Mathematical model of flagella gene expression dynamics in *Salmonella enterica* serovar *typhimurium*

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Received: 13 June 2014/Revised: 11 October 2014/Accepted: 21 January 2015/Published online: 4 February 2015
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Abstract Flagellar assembly in *Salmonella* is controlled by an intricate genetic and biochemical network. This network comprises of a number of inter-connected feedback loops, which control the assembly process dynamically. Critical among these are the FliA–FlgM feedback, FliZ-mediated positive feedback, and FliT-mediated negative feedback. In this work, we develop a mathematical model to track the dynamics of flagellar gene expression in *Salmonella*. Analysis of our model demonstrates that the network is wired to not only control the transition of the cell from a non-flagellated to a flagellated state, but to also control dynamics of gene expression during cell division. Further, we predict that FliZ encoded in the flagellar regulon acts as a critical secretion-dependent molecular link between flagella and *Salmonella* Pathogenicity Island 1 gene expression. Sensitivity analysis of the model demonstrates that the flagellar regulatory network architecture is extremely robust to mutations.

Keywords Flagella · *Salmonella* · T3SS · Gene regulation

Introduction

Salmonella enterica serovar *typhimurium* (*S. typhimurium*) is a common food-borne pathogen that uses flagella to swim in liquid media and swarm on solid surfaces (Berg 2003). Flagella are located peritrichously on the bacterium's surface, and number between 6 and 8 per cell (Karlinsey et al. 2000b). Assembly of a functional flagellum involves expression from more than 50 genes located at different regions on the chromosome (Chilcott and Hughes 2000).

Structurally, a flagellum is divided into three parts: basal-body, hook, and the filament (Macnab 1999). The basal body anchors the flagellum to the cell membrane; hook is the flexible joint that links the rigid basal-body with the filament; and the filament, a rigid helical structure which rotates to drive the cell forward (Yonekura et al. 2003). Flagellum synthesis starts from the assembly of basal body first, followed by the hook and the filament. In addition to these parts, the flagellum also needs the chemotaxis and motor proteins to sense gradients and power movement in a particular direction (Mariconda et al. 2006).

The assembly of a flagellum is linked with gene expression. Depending on their timing of activation, the promoters in the flagellar regulon are divided into three classes, namely class 1, 2, and 3 (Fig. 1) (Wada et al. 2011). Environmental and cellular signals are fed into the class 1 promoter which controls expression of two proteins, FlhD and FlhC (Chilcott and Hughes 2000; Kutsukake 1997; Yanagihara et al. 1999). The resulting proteins form a complex FlhD₄C₂, which activates transcription from class 2 promoters (Ikebe et al. 1999a; Wang et al. 2006). The class 2 promoters control expression of all the proteins required for the assembly of a functional hook-basal body (HBB) structure. Once the HBB assembly is complete and

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Electronic supplementary material The online version of this article (doi:10.1007/s11693-015-9160-3) contains supplementary material, which is available to authorized users.

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Fig. 1 Regulatory network controlling flagella gene expression in *S. typhimurium*. FlhD₄C₂ (the master regulator) is expressed after combinatorial inputs from a large number of cellular and environmental signals. FlhD₄C₂ activates genes responsible for HBB, FlhA (flagella-specific sigma factor), FlgM (anti-sigma factor), FlhZ (regulatory protein), FliD (cap protein), and FliT (cap chaperone, and anti- FlhD₄C₂ factor). FlhZ represses expression of YdiV transcriptionally. YdiV, whose expression is controlled in response to the nutritional status of the cell, controls FlhD₄C₂ amounts in the cell through control via proteolysis by ClpXP

the resulting structure capable of secretion, expression from class 3 promoters takes place resulting in expression and assembly of filament, chemotaxis, and motor proteins. If the HBB structure is not functional, there is no class 3 expression (Hughes et al. 1993).

An important feature of the flagellar biogenesis is that expression of class 3 is contingent on successful assembly of HBB structure. This logic is encoded via the following interactions. In addition to controlling expression of class 2 proteins, which form components of the basal-body structure, the FlhD₄C₂ complex also activates expression of FlgM and FlhA. FlhA is the flagella-specific sigma factor (σ^{28}) which is essential for class 3 promoter activation (Ohnishi et al. 1992). FlgM is the anti-sigma factor for FlhA. Prior to completion of HBB, FlgM binds FlhA and prevents FlhA-dependent activation of class 3 promoters (Chadsey and Hughes 2001; Chadsey et al. 1998). On HBB completion, the substrate specificity for export from the HBB structure changes, and FlgM is secreted from the cell (Hughes et al. 1993). The secretion of FlgM frees FlhA inside the cell, which leads to activation of class 3 promoters. Interestingly, expression of both, FlhA and FlgM, is also controlled by a FlhA-dependent class 3 promoter (Ikebe et al. 1999b).

In addition to FlhA, FlgM, and the HBB proteins; FlhD₄C₂ also activates expression of several other regulators. These include FlhZ, FliD, and FliT (Karlinsky et al. 2000a; Kutsukake et al. 1999). All three regulators are under the dual control of class 2 (activated by FlhD₄C₂) and class 3 (activated by FlhA) promoters. FlhZ is a protein of unknown homology which positively regulates flagella gene expression (Kutsukake et al. 1999; Mytelka and Chamberlin 1996; Saini et al. 2008). FlhZ is expressed in

the same operon as FlhA, and controls class 2 gene expression via a protein YdiV (Wada et al. 2011). YdiV controls the amounts of FlhD₄C₂ amounts in the cell by controlling its exposure to ClpXP protease (Takaya et al. 2012). In addition, FlhZ is also known to activate expression of the genes located on the Salmonella Pathogenicity Island-1 (SPI1) (Chubiz et al. 2010).

FliD is the cap protein of the flagella, and its point of assembly defines the distal end of the flagellum. FliT is the chaperone for FliD and is expressed from the same operon as *fliD* (Kutsukake et al. 1999; Yamamoto and Kutsukake 2006). Prior to HBB completion, FliT is bound to the intracellular FliD (Aldridge et al. 2010). However, on completion of HBB, FliD is secreted from the flagellum to be assembled at its distal end. This frees the intracellular FliT, which feeds back and interacts with the FlhD₄C₂ complex, resulting in formation of a FlhD₄C₂:FliT complex. This FlhD₄C₂:FliT complex is unable to activate expression from class 2 promoters (Aldridge et al. 2010). Thus, FliT forms a secretion dependent negative feedback loop controlling expression of class 2 genes in the flagella regulon (Fig. 1).

Interestingly, none of FlgM, FlhZ, or FliT is essential for assembly of a functional flagellum (or swimming) in *Salmonella* (Aldridge et al. 2010; Saini et al. 2008, 2011). This leads us to a question that what role do feedback loops encoded by these regulators play in the flagella regulatory network? To answer this question, we developed a mathematical model describing regulation and dynamics of gene expression in the flagellar network. Our model predicts that the feedback loops encoded by FlgM, FlhZ, and FliT are essential for correct timing of expression of genes. This is true not only for transition from non-flagellated to a flagellated state, but also when a cell with existing flagella divides. We also show that FlhZ likely links flagellar gene expression with SPI1 gene expression in a secretion-dependent manner. SPI1 encodes for a Type 3 Secretion System (T3SS) which is essential for the bacterium gaining entry into the host cell. Collectively, we show that the flagellar regulatory network comprises of many non-trivial interactions, and each is designed for robustness and control over the assembly and function of flagella. Our model also exhibits a role for interlinked feedback loops in regulatory networks, where feedback loops are activated (or deactivated) in response to secretion status of the cell (which corresponds to the flagellar abundance on the cellular surface).

Development of the mathematical model

Mathematical model was developed using a deterministic formulation of flagellar gene regulation. The following

species were modeled in our simulations: FlhD₄C₂ (represented as FlhDC in equations for simplicity), HBB (representing all class 2 proteins), FlgM, FliA, FlgM–FliA complex, FliD, FliT, FliD–FliT complex, FliZ, YdiV, FlhD₄C₂–FliT complex, and class 3 proteins. All parameter values used in the equations are listed in Table 1. Many of the biochemical interactions in the flagellar network are well established, hence, we have accurate estimates of biochemical parameters. Particularly, the parameters associated with FliA–FlgM interactions are taken from Barembuch and Hengge's work (2007) the association and disassociation constants from Chadsey et al.'s work (1998) and from a previous mathematical study on flagellar regulation (Saini et al. 2011). For all remaining parameters, there are no quantitative measurements available. However, considerable work on biochemistry of the interactions provides us with inputs regarding the relative magnitudes of parameters. Hence, the remaining parameters are estimated to best fit the

data from a number of experimental studies on the flagella system (Aldridge et al. 2003, 2010; Saini et al. 2008, 2011). The model was developed with the following assumptions:

1. Expression from the class 1 promoter is known to be controlled via a large number of global regulators, via unknown mechanisms (Clarke and Sperandio 2005; Ko and Park 2000; Teplitski et al. 2003; Tomoyasu et al. 2002; Wei et al. 2001). It is also not clear how these inputs are integrated at the class 1 promoter (or post-transcriptionally) leading to the control of FlhD₄C₂ production. In the absence of these details, these effects have been lumped together as a step function that feeds into the class 1 promoter (Saini et al. 2011). FlhD₄C₂ autoregulation has been neglected. FlhD₄C₂ has been observed to auto regulate its expression, (Kutsukake 1997) but this effect has been found to be relatively weak and hence, has been left out from our equations.

Table 1 List of parameters used in the model and their values

Parameter	Value	Description
K ₁	3	Maximal class 1 promoter activity
K _{DCT} /K _{DC.T}	1,000	Rate constant FlhDC. FliT formation/break
B	10	Negative regulation by YdiV
K ₂	5	Activation of HBB expression by FlhDC
K _{2m}	10	Michaelis constant for HBB activation by FlhDC
K ₃	9	Activation of FlgM by FlhDC
K _{3m}	3	Michaelis constant for FlgM activation by FlhDC
K ₇	5	Activation of FlgM by FliA
K _{7m}	1	Michaelis constant for FlgM activation by FliA
K _{A.M} /K _{AM}	333	Ratio of rate constant of FliA. FlgM complex formation/break
K ₄	3	Activation of FliAZ by FlhDC
K _{4m}	8	Michaelis constant for FliAZ activation by FlhDC
K ₈	10	Activation of FliAZ by FliA
K _{8m}	6	Michaelis constant for FliAZ activation by FliA
K _{sec}	0.1	Secretion rate constant from HBB
K ₆	8	Activation of class 3 by FliA
K _{6m}	40	Michaelis constant for class 3 activation by FliA
K ₉	1	Activation of FliDT by FlhDC
K _{9m}	1	Michaelis constant for FliDT activation by FlhDC
K ₁₀	1	Activation of FliDT by FliA
K _{10m}	1	Michaelis constant for FliDT activation by FliA
K ₁₂	0.1	Activation of FliT by FlhDC
K _{12m}	10	Michaelis constant for FliT activation by FlhDC
K _{13m}	50	Michaelis constant for FliT activation by FliA
K _{D.T} /K _{DT}	1,080	Ratio of rate constant of FliD.FliT complex formation/break
K	0.35	Maximal rate of expression of YdiV
Γ	30	Repression coefficient of YdiV by FliZ
X	Between 0 and 1	Starvation status of the cell
K _d	0.1	Degradation constant
K _{d2}	1	Degradation constant

Parameters without references in the description column were approximated to best represent the experimental data

2. FliZ has been assumed to control YdiV expression, which, in turn controls the amounts of FlhD₄C₂ inside the cell. FliZ has been shown to control protein amounts of FlhD₄C₂ previously, and we assume in our model, that this happens via YdiV (Wada et al. 2011).
 3. Flagella network has recently been shown to exhibit bistability at class 2 gene expression level (Koirala et al. 2014). This bistability is presumably due to heterogeneity in the induction of the *ydiV* promoter (as a result of the availability of nutrients in the surrounding environment). However, as there are no details available regarding expression from the *ydiV* promoter, our model does not capture this bistability, or the cell-to-cell heterogeneity, as observed previously in the flagellar network (Saini et al. 2010a).
 4. FliZ and FliA (and similarly FliD and FliT) are expressed from the same operon (Ikebe et al. 1999b; Kawagishi et al. 1992; Mytelka and Chamberlin 1996; Yokoseki et al. 1995) and the model assumes that their production rates, both from the class 2 and class 3 transcripts, are identical. In addition, we have assumed that the production rates for FliA and FliZ (and similarly, FliD and FliT) are additive with respect to expression from class 2 and class 3 transcripts. This is supported by evidence that the binding sites of FlhDC complex and FliA in both, the *fliAZ* and *fliDST* operons do not overlap (Kutsukake and Ide 1995; Tanabe et al. 2011). However, real time data regarding interaction of the two promoters is not available. In addition, there is no known effects of translational coupling on the *fliAZ* or *fliDST* operons.
 5. FlgM is assumed to be only exported when bound to FliA. Absence of FliA, reduces secretion of FlgM significantly, and has been ignored in the model (Aldridge et al. 2006). Similarly, it has been assumed in the model that FliD is only secreted from the cell when bound with its chaperone FliT. Free FliD has been assumed to be incapable of secretion (Aldridge et al. 2010).
 6. HBB [which comprises of more than ten proteins (Chilcott and Hughes 2000)] has been assumed as a lumped variable, and its expression has been assumed to be solely dependent on FlhD₄C₂. Moreover, HBB expression has been assumed to be a continuous variable. In reality, a functional HBB will be a discrete event in the cell, and secretion from completed HBBs will increase in steps. Rather, our model assumes a continuous increase/decrease in HBB amounts, and consequently secretion. While this assumption is clearly an over-simplification of the assembly process, there is little data available on dynamics of HBB assembly inside a cell (Karlinsky et al. 2000b). In the absence of these details, our model proceeds with this over-simplified assumption.
 7. Our model assumes that secretion of regulatory proteins from the HBBs is linear to the amount of HBB produced in the cell (Saini et al. 2011). In reality, the amount of protein secretion would likely reach a saturated state, once the cell has assembled sufficient HBBs. However, there is no experimental data available to provide evidence for this claim. In addition, we also assume that the secretion from a HBB is independent of the developmental stage of an HBB (Rosu and Hughes 2006).
 8. FliS and FlgN, chaperones for filament FliC and hook-associated proteins (HAPs) FlgKL respectively, have also been reported to play a regulatory role in the flagellar cascade (Aldridge et al. 2003; Muskotal et al. 2006). However, these effects are relatively minor and thought to be mechanisms for fine-tuning gene expression. Recently, FliS has also been shown to interact with FlgM, and sequester its binding with FliA (Galeva et al. 2014). Hence, FliS plays a dual role in the cell, where it is interacting with FliC as well as FlgM. In the absence of any data as to how FliS interacts with FlgM in presence of FliC, these effects have not been included in the model.
 9. The FliA. FlgM complex is considered to be stable as compared to FliA and FlgM inside the cell. Hence, in our model, the degradation of FliA. FlgM complex has been ignored (Barembuch and Hengge 2007; Chadsey and Hughes 2001).
 10. Unbound FliA, FlgM, and FliD are unstable, and hence have a fast turnover. This is represented in the model by choosing a high degradation rate constant (same for all three). All other proteins have been assumed to be relatively more stable, and hence a lower degradation rate has been chosen for all other protein species in the system (Table 1). By choosing only two degradation rates in our model, we minimize the number of free parameters used.
 11. We assume that gene expression dynamics is governed by protein numbers per cell rather than protein numbers per volume. Typical size of *E. coli* is small enough that the time for a protein to diffuse from one to the other is of the order of mseconds (Burton 2000). As a result, time for the proteins to find their targets is small compared to the other processes like transcription, translation (Paulsson 2005). As a result, the effect of dilution in terms of the time required for proteins to find their regulatory targets has been ignored.
- With the above listed assumptions, the equations governing the kinetics of expression of flagella genes were developed as follows:

$$\frac{d(\text{FlhDC})}{dt} = \frac{K_1}{1 + \beta(\text{YdiV})} - K_d(\text{FlhDC}) - K_{\text{DC-T}}(\text{FlhDC})(\text{FliT}) + K_{\text{DCT}}(\text{FlhDC} \cdot \text{FliT}) \quad (1)$$

$$\frac{d(\text{HBB})}{dt} = \frac{K_2(\text{FlhDC})}{K_{2m} + (\text{FlhDC})} - K_d(\text{HBB}) \quad (2)$$

$$\begin{aligned} \frac{d(\text{FlgM})}{dt} = & \frac{K_3(\text{FlhDC})}{K_{3m} + (\text{FlhDC})} - K_{d2}(\text{FlgM}) \\ & - K_{A-M}(\text{FlgM})(\text{FliA}) + K_{AM}(\text{FlgM} \cdot \text{FliA}) \\ & + \frac{K_7(\text{FliA})}{K_{7m} + (\text{FliA})} \end{aligned} \quad (3)$$

$$\begin{aligned} \frac{d(\text{FliA})}{dt} = & \frac{K_4(\text{FlhDC})}{K_{4m} + (\text{FlhDC})} - K_{d2}(\text{FliA}) \\ & - K_{A-M}(\text{FlgM})(\text{FliA}) + K_{AM}(\text{FlgM} \cdot \text{FliA}) \\ & + K_{\text{sec}}(\text{HBB})(\text{FlgM} \cdot \text{FliA}) + \frac{K_8(\text{FliA})}{K_{8m} + (\text{FliA})} \end{aligned} \quad (4)$$

$$\frac{d(\text{FliZ})}{dt} = \frac{K_4(\text{FlhDC})}{K_{4m} + (\text{FlhDC})} - K_d(\text{FliZ}) + \frac{K_8(\text{FliA})}{K_{8m} + (\text{FliA})} \quad (5)$$

$$\begin{aligned} \frac{d(\text{FlgM} \cdot \text{FliA})}{dt} = & K_{A-M}(\text{FlgM})(\text{FliA}) - K_{AM}(\text{FlgM} \cdot \text{FliA}) \\ & - K_{\text{sec}}(\text{HBB})(\text{FlgM} \cdot \text{FliA}) \end{aligned} \quad (6)$$

$$\begin{aligned} \frac{d(\text{FliD})}{dt} = & \frac{K_9(\text{FlhDC})}{K_{9m} + (\text{FlhDC})} - K_{d2}(\text{FliD}) \\ & - K_{D-T}(\text{FliD})(\text{FliT}) + K_{DT}(\text{FliD} \cdot \text{FliT}) \\ & + \frac{K_{10}(\text{FliA})}{K_{10m} + (\text{FliA})} \end{aligned} \quad (7)$$

$$\begin{aligned} \frac{d(\text{FliT})}{dt} = & \frac{K_{12}(\text{FlhDC})}{K_{12m} + (\text{FlhDC})} - K_d(\text{FliT}) \\ & - K_{D-T}(\text{FliD})(\text{FliT}) + K_{DT}(\text{FliD} \cdot \text{FliT}) \\ & + K_{\text{sec}}(\text{HBB})(\text{FliD} \cdot \text{FliT}) \frac{K_{13}(\text{FliA})}{K_{13m} + (\text{FliA})} \\ & - K_{\text{DC-T}}(\text{FlhDC})(\text{FliT}) + K_{\text{DCT}}(\text{FlhDC} \cdot \text{FliT}) \end{aligned} \quad (8)$$

$$\begin{aligned} \frac{d(\text{FliD} \cdot \text{FliT})}{dt} = & K_{D-T}(\text{FliD})(\text{FliT}) - K_{DT}(\text{FliD} \cdot \text{FliT}) \\ & - K_{\text{sec}}(\text{HBB})(\text{FliD} \cdot \text{FliT}) \end{aligned} \quad (9)$$

$$\frac{d(\text{FlhDC} \cdot \text{FliT})}{dt} = K_{\text{DC-T}}(\text{FlhDC})(\text{FliT}) - K_{\text{DCT}}(\text{FlhDC} \cdot \text{FliT}) \quad (10)$$

$$\frac{d(\text{YdiV})}{dt} = \frac{K}{1 + \gamma(\text{FliZ})} - K_d(\text{YdiV}) \quad (11)$$

$$\frac{d(\text{class3})}{dt} = \frac{K_6(\text{FliA})}{K_{6m} + (\text{FliA})} - K_d(\text{class3}) \quad (12)$$

The equations were solved in MATLAB 7.10.0. To simulate mutant behavior, we substituted the formation term of the appropriate gene's equation by a zero. For instance, to capture dynamics in a mutant where FliZ is only produced from the class 2 promoter, we replaced the FliA-dependent formation of FliZ (Eq. 5) by zero. Unless otherwise mentioned, the simulations were performed with initial number of all flagellar proteins equal to zero (Saini et al. 2011).

Cell division, and the consequent gene expression dynamics were modeled in the following manner. Under a given nutritional condition, the equations were simulated to steady state expression levels. At steady state, cell division was assumed to have occurred which would reduce all protein numbers in the cell by a factor of two. This reduced number of protein molecules in the cell was used as the initial condition for simulating dynamics post division. Kinetics of class 2 and class 3 promoters were observed.

Results and discussion

Hierarchical expression of class 2 and class 3 genes in flagellar regulon (WT and mutants)

A characteristic feature of flagellar gene expression is hierarchical expression of class 2 and class 3 genes. In wild-type *Salmonella*, expression of class 3 genes only starts after the HBB assembly has been successfully completed (Saini et al. 2008, 2011). Consistent with these experimental observations, our model captures this qualitative aspect of flagellar regulation (Fig. 2). In addition, simulations of our model in a ΔflgM mutant leads to elimination of the lag in expression of class 3 genes. In a HBB mutant, since no secretion is possible, class 3 expression is eliminated (Kutsukake and Iino 1994). However, there is no significant impact on class 2 gene expression. Similarly, in a ΔfliA mutant, there is no significant effect on class 2 gene expression (either end-point or kinetic) and class 3 expression is eliminated (Kutsukake 1997) (Supplement Fig. 1). Similarly, kinetic experiments from previous reports were used to calibrate and validate our model (Aldridge et al. 2010; Saini et al. 2008, 2011).

In addition, our model is able to capture successfully the dynamics of gene expression in *fliZ*, *fliT*, and *fliD* mutants. A ΔfliZ mutant leads to slower and lower expression of class 2 genes, as observed previously (Saini et al. 2008). In

addition, impact of *FliZ* on flagella gene expression was found to be dependent on the nutritional status of the cell, as observed previously (Wada et al. 2011). Specifically, the impact of a *fliZ* mutation was minimal when the starvation levels are low (i.e., the cells are grown in rich media) but the impact increases as the starvation increases. A $\Delta fliZ$ mutation was also found to lead to lower class 3 expression levels, as has been observed previously (Saini et al. 2008). In absence of *FliT*, our model validates that the expression of both class 2 and class 3 genes is up regulated as compared to wild type, where as in absence of *FliD*, lower levels of class 2 and class 3 proteins (compared to wild-type) are observed (Aldridge et al. 2010) (Supplement Fig. 1).

We also note that the free-*FlhD₄C₂* (the class 1 product) reaches its maximal value at the time when secretion is just about to begin. Post-secretion, the amount of free class 1 product decreases inside the cell because of sequestration by *FliT*.

Previous modeling efforts regarding flagella gene regulation have only focused on the *FliA*–*FlgM* interaction, or the hook-length control in growing flagella (Barembach and Hengge 2007; Chadsey and Hughes 2001; Chilcott and Hughes 2000; Saini et al. 2011). There has been no published attempt to capture the dynamics of gene expression with the multiple and coupled feedback loops driving the flagella gene expression. In addition, all earlier gene expression dynamics modeling efforts have focused on studying the transition from a non-flagellated to a flagellated state in *Salmonella*. However, experimental evidence

Fig. 2 Hierarchical expression of flagella genes. Dynamics of expression of class 1, class 2, and class 3 genes in flagella regulatory network in wild-type *S. typhimurium*. Characteristic of the expression pattern is the delay in induction of class 3. This delay corresponds to the time required by the bacterium to assemble a sufficient number of HBBs to relieve *FlgM* repression of *FliA*. The class 1 product in the graph represents the free *FlhD₄C₂* present in the cell, and does not include the *FlhD₄C₂* bound with *FliT*. Class 2 proteins represent the HBB proteins lumped together. Class 3 proteins represent the filament, chemotaxis, and motor proteins

suggests that the class 1 promoter in *Salmonella*, unlike in *E. coli*, is never in the OFF state, (Karlinsky et al. 2000b) and hence, a typical OFF to ON transition does not capture the physiological state of the organism.

Steady state class 2 and class 3 expressions at various nutritional levels

Since flagellar gene expression (via the *FliZ*-dependent feedback loop) in *Salmonella* is strongly linked with the nutritional status of the cell, we explored the relationship by studying the steady state class 2 and class 3 gene expression under a variety of nutritional status of the cell. Our model predicts that the flagella assembly is linked with the expression kinetics of the gene *ydiV*. In our model, we have assumed *YdiV* expression to be linearly proportional to the nutritional status of the cell. However, in terms of the steady state gene expression of class 2 and class 3 levels, this leads to two distinct regimes. In the first phase (at low starvation levels), the class 2 expression decreases rapidly with increase in starvation (Fig. 3). However, after reaching a certain critical value, the repression from increased starvation shuts down flagella gene expression. This has previously been observed experimentally where in conditions of poor nutrition, a majority of the cells across a population do not have flagella (Aldridge et al. 2010). Thereafter, increasing the starvation levels of the cell do not have any effect on the steady state expression levels of class 2 and class 3 proteins. Interestingly, the mutants exhibit different sensitivities to the nutritional status of the cell. For instance, as compared to the *fliD* mutant, the *fliZ* mutant exhibits higher class 2 and class 3 expression levels in conditions of no nutritional stress. However, as stress is introduced in the surroundings, the *FliZ*-dependent feedback is much more sensitive and as a result, the *fliZ* mutant exhibits a much steeper decline in class 2 and class 3 expression (Fig. 3a, b). This demonstrates that the relative strength of the various feedback loops in the network is contingent on the precise environmental conditions under which the bacteria are grown. Recently, *YdiV* has also been shown to be involved in causing bistability in flagellar network in *Salmonella* (Koirala et al. 2014). However, regulation and kinetics of the *ydiV* promoter are not yet understood. Hence, the precise cause of this bistability is difficult to comment on.

From this, we note that the two feedback loops in the network (*FliZ*- and *FliT*-dependent) likely serve two distinct purposes. In the absence of *FliZ*, the rate of decrease in class 2 gene expression (with increasing starvation) is much more rapid as compared to wild type. Thus, the *FliZ*-phenotype becomes much more significant at low levels of starvation than in poor media. A similar trend is observed for class 3 gene expression. Thus, *FliZ* helps the cell

Fig. 3 Steady State class 2 (left) and class 3 (right) gene expression in wild type, $\Delta fliZ$, $\Delta fliD$, and $\Delta fliT$ mutants. At high starvation levels, flagella gene expression is repressed in *Salmonella*. Between the two feedback loops, FliZ's impact on gene expression is the strongest in response to nutritional conditions

assemble flagella in rich environmental conditions. On the other hand, FliT and FliD mutants are likely involved in only tuning the gene expression. Our results show that in $\Delta fliT$ and $\Delta fliD$ mutants, class 2 and class 3 gene expression is up- and down-regulated as compared to the wild type, respectively. This effect is most prominent at low starvation levels. As starvation levels increase, the mutant profile approaches that of the wild-type. This is qualitatively different from the wild type and the $\Delta fliZ$ curves, where the difference between the steady state expression curves grows till the curve for the mutant reaches zero expression values.

Response to cell division in WT and mutants

An open question regarding flagellar regulation in *Salmonella* is how the cell responds to cell division and regulate expression (Aizawa and Kubori 1998). In our model, we have linked gene expression of the flagella genes with protein secretion. On division, the existing flagella are divided among the progenies randomly, which leads to a decrease in the total protein secretion per unit time from a cell. This leads to realignment of the regulatory network in the cell, leading to changes in rate of expression of the different species. Specifically, change in protein secretion rate from the cell changes the amount and ratio of regulators present inside the cell. The changed relative ratios then exert altered control over the network. To check how the cell re-adjusts gene expression after division, we performed simulations where after cell division, the protein amounts present in the cell were halved, and the dynamics of class 2 and class 3 gene expression studied.

Our simulations show that in wild type, post-division, the cell readjusts its gene expression rates to assemble additional flagella. In this readjustment phase, the hierarchy of gene expression is again maintained (Fig. 4a). This hierarchy is likely important as premature expression of class 3 genes is known to impact the growth of the cell (Chilcott and Hughes 2000). As against this, in $\Delta fliG$ M,

$\Delta fliZ$, and $\Delta fliD$ mutants, the hierarchy of gene expression is not maintained post cell-division. In particular, in these mutants, upon division, class 3 gene expression leads class 2 gene expression. Thus, we propose that an additional role of the feedback loops associated with FlgM, FliZ, and FliD is to control the hierarchical expression of genes post cell-division. In $\Delta fliT$ mutant, the expression of the three classes of genes is hierarchical, implying that absence of FliT does not directly control hierarchy of expression post division. However, all classes of genes were upregulated as compared to the wild type, suggesting FliT's role in negatively controlling number of flagella per cell (Fig. 4b–f) (Aldridge et al. 2010).

We consider this to be a significant input and prediction from our model. Why the flagella gene expression system needs a large number of feedback loops has been an open question, especially because many of these auxiliary regulators are non-essential for motility and only have a limited to no impact on swimming speed. Our model predicts that there is an important physiological role served by these regulators, which is only apparent when studying cell division and the associated regulation of flagella number and assembly.

Secretion dependent FliZ-positive feedback loop is not required for flagellar gene expression regulation

An additional feature of the flagellar gene regulatory network architecture is the presence of different feedback loops at different secretion rates during assembly. At low/no secretion, the FliZ-dependent positive feedback loop is active, whereas at high secretion rates, the FliT-dependent negative feedback loop is active (Aldridge et al. 2010; Saini et al. 2010a). However, at high secretion, another FliZ-dependent positive feedback loop is active. This includes FlhD₄C₂-dependent activation of FliA. FliA autoregulation and activation of FliZ, and FliZ-dependent positive regulation of FlhD₄C₂. This implies that at high

Fig. 4 Dynamics of flagella gene expression post cell-division. Class 1, class 2, and class 3 gene expression dynamics after cell division in **a** Wild Type, **b** $\Delta fliA$, **c** $\Delta flgM$, **d** $\Delta fliZ$, **e** $\Delta fliT$, and **f** $\Delta fliD$ mutants. Note that in $\Delta flgM$, $\Delta fliZ$, and $\Delta fliD$ mutants, class 3 (red) gene

expression leads class 2 (green) gene expression. Class 2 proteins represent the HBB proteins lumped together. Class 3 proteins represent the filament, chemotaxis, and motor proteins

secretion rates there are two antagonistic feedback loops controlling FlhD₄C₂ amounts/activity.

To ascertain the role of these feedback loops we performed a simulation where we set production of FliZ independent of FliA. That is, FliZ expression was solely under the control of the FlhD₄C₂-dependent promoter. Experimentally, this is equivalent to removing FliZ from its chromosomal locus, and placing it under a class 2 promoter elsewhere on the chromosome. Mathematically, in our model, this is equivalent to replacing the FliA-dependent FliZ production term by zero. Note that in this mutant, FliA is still under both class 2 and class 3 promoters. Class 2 and class 3 dynamics for this mutant show that there is no discernible class 2 or class 3 gene expression difference between the mutant and the wild type (Fig. 5). This suggests that the late FliZ (expressed from class 3) does not have any role in control of flagella gene expression. This likely implies that post-secretion, FliZ made from the class 3 transcript is responsible for activation of SPI1 and SPI4 gene expression. The role of FliZ in activating SPI1 and SPI4 gene expression has been previously reported (Chubiz et al. 2010; Kage et al. 2008; Saini and Rao 2010; Saini et al. 2010b).

Hence, our modeling results suggest that late expression of FliZ dictates timely activation of SPI1 and SPI4 in the *Salmonella* infection cycle, and that FliZ acts as a molecular link between these systems involved in infection.

Physiologically, this prediction from our model has important implications for the infection process. Our prediction suggests that the initial role of FliZ in the cell is to aid assembly of sufficient number of HBBs, presumably to help the bacterium locate its preferred site of infection (Iyoda et al. 2001). However, once that is accomplished,

Fig. 5 FliZ from the class 2 transcript controls class 2 gene expression dynamics. Gene expression dynamics in Wild type and a mutant where FliZ production from the class 3 transcript has been set to zero. FliZ is only being produced from the class 2 transcript. Class 2 proteins represent the HBB proteins lumped together. Class 3 proteins represent the filament, chemotaxis, and motor proteins

late *FliZ* (from the class 3 promoter) is used by the cell as a signal to prepare for the next phase of infection, i.e. expression of the T3SS encoded on the SPI1 locus (Lostroh and Lee 2001).

FliA-dependent activation of *FlgM* ensures an effective “off” switch for class 3 gene expression as cells transition from high- to low-secretion regime

Another curious regulatory interaction in the flagella network is activation of *FlgM* by *FliA* (post-secretion). Post-secretion, *FlgM* is constantly secreted from the cell, and hence plays no role in regulation. However, the network not only activates *FlgM* production via *FliA* but also stabilizes the class 3 *flgM* transcript via *FlgN* (which is the chaperone for the hook-associated proteins, *FlgKL*) (Karlinsey et al. 2000a). This post-secretion activation of *FlgM*, intuitively, seems wasteful. To test the possible role for this regulation, we performed simulations in a mutant where post-secretion *FlgM* production is absent. This is equivalent to making *FlgM* expression only dependent on *FlhD₄C₂*.

In our simulations, we tested the dynamics of class 2 and class 3 gene expression in wild-type and this regulatory mutant (Fig. 6). Our results show that in wild type, *FlgM* resulting from class 3 transcript is only incremental in halting class 3 expression. In both the wild type and the mutant, post cell-division, class 3 expression is halted for some time, and increases thereafter. The increase in the mutant is however, greater than in the wild type (Fig. 6b). The delay in class 3 expression could be important for the cell. Upon division, the secretion rate from the flagella decreases, and the cell needs to build more HBBs. During this time, when the secretion is low, aggressive expression of class 3 genes could be deleterious to the cell. This is because class 3 products include *FliC* (flagellin) and if not

secreted effectively, *FliC* polymerizes inside the cell leading to death (Auvray et al. 2001; Yokoseki et al. 1995). Hence, from a cellular perspective it is very important that class 3 expression is commensurate with the secretion capability of the cell. And our results show that the *FlgM* activation by *FliA* is designed to provide a check for this effect.

Fraction of free-*FlhD₄C₂* complex as a function of class 3 expression (or secretion) in WT, *fliZ* mutant, *fliD* mutant, and a *fliT* mutant

The modes of regulation of *FlhD₄C₂* by *FliZ* and *FliT* are different. While *FliZ* controls *FlhD₄C₂* amounts in the cell, *FliT* controls the activity of the *FlhD₄C₂* transcription factor complex. However, both control the relative ratio of the *FlhD₄C₂* complex in the active form. By active form, we mean the fraction of *FlhD₄C₂* which is not bound to *FliT*. Our model demonstrates that there is a significant difference in the fraction of the *FlhD₄C₂* present as free-*FlhD₄C₂* between wild type, $\Delta fliD$, $\Delta fliT$, and a $\Delta fliZ$ mutant (Fig. 7).

As expected, as compared to the wild type, the fraction of *FlhD₄C₂* which is free is reduced in a $\Delta fliD$ and is always equal to one in a $\Delta fliT$ mutant. This temporal control of free *FlhD₄C₂* is as shown in Fig. 8. However, contrary to expectation, we also note that the fraction is enhanced in a $\Delta fliZ$ mutant (compared to wild type). The absence of *FliZ* leads to a fall in the intracellular levels of *FlhD₄C₂*. As a result, class 2 gene expression (and consequently HBB) is considerably lowered as well. Lower expression of HBB leads to slow secretion, which implies that *FliD* secretion from the cell is less than as compared to the wild type. This reduced secretion of *FliD* sequesters the intracellular *FliT*, and negatively controls the *FliT*-mediated negative feedback loop in the network. Hence, *FliD*

Fig. 6 *FlgM* from the class 3 transcript controls the dynamics of class 3 gene expression. (Left) class 3 gene expression in Wild Type (solid) and a mutant where *FliA* does not regulate *FlgM* expression

(dash). (Right) class 3 gene expression in WT (solid) and mutant (dash) upon cell division. Class 3 proteins represent the filament, chemotaxis, and motor proteins

Fig. 7 Feedback loops control the amount and fraction of free FlhD₄C₂ inside the cell. Fraction of free FlhD₄C₂ complex inside the cell in wild type, $\Delta fliZ$, $\Delta fliT$, and $\Delta fliD$ mutant as a fraction of time. FlhD₄C₂ is assumed to exist either freely or bound with FliT. All values are normalized with respect to the wild type levels of total FlhD₄C₂ amounts inside the cell

acts as an anti-anti- FlhD₄C₂ factor, as proposed before (Aldridge et al. 2010). However, we propose that the strength of the FliT- FlhD₄C₂ loop is controlled by FliZ present in the cell. The absence of FliZ is “sensed” by the cell in terms of reduced HBB expression, and the cell tried to compensate for this loss of HBB expression by retaining more of FlhD₄C₂ in the cell in its free form.

Sensitivity analysis of model parameters

Salmonella has been a model organism to study regulation and assembly of flagella in a number of studies. This has led to characterization of a number of biochemical and genetic interactions in the system, which has significantly aided in modeling the system. To characterize the relative

Fig. 8 Sensitivity analysis. Class 2 (left) and class 3 (right) expression values on making 10 % change in the parameter values. Red bars indicate change in steady state value after increasing the parameter value by 10 %, and green bars indicate the change in steady state value after decreasing the parameter value by 10 %.

significance of the various parameters associated with the network, we simulated the network after having changed the value of each parameter (one at a time) by $\pm 10\%$, and observed the change in the difference in the steady state values of class 2 and class 3 gene expression levels. Our results identified two sets of eight parameters which were found to be significant in controlling flagella gene expression. The parameters are as presented in (Fig. 8). All other parameters were found to result in a percent change $< 5\%$ in our analysis (data not shown).

Our results show that for class 2 gene expression, there are only two model parameters which exhibit a more than 10 % change in steady state values (on changing the parameter value by $\pm 10\%$). These two are FlhD₄C₂-dependent parameter K_2 , and the degradation rate of class 2 products, K_{d2} . In the case of class 3 gene products, the expression is sensitive to five parameters, which include: degradation constants of FliA and class 3 products, and constants associated with FliA-dependent activation of Class 3 genes and FliA (Fig. 8). Despite being a complex network, change in value of no other parameter (one at a time) exhibited a significant change in steady state expression of class 2 and class 3 products. From a networks perspective, this indicates that the flagellar regulatory network in *Salmonella* is extremely robust against mutations.

Conclusions

In this work, we have developed a mathematical model of the flagellar regulatory network in the model organism *S. typhimurium*. Flagellar gene regulation has been the subject of interest in a large number of experimental studies (Chilcott and Hughes 2000; Jones and Aizawa 1991; Macnab 1977, 1999, 2003; Silverman and Simon 1974).

Changing values of all other model parameters resulted in $< 10\%$ change (data not shown). K_{d2} is the degradation constant of class 2 genes. Kd4 and Kd6 are the degradation constants of FliA and Class 3 proteins respectively

Fig. 9 Flagella regulatory network tunes in response to the secretion from the assembled HBBs. (*Left* at low secretion rates): FliD sequesters FliT, and FlgM sequesters FliA. As a result, all interactions regulated by free FliA and FliT are inactive (in grey). The active interactions in this regime are highlighted in black. (*Right* at high secretion rates): FlgM and FliD are actively secreted from the cell.

Free FliA activates expression of class *fliAZ*, *fliDST*, *flgM*, and class 3 promoters. FliT interacts with FlhD₄C₂ to repress FlhD₄C₂-dependent activation of class 2 promoters. In both figures, *black arrows* represent active interactions, and grey represent inactive interactions. The transition from *black* to *grey* (or vice versa) is gradual

The regulatory network includes a number of coupled feedback loops, which makes it difficult to decouple the role of each interaction experimentally. The modeling efforts in this direction have been limited to only the FliA–FlgM interaction, which has been the most well-understood interaction in the network, and is thought to be the key check-point in the process (Chevance and Hughes 2008; Chilcott and Hughes 2000; Hughes et al. 1993). However, important differences exist between flagellar regulation in *E. coli* and *Salmonella*. One, both respond differently to stress. Second, unlike *E. coli*, in *Salmonella*, the flagellar regulon is never truly in the OFF state. And last, in *Salmonella*, motility is a virulence factor, and proteins from the flagellar network have been shown to regulate genes on Pathogenicity Island of the organism (Chubiz et al. 2010; Iyoda et al. 2001; Kage et al. 2008). To study these questions, we develop a model taking into account all major feedback loops in the network, and study the impact of different interactions on the dynamics of gene expression.

Through the aid of the model, we demonstrate that the number of feedback loops encoded in the network play a role of precisely timing the hierarchical expression of genes in this network. Our model is the first mathematical expression of the flagellar network where we demonstrate that the relative strength of these loops changes with the secretion status of the flagella in a cell. In a non-flagellated state (or in absence of secretion), the regulatory interactions which predominantly drive gene expression are as shown in Fig. 9 (left panel). As the cell assembles more and more number of HBBs capable of secretion, the regulatory network moves towards Fig. 9 (right panel). This transition is continuous as the cell assembles more and more HBBs, but also discrete since completion of a HBB is a discrete event. In addition, we show that one of the major roles of the accessory proteins in the network (FlgM, FliZ,

and FliD) could be to ensure hierarchy of expression of class 2 and class 3 genes upon cell division. This is likely important as premature expression of class 3 products is known to be detrimental to cell growth. Another insight from the model is the prediction that FliZ is the secretion-dependent link between flagella and the SPI1 regulatory network. FliZ has been previously shown to be activator of SPI1 gene expression, and our model predicts that this happens post-secretion (Chubiz et al. 2010; Iyoda et al. 2001; Lucas et al. 2000). Previous modeling efforts of the flagellar regulon have been limited to the FliA–FlgM interaction only (Kalir et al. 2005; Saini et al. 2011). As a result, these models have been unable to capture the additional roles of regulators and feedback loops that our model predicts in this study.

From a networks perspective, more than 50 genes come together leading to a functional flagella (Chilcott and Hughes 2000). In addition to their timely activation and assembly, it is also desirable that the network is immune to mutations and changes in biochemical parameters. We show that the regulatory structure of the flagella network is such that it renders steady state expressions of class 2 and 3 genes dependent on a minimal number of parameters.

From a networks perspective, feedback has been demonstrated to play an important role in control of biological processes, including virulence (Brandman et al. 2005; Freeman 2000; Kim et al. 2007; Mitrophanov and Groisman 2008; Saini and Rao 2010; Shin et al. 2006; Thomas et al. 1995). In this direction, our work adds to the number of behaviors known to be exhibited by living cells using feedback in networks. Among this, the role of secretion to tune the strength of the various feedback loops is a critical and novel one. From the perspective of *Salmonella* pathogenesis, further work needs to be done to validate and test predictions from our model, and shed more light on the

multi-process control of the *Salmonella* infection cycle (Clegg and Hughes 2002; Ellermeier and Slauch 2003; Iyoda et al. 2001; Saini et al. 2010b; Wu et al. 2012).

Acknowledgments This work was funded by the Innovative Young Biotechnologist Award (IYBA) 2010 program of the Department of Biotechnology, Ministry of Science and Technology, Government of India.

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