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Genetic dissection of the consensus sequence for the class 2 and class 3 flagellar promoters

Christopher E. Wozniak[1,†*] and **Kelly T. Hughes**

1*Department of Microbiology, University of Washington, Seattle, WA 98195*

2*Department of Biology, University of Utah, Salt Lake City, UT 84112*

Summary

Computational searches for DNA binding sites often utilize consensus sequences. These search models make assumptions that the frequency of a base pair in an alignment relates to the base pair's importance in binding and presume that base pairs contribute independently to the overall interaction with the DNA binding protein. These two assumptions have generally been found to be accurate for DNA binding sites. However, these assumptions are often not satisfied for promoters, which are involved in additional steps in transcription initiation after RNA polymerase has bound to the DNA. To test these assumptions for the flagellar regulatory hierarchy, class 2 and class 3 flagellar promoters were randomly mutagenized in *Salmonella*. Important positions were then saturated for mutagenesis and compared to scores calculated from the consensus sequence. Double mutants were constructed to determine how mutations combined for each promoter type. Mutations in the binding site for $FlhD_4C_2$, the activator of class 2 promoters, better satisfied the assumptions for the binding model than did mutations in the class 3 promoter, which is recognized by the σ^{28} transcription factor. These *in vivo* results indicate that the activator sites within flagellar promoters can be modeled using simple assumptions but that the DNA sequences recognized by the flagellar sigma factor require more complex models.

Keywords

consensus sequence; gene expression; sigma 28; transcription

Introduction

Flagella provide a competitive advantage to many bacteria by enabling them to swim toward nutrients and away from harmful substances. About sixty genes in *Salmonella* and *E. coli* are involved in the construction, function, and regulation of flagella.[1] Flagellar genes are activated in a specific order, which may limit the expression of flagellar proteins to when they can be incorporated into the flagellar structure.[2,3] This coordination between expression and assembly is in part due to the organization of flagellar promoters into a transcriptional hierarchy of three classes.[1] The single class 1 promoter integrates multiple signals concerning the metabolic state of the cell to decide when to transcribe the *flhDC* genes. Together with the

*Corresponding author, Telephone: 801-585-6950, Fax: 801-585-9735, E-mail: wozniak@biology.utah.edu.
†Current Address: Department of Biology, University of Utah, Salt Lake City, UT 84112

housekeeping sigma factor (σ^{70}), the $FlhD_4C_2$ activator complex recognizes and initiates transcription from the class 2 promoters. The class 2 promoters transcribe genes involved in building the flagellar motor, which anchors the flagellum to the membranes and peptidoglycan, and in assembling a flexible, external linker called the hook. One of the class 2 promoters transcribes the gene for the alternative sigma factor σ^{28}, which recognizes flagellar class 3 promoters. Class 3 promoters express the genes for the long external filament, the motor force generators, and the chemosensory system. These late components can then be added onto or interact with the motor and the hook.[1]

Many class 2 and class 3 flagellar promoters have been characterized through the identification of their transcriptional start sites and by DNA footprint analysis.[4–9] This experimental data has facilitated the alignment of flagellar promoters for building consensus sequences. Class 3 promoters have conserved −10 and −35 regions that are recognized by the flagellar sigma factor (σ^{28}).[5] At class 2 promoters, $FlhD_4C_2$ binds upstream of σ^{70} to a palindromic DNA site that is not well conserved but has been verified experimentally through footprinting experiments.[4,9] $FlhD_4C_2$ is unable to activate transcription in strains lacking the C-terminal domain of the alpha subunit of RNA polymerase.[10] This suggests that $FlhD_4C_2$ contributes to the initial binding of polymerase to class 2 promoters through a direct interaction between $FlhD_4C_2$ and the alpha subunit. While a crystal structure of $FlhD_4C_2$ has been solved, this structure does not include the DNA to which $FlhD_4C_2$ binds.[11]

Using consensus sequences, several groups have used simple matching[5] or more complicated PSSM's (position specific score matrix)[12–14] to search for new flagellar promoters. As has been seen for other promoters and binding sites, these searches detect a large number of false positives.[15] This high background is often a result of low information content in a binding site combined with a large DNA search space. However, the large number of false positives may also be due to the assumptions used in building the search models that are intended for simple DNA binding proteins.

A DNA binding site is frequently defined through an alignment of sequences from several closely related genomes, and the consensus sequence derived from this alignment is then used for genome-wide searches.[15] Assumptions are usually made about the sequence alignment and the manner in which each nucleotide contributes to a binding site. While the sequences used in an alignment should have arisen independently and be experimentally verified, binding sites for the same operons from closely related species are often used to increase the sample size. Also, the frequency of each nucleotide in an alignment may not be directly related to its importance in the binding site. Stronger binding sites may show greater conservation of bases than weaker binding sites. Finally, it is frequently assumed that each base contributes to the binding site independently from all the others.[16] Several groups have provided evidence that most of the contributions to a binding site from individual bases are independent,[17–19] but this assumption may not be valid for all binding proteins.

In particular, these assumptions are not generally valid for promoters, which have additional roles to play after binding the DNA. Promoters go through multiple steps during transcription initiation including the binding of RNA polymerase, open complex formation, initiation of RNA synthesis, and promoter clearance. These different steps can be at odds with each other when determining the overall activity of a promoter.[20] For example, a promoter that matches the consensus sequence may have low activity because strong binding of RNA polymerase can interfere with promoter clearance.[21] Additionally, the strength of a promoter can be affected by poorly conserved DNA outside of the consensus sequences. For σ^{70}-dependent promoters, less conserved sequences like the UP element,[22,23] extended −10 element,[24,25] discriminator region,[26] and the initial transcribed sequence[27,28] can affect promoter activity by 10- to 100-fold. Because of the complex kinetics and multiple factors involved in promoter activation,

flagellar promoters may not be modeled well using the assumptions for simple DNA binding proteins. Here we test these assumptions for the activator protein of the class 2 flagellar promoters and the sigma factor for the class 3 flagellar promoters through extensive mutational analysis.

In this paper, we used genetics to dissect the consensus sequences for flagellar promoters. Four *Salmonella* promoters were randomly mutagenized to identify elements important for flagellar transcription. Seventeen positions in a class 3 promoter and 29 positions in a class 2 promoter were then saturated for mutation to all three possible base substitutions. To assess the accuracy of the consensus sequence for flagellar promoters, the activities of these mutants were compared to scores generated from sequence alignments. Additionally, pairs of mutations were constructed to determine how individual mutations combine to affect the overall activity of the promoter. This *in vivo* study revealed differences between an activator protein and a sigma factor in how transcription was affected by mutations in the promoter sequence. This mutagenesis data illustrates the suitability of these models for flagellar promoters.

Results

Promoter random mutagenesis

To identify bases in the flagellar promoters that are important for transcription, random mutations generated through error-prone PCR were introduced into the promoter regions for four flagellar operons (*flgKL*, *flgMN*, *fliAZY*, and *fliDST*) in *Salmonella typhimurium*. These four operons are transcribed from both class 2 and class 3 promoters. While two of the promoter regions that were mutagenized contained both the class 2 and class 3 promoters (*fliAZY* and *fliDST*), the two other promoter regions contained only class 3 promoters (*flgKL* and *flgMN*). The class 2 promoters for the *flgKL* and *flgMN* operons are located one or more genes upstream of the class 3 promoters and were not mutagenized. Fusion of the *lac* genes to these operons facilitated screening for mutants with altered transcriptional activity (Figure 1).

For all four promoter regions mutagenized, a total of 151 changes in transcription were detected among the 6,500 colonies that were screened. Seventeen unique *flgKL* promoter mutants were detected out of 1,500 colonies screened. Twenty unique *fliDST* promoter mutants were found among 1,300 colonies screened. One *flgMN* promoter mutant was detected among 1,000 colonies screened. Forty-seven unique *fliAZY* promoter mutants were found among 2,700 colonies screened. Transitions (A:T <–> G:C) accounted for 76% of the substitution mutants isolated.

Altogether, these recombinants contained 85 unique mutations. Eighty were single base pair substitutions or deletions in the promoter region (Figure 2). Four colonies had multiple mutations, and one colony contained a 123 bp deletion. Most of the single mutations were located in regions of the promoter that matched the consensus sequence for either $FlhD_4C_2$ (19 mutations), σ^{70} (7 mutations), or σ^{28} (32 mutations). Fourteen mutations were also found near these conserved sequences. This localization of mutations within sequences that match the consensus is most striking in the *fliAZY* promoter where the class 2 and class 3 promoters overlap (Figure 2c). Moving along the DNA in the direction of transcription, mutations alternate between affecting class 2 transcription and class 3 transcription depending on which consensus sequence is matched in the DNA. Additionally, 10 mutations were in the untranslated region (UTR) of the downstream mRNA, and one mutation was 4 bp after the stop codon for the gene upstream of the *flgKL* promoter. The mutations in the UTRs and after the stop codon probably affect mRNA stability, introduce pause sites into the mRNA, or interfere with promoter escape. All mutations resulted in decreased transcription except for one mutation in the *fliAZY* $FlhD_4C_2$ binding site and two mutations in the *fliDST* 5' UTR. Transcription was decreased by as much as 99% for any single substitution.

Of 1,000 colonies screened for mutations in the *flgMN* class 3 promoter, only one colony (a −65 C:T mutant, in the −10 region) showed decreased transcription. The rarity of mutations isolated at this promoter is most likely the result of the *lac* reporter being inserted in the gene for the anti-sigma factor FlgM. Without FlgM, class 3 transcription levels are very high, and small decreases in activity probably would be missed on the indicator plates used. Also, high levels of free σ^{28} could compensate for binding defects in the *flgMN* promoter to reduce the number of mutants affecting transcription.

Saturation mutagenesis of the *flgKL* class 3 promoter

The random mutagenesis demonstrated that most of the point mutations affecting transcription from the class 3 promoter are located in sequences matching the consensus. Therefore, primers were designed to construct all three possible nucleotide changes at each conserved position in the *flgKL* class 3 promoter. For eight conserved positions in the −35 region and nine conserved positions in the −10 region, 39 additional mutations were generated that primarily decreased class 3 transcription (Figure 3b).

The mutagenesis data reveals that some positions in the promoter are more important for activity than would be expected from the consensus sequence. The 'CGA' in the center of the −10 region (gcCGAtaa) seems to be most important for activity, even though other bases in the −10 and −35 regions are nearly as conserved (−59T, −56A, −40G, −39C, −35T, −34A). Mutations at −59T and −57A result in the largest decreases in transcription in the −35 hexamer (TaAagttt), despite similarly well-conserved bases at −56A and −55G. Several bases show some conservation but contribute very little to the activity of the promoter (−58A, −54T, −53T, −52T, −41T, and −33A).

Similar results were observed in Yu, *et. al.*, where a chlamydial class 3 promoter was saturated for mutagenesis and assayed for transcriptional activity *in vitro*.[29] While both their *in vitro* and our *in vivo* data assigned similar importance to positions within the class 3 promoter (*e.g.*, the 'CGA' in the middle of the −10 hexamer), we were unable to directly correlate the data sets (data not shown). Changes in activity in our *in vivo* data were generally reflected by much larger changes in their *in vitro* data. These discrepancies probably resulted from a combination of studying different class 3 promoters that have spacers of different lengths under different conditions (*in vitro* vs. *in vivo*).

One explanation for this pattern is that σ^{28} is overexpressed in our reporter system. As demonstrated later, these high σ^{28} levels probably compensate for binding defects in the promoter. Bases involved in initial binding of σ^{28} would not be as important for activity with these high levels of σ^{28}. Therefore, those bases that are more important for activity than expected from the consensus sequence (*e.g.*, the 'CGA' in the middle of the −10 hexamer) are likely to be important for steps in transcription initiation after initial binding.

Insertions and deletions in the spacer for the *flgKL* class 3 promoter

In *E. coli* and *Salmonella*, the well-conserved octamers of the −10 and −35 regions in class 3 promoters are separated by an 11 bp spacer.[5] However, in the *in vitro* study of Yu, *et. al.*,[29] spacers of 11 bp or 12 bp worked equally well for class 3 transcription using *E. coli* σ^{28} and RNA polymerase. Also, a 10 bp spacer showed only a 40% decrease in promoter activity. To determine whether transcription *in vivo* shows the same tolerance for different spacer lengths, insertions or deletions were introduced into the spacer for the *flgKL* class 3 promoter (Figure 4). The pattern of transcription *in vivo* was nearly identical to the Yu, *et. al. in vitro* data.[29] The only major difference was that the promoter with the 10 bp spacer was not transcribed well *in vivo* but exhibited about 60% of wildtype activity *in vitro*. Given the nearly equal

activities for class 3 promoters with 11 bp and 12 bp spacers, it is unclear why only 11 bp spacers are observed in *Salmonella* and *E. coli*.

Construction of a class 2 promoter consensus sequence

A consensus sequence for the palindromic $FlhD_4C_2$ binding site in the class 2 promoter was constructed to help determine which positions were likely to be important for transcription. Using the $FlhD_4C_2$ consensus sequence from Stafford, *et. al.* as an initial search pattern,[13] promoter regions from *Salmonella*, *E. coli*, and *Proteus* that have experimental evidence for containing a class 2 promoter were searched. To increase the sample size, promoter regions for flagellar genes in related organisms (*Erwinia*, *Photorhabdus*, *Shigella*, and *Yersinia*) were also searched. Both halves of the palindromic binding site for each of the 43 matches were aligned to construct a consensus sequence. As has been observed before,[6] the $FlhD_4C_2$ binding site is not as well conserved as the σ_{28} binding site (Figure 3a and Figure 5a). This conservation can be quantified by determining how much information or nonrandomness is present in the alignment. Based upon this entropy measurement, the σ^{28} site (12.4 bits entropy) contains 15 times more information than the $FlhD_4C_2$ half-site (8.5 bits entropy).

In reality, the class 3 promoter likely contains much more entropy than reported here. Because each of the four nucleotides might not be represented at each position in a sequence alignment, “pseudocounts” are added into the regular counts of nucleotides at each position. Pseudocounts introduce flexibility into the model to correct for small sample sizes. Pseudocounts allow potential binding site matches to contain nucleotides that were not observed in the alignment. Because 17 experimentally verified promoters were used in the class 3 promoter alignment, the four pseudocounts that were added greatly reduced the entropy of the overall site. These four pseudocounts had a much smaller effect on the 86 half-sites in the $FlhD_4C_2$ alignment. If only one pseudocount had been added, the σ^{28} site (19 bits entropy) would contain twice the entropy or over 700 times more information than the $FlhD_4C_2$ half-site (9.5 bits entropy). $FlhD_4C_2$ likely recognizes class 2 promoters specifically by interacting with two of these less conserved DNA sites.

Since some of the 43 binding sites in the alignment are shared between divergent promoters (*flgB-L* and *flgAMN*, and *fliE* and *fliF-K*), an alignment including these binding sites might obscure directional information in the promoter. Therefore, the consensus sequence for the alignment of the “unidirectional” binding sites that activate a single promoter (30 sites) was constructed (Figure 5b,c). We refer to the half of the palindromic binding site closest to the −10 hexamer as the “proximal” site and the half-site farther upstream as the “distal” site. While the proximal and distal sites have much in common, several positions have different conserved bases (positions 1, 4, 6, 9, 10, 15, 16, and 17). Finally, several T’s are conserved in the center of the spacer when oriented to the distal site, whereas A’s are conserved at those same positions when looking at the proximal site.

Saturation mutagenesis of the *fliAZY* class 2 promoter

Most point mutations that affected class 2 transcription were either in the −10 hexamer, the sequences matching the $FlhD_4C_2$ binding site, or in the spacer between the $FlhD_4C_2$ proximal and distal sites. Since the −10 hexamer for σ^{70} has already been well characterized, we further investigated the class 2 promoter by saturating mutagenesis for the proximal $FlhD_4C_2$ site. The mutagenesis data for the proximal site tended to reflect the conservation of bases in the consensus sequence (Figure 5e). The most important base pairs in the proximal site were grouped into two segments (YaATCg---GAATAarr; where Y = C/T and R = A/G) that were separated by three base pairs that had little effect on activity when mutated. Although the structure of the $FlhD_4C_2$ complex was recently solved in the absence of its DNA binding site,[11] the authors proposed a model where each DNA half-site made two major contacts with an

FlhD dimer. These two contacts were separated by about 4 bp of noninteracting DNA, which is very similar to the 3 bp of nonessential DNA that we see here.

Five of the positions in the distal site that were predicted by the sequence alignments to be different from the corresponding positions in the proximal site were also saturated for mutagenesis. Three of these positions (10, 15, and 17) showed similar nucleotide preferences for both the proximal and distal sites when mutated (Figure 5d,e). At position 6, the distal site preferred a G/C and the proximal site preferred a G as predicted by the consensus sequences. At position 16, the distal site preferred a G and the proximal site preferred an A, which was not predicted by the consensus sequences. These differences could reflect a different requirement for binding where $FlhD_4C_2$ contacts RNA polymerase. This mutagenesis data also suggests that most of the differences in the consensus sequences were not significant, and it is not surprising that these poorly conserved consensus sequences were not entirely reliable.

Additionally, another group has suggested that the −35 hexamer for σ^{70} (TTGACA) overlaps the outermost three bases in $FlhD_4C_2$'s proximal site (TTG and TTA on the nontemplate strand).[13] Therefore, we saturated mutagenesis for these positions in both the proximal and distal sites. If these three positions in the proximal site are important for interacting with σ^{70}, then mutating these same bases in the upstream distal site should not affect promoter activity. The mutagenesis revealed that both sites prefer TTG and TTA (Figure 5d,e), suggesting that these nucleotides are conserved to allow binding of $FlhD_4C_2$. However, the *fliAZY* promoter that was mutagenized might not utilize this potential −35 hexamer due to a short 14 bp spacer for σ^{70}.

Finally, the sequence alignments indicated that the center of the spacer between the $FlhD_4C_2$ proximal and distal sites is enriched for T's. Individual mutations to A, C, or G at these positions reduced the activity of the promoter, which suggests that there is a preference for T's (Figure 5d). Since a run of A's or T's can put a bend in the DNA,[30] these four T's may be needed for DNA curvature and not for protein interactions. To test this, these four T's were replaced with four A's, and this new *fliAZY* promoter initiated transcription at near wildtype levels (Figure 6). In the model for $FlhD_4C_2$ interaction with its binding site,[11] a bend is located in this spacer, which agrees with our data suggesting a preference for a run of A's or T's.

Insertions and deletions in $FlhD_4C_2$'s spacer

To determine which spacers are compatible with a functional $FlhD_4C_2$ binding site, insertions and deletions were constructed in the 12 bp spacer of the *fliAZY* promoter (Figure 6). The promoter does well with an 11 bp spacer and tolerates some 10 bp spacers. A 13 bp spacer, however, does not function well. This mutagenesis data somewhat agrees with our sequence alignments, where the $FlhD_4C_2$ spacer is predicted to be 11 or 12 bp in length. Additionally, DNA encompassing one turn of the DNA helix (10 or 11 bp) from either the *flgB* or *fliE* spacer was inserted into the *fliAZY* spacer. These mutants showed low transcriptional activity (Figure 6). This suggests that the $FlhD_4C_2$ sites need to be close to each other and not merely positioned on the proper face of the DNA.

Comparison of log-odds scores to the activities of mutated promoters

The changes in promoter activity due to mutations were compared to the corresponding changes in log-odds scores calculated from the sequence alignments (Figure 7). Log-odds scores assigned to individual nucleotides are derived from the frequency of a given nucleotide within an alignment. The odds that a nucleotide at a certain position is not there by chance is the "odds" in the log-odds score. For example, there are 2:1 odds if the nucleotide is present at twice the normal frequency. The unit for a log-odds score is the bit since the logarithm (base

2) of the "odds" is used. A change in a log-odds score for a binding site is often assumed to directly correspond to the change in binding energy or the change in promoter activity.[15]

To test this assumption for the $FlhD_4C_2$ binding site and the class 3 promoter, the changes in promoter activities due to mutations were plotted against the changes in log-odds scores (Figure 7a,b). While there was an overall linear relationship between activity and log-odds score for the mutations in the $FlhD_4C_2$ binding site, there was a wide range of observed activities for each score (correlation coefficient of r = 0.74). Therefore, the log-odds score for the $FlhD_4C_2$ binding site would likely be an adequate predictor for transcription from class 2 promoters. In contrast, there was a nonlinear relationship between activities and log-odds scores for the class 3 promoter. Almost half the mutations resulted in activities that were within 15% of the wildtype promoter activity but had a wide range of log-odds scores. Most of the remaining mutations had log-odds scores between −3 and −4 but had a wide range of activities.

An explanation for this pattern is that σ^{28} was overexpressed in our strains and these high σ^{28} levels compensated for small binding defects in the promoter. These same σ^{28} levels may not be high enough to compensate for larger binding defects. To determine whether overexpression of σ^{28} was responsible for this pattern, we reduced σ^{28} levels by mutating the arabinose promoter that transcribes σ^{28} in our reporter system. As judged by a transcriptional fusion to the class 3 *motAB* promoter, the base changes introduced in the arabinose promoter were able to reduce class 3 transcription from 162% of wildtype levels in the overexpression strain to either 110% or 64% of the wildtype level. A subset of our class 3 *flgKL* promoter mutants were transferred to these backgrounds that expressed reduced levels of σ^{28} (Figure 7c). If the initial binding of σ^{28} at the *flgKL* promoter was not a rate-limiting step in transcription initiation, the activities of all the mutants should have been reduced by the same percentage when σ^{28} levels were reduced. Since mutants with intermediate levels of class 3 transcription (*e.g.*, 50% of wildtype) decreased their activity the most when σ^{28} levels were reduced, the pattern in Figure 6b is likely an artifact of overexpressing σ^{28}. The correlation coefficient for the medium level of class 3 transcription (r = 0.77 for 15 mutants) revealed a somewhat more linear relationship between activity and log-odds score than under the original high levels of class 3 transcription (r = 0.60 for 71 mutants). These results underscore the importance of using physiologically relevant levels of transcription factors when examining promoters.

Interestingly, only a 62% increase in class 3 transcription levels was enough to compensate for binding defects in the *flgKL* promoter. σ^{28} may normally be expressed inside the cell at levels where it is sensitive to small changes in binding affinity in potential class 3 promoters. The binding affinity of the class 3 promoters may be optimized so that the level of σ^{28} in the cell increases and decreases past the steepest part of a Michaelis-Menton curve to produce the largest changes in promoter activity.

Comparison of predicted and observed activities for double mutants

Another assumption frequently made in computational models is that individual base pairs exert independent effects on the overall ability of a protein to bind DNA.[15] When all activities are expressed as a fraction of the activity of the wildtype promoter and two mutations are combined into one binding site, the activity of the double mutant is expected to equal the activity of the first mutant multiplied by the activity of the second mutant. To test this assumption for flagellar promoters, pairs of mutations were combined for certain positions within the promoters. To minimize the work and cost involved, double mutants were constructed for selected pairs of positions within the binding sites. These pairs were chosen in order to sample many positions and a large range of predicted activities. For the $FlhD_4C_2$ binding site in the *fliAZY* promoter, mutations for positions within the proximal site and for positions between the distal and proximal sites were combined (Figure 8a). The activities of the double mutants match well with the predicted activities. For the σ^{28}-dependent promoter for the *flgKL* operon,

mutations for positions within the −10 region, within the −35 region, and between the −10 and −35 regions were combined (Figure 8b). The activities of these double mutants do not directly match with the predicted activities. In most cases for the class 3 promoter, when two mutations of decreased activity were combined, the activity of the resulting double mutant was lower than what was predicted.

Once again, this nonlinear pattern could be the result of high σ^{28} levels compensating for small defects in binding to the promoter but not compensating for large defects in binding (*i.e.*, the double mutant). Therefore, eight of the double mutants were moved into the backgrounds with reduced σ^{28} levels (Figure 8c). Since the defects in transcription for the single mutants became more pronounced when σ^{28} levels were reduced, it is not surprising that the predicted activities of the double mutants (lower activity #1 × lower activity #2) decreased when σ^{28} levels were reduced. However, the same pattern of expression for the double mutants under high σ^{28} levels is repeated when there are medium or low levels of σ^{28}. For all three σ^{28} levels, the predicted activity matches the observed activity for the two most highly expressed double mutants and perhaps a few of the lowest expressed mutants. For the other double mutants, the observed activity is about 40% of the predicted activity for all three σ^{28} levels. Therefore, the pattern for the activity of the double mutants is not an artifact of overexpressing σ^{28}. The repetition of the pattern for a range of σ^{28} levels indicates that steps in transcription initiation after binding are being affected.

Introduction of elements of other class 3 promoters into the *flgKL* promoter

To determine how well alignment data could predict the activity of real class 3 promoters, elements of the *tar*, *motA*, *cheV*, and *aer* promoters were introduced into the *flgKL* class 3 promoter. Since the alignment data showed significant conservation in only the −10 and −35 regions, these elements were first substituted into the *flgKL* promoter (Figure 9, gray circles). As the modified promoters increasingly matched the consensus sequence, the activities generally increased. The exception to this trend is that the introduced −10 and −35 regions of the *motA* promoter increased activity more than the similarly scored wildtype *flgKL* promoter and the higher scoring *tar* promoter. To determine how sequences that are not conserved affect class 3 transcription, the intergenic region upstream of the transcription start site of the *flgKL* promoter was replaced by sequences from other class 3 promoters comprising the DNA upstream of the transcription start site through 25 bp upstream of the −35 region (Figure 9, black circles).The additional sequences increased the activity from promoters containing the less-conserved *aer* and *cheV* −10 and −35 regions and reduced activity from promoters containing the better-conserved *motA* and *tar* −10 and −35 regions. For the four promoters examined, these nonconserved sequences had a significant moderating effect on overall promoter activity. While the data for these modified promoters did not reveal any strong relationship between conservation and activity, the two less-conserved promoters did exhibit lower activities than the two better-conserved promoters.

Discussion

The mutagenesis data in this paper illustrates some of the difficulties involved in predicting flagellar promoters within a genome sequence. While the location of most of the point mutations affecting transcription from the class 2 and class 3 promoters could be predicted from the consensus sequences, a few mutations were isolated near these conserved regions or in the UTR. These nonconserved regions played a significant role in determining the overall activity when substituted into a class 3 promoter. Additionally, sites for the activator $FlhD_4C_2$ (the class 2 promoter) better satisfied assumptions about protein binding than did σ^{28} sites (the class 3 promoter). The activities for mutations in an $FlhD_4C_2$ binding site correlated with the frequency of nucleotides in an alignment and combined independently with

other mutations. The σ^{28} site did not satisfy either assumption as well. This may reflect the sensitivity of class 3 promoters to σ^{28} levels as well as the multiple roles that σ^{28} plays in transcription initiation (*e.g.*, initial binding, open complex formation, and promoter clearance). This genetic data suggests that the binding sites for flagellar transcription factors and sigma factors should not be modeled the same way.

The saturation mutagenesis for the class 2 promoter revealed the nucleotide preferences for the poorly conserved $FlhD_4C_2$ binding site. This study systematically mutated each position in one half of the palindromic $FlhD_4C_2$ binding site. This saturation mutagenesis highlighted two segments separated by three nonessential base pairs in the half-site that were important for activity. In a model for $FlhD_4C_2$ binding, two contacts were formed between an FlhD dimer and each half-site in the DNA.[11] Our data provides some support for this model since these two contacts formed with the DNA is similar to the two important segments identified by the mutagenesis data. Additionally, this model proposed a 110° bend in the spacer DNA that separates the two half-sites. Our mutagenesis data demonstrated that a run of A's or T's is preferred in the spacer. Since a run of A's or T's can put a bend in the DNA, this data provides some further support for the model. To definitively test this model, crosslinking experiments or mutagenesis of FlhD and FlhC would need to be performed.

The saturation mutagenesis for the class 3 promoter revealed that some base pairs were far more important for activity than would be expected from the consensus sequence. In particular, the 'CGA' in the middle of the −10 region was as well conserved as other bases in the alignments but had much larger effects on transcription when mutated. Similar results were obtained by *in vitro* transcription for a different class 3 promoter.[29] However, when the amount of σ^{28} in our reporter system was decreased to better approximate the wildtype level, mutations that previously had little effect on class 3 transcription now exhibited greater defects in transcription. This indicated that overexpressing σ^{28} could compensate for small binding defects in the promoter. Therefore, the consensus sequence is likely to be a better approximation for activity than was revealed by our class 3 promoter mutagenesis data.

Surprisingly, this compensation for binding defects occurred when overexpressed σ^{28} increased transcription from wildtype class 3 promoters by only 62%. This suggests that σ^{28} is normally present in the cell at levels that are responsive to small changes in binding affinity at class 3 promoters. Conversely, when σ^{28} levels increase after the flagellar regulon is induced, initially low σ^{28} levels may turn on strong class 3 promoters first as proposed by Kalir, *et. al.* [3] These strong promoters could express genes for adaptor proteins (FlgK, FlgL, and FliD) needed earlier in flagellar assembly than other class 3 expressed genes (*e.g.*, the flagellin subunits).

The lack of normal flagellar regulation in our reporter strain might also contribute to the nonlinear relationship between activity and log-odds score for the class 3 promoter. By expressing σ^{28} from the arabinose promoter in the absence of class 1 and class 2 transcription, σ^{28} was always expressed despite the lack of both the motor and hook. Class 3 transcription normally occurs only after completion of these two structures. As a result, σ^{28} expression was not coordinated with flagellar assembly and lacked the normal negative feedback through FlgM that moderates its activity. The activity of flagellar promoters in the context of normal flagellar regulation might exhibit a better correlation to the log-odds score.

An assumption in binding models is that each base pair affects binding independently from other base pairs in the binding site. Double mutations in the $FlhD_4C_2$ binding site satisfied this assumption. For the class 3 promoter, most mutations did not combine independently. Most mutants had an observed activity that was 40% of what was predicted. This pattern held even when σ^{28} levels were reduced. The unusual behavior of σ^{28} could be due to the additional

complexity of a sigma factor. Besides recognizing its binding site, sigma factors help RNA polymerase to melt the DNA strands, initiate RNA synthesis, and aid in promoter clearance.[31] While initial binding of σ^{28} was revealed to be a critical stage for class 3 transcription in this study, the double mutant analysis demonstrated that other steps in transcription initiation contribute to the strength of a class 3 promoter. The 'CGA' in the −10 region that was so important for activity most likely plays a role in these later steps. Since mutating the 'CGA' nucleotides resulted in severe transcription defects even under high σ^{28} levels, these mutations probably affect steps after binding. In contrast, $FlhD_4C_2$ most likely activates transcription simply by binding upstream of RNA polymerase and contacting the alpha subunit.[10] The extra roles that σ^{28} plays may alter the manner in which bases in the promoter contribute to transcription initiation.

Genome-wide searches for DNA binding sites tend to be difficult due to large numbers of false positives.[15] This high background is often a result of low information content in a binding site combined with a large DNA search space. Also, not all proteins may satisfy assumptions that relate binding ability to the frequency of a base pair in an alignment and presume that base pairs contribute independently to the overall site. In this study, we demonstrated *in vivo* that an activator for the class 2 flagellar promoters satisfied these assumptions and that the alternate sigma factor for the class 3 promoters perhaps satisfied only one assumption. These results suggest that simple models for DNA binding can be applied to the activator proteins for promoters but are not as appropriate for the sigma factors. Allowing for this alternate behavior presents additional challenges in designing computational models for promoters. However, these insights may enable better recognition of DNA binding sites and promoters.

Materials and Methods

Bacterial strains

The strains used in this study were derived from *Salmonella typhimurium* strain LT2 and are listed in Table 1. Promoter mutants are listed in Supplemental Table S9.

Media and general techniques

Cultures of bacterial strains and phage P22 lysates were prepared as described,[32] except that X-gal was added to plates at a concentration of 40 μg/ml and LB (per liter: 10 g Bacto tryptone, 5 g Bacto yeast extract, 5 g NaCl) was used as a rich medium for growing bacteria. Chlortetracycline (50 μg/ml, autoclaved) or 0.2% arabinose (Ara) were used to induce transcription from the *tetA* and *araBAD* promoters, respectively. Tetracycline-sensitive (Tet^S) selections and transductions were performed as described,[33,34] except that LB was used instead of NB. Primers were synthesized by Integrated DNA Technologies (Coralville, IA). PCR products were sequenced at the DNA Sequencing Facility (Department of Biochemistry) at the University of Washington or at the DNA Sequencing Core Facility at the University of Utah.

Using the λ-red recombination proteins expressed from plasmid pKD46, PCR products were recombined into the chromosome.[35] Each 25 ml LB-Ampicillin-Ara culture was inoculated with a single colony and grown with shaking at 30°C until the culture reached an OD_{600} of 0.6. The cells were washed twice in cold, sterile water and resuspended to a final volume of 100 μl to 500 μl with water. An ethanol-precipitated PCR product was electroporated into 50 μl of cells, incubated at room temperature for 1 hour, and spread on selective media. Recombinants were selected at 37°C or above to ensure loss of the temperature-sensitive pKD46 plasmid.[35]

Overview of random mutagenesis of flagellar promoters

Random mutations were introduced into the promoter regions for the four flagellar operons of *Salmonella* that are transcribed from both class 2 and class 3 promoters. Random mutations in the promoter DNA sites were generated by amplifying the promoter regions using high concentrations of Taq polymerase (5U Promega Taq polymerase per 20 μl reaction)[36]. These PCR products were moved into the chromosome by using λ-red recombination to replace a Tet^R cassette in the promoter region. Tet^S recombinants were isolated on Tet-sensitive selective media. Individual colonies were purified by single colony isolation on LB-plates before being screened on lactose indicator media (LB-X-gal, MacConkey-lactose, and Triphenyl Tetrazolium Chloride (TTC)-lactose plates) for changes in transcription from *lac* fusion reporters inserted downstream of the mutagenized promoters.

To assay class 2 transcription independent of class 3 transcription, a strain that expresses the *flhDC* operon from a tetracycline-inducible promoter and *fliA* (σ^{28}) from an arabinose-inducible promoter was constructed (Figure 1). When this strain (TH8927) is grown in the presence of chlortetracycline, *flhDC* is transcribed and class 2 promoters are activated. When the strain is grown in media containing arabinose, *fliA* (σ^{28}) is transcribed and class 3 (σ^{28}-dependent) promoters are activated. Mutants could immediately be screened for changes in class 2 transcription independent of changes in class 3 transcription by checking the Lac phenotype on indicator plates containing chlortetracycline or arabinose, respectively.

Construction of strains used in random mutagenesis

The Δ*araBAD956*::*fliA*$^+$ allele in TH8927 was constructed by amplifying the *fliA* coding sequence with primers araBfliAF and araDfliAR (primers are listed in Table 2) and then using λ-red recombination to replace the Δ*araBAD*::*tetRA* in TH8006 with the amplified *fliA*$^+$ DNA. The *tetRA* cassette encodes the tetracycline resistance determinants from transposons Tn*10* and Tn*10d*Tc. This Δ*araBAD956*::*fliA*$^+$ allele replaces the *araB* start codon through the *araD* stop codon with the start codon (ATG) through the stop codon of *fliA*. The P*flhDC5451*::Tn*10d*Tc[Δ*25*] allele from TH3730 was transduced into TH8925 to give TH8926. The P*flhDC5451*::Tn*10d*Tc[Δ*25*] allele is an operon fusion of *tetA* to *flhDC* that is expressed from the *tetA* promoter. A spontaneous mutant of TH8926 that was no longer Tet^R but could induce class 2 transcription was selected for on Tet-sensitive plates (TH8927). This Tet^S mutant is presumably defective in the tetracycline resistance gene (*tetA*) but still transcribes the *flhDC* operon from P*tetA*.

MudJ and MudK vectors were used for the construction of *lac* transcriptional and translational fusions, respectively, to operons of interest.[37] The *lac* reporter fusions used in this study were *flgM5222*::MudJ (TH2779), *fliS5480*::MudK (TH4212), *flgK5396*::MudJ (TH4721), and *fliZ6591*::MudJ (TH10049). The *fliZ6591*::MudJ insertion allele was isolated in this study and is described in a later section. The Tet^R markers used in the Tet-sensitive selections were P*fliD5744*::Tn*10d*Tc (TH5794), *flgJ5964*::*tetRA* (TH7270), *flgA6093*::*tetRA* (TH8091), *fliA6399*::*tetRA* (TH9250), *fliA6785*::*tetRA* (TH10826), and *fliA7226*::*tetRA* (TH11808). In order to construct the *tetRA* cassette insertion alleles, primers flgJtetR and flgKtetA (TH7270), flgAtetR and flgMtetA (TH8091), fliAPtetR and fliAPtetA (TH9250), fliAtetR2 and fliAPtetA (TH10826), and fliAPtetR and fliAPtetA2 (TH11808) were used to amplify *tetRA* cassettes from TH5794. The λ-red system was used to recombine these PCR products into the chromosome, and Tet^R colonies were selected. These *lac* reporters and Tet^R markers were moved by transduction into TH8927.

Random mutagenesis of promoters

The *flgMN* class 3 promoter was mutagenized (replacing −132 to +24 bp relative to the *flgM* start codon) by recombining an error prone PCR product (primers flgM5'UP and flgM+24/

+5R, 45°C annealing) into TH8938. The *flgMN* promoter regions of recombinants were sequenced using primer flgA+417/+436 and the amplified promoter (primers flgA+417/+436 and flgM+61/+45, 45°C annealing).

The *flgKL* class 3 promoter was mutagenized (replacing −105 to +42 bp relative to the *flgK* start codon) by recombining an error prone PCR product (primers flgK−105/−86F and flgK +42/+23R, 60°C annealing) into TH8315 or TH8936. The *flgKL* promoter region of recombinants was sequenced using primer flgJ5'RTPCR and the amplified promoter (primers flgJ5'RTPCR and flgK+100/+81, 45°C annealing).

The *fliDST* class 2 and class 3 promoter region was mutagenized (replacing −196 to +75 bp relative to the *fliD* start codon) by recombining an error prone PCR product (primers fliD−196/−177F and fliC13, 50°C annealing) into TH8321. Alternatively, the *fliDST* promoter was mutagenized (replacing −196 to +4 bp relative to the *fliD* start codon) by recombining an error prone PCR product (primers fliD−196/−177F and fliD+4/−16, 60°C annealing) into TH8937. The *fliDST* promoter regions of recombinants were sequenced using primer fliC+39R and the amplified promoter (primers fliD+190R and fliC+39R, 60°C annealing).

The *fliAZY* class 2 and class 3 promoter region was mutagenized (replacing −156 to +5 bp relative to the *fliA* start codon) by recombining an error prone PCR product (primers fliA+5/−15 and fliA−156/−137, 55°C annealing) into TH9252 or TH10151. The *fliAZY* promoter region of recombinants was sequenced using primer fliA+720/+697 and the amplified promoter (primers fliA#4 and fliA−225/−209, 60°C annealing).

Twelve *flgKL* and 12 *fliDST* promoter mutants were isolated using TH8315 and TH8321. It was then discovered that the Δ*araBAD943*::*fliA* allele in these strains contained a mutation (P190S). These promoter mutants were then transduced to backgrounds containing wildtype *fliA*$^+$ (Δ*araBAD956*::*fliA*$^+$). All transcriptional activities were quantified in this wildtype *fliA*$^+$ background. No significant difference in Lac activity for these strains was observed between wildtype *fliA* and the *fliA*(P190S) allele.

Eleven *fliAZY* promoter mutants were isolated using TH9252, which contains a *lac* fusion to the *fliAZY* promoter on a P22 prophage. When β-galactosidase assays were attempted on these strains, the β-galactosidase activities disappeared quickly after the cells were lysed (~2 minute half-life). Therefore, the *fliZ6591*::MudJ was isolated (see below) and used for all additional screens of *fliAZY* promoter mutants. The 11 previously isolated mutations were amplified, moved into TH10151, and quantified.

Isolation of *fliZ*::MudJ

In order to detect changes in transcription from the *fliAZY* promoter, an insertion of a MudJ transcriptional reporter in this operon was isolated. Random MudJ insertions were generated[38] in a strain containing the *fliAZY* operon transcribed from P*tetA* (TH10022). Eighty thousand colonies were pooled, and a phage stock was prepared on the pooled colonies. This pool of random insertions was transduced into TH8925 (Δ*araBAD*::*fliA* Δ*fliA*::*FRT*) and spread onto LB-Tet-Kan-EGTA plates to select for transductions that had brought in the *fliAZY* region (selecting Tet^R) and nearby MudJ insertions (selecting for MudJ-encoded kanamycin resistance). Four hundred transductants were patched to X-gal, MacConkey-lactose, and TTC-lactose plates containing either arabinose, chlortetracycline, or no inducer. Five colonies exhibited a Lac$^+$ phenotype on the chlortetracycline plates and were Lac$^-$ on the arabinose and no inducer plates. This pattern would be expected for an insertion in the P*tetA*-*fliAZY* operon but not in other class 3 transcribed genes. One out of the five insertions was located in *fliZ* by PCR. By sequencing into the right end, the MudJ was confirmed to be inserted after V38 in *fliZ*. In TH10049, this *fliZ6591*::MudJ was transduced into a wildtype background (TH437).

Construction of specific promoter mutations

Mutations in the *fliAZY* $FlhD_4C_2$ spacer (the DNA between the distal and proximal halves of the $FlhD_4C_2$ binding site) and for the saturation mutagenesis were generated using primers containing the desired mutations. These primers were designed with about 40 bp of homology to the promoter (for recombination), then the mutation, and about 20 bp of homology to the promoter (for amplification; ~55°C T_m). These primers were then used with an appropriate primer from the random mutagenesis experiment to amplify the promoter. λ-red recombination was used to move these mutations into the chromosome. When two or three mutations were desired at a certain position, a mixed base was used in the primer. After recombination, 16 or 32 colonies, respectively, were checked for Lac activity on lactose indicator plates, and representative colonies were sequenced. Double mutants were constructed by designing both mutations into a primer or by amplifying a mutant promoter with a primer containing a second mutation (Supplementary Tables S10 and S11).

Measuring the levels of class 2 and class 3 transcription in the reporter strains

Transcriptional fusions to class 2 and class 3 promoters were utilized to determine whether $FlhD_4C_2$ and σ^{28} were being overexpressed in the reporter strains. When $FlhD_4C_2$ was expressed from the *tet* promoter in the absence of σ^{28}, *lac* fusions inserted in the class 2 expressed genes *flgA flgC*, and *fliG* exhibited transcription levels that were 71%, 92%, and 97% of the wildtype level, respectively. Averaging these three measurements, class 2 transcription was 87% of the wildtype level in the reporter strains. When σ^{28} was expressed from the arabinose promoter in the absence of *flhDC* transcription, a *lac* fusion inserted in the class 3 gene *motA* was transcribed at 162% of the wildtype level.

Construction of mutations in the arabinose promoter

To reduce σ^{28} levels, mutations were constructed in the arabinose promoter region that expresses σ^{28} in the reporter strains. First, a *tetRA* cassette was amplified from TH5794 using primers ParaBtetR and ParaBtetA (49°C annealing temperature) and inserted into the arabinose promoter using λ-red recombination (TH12906). This *tetRA* was transferred to a strain containing a transcriptional fusion to the *motA* class 3 promoter (TH12990). Next, two promoter mutations that were initially isolated in Aldridge, *et. al.*[36] to lower FlgM levels in a P_{ara}-*flgM* strain were tested. Primers araC+5R and ara5' were used to amplify these mutations from strains TH7395 (−85 A:T) and TH7396 (−41 T:C). λ-red recombination was used to move these PCR products into the chromosome to replace the *tetRA* in the arabinose promoter. TH13035 expressed the *motA* class 3 transcriptional fusion at 110% of wildtype levels, and TH13036 expressed the fusion at 64% of wildtype levels. These mutated arabinose promoter alleles were transferred to a strain containing a *tetRA* insertion in the *flgKL* promoter and a wildtype *motA* gene (TH13127 and TH13128). *flgKL* promoter mutants were transduced into these backgrounds by selecting for the Kan^R *flgK*::MudJ allele and screening for loss of the *tetRA* insertion.

Formation of consensus sequences

In order to construct consensus sequences, a position specific score matrix algorithm (PSSM) [16] was implemented in Java. The PSSM uses the frequency of nucleotides at each position in an alignment to assign log-odds scores. The log-odds score is equal to the logarithm (base 2) of the frequency of a nucleotide at a position in an alignment divided by the background frequency of that nucleotide. The unit for a log-odds score is the bit since the logarithm (base 2) of the "odds" are used. The PSSM evaluates potential binding sites by adding up log-odds scores for each position in the binding site.

This "log-odds" method was utilized instead of the closely related information theory approach. The difference between these two techniques arises from the choice of background frequencies for calculating the significance of each nucleotide in a sequence alignment. If a nucleotide appears more frequently in the alignment than observed in the background distribution of nucleotides, a positive score is calculated. For the log-odds method that was used, the frequency of nucleotides in the intergenic regions were used for the background frequencies.[16] In information theory, each nucleotide is given the same background frequency (*i.e.*, 0.25).[39]

The argument for using information theory is that there is no "external observer" of the genome composition involved in the binding process. The information gained by the system would only depend on the change in entropy from the regulatory protein being unbound in the *before* state to being bound to the DNA site in the *after* state. Since the regulatory protein "does not make physical contact with the nucleic acid bases in the *before* state the composition of the genome should not matter".[39] We argue that the log-odds method is a valid technique because the evolutionary process is an observer of the system. In order for a regulatory protein to specifically recognize its DNA binding sites in the genome, the DNA binding sites must contain enough information to be distinguished from random sequences. If the genome is GC rich and the sites the regulatory protein binds to are GC rich, there are more random sequences to which the regulatory protein can bind than if the genome were AT rich. The regulatory protein will be selected against if it improperly regulates essential genes or important pathways through binding of too many of these random sequences. Therefore, the evolutionary process likely takes into account the background frequencies of nucleotides in the genome when adapting a regulatory protein for that genome. Since the DNA binding sites for the regulator are then used in sequence alignments, we argue that it is valid for the log-odds method to take into account these background frequencies.

The nucleotide frequencies in the intergenic regions of a genome were therefore used as the background frequencies for calculating log-odds. Moreover, essentially the same results were obtained when the figures were prepared using information theory (data not shown). Few differences were observed because the frequency of A's or T's in the intergenic regions of the organisms we studied (0.28 in *Salmonella*, for example) are close to the 0.25 frequency used in information theory.

Sequences used in alignments were weighted according to similarity using a neighbor-joining algorithm.[16] Four pseudocounts (multiplied by the background frequencies) were added into the observed counts for each nucleotide at each position.

The consensus sequence for the $FlhD_4C_2$ binding sites was constructed through multiple rounds of PSSM searching. Only the upstream regions for genes (*flgA, flgB, flhB, fliA, fliB, fliD, fliE, fliF, fliL, fepE, narK, rpiA, serA, srfA, yecR, yffO*, and *yqfE*) that have some experimental evidence for an $FlhD_4C_2$ binding site[4,6,9,13,40,41] were searched. The search was limited to 20 bp upstream of the start codon through 100 bp of the gene immediately upstream. After each round of searching, the top matches were used to form a new PSSM. Proximal and distal sites were combined so as not to bias the PSSM toward either site. Matches were not used in the new PSSM if they were nowhere near where the biological data suggested they should be.

The simple consensus sequence identified in Stafford, *et. al.*[13] was used for an initial search. Since simple consensus sequences are not very effective PSSM's, matches with scores above 4 bits were considered. All later searches used a more restrictive cutoff of 10 bits. *Salmonella, E. coli*, and *Proteus mirablis* (gi numbers **16763390**, **49175990**, **6959881**, and **1857436**; *Proteus flhB* sequence in Claret and Hughes[42]), for which biological data on flagellar promoters is available, were iteratively searched until no new matches were detected. To increase the sample size, we then searched upstream of flagellar genes in related organisms

(*Erwinia*, *Photorhabdus*, *Shigella*, and *Yersinia*; gi numbers **50118965**, **37524032**, **30061571**, and **51594359**) until no new matches were detected. Spacers of 10, 11, and 12 bp were tried in each search. However, the final iterations removed all but one of the promoters that matched best with the 10 bp spacer.

β-galactosidase assays

10 μl of an overnight culture (LB) was subcultured into 3 ml of LB + arabinose or LB + chlortetracycline. Tubes were incubated with shaking at 37°C until they reached a mid-log density of 70 Klett units, which corresponds to an OD_{600} of about 0.7. Cultures were put on ice, spun down, and resuspended in 3 ml of cold, buffered saline. Between 50 μl and 0.5 ml of culture was added to 0.55 ml of complete Z-buffer[34] (Z-buffer plus 5 μl 10% SDS and 100 μl chloroform) and buffered saline to give a total aqueous volume of 1.05 ml. The assay was continued as described.[34,43–46] For each strain, assays were performed for three independent biological replicates.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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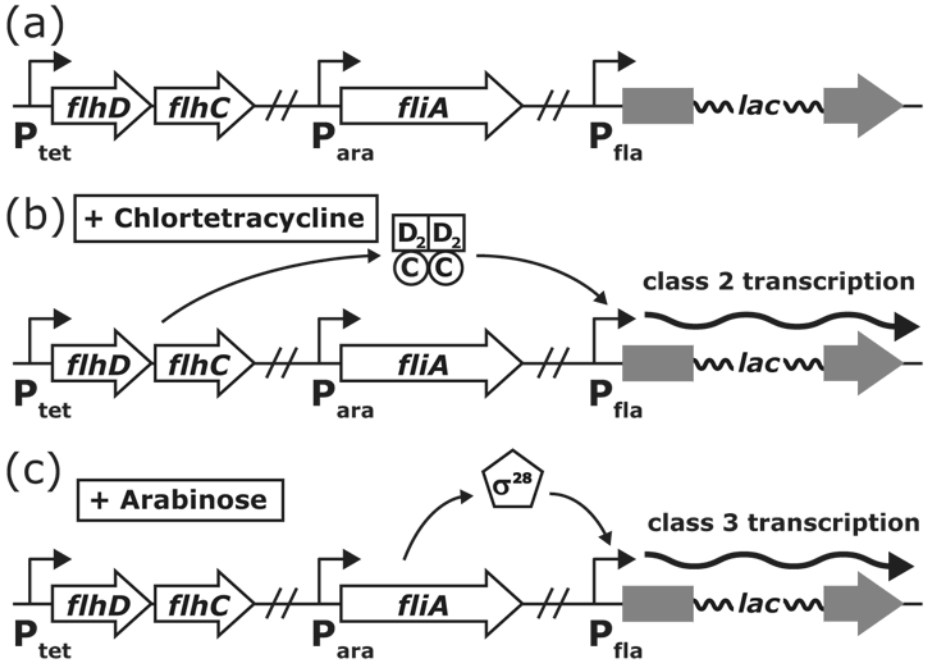


Figure 1.

Reporter constructs for the isolation of class 2 and class 3 promoter mutants. (a) Class 2 transcription was assayed separately from class 3 transcription by putting $FlhD_4C_2$ and σ^{28} expression under the control of inducible promoters. (b) When autoclaved chlortetracycline was added, *flhDC* was expressed from its tetracycline-inducible promoter and resulted in activation of class 2 transcription at 87% of the wildtype level. (c) When arabinose was added, *fliA* (σ^{28}) was expressed from its arabinose-inducible promoter and activated class 3 transcription at 162% of the wildtype level. Transcription was assayed using a *lac*-fusion inserted within the first or second genes of the flagellar operon.

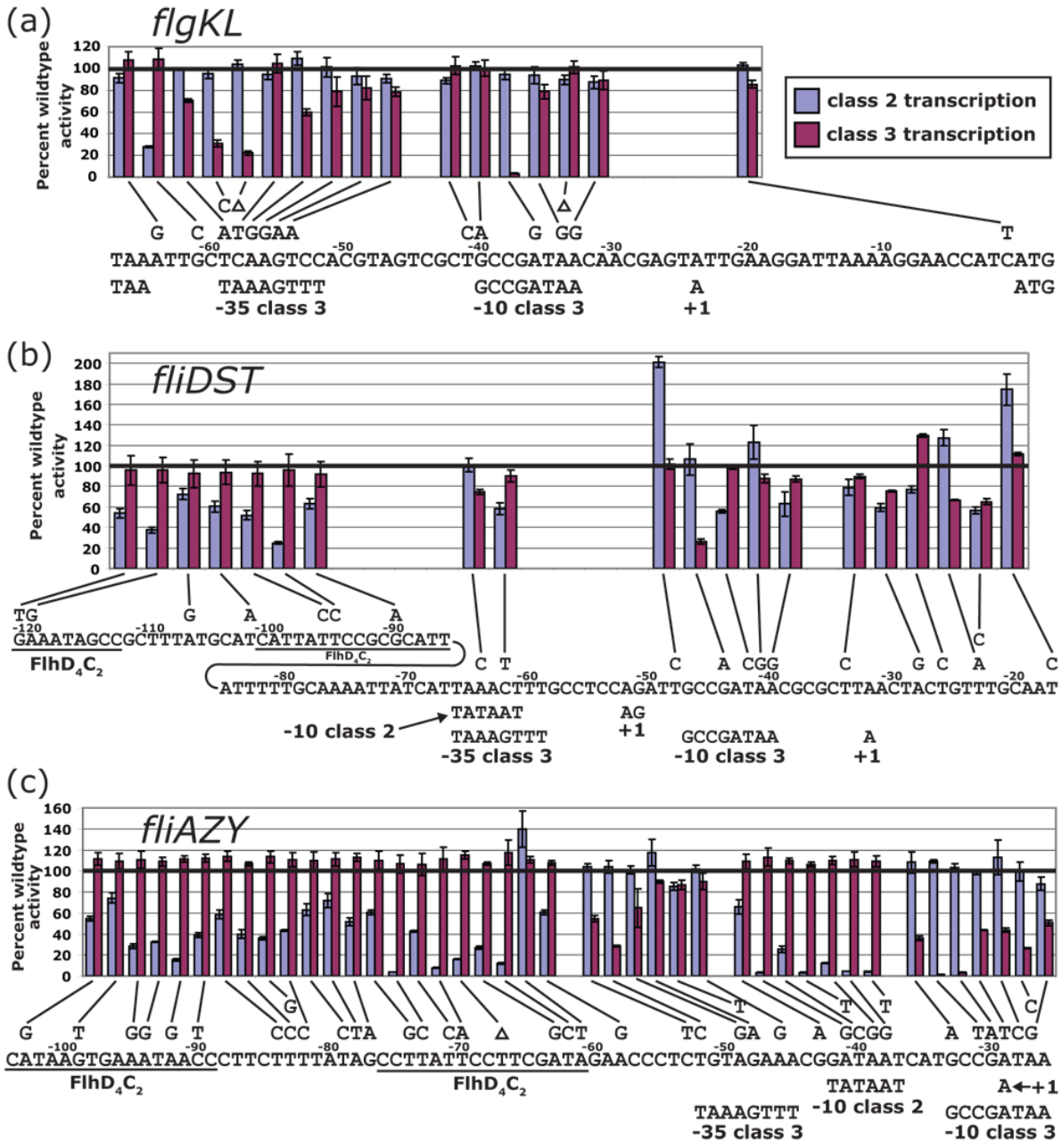


Figure 2.

Locations of random mutations and their affects on class 2 and class 3 flagellar transcription. The *flgKL* (a), *fliDST* (b), and *fliAZY* (c) promoters were screened for mutations affecting class 2 or class 3 transcription. The single base pair mutants that were isolated are displayed above the promoter sequences, and transcriptional start sites[47,48] and consensus sequences are below. $FlhD_4C_2$ binding sites are underlined. When either class 2 transcription ($FlhD_4C_2$) or class 3 transcription (σ^{28}) was induced, the activities of *lac*-fusions to these mutant promoters were quantified and normalized to the wildtype promoter (100%). These β-galactosidase activities are connected by a line to their respective mutations. The numbers above the DNA indicate the distance from the start codon.

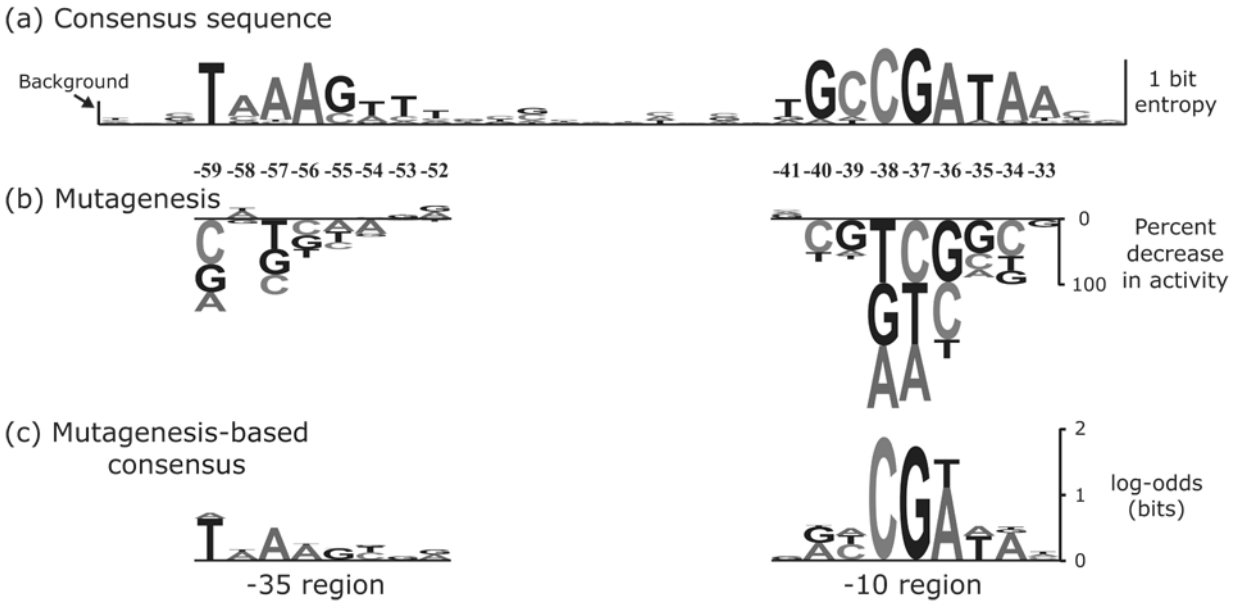


Figure 3.

Comparison of the class 3 consensus sequence to the activities of *flgKL* promoter mutants.(a) The class 3 consensus sequence was constructed from the alignment of 17 flagellar promoters in Table 2 of Ide, *et. al.*[5] The height of each WebLogo-like nucleotide stack[49] corresponds to that position's entropy,[16] which is a measure of the conservation of nucleotides at that position. The frequency of each nucleotide in the alignment is reflected by the height of that nucleotide within the stack. On the left side of the figure, the height of the bar labeled background is twice the small sample correction as calculated by WebLogo.[49] The small sample correction gives the average entropy value for a random alignment of sequences.[50] (b) All single base pair mutations for eight positions in the −35 region and nine positions in the −10 region of the *flgKL* class 3 promoter were constructed. The height of each letter corresponds to the percent change in promoter activity as quantified by β-galactosidase assays. Letters above or below the line indicate an increase or decrease, respectively, in promoter activity. The numbers above the DNA indicate the distance from the start codon. (c) The measured activities in (b) were used as counts in a new "alignment" in order to generate a new consensus sequence. The log-odds scores displayed were calculated using these counts and are a measure of the conservation of nucleotides in the alignment.

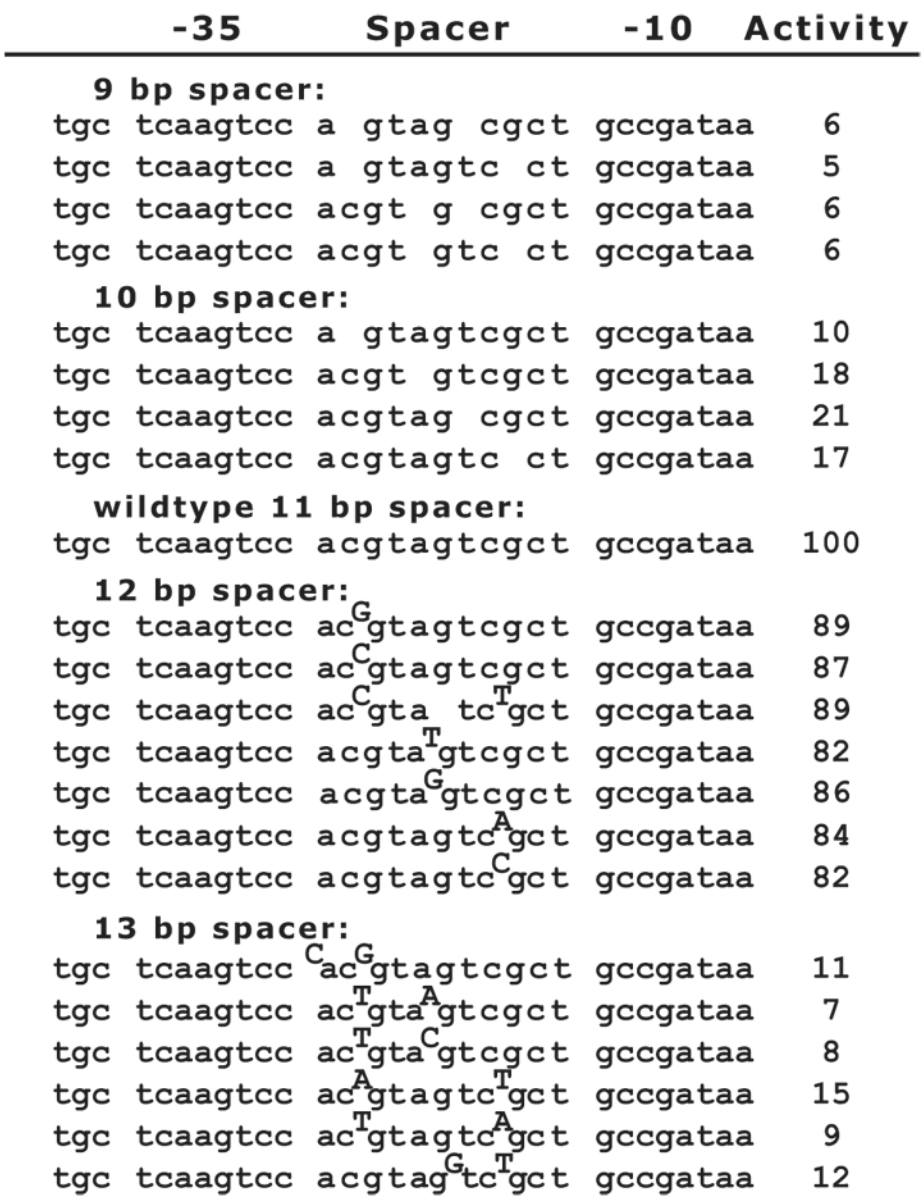


Figure 4.

Insertions and deletions were constructed in the spacer of the *flgKL* class 3 promoter.β-galactosidase activities for these mutants were measured and normalized to the activity of the wildtype promoter (100%). These mutants were assayed in a background containing nearly wildtype levels of class 3 transcription (110% of wildtype).

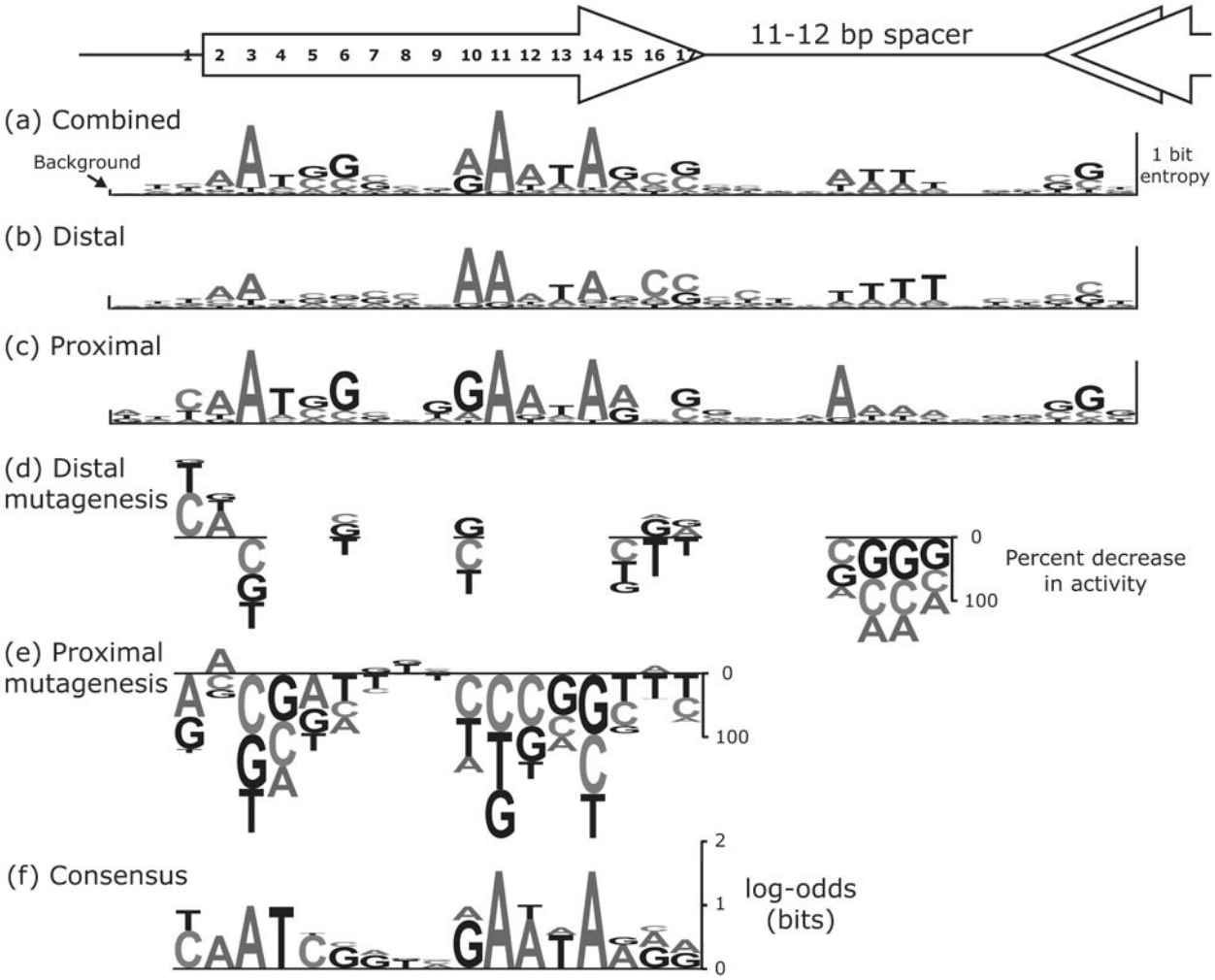
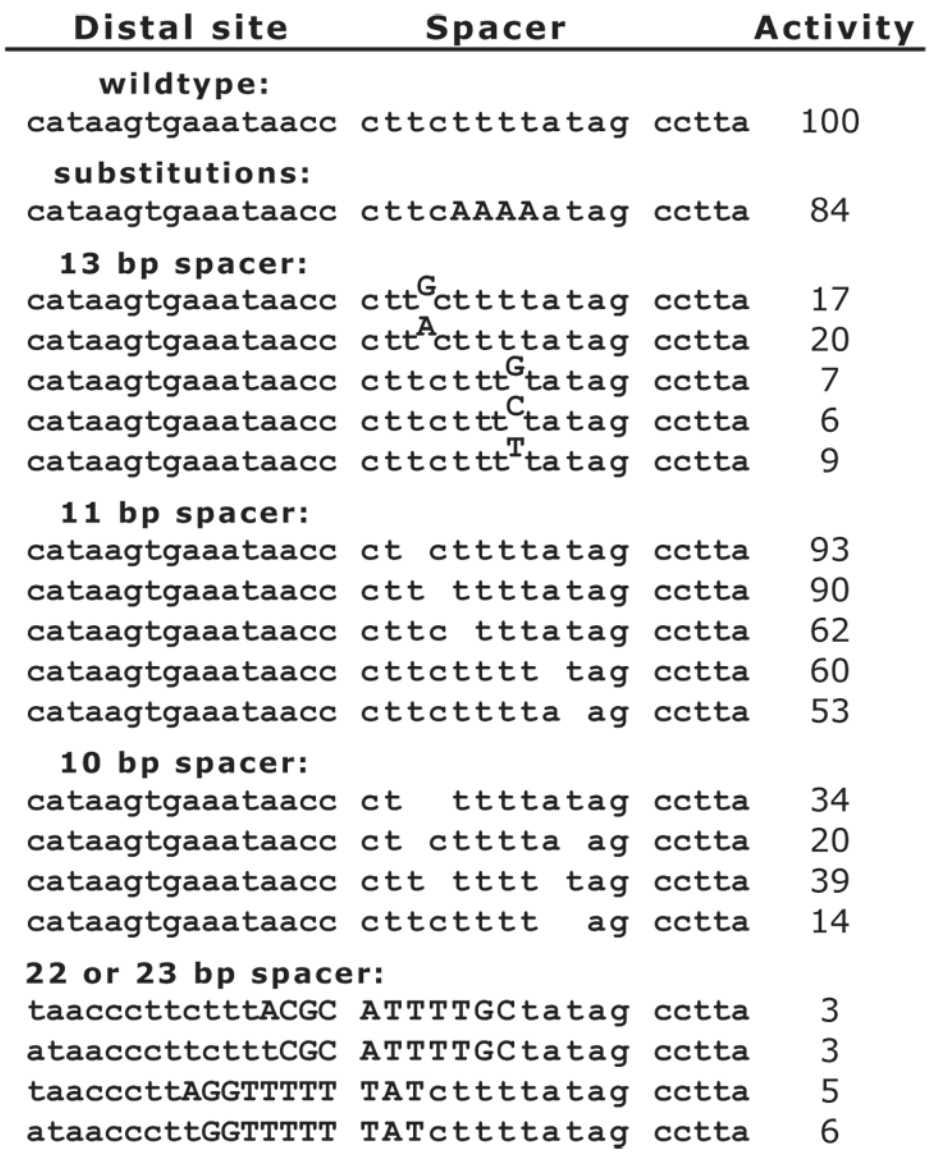


Figure 5.

Comparison of the class 2 consensus sequence to the activities of *fliAZY* promoter mutants.Eighty-six proximal and distal $FlhD_4C_2$ sites were aligned to give the entropies in (a). The entropies for distal sites (b) or proximal sites (c) from an alignment of 30 “unidirectional” promoters are also displayed. The height of each WebLogo-like nucleotide stack[49] corresponds to that position’s entropy,[16] which is a measure of the conservation of nucleotides at that position. The frequency of each nucleotide in the alignment is reflected by the height of that nucleotide within the stack. On the left side of the figure, the height of the bar labeled background is twice the small sample correction as calculated by WebLogo.[49] The small sample correction gives the average entropy value for a random alignment of sequences.[50] All single base pair mutations at eight positions in the distal site (d), at four positions in the spacer (d), and at 17 positions in the proximal site (e) of the *fliAZY* promoter were constructed. The height of each letter corresponds to the percent change in promoter activity as quantified by β-galactosidase assays. Letters above or below the line indicate an increase or decrease, respectively, in promoter activity. (f) The measured activities for 17 positions in the distal and proximal sites were used as counts in order to generate a new consensus sequence. The activities for equivalent positions in the distal and proximal sites were combined, and the log-odds scores that are displayed were calculated using these counts. The log-odds scores are a measure of the conservation of nucleotides in the alignment.



Distal site	Spacer	Activity
wildtype:		
cataagtgaaataacc	cttcttttatag cctta	100
substitutions:		
cataagtgaaataacc	cttcAAAAatag cctta	84
13 bp spacer:		
cataagtgaaataacc	ctt^{G}ctttatag cctta	17
cataagtgaaataacc	ctt^{A}ctttatag cctta	20
cataagtgaaataacc	cttcttt^{G}tatag cctta	7
cataagtgaaataacc	cttcttt^{C}tatag cctta	6
cataagtgaaataacc	cttcttt^{T}tatag cctta	9
11 bp spacer:		
cataagtgaaataacc	ct ctttatag cctta	93
cataagtgaaataacc	ctt ttttatag cctta	90
cataagtgaaataacc	cttc tttatag cctta	62
cataagtgaaataacc	cttctttt tag cctta	60
cataagtgaaataacc	cttctttta ag cctta	53
10 bp spacer:		
cataagtgaaataacc	ct ttttatag cctta	34
cataagtgaaataacc	ct ctttta ag cctta	20
cataagtgaaataacc	ctt tttt tag cctta	39
cataagtgaaataacc	cttctttt ag cctta	14
22 or 23 bp spacer:		
taacccttctttACGC	ATTTTGCtatag cctta	3
ataacccttctttCGC	ATTTTGCtatag cctta	3
taacccttAGGTTTTT	TATcttttatag cctta	5
ataacccttGGTTTTT	TATcttttatag cctta	6

Figure 6.
Insertions, deletions, and multiple substitutions were constructed in the $FlhD_4C_2$ spacer of the *fliAZY* promoter. β-galactosidase activities for these mutants were measured and normalized to the activity of the wildtype promoter (100%).

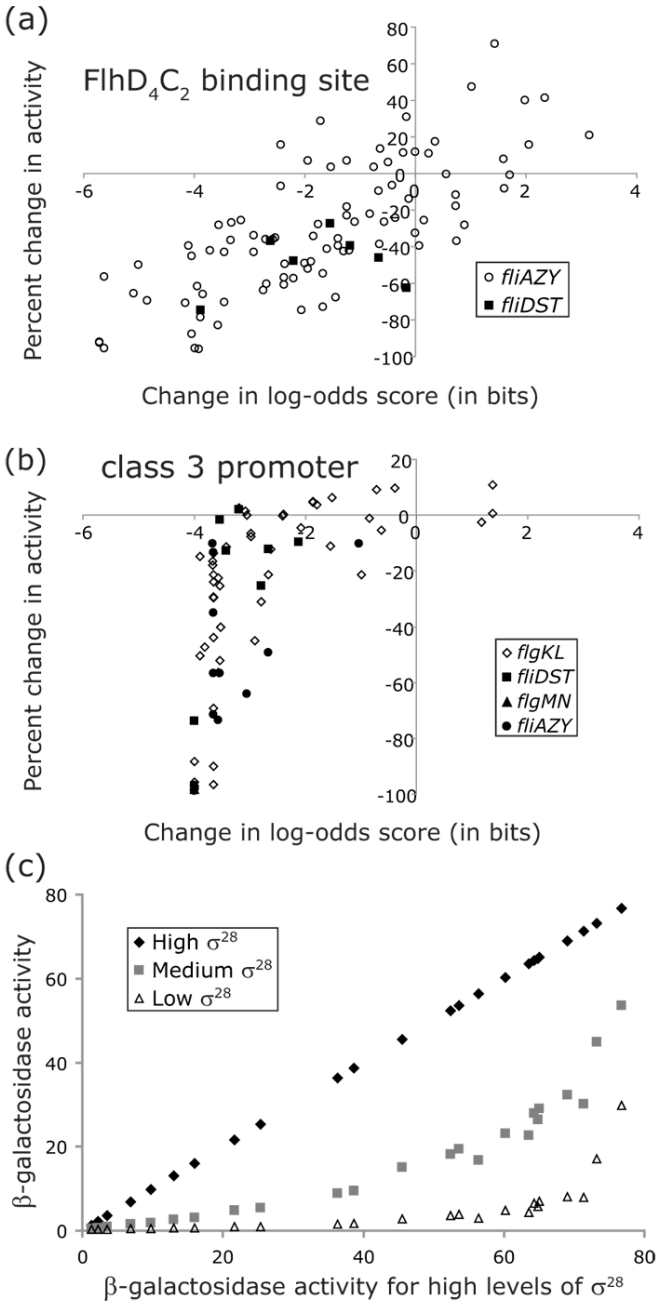


Figure 7.

Comparison of log-odds scores to the activities of mutated promoters. For mutations in the $FlhD_4C_2$ binding sites (a) or the class 3 promoters (b), the change in log-odds scores calculated from the consensus sequence was plotted against the observed change in promoter activity. (c) The activities of a subset of the class 3 *flgKL* promoter mutants were assayed in backgrounds expressing high (162% of wildtype), medium (110% of wildtype), or low (64% of wildtype) levels of class 3 transcription. The activities of the mutants in these three backgrounds were plotted against their activity in the high class 3 transcription background. The β-galactosidase activities in (c) are expressed in units of nmol/min/OD650/ml.

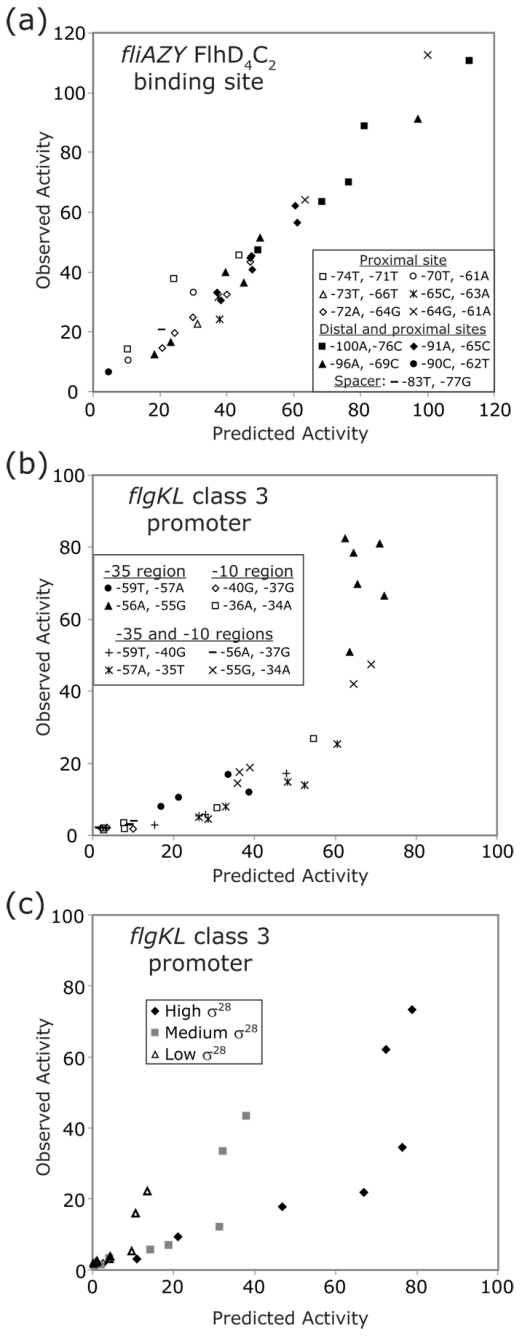


Figure 8.

Comparison of the predicted and observed activities for double mutants. The predicted activity for a double mutant is equal to the activity of the first mutant multiplied by the activity of the second mutant. The observed activity is the quantified β-galactosidase activity of the double mutant. All these activities are normalized to the wildtype promoter. The wildtype nucleotides for the mutated positions are listed in the keys. (a) Double mutants within the proximal site, in both the distal and proximal sites, and within the spacer for the *fliAZY* $FlhD_4C_2$ binding site were constructed. (b) Double mutants within the −35 region, within the −10 region, and in both the −35 and −10 regions for the *flgKL* class 3 promoter were constructed. (c) Eight of the class

3 double mutants were assayed in backgrounds expressing high (162% of wildtype), medium (110% of wildtype), or low (64% of wildtype) levels of class 3 transcription.

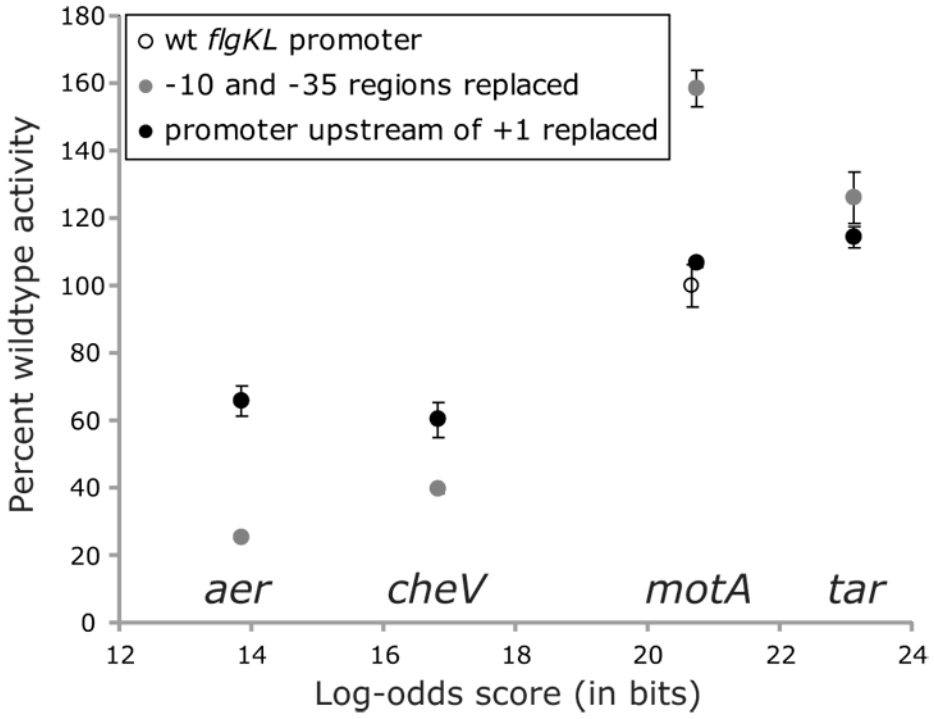


Figure 9.

Effect of replacing elements of the *flgKL* class 3 promoter with elements of other class 3 promoters. For each of four class 3 promoters, the −10 and −35 regions alone were substituted into the *flgKL* class 3 promoter (gray circles). Also, the sequence upstream of the transcription start site through 25 bp upstream of the −35 region for each of the four promoters was used to replace the *flgKL* promoter upstream of the transcription start site (black circles). The activities of these hybrid promoters were plotted against the total log-odds score for their −10 and −35 regions. A high log-odds score indicates a close match to the consensus sequence. All activities are normalized to the level of transcription from the wildtype *flgKL* promoter (empty circle).

Table 1

Salmonella strains used in this study.

Strain	Genotype	Reference[a]
TH437	LT2	J. Roth
TH2090	*fliG5101*::MudJ	lab collection
TH2142	*hisD9953*::MudJ *his-9944*::MudI	Hughes and Roth[29]
TH2534	*flgA5211*::MudJ	Gillen and Hughes[33]
TH2570	*flgC5215*::MudJ	Gillen and Hughes[33]
TH2779	*flgM5222*::MudJ	Gillen and Hughes[33]
TH2856	*fliA5059*::Tn*10d*Cm	Karlinsey, *et. al.*[34]
TH2945	*ataA*::[P22 Kn9 P*fliA*(−600)-*lacZYA*-'9]	M. Chadsey and G. Chilcott
TH3730	P*flhDC5451*::Tn*10d*Tc[Δ*25*]	Karlinsey, *et. al.*[35]
TH4212	*fliS5480*::MudK	H. Bonifield
TH4702	pKD46 (λ-red recombinase plasmid, Amp^R, temperature sensitive replication)	Datsenko and Wanner[26]
TH4721	*flgK5396*::MudJ	Aldridge, *et. al.*[36]
TH5504	Δ*fliA5647*::FRT	Aldridge, *et. al.*[27]
TH5794	P*fliD5744*::Tn*10d*Tc Δ*flgHI958 fljB* ^{e,n,x} *vh2*	lab collection
TH6701	Δ*araBAD925*::*tetRA*	P. Aldridge
TH7023	ΔIS*200*IV::*cat*(*fli-5583*)	lab collection
TH7270	*flgJ5964*::*tetRA*(inserted after stop codon) n	
TH7395	Δ*flgM5628*::FRT *motA5461*::MudJ Δ*araBAD923*::*flgM*-FKF P*araB935*	Aldridge, *et. al.*[27]
TH7396	Δ*flgM5628*::FRT *motA5461*::MudJ Δ*araBAD923*::*flgM*-FKF P*araB936*	Aldridge, *et. al.*[27]
TH8006	pKD46 / Δ*araBAD925*::*tetRA* Δ*fliA5647*::FRT	
TH8082	P*flhDC5451*::Tn*10d*Tc[Δ*25*] Δ*araBAD943*::*fliA* Δ*fliA5647*::FRT	
TH8083	CRR*4108*[P*flhDC5451*::Tn*10d*Tc[Δ*25*](Tc^S)] Δ*araBAD943*::*fliA* Δ*fliA5647*::FRT	
TH8091	*flgA6093*::*tetRA* (inserted after stop codon)	
TH8239	Δ*araBAD943*::*fliA* Δ*fliA5647*::FRT	
TH8315	pKD46 / CRR*4108*[P*flhDC5451*::Tn*10d*Tc[Δ*25*](Tc^S)] Δ*araBAD943*::*fliA* Δ*fliA5647*::FRT *flgK5396*::MudJ *flgJ5964*::*tetRA*	
TH8321	pKD46 / CRR*4108*[P*flhDC5451*::Tn*10d*Tc[Δ*25*](Tc^S)] Δ*araBAD943*::*fliA* Δ*fliA5647*::FRT *fliS5480*::MudK *fliD5744*::Tn*10d*Tc	
TH8922	*fliA5890*:Tn*10d*Tc[Δ*25*, IS10R(Δ2808–2894)] Δ*tar-flhD2039 fljB*^{e,n,x} *vh2*	
TH8925	Δ*araBAD956*::*fliA* Δ*fliA5647*::FRT	
TH8926	Δ*araBAD956*::*fliA* Δ*fliA5647*::FRT P*flhDC5451*::Tn*10d*Tc[Δ*25*]	
TH8927	Δ*araBAD956*::*fliA* Δ*fliA5647*::FRT CRR4107[P*flhDC5451*::Tn*10d*Tc[Δ*25*](Tc^S)]	
TH8928	Δ*araBAD956*::*fliA* Δ*fliA5647*::FRT CRR4107[P*flhDC5451*::Tn*10d*Tc[Δ*25*](Tc^S)] *ataA*::P22[*sieA*'-Kn6-P*fliA*(−583 to +1) - *lacZYA*'-'9]	
TH8929	Δ*araBAD956*::*fliA* Δ*fliA5647*::FRT CRR4107[P*flhDC5451*::Tn*10d*Tc[Δ*25*](Tc^S)] *flgK5396*::MudJ	
TH8931	Δ*araBAD956*::*fliA* Δ*fliA5647*::FRT CRR4107[P*flhDC5451*::Tn*10d*Tc[Δ*25*](Tc^S)] *fliS5480*::MudK	
TH8933	Δ*araBAD956*::*fliA* Δ*fliA5647*::FRT CRR4107[P*flhDC5451*::Tn*10d*Tc[Δ*25*](Tc^S)] *flgM5222*::MudJ	
TH8936	pKD46 / CRR*4107*[P*flhDC5451*::Tn*10d*Tc[Δ*25*](Tc^S)] Δ*araBAD956*::*fliA* Δ*fliA5647*::FRT *flgK5396*::MudJ *flgJ5964*::*tetRA*	
TH8937	pKD46 / CRR*4107*[P*flhDC5451*::Tn*10d*Tc[Δ*25*](Tc^S)] Δ*araBAD956*::*fliA* Δ*fliA5647*::FRT *fliS5480*::MudK *fliD5744*::Tn*10d*Tc	
TH8938	pKD46 / CRR*4107*[P*flhDC5451*::Tn*10d*Tc[Δ*25*](Tc^S)] Δ*araBAD956*::*fliA* Δ*fliA5647*::FRT *flgM5222*::MudJ *flgA6093*::*tetRA*	
TH8939	pKD46 / CRR*4107*[P*flhDC5451*::Tn*10d*Tc[Δ*25*](Tc^S)] Δ*araBAD956*::*fliA* Δ*fliA5647*::FRT *ataA*::P22[*sieA*'-Km6-P*fliA*(−583 to +1)-*lacZYA*'-'9]	
TH9250	*fliA6399*::*tetRA* (replaces −79 to −44 bp from GTG with *tetRA*)	
TH9252	pKD46 / CRR*4107*[P*flhDC5451*::Tn*10d*Tc[Δ*25*](Tc^S)] Δ*araBAD956*::*fliA* Δ*fliA5647*::FRT *ataA*::P22[*sieA*'-Km6-P*fliA*(−583 to +1) *6400*::*tetRA-lacZYA*'-'9]	
TH10022	Δ*fli-5583*::*cat*(ΔIS*200*IV) *fliA5890*::Tn*10d*Tc*[Δ25*, IS*10*R(Δ2808–2894)] Δ*tar-flhD2039 fljB*^{e,n,x}*vh2*	
TH10032	Δ*araBAD956*::*fliA fliZ6591*::MudJ *fliA5890*::Tn*10d*Tc[Δ*25*, IS*10*R(Δ2808–2894)]	
TH10049	*fliZ6591*::MudJ	
TH10132	CRR*4107*[P*flhDC5451*::Tn*10d*Tc[Δ*25*](Tc^S)] Δ*araBAD956*::*fliA fliZ6591*::MudJ Δ*fliA5647*::FRT	
TH10151	pKD46 / CRR*4107*[P*flhDC5451*::Tn*10d*Tc[Δ*25*](Tc^S)] Δ*araBAD956*::*fliA fliZ6591*::MudJ Δ*fliA5647*::FRT *fliA6399*::*tetRA*	
TH10826	*fliA6785*::*tetRA* (inserted −43 bp from GTG)	
TH10896	pKD46 / CRR*4107*[P*flhDC5451*::Tn*10d*Tc[Δ*25*](Tc^S)] Δ*araBAD956*::*fliA fliZ6591*::MudJ Δ*fliA5647*::FRT *fliA6785*::*tetRA*	
TH11808	*fliA7226*::*tetRA* (inserted −79 bp from GTG)	
TH12065	pKD46 / CRR*4107*[P*flhDC5451*::Tn*10d*Tc[Δ*25*](Tc^S)] Δ*araBAD956*::*fliA fliZ6591*::MudJ Δ*fliA5647*::FRT *fliA7226*::*tetRA*	
TH12359	CRR4107[P*flhDC5451*::Tn*10d*Tc[Δ*25*](Tc^S)] Δ*araBAD956*::*fliA* Δ*fliA5647*::FRT *motA5461*::MudJ	
TH12906	P*ara998*::*tetRA* Δ*araBAD956*::*fliA* Δ*fliA5647*::FRT	
TH12927	CRR4107[P*flhDC5451*::Tn*10d*Tc[Δ*25*](Tc^S)] Δ*araBAD956*::*fliA fliA5059*::Tn*10d*Cm *flgC5215*::MudJ	
TH12930	CRR4107[P*flhDC5451*::Tn*10d*Tc[Δ*25*](Tc^S)] Δ*araBAD956*::*fliA fliA5059*::Tn*10d*Cm *flgA5211*::MudJ	
TH12933	CRR4107[P*flhDC5451*::Tn*10d*Tc[Δ*25*](Tc^S)] Δ*araBAD956*::*fliA fliA5059*::Tn*10d*Cm *fliG5101*::MudJ	
TH12990	pKD46 / CRR4107[P*flhDC5451*::Tn*10d*Tc[Δ*25*](Tc^S)] Δ*araBAD956*::*fliA* P*ara998*::*tetRA* Δ*fliA5647*::FRT *motA5461*::MudJ	
TH13035	CRR4107[P*flhDC5451*::Tn*10d*Tc[Δ*25*](Tc^S)] Δ*araBAD956*::*fliA* P*araB935*(−85 A:T) Δ*fliA5647*::FRT *motA5461*::MudJ	
TH13036	CRR4107[P*flhDC5451*::Tn*10d*Tc[Δ*25*](Tc^S)] Δ*araBAD956*::*fliA* P*araB936*(−41 T:C) Δ*fliA5647*::FRT *motA5461*::MudJ	
TH13127	CRR4107[P*flhDC5451*::Tn*10d*Tc[Δ*25*](Tc^S)] Δ*araBAD956*::*fliA* P*araB935* Δ*fliA5647*::FRT *flgJ6310*::*tetRA*	
TH13128	CRR4107[P*flhDC5451*::Tn*10d*Tc[Δ*25*](Tc^S)] Δ*araBAD956*::*fliA* P*araB936* Δ*fliA5647*::FRT *flgJ6310*::*tetRA*	
TH13161	CRR4107[P*flhDC5451*::Tn*10d*Tc[Δ*25*](Tc^S)] P*araB*936 Δ*araBAD956*::*fliA* Δ*fliA5647*::FRT *flgK5396*::MudJ	
TH13237	CRR4107[P*flhDC5451*::Tn*10d*Tc[Δ*25*](Tc^S)] P*araB*935 Δ*araBAD956*::*fliA* Δ*fliA5647*::FRT *flgK5396*::MudJ	

[a] Strains given no reference were constructed for this study.

Table 2

Primers used in this study.

Primer name	Sequence
ara5’	cgtcttactccatccagaaaaacagg
araC+5R	ccatgatttctctacccc
flgA +417/+436	cgtcagtttgcgcgatctcg
flgJ 5’ RT-PCR	gatcaccaccactgaatacg
flgK +100/+81	aacccgcaacgttataattg
flgK +42/+23 R	ggcgttaagtccgctcatgg
flgK −105/−86 F	tcagcaaaacctacagcgcg
flgM +24/+5 R	aggtgaggtacggtcaatgc
flgM +61/+45	tggtttcgcgcgtctgg
flgM5′-UP	gaaccgtcgattctgatg
fliA +27/+8	accttcagcggtatacagtg
fliA +5/−15	ttcacgataaacagccctgc
fliA +720/+697	ctataacttacccagtttggtgcg
fliA −156/−137	tctggctgattttattctgc
fliA −225/−209	gcggctggtaagagagc
fliA#4	tagtctatacgttgtgcggc
fliC+39R	caacagcgacaggctgtttg
fliC13	gttctttgtcaggtctgtc
fliD +4/−16	ccatgccttcttccttttg
fliD −196/−177 F	gtaacccttgtatcggcacc
fliD+190R	aacgcggtatttgccgtc
araBfliA F	actgtttctccatacctgtttttctggatggagtaagacgatgaattcactgtataccgc
araDfliA R	ttcatcaacgcgccccccatgggacgcgtttttagaggcactataacttacccagtttgg
flgAtetR	aaccgtcgattctgatgggaatattcttattaacctataattaagacccactttcacatt
flgJtetR	cagcaaaacctacagcgcgaatctcgacaatctcttttaattaagacccactttcacatt
flgKtetA	cgttaagtccgctcatggcgtgattaatcaagctggacatctaagcacttgtctcctg
flgMtetA	tgaatatctcatcggcagccgcgacaaaatctttacacaactaagcacttgtctcctg
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