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Using a Quantitative Blueprint to Reprogram the Dynamics of the Flagella Gene Network

Shiraz Kalir and Uri Alon*
Department of Molecular Cell Biology and
Department of Physics of Complex Systems
Weizmann Institute of Science
Rehovot, Israel 76100

Summary

Detailed understanding and control of biological networks will require a level of description similar to that of electronic engineering blueprints. Currently, however, even the best-studied systems are usually described using qualitative arrow diagrams. A quantitative blueprint requires in vivo measurements of (1) the relative strength of the interactions (numbers on the arrows) and (2) the functions that integrate multiple inputs. Here, we address this using a well-studied system, the flagella biosynthesis transcription network in *Escherichia coli*. We use theory and high-resolution experiments to obtain a quantitative blueprint with (1) numbers on the arrows, finding different hierarchies of activation coefficients for the two regulators, FlhDC and FliA; and (2) *cis*-regulatory input functions, which summate the input from the two regulators (SUM gates). We then demonstrate experimentally how this blueprint can be used to reprogram temporal expression patterns in this system, using controlled expression of the regulators or point mutations in their binding sites. The present approach can be used to define blueprints of other gene networks and to quantitatively reprogram their dynamics.

Introduction

A major goal of biology is to obtain quantitative blueprints of gene networks, which can be used to control network behavior based on mathematical understanding of their dynamics (Bolouri and Davidson, 2002; Bray, 1995; Buchler et al., 2003; Elowitz and Leibler, 2000; Gardner et al., 2003; Hartwell et al., 1999; Hoffmann et al., 2002; Ideker et al., 2001; Lazebnik, 2002; Lee et al., 2003; Liao et al., 2003; McAdams and Shapiro, 1995; Ozbudak et al., 2004; Pomerening et al., 2003; Ronen et al., 2002; Savageau, 1976; Setty et al., 2003; Tyson et al., 2003; Wolf and Arkin, 2002). To address this, we employ a classic system, the *E. coli* flagella biosynthesis transcription network (Aldridge and Hughes, 2002; Kutsukake et al., 1990; Macnab, 1999). This system regulates the transcription of 14 operons that encode for the flagella motor and filament, the equipment that allows *E. coli* to swim. The flagella genes are arranged in a transcription hierarchy of three stages in which the master regulator FlhDC activates the seven class 2 operons, which encode proteins that make up the motor (Figure 1A). One class 2 gene encodes the sigma factor FliA that transcriptionally activates class 3 promoters and also further activates class 2 promoters, including its own promoter (Ikebe et al., 1999a, 1999b; Kutsukake et al., 1990; Liu and Matsumura, 1996). It is not known whether FliA can activate class 2 genes in vivo in the absence of FlhDC, though in vitro FliA can bind RNA polymerase to activate some class 2 promoters without need of FlhDC (Liu and Matsumura, 1996). FliA is inhibited by the anti-sigma factor FlgM, which is expressed from both class 2 and class 3 promoters. When a functional motor is assembled, FlgM is exported from the cell, and FliA becomes active as a transcription factor. The class 2 promoters are activated in a temporal order, with differences on the order of minutes (Laub et al., 2000; Kalir et al., 2001). This activation order corresponds to the order in which the gene products are assembled to form the flagellar motor. It has recently been found using in vitro biochemical measurements that FlhDC binds the early expressed class 2 promoters more strongly than late expressed promoters (Claret and Hughes, 2002), supporting a theory that the temporal order is produced by a hierarchy of activation strengths of these promoters (Kalir et al., 2001; Shen-Orr et al., 2002).

Results and Discussion

To quantitatively map this system in vivo, we obtained high-resolution expression dynamics from living cells using GFP reporter strains (Kalir et al., 2001). Each strain carries a low-copy plasmid in which one of the class 2 promoters governs expression of a fast-folding green fluorescent protein (Cormack et al., 1996). The strains were grown in parallel in an automated multiwell fluorometer that measured GFP fluorescence and cell density (OD) at a temporal resolution of about 5 min. We calculated the promoter activity (relative rate of transcription) as the rate of GFP accumulation per cell:

$$Pi(t) = dGFP/dOD \quad (1)$$

The flagella system is induced following dilution of the cells into fresh defined medium (Kalir et al., 2001). We find that the promoter activity dynamics show two phases (Figure 1B). In the first phase, the promoter activity is approximately constant, with different promoter activity for each operon. The promoter activity in this phase, β_i, is ranked from high to low in the order *fliL, fliE, fliF, flgB, flgA, flhB*, and *fliA* (we name each operon by its first gene; for example, *fliL* represents the *fliLMNOPQR* operon). The highest promoter activity, $\beta_1 = 1200 \pm 100$ GFP/OD units for the *fliL* operon, is more than 20-fold stronger than the weakest promoter, *fliA*, which has $\beta_7 = 50 \pm 20$. The order of promoter activity in this phase matches the temporal order in which the genes were found to be expressed (Kalir et al., 2001). The ranking of these in vivo β_i values qualitatively agrees with in vitro measurements of FlhDC binding affinity to the class 2 promoters (Claret and Hughes, 2002). In the second phase, beginning at OD $\sim$0.06, the promoters show a peak of activation with similar promoter activi-

*Correspondence: urialon@weizmann.ac.il

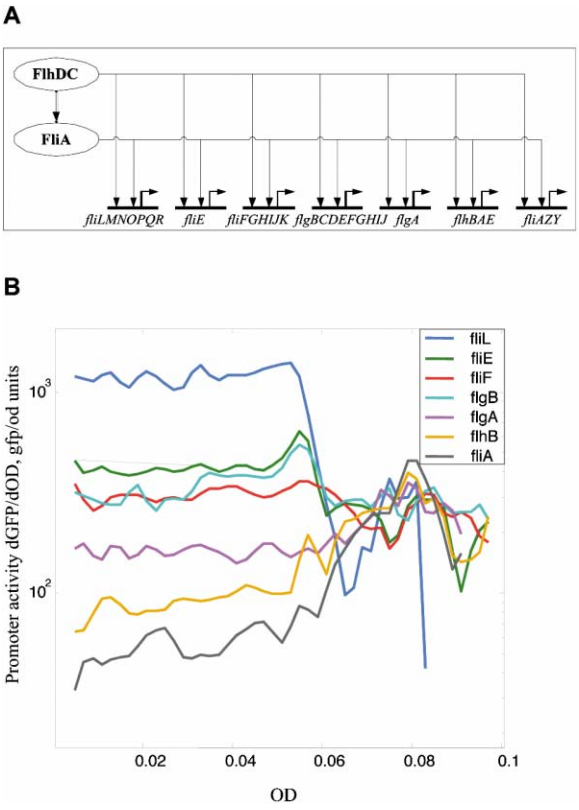

Figure 1. Arrow Diagram and Dynamics of the Flagella Class 2 Transcription Network

(A) Qualitative arrow diagram of the flagella class 2 gene network. (B) Promoter activity (rate of GFP production per cell, dGFP/dOD) of the seven class 2 promoters as a function of OD. Measurements were performed during exponential growth after dilution from overnight cultures into defined medium in a multiwell fluorometer (Kalir et al., 2001; Ronen et al., 2002).

ties, $\beta'_i = 300 - 400$ GFP/OD units for all promoters. The second phase corresponds also to the onset of expression of the class 3 promoters and results from activation of the FliA transcription factor.

Next, we studied the *cis*-regulatory input function (Bolouri and Davidson, 2002; Buchler et al., 2003; Setty et al., 2003) that integrates the inputs from the two transcription activators, FlhDC and FliA. We constructed strains in which the *flhD*, *fliA*, and *flgM* genes are deleted. Expressing FliA from an exogenous promoter in this strain shows that the class 2 genes can be activated by FliA in the absence of FlhDC (Figure 2A). Thus, in a Boolean approximation, the input functions can be described as OR gates over the activity of their two inputs, FliA and FlhDC.

To investigate the additivity of the two inputs, we constructed strains in which both FliA and FlhDC can be exogenously coexpressed in a controlled fashion. In this strain, the *flhD*, *fliA*, and *flgM* genes are deleted. The strain bears three compatible plasmids: one with *flhDC* under the lac promoter; another with *fliA* under the tet promoter, allowing controlled induction using the inducers IPTG and aTc, respectively; and a third reporter plasmid in which one of the flagella promoters controls GFP. This strain also had a chromosomal cassette expressing the repressors TetR and LacIq (Lutz and Bujard, 1997). We find no measurable *fliL* promoter activity in the absence of the inducers IPTG and aTc. We measured promoter activity at 96 different combinations of IPTG and aTc to find that the promoter activity was activated in a graded manner by both IPTG and aTc (Figure 2B). Figure 2B represents the *cis*-regulatory input function of the fliL promoter (Setty et al., 2003). We find that the effects of FliA and FlhDC are additive: denoting the promoter activity at concentration x of IPTG and concentration y of aTc by f(x,y), we find that

$$f(x,y) = f(x,0) + f(0,y)$$

as shown in Figure 2C. This type of input function is best described as a SUM gate, which may be considered as a graded version of an OR gate.

These findings were used to construct a mathematical model of the flagella class 2 gene network, represented by a quantitative blueprint (Figure 3). The activity of each promoter is

$$Pi(t) = \beta_i X(t) + \beta'_i Y(t) \quad (2)$$

where X(t) and Y(t) are the effective protein-level activity profiles of FlhDC and FliA, respectively (in dimensionless units). According to this model, the promoter activities of the seven class 2 operons are explained by the two "hidden functions," X(t) and Y(t). The β_i and β'_i correspond to the in vivo activation coefficients that result from the action of each of the two input regulators (Figure 1). Since Equation 2 is bilinear, one can find the best-fit values of the parameters β_i, β'_i, and the functions X(t) and Y(t), using an algebraic procedure termed singular value decomposition (Alter et al., 2000; Ronen et al., 2002) (see Experimental Procedures). We find that the dynamics of all of the class 2 promoters can be well described using this model (compare full and dashed lines in Figure 4B).

The fitting procedure produces predicted activity profiles X(t) and Y(t). Due to the linear form of the regulation, any linear combination of X and Y can, in principle, fit the data equally well. One can find specific predictions for X and Y by using an additional constraint, based on the fact that FlhDC activates FliA transcription, and, hence, X activity should preceed Y activation. This leads to predicted activity profiles that suggest that, under the present experimental conditions, FlhDC activity is first constant and then drops and that FliA activity begins to rise at about the same time as the drop in FlhDC activity (Figure 4C). These dynamics are in reasonable agreement with direct measurements: we measured FliA activity using promoters responsive to FliA but not to FlhDC (class 3 flagella promoters *mecha* and *mocha*) and measured FlhDC activity using a promoter responsive to FlhDC but not to FliA (*fliL* promoter in which the FliA binding site was mutated, termed *fliL**). These reporter strains indicate that FlhDC activity is approximately constant at early times and then turns off and that FliA activity begins to rise when FlhDC activity begins to decrease (Figure 4D).

The present model provides an explanation for the previously observed temporal order in the GFP dynamics of the class 2 reporter strains (Kalir et al., 2001). In

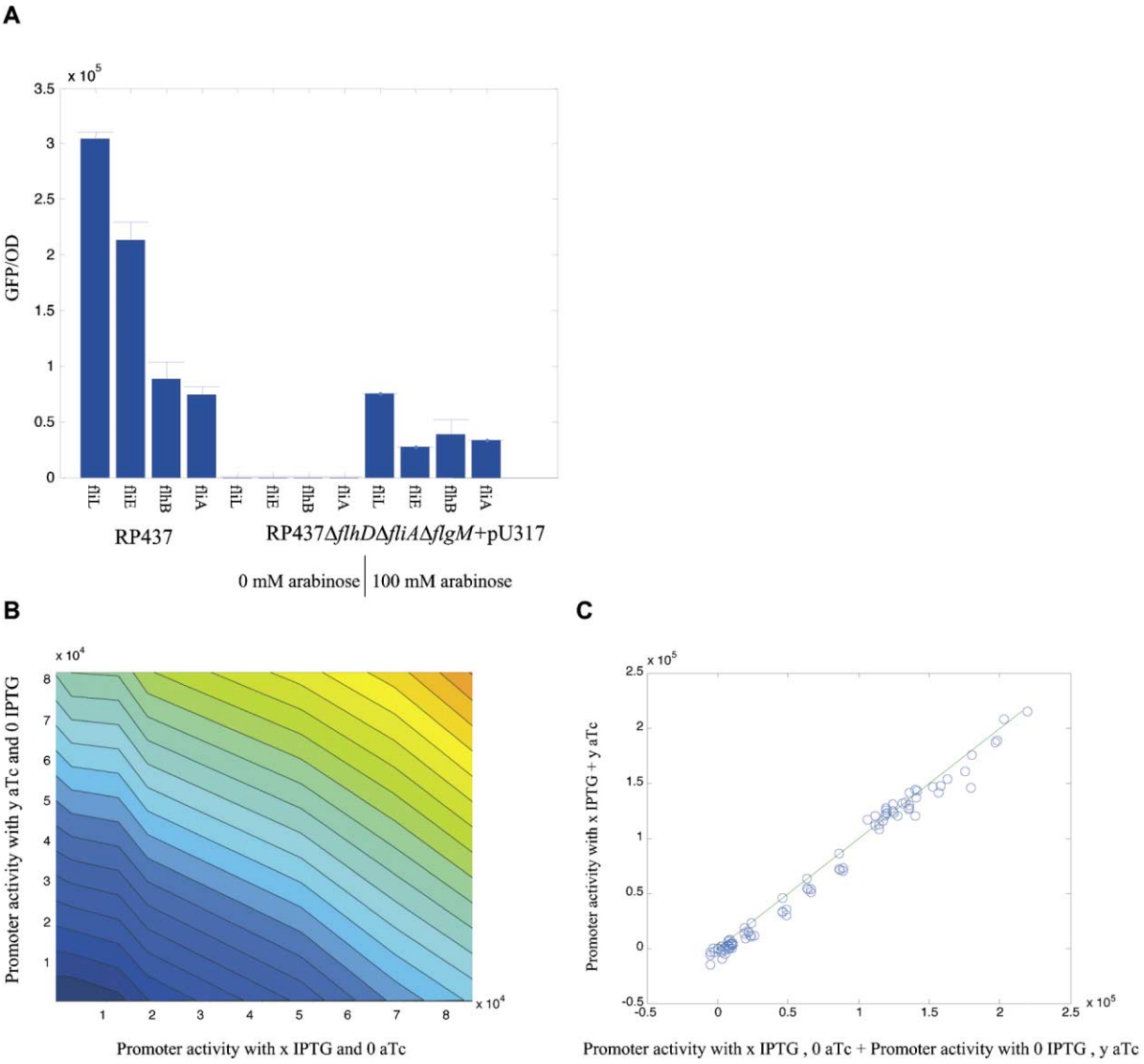

Figure 2. *cis*-Regulatory Input Function of Class 2 Flagella Operons

(A) Maximal GFP/OD of class 2 flagella reporter strains in wild-type background (RP437) and with exogenous FliA in RP437Δ*flhD*Δ*fliA*Δ*flgM* deletion strains U308 + pU317. FliA was expressed from the arabinose-inducible promoter using no or saturating amounts of L-arabinose. For clarity, data for *fliL* in the wild-type strain were multiplied by 0.5.

(B) Contour plot of the *cis*-regulatory input function (Setty et al., 2003) of the *fliL* promoter as a function of various combinations of FlhDC and FliA levels. *fliL* promoter activity was measured at midlog phase in RP437Δ*flhD*Δ*fliA* Δ*flgM* + pU320 + p321 + pJM35, grown with 96 different combinations of IPTG (ranging from 0 to 2.4 mM) and aTc (ranging from 0 to 70 ng/ml). In this strain, IPTG and aTc control the exogenous expression of FlhDC and FliA, respectively. Note that the axes represent promoter activities and not inducer concentrations. Promoter activity was not linear as a function of the concentration of the inducers aTc or IPTG.

(C) f(x,y) versus f(x,0) + f(0,y), where f(x,y) is the promoter activity and x and y are the ITPG and aTc concentrations. The straight line represents perfect additivity of the two inputs. Note that RP437 is *lacY*$^-$, allowing graded induction with IPTG.

the first phase of growth, only FlhDC is active, and the class 2 promoters are activated in a hierarchy of strengths according to the β_i parameters. If this were all, the GFP dynamics normalized to maximal levels would be identical for all promoters. However, in the second phase, FliA becomes activated and contributes an additional dose of GFP, Ga, which is approximately equal for all promoters (Figure 1B). The relative effect of this late dose is smallest for *fliL* because of its strong activation by FlhDC and results in a small convex flattening out of the normalized GFP dynamics (Figure 4A). However, for operons with weaker promoter activity, such as *flhB* and *fliA*, the extra expression dose makes a large contribution to the dynamics, resulting in a concave late rise in the GFP curve and a longer response time (Figure 4A). The response time (or, more precisely, the "response OD"), termed Nqi, can be obtained by drawing a horizontal line in Figure 4A at normalized GFP = q and determining the OD at which it intersects each promoter curve. In the model, the response time

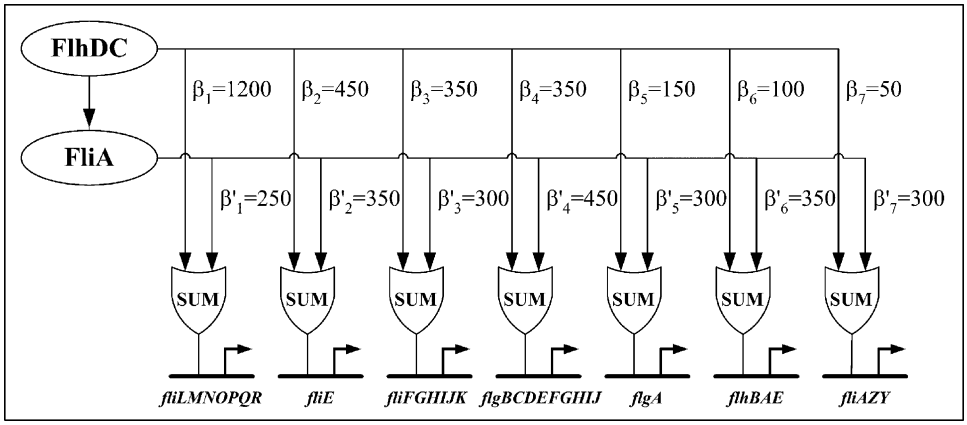

Figure 3. Quantitative Blueprint of the Flagella Class 2 System

β_1, β_2, ... β_7 correspond to the FlhDC activation coefficient for the *fliL, fliE, fliF, flgB, flgA, flhB, and fliA* promoters, and β'_1, β'_2, . . . β'_7 are the activation coefficients of FliA for these promoters, with units of GFP/OD. The *cis*-regulatory input function is a SUM gate at each promoter. The estimated errors for βi and β'i are at most $\pm$20%, and $\pm$20 GFP/OD units for β_7.

is predicted to decrease with increasing maximal expression (maximal GFP reading), Gmax,i:

$$Nqi = q\ Nf\ Gmax,i/(Gmax,i-Ga) \quad (3)$$

where Nf is the OD at which FlhDC activity declines. The measured response times for the seven class 2 promoters (q = 0.1) agree with this equation reasonably well (Figure 5A).

Can the present blueprint model be used to design experiments to reprogram the temporal order of flagella gene expression? The model suggests that, in order to change the response time, one can change the numbers on the arrows. For the earliest promoter, *fliL*, decreasing β_1 should make the rise time longer and the maximal expression lower compared to wild-type promoter. Decreasing β'_1, on the other hand, should have only a small effect on the timing, because β'_1 is much smaller than β_1.

To experimentally change β_1, we constructed reporter plasmids with 1–3 point mutations in the FlhDC binding site of the *fliL* promoter (Ikebe et al., 1999a). We find that these mutations make promoter activity later and weaker (Figure 5B). The more mutations were made in the FlhDC binding site, the larger this effect. Furthermore, the mutant reporter dynamics fall on the same curve as the wild-type class 2 promoters and Equation 3, suggesting an internal consistency in our understanding of the dynamics. In contrast, mutating the FliA binding site of the *fliL* promoter by means of point mutations resulted in weakening of the maximal expression but no significant effect on the response time, as expected based on the model (data not shown). In control experiments, the *lacZ* promoter was induced to various levels using IPTG. The response time did not significantly depend on the maximal expression level (Figure 5C).

An additional way to affect all of the β_i X(t) terms at once is to change the expression level of FlhDC. The model predicts that the stronger β_i X(t) is relative to β'_i Y(t), the smaller the delays between genes (in the limit when the former is very strong, the normalized dynamics of all of the genes should overlap with no timing differences). We used an *flhD* deletion strain and exogenously expressed FlhDC under an inducible promoter using various levels of the inducer. We find that the higher the induction of FlhDC, the smaller the delays between the various class 2 promoters (Figure 6).

The class 2 flagella system has a multioutput feedforward loop (FFL) architecture (Kashtan et al., 2004; Mangan and Alon, 2003; Shen-Orr et al., 2002) (Figure 2). The multioutput FFL is a recurring network motif in transcription networks. We find that the activation coefficients for the master regulator (FlhDC) show a hierarchy, whereas the coefficients for the downstream regulator (FliA) are quite similar for the different promoters. This results in a hierarchy of promoter activity and timing in which the earlier the gene products participate in flagella assembly, the stronger and earlier its promoter is activated. Similar dynamical principles, generated by a different mechanism, were recently found in metabolic pathway regulation (Zaslaver et al., 2004). It would be interesting to find whether this design can be found in other feedforward loop systems. More generally, it would also be interesting to discover whether the input functions and parameter hierarchies are crafted in a specific manner for each system or whether there exist for each type of network motif only a limited number of "standard" designs.

The present approach can be used to provide blueprints of other gene regulation networks in *E. coli* and in other organisms. The present study demonstrated, on a small scale, how a blueprint can be used to design interventions that reprogram the network dynamics. It would be important to use this approach to parameterize the effects of drugs or targeted gene therapy (adding, modifying, or removing interactions) on various network components to approach the goal of future improved quantitative design in medicine.

Experimental Procedures

Strains and Plasmids

GFP reporter plasmids in *E. coli* K12 RP437 (wild-type for flagella and chemotaxis) were described (Kalir et al., 2001). Deletion strains

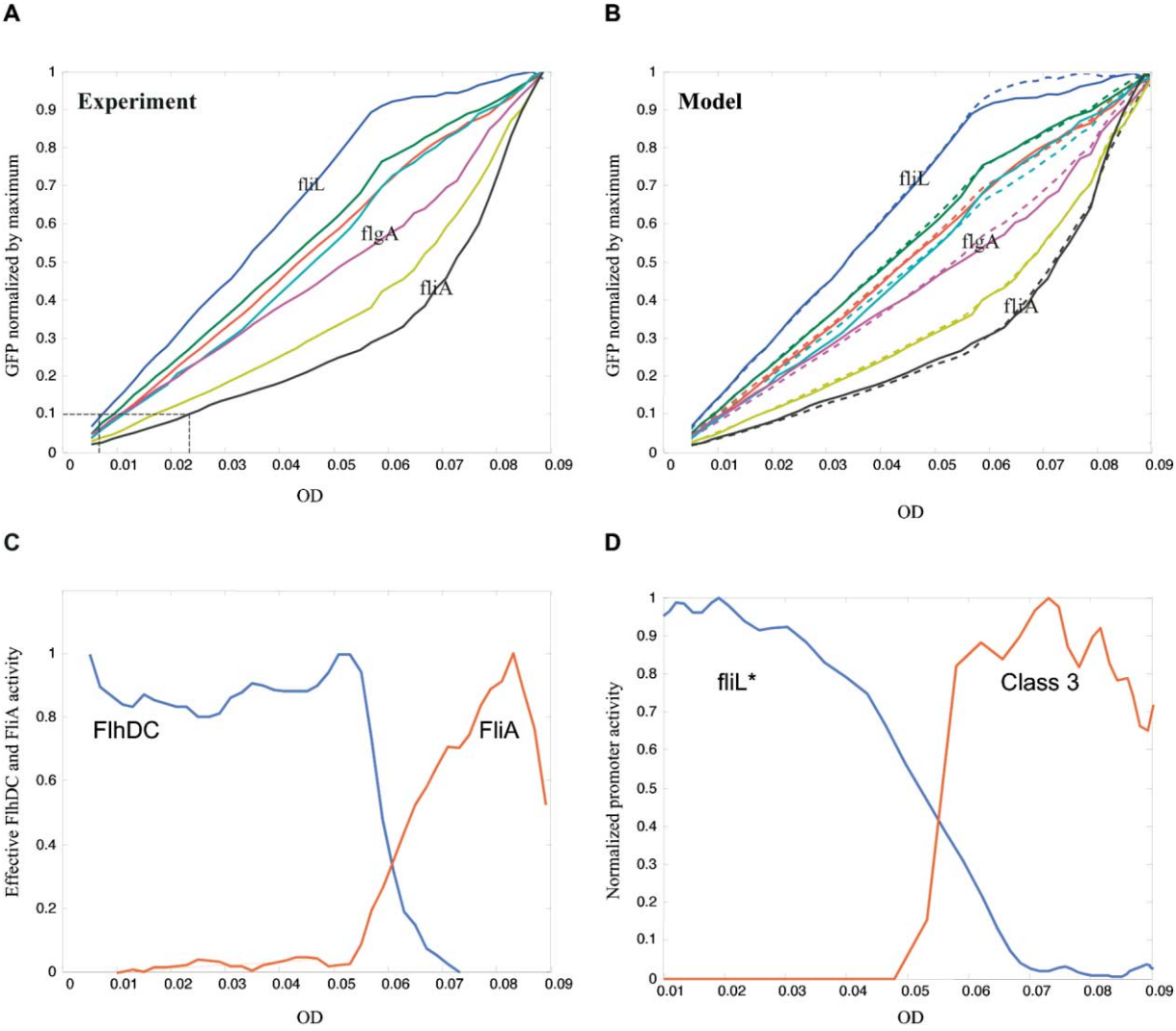

Figure 4. Experiment and Model for GFP Fluorescence as a Function of OD for the Class 2 Operon Reporter Strains

(A) GFP versus OD, normalized by maximal GFP level, during exponential growth in a multiwell fluorometer.

(B) Model GFP normalized by maximal level versus cell number (dashed lines) together with experimental data (full lines).

(C) The FlhDC and FliA effective protein-level activity profiles X(t) and Y(t).

(D) Measured promoter activities of *fliL** and late class 3, normalized to their maximal values, as a function of OD. *fliL** is the *fliL* promoter with inactivating point mutations in its FliA binding site, which is responsive to FlhDC but not FliA. The class 3 late promoters (mean of *meche* and *mocha* promoter data) are regulated by FliA but not FlhDC. The OD for these strains was normalized to maximum value of OD = 0.1.

were constructed by replacing the entire deleted ORF from start to stop codon with a Cm antibiotic cassette, which was then removed by FRT recombination using the method of Datsenko and Wanner (2000) and confirmed by PCR and sequencing. All deletion strains were based on RP437: U306 (RP437 Δ *flhD*), U309 (RP437 Δ *fliA*), U307 (RP437 Δ *flhD* Δ *fliA*), and U308 (RP437 Δ *fliA* Δ *flhD* Δ *flgM*). In a Δ *flhD* Δ *fliA* strain not deleted for *flgM*, exogenously expressed *FliA* is not detectibly active in causing expression of flagella promoters, presumably because it is inhibited by FlgM present in the cells. For that reason, the triple deletion Δ *flhD* Δ *fliA* Δ *flgM* was constructed. Primers for deleting *fliA* were R: CCCAGTTTAGT GCGTAACCGTTTAATAGCCTGGCTGTGTAACTGACTGACCCGCG GTGTAGGCTGGAGCTGCTTC, L: CACTCTATACCGCTGAAGGTGT AATGGATAAACACTCGCTGTGGCAGCGCATATGAATATCCTCCT TAG; for *flhD* were R: CAGGCCCTTTTCTTGCGC AGCGCTTCTT CAGGCTGATTAACATCGTGTAGGCTGGAGCTGCTTC, L: GTGGGA ATAATGCATACCTCCGAGTTGCTGAAACACCATATGAATATCCTC CTTAG; and for *flgM* were R: CAGTTACTCTGCAAGTCTTGCTGCG CTTCGTTGATCAGCGCATCGGCGTGTAGGCTGGAGCTGCTTC, L: GAGTATTGATCGCACTTCGCCTCTGAAGCCTGTAAGCACCGTT CAACCGCCATATGAATAT CCTCCTTAG.

Exogenous expression of FliA was from pU317, constructed by subcloning the *fliA* gene into the NheI-HindIII site of pBAD18. Exogenous expression of FlhDC was from pJM45 (gift of M.G. Surette), in which *flhDC* operon was subcloned into pBAD18. The FlhDC binding site in the *fliL* promoter, which begins at −80bp from transcription start, is CGCCTAA…N_{16}…GTAATCC (Ikebe et al., 1999a). Point mutations (Stratagene QuikChange Site-Directed Mutagenesis Kit) were (changed bp are in bold) fliL-1, CGCC**G**AA…N_{16}…GTAATCC; fliL-2, CGCCTAA…N_{16}…GTAA**C**CC; fliL-3, CGCC**GC**A…N_{16}…GTA ATCC; and fliL-4, CGCC**GC**A…N_{16}…GTAA**C**CC.

The FliA binding site in the *fliL* promoter (Liu and Matsumura, 1996), TCAA-N15-GCCGATAA-N29-ATG, was mutated as follows: *fliL**, TCAA-N15-**T**CCGAT**T**A-N29-ATG. The *fliL** promoter was acti-

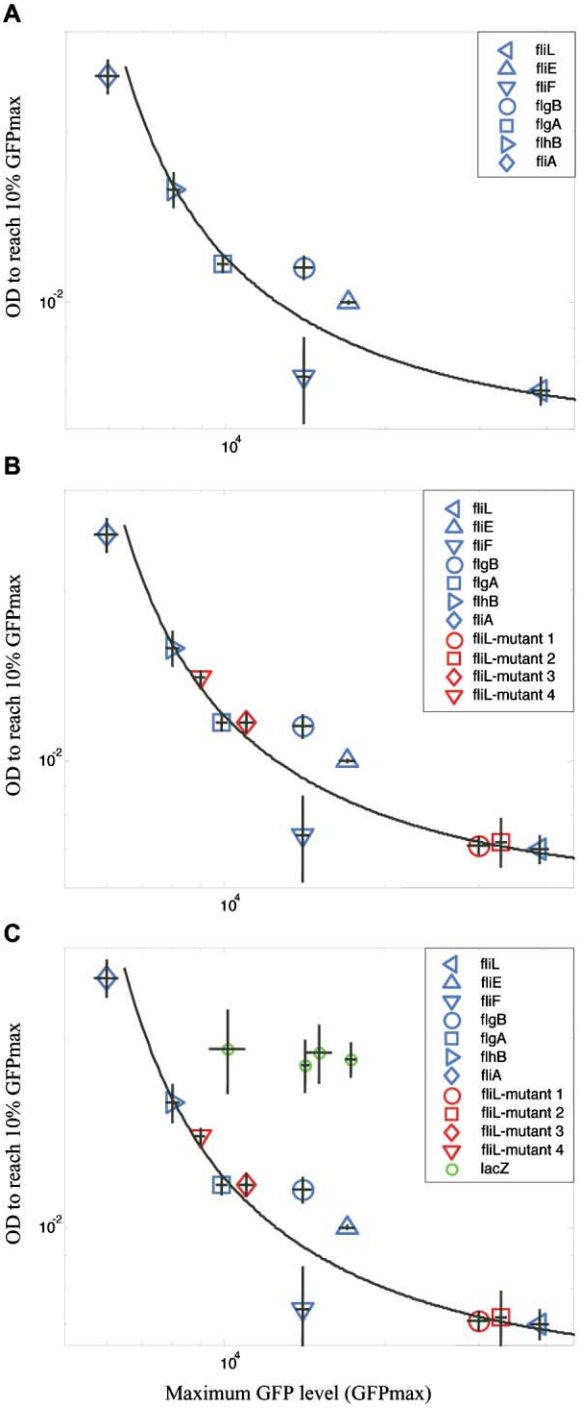

Figure 5. Reprogramming the Temporal Expression Dynamics in the Flagella System by Means of Point Mutations in the Regulator Binding Sites

(A) Response time (OD to reach 10% of maximal GFP level) versus maximal GFP level for the seven class 2 operons. Line, model results, Equation 3.

(B) Response time of *fliL* promoters with 1–3 point mutations in the FlhDC binding site.

(C) Response time of *LacZYA* promoter with different concentrations of IPTG.

vated by exogenous FlhDC. Exogenous FliA produced from pZE11-fliA (pU320) in the Δ*flhD*Δ *fliA*Δ *flgM* strain resulted in significant GFP production from the wild-type *fliL* reporter but undetectable GFP production from the *fliL** reporter (data not shown).

We find that class 2 promoter activity from RP437Δ *flhD* + *pJM45* in the presence of arabinose (so that FlhDC is exogenously expressed from pJM45) is not higher than its activity in a corresponding strain deleted for *fliA*, RP437Δ *flhD* Δ *fliA* + pJM45, with the same level of exogenous FlhDC expression (data not shown), suggesting that FliA and FlhDC do not measurably compete in vivo.

The strain U319, allowing combinations of FliA and FlhDC to be coexpressed, was constructed using U308 + pJM35 + pU321 + pU320. pU321 was constructed by subcloning the *flhDC* coding region into the KpnI, HindIII sites after the *lac* promoter in pZA32, which has p15A origin and CholramphenicoI resistance cassette (Lutz and Bujard, 1997). pU320 was constructed by subcloning the *fliA* coding region into the KpnI, HindIII sites after the *tet* promoter in pZE11, which has a colE1 origin and an Ampicilin resistance cassette (Lutz and Bujard, 1997). The tetR-lacIq cassette of Dh5αZ1 was introduced into U308 using P1 phage transduction and Spectinomycin selection.

Dynamic Expression Measurements

Strains were diluted 1:600 from overnight culture into defined medium and assayed in 96-well plates in an automated Victor2 fluorometer as described (Kalir et al., 2001). In control experiments, a reporter strain for the *lacZYA* promoter UA0344 (Ronen et al., 2002) was induced to various levels using the inducer IPTG. We find that the response time of this operon (time to reach 10% or 50% of maximal expression) does not depend on the maximal strength of expression.

Mathematical Model of Class 2 Flagella Network

Given the additivity found in the action of FliA and FlhDC, we model the GFP produced by the reporter strain for each promoter by

$$Pi(t) = dGi(t)/dN = \beta_i X(t) + \beta'_i Y(t) \quad (4)$$

and

$$dN/dt = \alpha N \quad (5)$$

where N is the cell number (OD) and α is the exponential growth rate. To find the best fit functions X(t) and Y(t), as well as the parameters β_i and β'_i, we used singular value decomposition (SVD) (Alter et al., 2000; Ronen et al., 2002). SVD is an algebraic procedure that decomposes the promoter activities Pi(t) as a sum on coefficients that depend only on i times vectors that depend only on t: Pi (t) = β_i A(t) + β'_i B(t) + β''_i C(t) + …. We find that the first two SVD vectors, A(t) and B(t), capture more than 97% of the variation in the data. This agrees with the expectation that two independent regulators affect the flagella system during the presently studied phase of growth. In principle, any linear combination of A(t) and B(t) is an equally valid solution, and thus there are four free parameters for mixing the two vectors: a A(t) + b B(t), c A(t) + d B(t) (the β and β' parameters get mixed in an analogous way, depending on a, b, c, and d). We determined these four parameters, a, b, c, and d, to find X(t) = a A(t) + b B(t), Y(t) = c A(t) + d B(t), according to four conditions: max(X) = 1, max(Y) = 1, X(t = 0) = 1, Y(t = 0) = 0 (maximal values of one, X(t) starting at one and Y(t) starting at zero, according to the expectation that FlhDC activity precedes FliA activity). Small negative values of X and Y were set to zero. This determined X(t) and Y(t), shown in Figure 4C; and the coefficients β_i and β'_i listed in Figure 3. The late decrease in effective FliA activity may be due to the approach to stationary phase, since flagella synthesis in the present strain, RP437, is shut down in stationary phase (Amsler et al., 1993; Staropoli and Alon, 2000).

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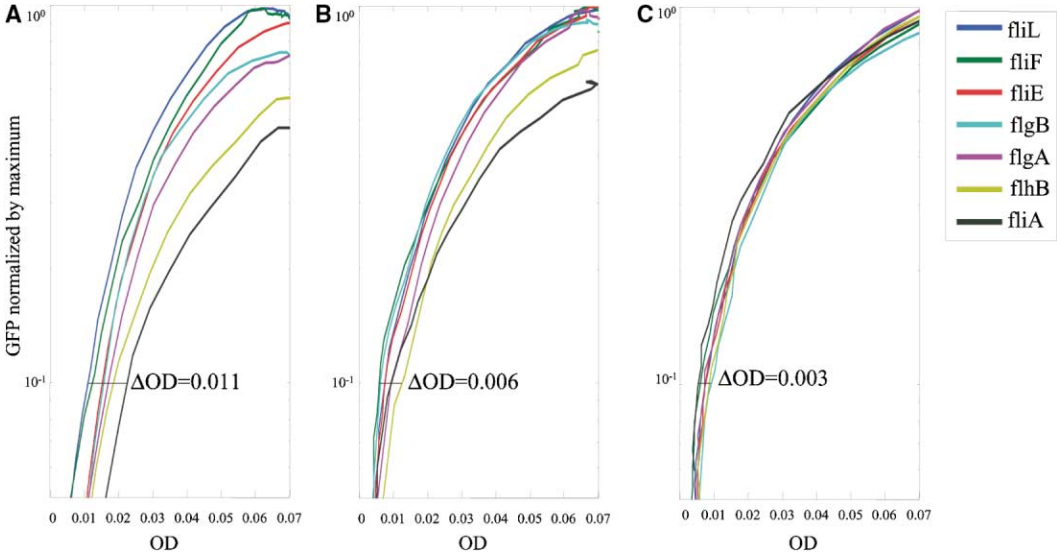

Figure 6. Reprogramming the Timing Differences between Promoters in the Flagella System by means of Controlled Expression of FlhDC to Different Levels in an *flhD* Deletion Strain (RP437 Δ*flhD*)

(A) Expression of FlhDC from an ara-inducible promoter with 200 uM L-arabinose, (B) 500 uM L-arabinose, (C) 10 mM L-arabinose. ΔOD is the response OD difference of the first and last promoter q = 0.1. Similar results were found using tet-inducible FlhDC (data not shown).

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