FliZ Induces a Kinetic Switch in Flagellar Gene Expression†‡

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FliZ is an activator of class 2 flagellar gene expression in Salmonella enterica. To understand its role in flagellar assembly, we investigated how FliZ affects gene expression dynamics. We demonstrate that FliZ participates in a positive-feedback loop that induces a kinetic switch in class 2 gene expression.

Flagellar assembly proceeds in a sequential manner beginning at the base along the inner plasma membrane and concluding at the distal tip of the filament (21). Over 50 genes divided among at least 17 operons are involved in assembling flagella (6). These genes are expressed in a temporal hierarchy that mirrors the assembly process itself (14, 15).

Briefly, the flagellar genes can be divided into three classes based on how they are temporally expressed. A single class 1 promoter region controls the expression of the FlhD4C2 master regulator (26). FlhD4C2 in turn activates the class 2 promoters, which control the expression of the genes encoding the hook-based body (HBB) complex (10, 18, 20). In addition, FlhD4C2 also activates the expression of two regulators, σ28 and FlgM. The flagellum-specific sigma factor σ28 activates the class 3 promoters, which control the expression of the genes encoding the filament, motor, and chemotaxis pathway (19, 22). Prior to HBB completion, however, FlgM binds to σ28 and prevents it from activating the class 3 promoters (4, 5, 8, 22). However, when the HBB complex is complete, FlgM gets secreted from the cell, freeing σ28 to activate the class 3 promoters (2, 9). This checkpoint couples class 3 gene expression with the completion of the hook-based body complex.

In addition to σ28 and FlgM, other flagellar proteins also regulate gene expression (1, 3, 16, 27). FliZ, the focus of this study, has previously been shown to be an activator of class 2 gene expression. Encoded in the same operon as σ28 (FliA) and expressed from the hybrid class 2/3 PflhA promoter, FliZ is thought to posttranslationally regulate FlhD4C2 in Salmonella enterica (3). Despite a growing list of investigations, little is known about FliZ’s role in regulating flagellar gene expression. In particular, null mutants of fliZ are motile and exhibit only modest reductions in the magnitude of flagellar gene expression (3), suggesting that FliZ plays only a minor role in flagellar assembly and may instead serve other functions such as coupling flagellar assembly with the expression of the S. enterica invasion genes (12, 17).

We hypothesized that FliZ does indeed play a role in flagellar assembly by regulating the dynamic expression of the flagellar genes. To test this hypothesis, we investigated flagellar gene expression dynamics at single-cell resolution using flow cytometry. We demonstrate that class 2 gene expression dynamics are heterogeneous and switch-like in the wild type but continuous and homogeneous in a ΔfliZ mutant. We also demonstrate that this kinetic switch results from FliZ’s participation in a secretion-dependent positive-feedback loop.

Flagellar gene expression dynamics are heterogeneous. We first compared expression from the class 2 PflhB and class 3 PmocA promoters at population resolution in wild-type cells and a ΔfliZ mutant, using a bulk assay. To measure gene expression, we employed chromosomally integrated transcriptional fusions to the Venus fluorescent protein as indirect measures of promoter activities (25). In these experiments, we diluted stationary-phase cultures to an optical density at 600 nm (OD600) of 0.05 in fresh Luria-Bertani medium and then measured the fluorescence and absorbance of the culture every 15 min during growth at 30°C. Consistent with previous reports (3), we observed that expression from the PflhB and PmocA promoters was reduced roughly 25% in a ΔfliZ mutant compared to that of the wild type (Fig. 1A and 2A).

We next compared expression from the PflhB and PmocA promoters at single-cell resolution, using flow cytometry. In these experiments, we employed a protocol similar to that used before, except that we took samples once every hour as opposed to continuously monitoring the same samples. Flow cytometry identified dynamic heterogeneity in the expression dynamics of both promoters in wild-type cells (Fig. 1B and 2B).

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In particular, we observed a bimodal distribution of expression states, suggesting that two populations of cells coexist during the first few hours of growth following dilution. One population likely corresponds to an “off” state, where cells are not expressing the flagellar genes. The other likely corresponds to an “on” state, where the cells are expressing the flagellar genes. The expression states, however, are not strictly binary, as a range of expression values are observed in the “on” state (2a).

Interestingly, in a ΔfliZ mutant, the expression dynamics of the class 2 P\_flhB promoter are homogeneous and continuous (Fig. 1C). In particular, only a single population is observed, suggesting that the cells are all behaving in roughly the same manner. We could restore the transient heterogeneity in the expression dynamics of the class 2 P\_flhB promoter by expressing FliZ from a plasmid in this mutant (see Fig. S1 in the supplemental material). With the class 3 P\_motA promoter, however, we still observed heterogeneous gene expression dynamics in a ΔfliZ mutant (Fig. 2C), indicating that the phenotype is specific to the class 2 genes.

To determine how FliZ affects flagellar abundance, we also measured the distribution of flagella, using transmission electron microscopy (Fig. 3). Consistent with a reduction in class 2 gene expression, we found that the average number of flagella per cell was reduced in a ΔfliZ mutant. However, we observed no significant change in the distribution of flagella among the population compared to that of the wild type, indicating that the changes in the distribution of gene expression do not translate to the changes in the distribution of flagella.

We also investigated gene expression dynamics in a strain in which the P\_flhDC promoter was replaced with the tetRA element from Tn\_10 (P\_flhDC::tetRA) (23). In this strain, flhDC expression is under the control of a tetracycline-inducible promoter. We have previously employed this strategy to decouple flhDC expression from its native regulation and synchronize assembly within the population (23). To measure gene expression in this strain, we employed the same experimental protocol as before, except that we added tetracycline (2.5 μg/ml) to induce flhDC expression upon dilution. Using this approach, we again observed heterogeneous gene expression dynamics (see Fig. S2 and S3 in the supplemental material). In addition, we found that the expression dynamics of the P\_flhB promoter were continuous in a ΔfliZ mutant. These results indicate that the heterogeneity is intrinsic to the flagellar gene circuit and not due to external factors affecting flhDC expression.

**Late protein secretion is required for heterogeneous gene expression dynamics.** Heterogeneity in gene expression is often associated with positive feedback. Consistent with such a model, FliZ is known to participate in two positive-feedback loops. In particular, FliZ expression is controlled by the hybrid
class 2/3 P_fliA promoter. As a consequence, FliZ can, in one loop, directly enhance its own expression through the activation of the class 2 component of the P_fliA promoter by posttranslationally regulating FlhD4C2. FliZ also participates along with FlgM in a second, secretion-dependent positive-feedback loop. In this loop, FlgM amplifies FliZ’s and its own expression through the activation of the class 3 component of the P_fliA promoter. However, this loop is only active when FlgM is being secreted from the cell. As the class 3 component of the P_fliA promoter is the strongest (11, 13), we hypothesized that this second, secretion-dependent loop may generate the heterogeneity.

To test this hypothesis, we measured flagellar gene expression dynamics in a /H9004 flgG— mutant at both population (Fig. 1A) and single-cell (Fig. 1D) resolutions. Note that a /H9004 flgG— mutant does not secrete FlgM as it is unable to build functional HBBs. In addition, the class 3 promoters are inactive in this strain (Fig. 2A). Consistent with the hypothesis that the heterogeneity is due to the /H9268 positive-feedback loop, we observed that the gene expression dynamics of the P_flhB promoter were homogeneous in a /H9004 flgG— mutant. Similar results were also observed in a P_flhDC::tetRA background (see Fig. S1D in the supplemental material).

We further explored this hypothesis by measuring flagellar gene expression dynamics in a strain in which the hybrid class 2/3 P_fliA promoter was replaced with the class 2 P_flhB promoter. As expression of the fliAZY operon is no longer under the control of σ28 in this strain (ΔP_fliA::P_flhB), the σ28 positive-feedback loop is broken. When we measured flagellar gene expression dynamics in this strain at single-cell resolution (Fig. 4), we found that the expression dynamics for both the P_flhB and P_motA promoters were homogeneous and continuous. These results indicate that the σ28 positive-feedback loop results in heterogeneous expression from both promoter classes.

Conclusions. FliZ has previously been shown to be an activator of class 2 gene expression. However, the effect is minor, suggesting perhaps that it may not have a direct role in regu-
Flagellar assembly. In this work, we found that FliZ induces dynamic heterogeneity in the expression of the class 2 flagellar genes. These results suggest that FliZ does indeed play an important role in regulating flagellar assembly, namely, by regulating gene expression dynamics.

Dynamic heterogeneity in gene expression is likely the consequence of a kinetic switch, where cells transition from an “off” state to an “on” state. As the transition times are most likely random, some cells will induce class 2 gene expression before others. If induction also results in a step increase in class 2 promoter activities, then there will be a gap in the levels of gene expression between the cells that induce class 2 genes early and those that induce them late. Such a model would explain the dynamic heterogeneity that we observe in class 2 gene expression dynamics. This model also suggests that the class 2 genes are induced more rapidly in individual cells than implied by bulk, population-level measurements, where a more gradual transition is observed (24). In the absence of FliZ, we imagine that the transition between the two expression states is less abrupt, leading to a more homogeneous response among individual cells within the population.

Why do cells employ this kinetic switch? We suspect that it provides a feedback mechanism whereby cells commit to strongly expressing the class 2 genes only when functional HBBs have been assembled (Fig. 2D), not unlike the class 3 checkpoint. A similar kinetic switch is employed with the class 2 genes only when functional flagellar assembly. A similar kinetic switch is employed with the class 2 genes only when functional flagellar assembly.

In conclusion, we have demonstrated that FliZ induces a kinetic switch in flagellar gene expression. Significantly, these results assign a specific phenotype to FliZ, namely, to further amplify the expression of the class 2 genes once functional HBBs are assembled.

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