* fimbriae expression

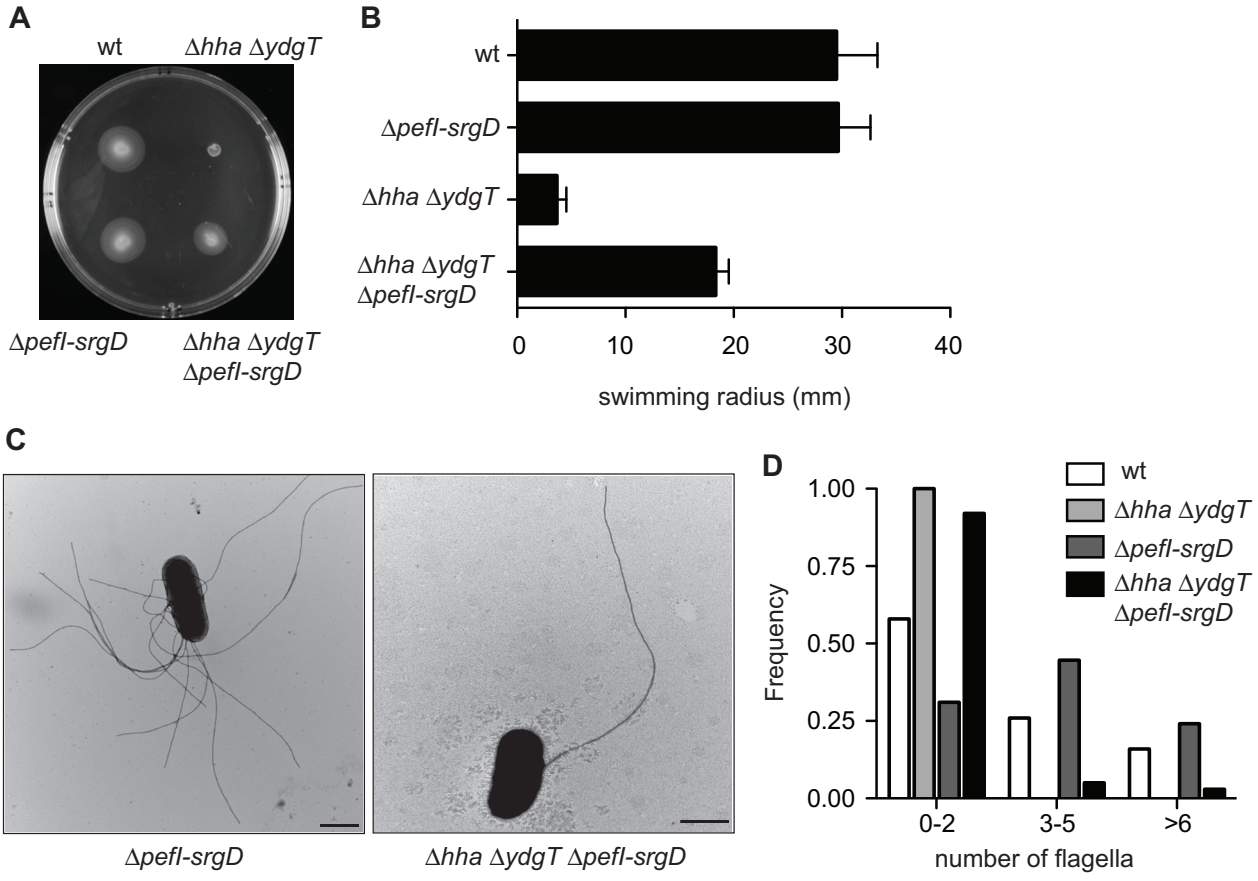

Figure 4 Loss of PefI-SrgD restores flagellar biosynthesis and flagellar-based motility in Δ*hha ydgT*. (A). Flagellar-based motility was determined in wild type, Δ*hha ydgT*, Δ*pefI-srgD* and Δ*hha ydgT* Δ*pefI-srgD* using a 0.25% soft agar motility assay. (B). The radius of the motility region was quantified after 6 h. (C). Bacteria and surface flagella were stained with 2% phosphotungstic acid and imaged using a transmission electron microscope. (D). Surface flagella were quantified for at least 100 bacteria cells for each strain. Motility assays are representative of three independent experiments and quantified as means and standard errors. TEM images are representative of two independent experiments.

[25,26]. Pef fimbriae are involved in bacterial adherence and fluid accumulation in the murine small intestine [27]. Phylogenetic data indicates that *S.* Typhimurium acquired *pef* as part of the serovar-specific virulence plasmid [28] which carries variable genetic elements required for virulence, fimbriae synthesis, plasmid transmission, innate immune resistance and antibiotic resistance [29,30].

The dual regulatory action of PefI-SrgD on both *pef* and flagellar promoters is similar to that seen for the regulation of fimbriae and flagella in other pathogens. PapX in uropathogenic *E. coli* acts to reciprocally regulate the expression of type 1 fimbriae and flagella during urinary tract infection [31]. MrpJ in *Proteus mirabilis*, an opportunistic urinary tract pathogen, activates MR/P fimbrial production while simultaneously repressing flagellar expression [32]. FimZ in *S.* Typhimurium coordinates reciprocal expression of type 1 fimbriae and flagella [33]. The existence of regulatory proteins able to dually control fimbriae and flagella production thus appears as an important evolutionary mechanism allowing tight modulation of adherence or motility phenotypes.

Although deletion of *pefI-srgD* in *hha ydgT* mutants de-represses the motility defect by re-establishing expression of surface flagella, it does not fully reconstitute class II/III and class III promoter activity to wild type levels suggesting the existence of other negative flagellar regulators. The protease ClpXP has been shown to degrade $FlhD_4C_2$ in *S.* Typhimurium [34], which may represent another negative regulatory mechanism in *hha ydgT* mutants.

The role of PefI-SrgD in the negative regulation of flagellar biosynthesis exemplifies the evolutionary significance of integrating horizontally acquired regulators into ancestral networks. For example, in *S.* Typhimurium, the horizontally acquired two-component regulatory system SsrA-SsrB regulates ancestral genes

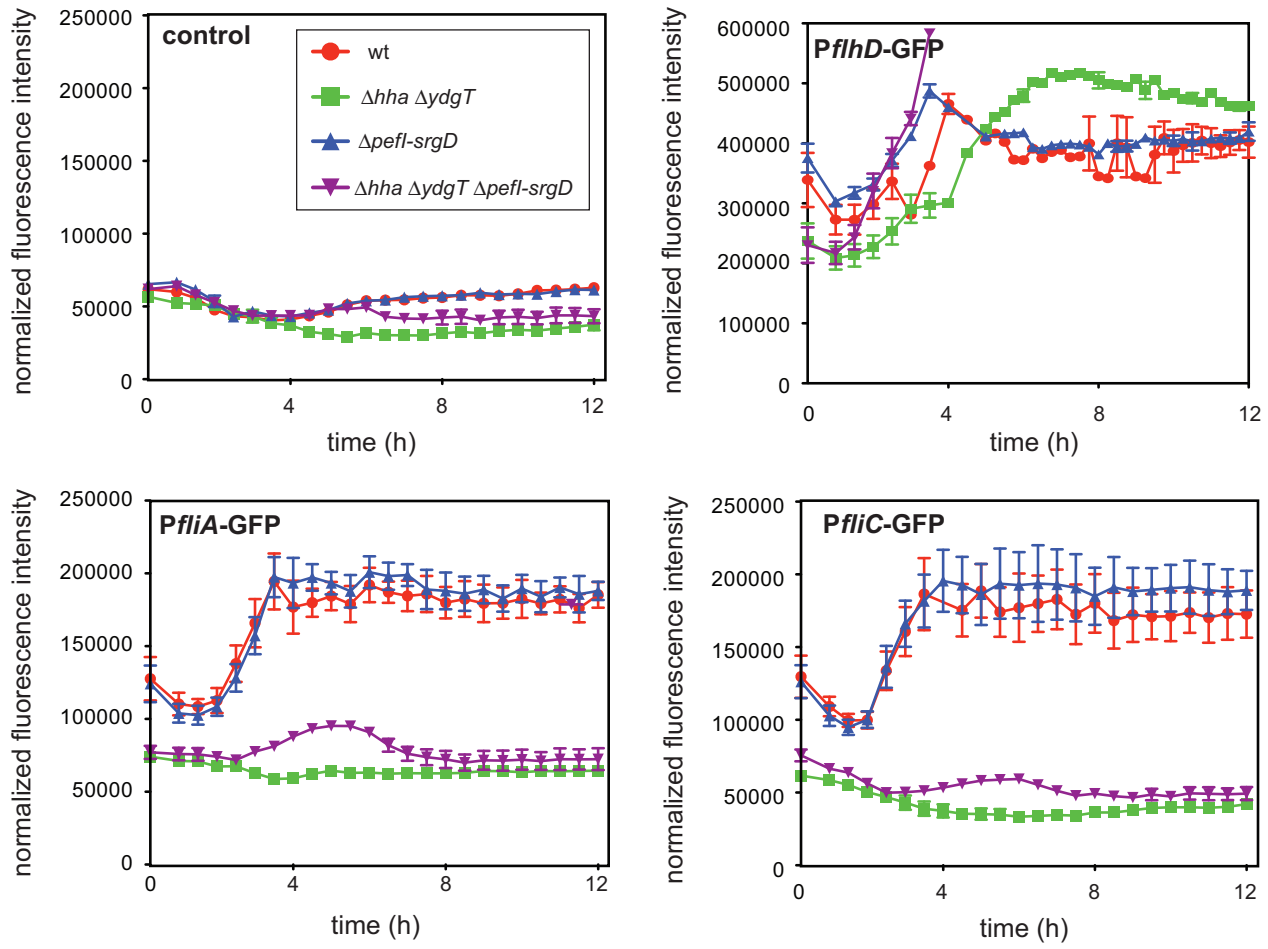

Figure 5 Loss of PefI-SrgD induces transient but sufficient Class II/III and III activation to restore flagellar biosynthesis in Δ*hha* Δ*ydgT*. Promoter activity at each transcriptional class in wild type, Δ*hha* Δ*ydgT*, Δ*pefI-srgD* and Δ*hha* Δ*ydgT* Δ*pefI-srgD* was measured as fluorescence intensity using plasmid-based GFP reporters. A promoterless GFP reporter construct was used as a negative control (first panel). Fluorescence intensity (485/525 nm) and OD_{600} was measured at 15 min intervals over 19 h. Data represents fluorescence intensity normalized to OD_{600} (RLU/OD_{600}). GFP transcriptional reporter assay data is representative of three independent experiments and quantified as means and standard errors (at the 3 h time point for P*flhD*, $P < 0.05$ for wt vs. Δ *pefI-srgD* and wt vs. Δ*hha* Δ*ydgT* Δ*pefI-srgD;* ANOVA, Newman-Keuls multiple comparison test). After 3-5 hours, P*flhD-gfp* activity in the quadruple mutant reached the maximum detection limit of the fluorescence reader. Data is shown for 12 hours rather than for 19 hours for the remaining flagellar reporters as there was no change in the fluorescence levels from 12-19 hours.

throughout the *Salmonella* genome [5,35]. In extraintestinal pathogenic *E. coli*, the horizontally acquired regulator Hfp interacts with the nucleoid-associated protein H-NS to regulate ancestral genes [36]. In *Shigella flexneri*, Sfh is located on a horizontally acquired virulence plasmid and regulates the expression of the ancestral proteins H-NS and StpA [37]. Thus, horizontal acquisition of regulatory proteins can have a significant impact on ancestral gene expression often by interacting with other regulatory pathways.

Conclusions

We have shown that the non-motile phenotype of Δ*hha* Δ*ydgT* requires the loss of both Hha and YdgT and that this phenotype is partially mediated through PefI-SrgD. These data contribute to our understanding of Hha-and YdgT-dependent flagellar biosynthesis regulation and demonstrate the integration of the horizontally acquired regulators PefI-SrgD into the flagellar biosynthesis network.

Methods

Bacterial Strains and Mutant Construction

Bacteria were propagated in Luria-Bertani (LB) broth at 37°C with aeration unless otherwise indicated. Marked, in-frame deletions of *clpXP* and *pefI-srgD* were made in *Salmonella enterica* serovar Typhimurium SL1344 using the λ Red Recombinase method [38]. Generation of Δ*hha* Δ*ydgT* was described previously [15] and this strain was used to generate mutants incorporating the

pefI-srgD deletion using the primers pefI-srgDF: GTG ATA CTT ATC CGG CCT CCG GTC CGC ATT CCA GGC CGG CCA TAT GAA TAT CCT CCT TAG and pefI-srgDR ATT CCG GTT TAT GAG TGA ATC CAT TGT TAC AAA AAT TAT TGT GTA GGC TGG AGC TGC TTC.

Soft Agar Motility Assay

Two μl of overnight culture was inoculated into 0.25% LB Agar motility plates with antibiotic and incubated at 37°C for 6 h.

Immunoblotting

Wild type and mutant strains were cultured until the optical density at 600 nm (OD_{600}) reached ~ 0.4-0.6. Whole cell lysates were collected and probed using anti-FlhC (1:5000), anti-FlhD (1:2500) and anti-DnaK (1:5000, Stressgen) antibodies. DnaK served as a loading control.

Transmission Electron Microscopy

Flagella were negatively stained using two different methods. In the first method, cells were cultured for 3-6 h. A carbon-stabilized Formvar support on 200-mesh copper TEM grid was floated for 30 seconds on a drop of culture, washed three times with water and stained for 10 seconds using 0.1% uranyl acetate. The second method involved staining copper grid-immobilized cells for 60 seconds with 2% phosphotungstic acid. Images were obtained using a JEOL-1200EX transmission electron microscope at the McMaster University Electron Microscopy Facility. For quantification, overnight cultures were diluted 1:50 or 1:100 in LB media with antibiotic and grown for at least 3 hours under static conditions. Flagella were stained as described above and quantified for at least 100 cells.

Transcriptional Reporter Assays

Wild type cells and the various mutants under study were transformed with the plasmid-based green fluorescent protein (GFP) reporter constructs pP_{flhD}-*GFP*, pP_{fliA}-*GFP*, pP_{fliC}-*GFP* and pP_{less}-*GFP* published previously [39]. For reporter experiments, strains were either sub-cultured into culture tubes and propagated for 6 h at which point fluorescence intensity and OD_{600} were measured or strains were sub-cultured into 96-well plates in M9 media containing 0.1% casamino acids and antibiotic and grown with shaking at 37°C at 1080 cycles per minute. Fluorescence intensity and OD_{600} were measured at 15 minute intervals for 19 h using a Synergy 2 Multi-Mode Microplate Reader (Fisher Scientific Co).

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Authors' contributions

LEW, AB and BKC conceived and designed experiments and analyzed data; LEW, AB and BKC performed experiments; LEW and BKC wrote the paper. All authors read and approved the final manuscript.

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