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The evolutionary impact of Intragenic FliA Promoters in Proteobacteria

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Abstract

In *Escherichia coli*, one Sigma factor recognizes the majority of promoters, and six "alternative" Sigma factors recognize specific subsets of promoters. The alternative Sigma factor FliA (σ^{28}) recognizes promoters upstream of many flagellar genes. We previously showed that most *E. coli* FliA binding sites are located inside genes. However, it was unclear whether these intragenic binding sites represent active promoters. Here, we construct and assay transcriptional promoter-*lacZ* fusions for all 52 putative FliA promoters previously identified by ChIP-seq. These experiments, coupled with integrative analysis of published genome-scale transcriptional datasets, strongly suggest that most intragenic FliA binding sites are active promoters that transcribe highly unstable RNAs. Additionally, we show that widespread intragenic FliA-dependent transcription may be a conserved phenomenon, but that specific promoters are not themselves conserved. We conclude that intragenic FliA-dependent promoters and the resulting RNAs are unlikely to have important regulatory functions. Nonetheless, one intragenic FliA promoter is broadly conserved, and constrains evolution of the overlapping protein-coding gene. Thus, our data indicate that intragenic regulatory elements can influence bacterial protein evolution, and suggest that the impact of intragenic regulatory sequences on genome evolution should be considered more broadly.

ABBREVIATED SUMMARY

Recent findings have identified thousands of bacterial promoters in unexpected locations, such as inside genes. Here, we investigate the functions of intragenic promoters for the flagellar sigma factor FliA. Our data suggest that most of these promoters are not functional, but that one intragenic FliA promoter is broadly conserved, and constrains evolution of the overlapping protein-coding gene. Our data suggest that intragenic regulatory sequences significantly impact bacterial genome evolution.

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ACCESSION NUMBERS

Raw ChIP-seq and RNA-seq data are available from the EBI ArrayExpress repository using accession numbers E-MTAB-6048 (RNA-seq) and E-MTAB-6049 (ChIP-seq).

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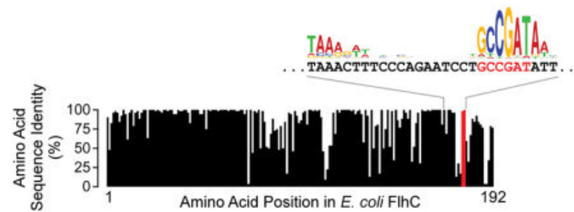

INTRODUCTION

In bacteria, RNA polymerase (RNAP) requires a transcription initiation factor, σ, to recognize promoter elements and initiate transcription. Bacteria encode one housekeeping σ factor that functions at most promoters, and multiple "alternative" σ factors that each recognize smaller sets of promoters. Historically, promoters were thought to be located solely upstream of annotated genes. However, widespread transcription initiation from inside genes has now been described in *Escherichia coli* and many other species (reviewed, (Lybecker *et al.*, 2014; Wade and Grainger, 2014)). Consistent with these observations, the *E. coli* housekeeping σ factor, σ^{70}, has been shown to bind many intragenic sites (Singh *et al.*, 2014). Similar findings have been reported for alternative σ factors, e.g. 40% of *Mycobacterium tuberculosis* SigF binding sites, 25% of *E. coli* σ^{32} binding sites, and 62% of *E. coli* σ^{54} binding sites are inside genes (Wade *et al.*, 2006; Hartkoorn *et al.*, 2012; Bonocora *et al.*, 2013; Bonocora *et al.*, 2015). The high degree of pervasive transcription involving multiple σ factors suggests that intragenic promoters have a substantial impact on global transcriptional networks.

Like σ factors, DNA-binding transcription factors often bind extensively within genes (Shimada *et al.*, 2008; J. Galagan *et al.*, 2013; J. E. Galagan *et al.*, 2013; Bonocora *et al.*, 2013; Wade and Grainger, 2014; Grainger, 2016). The regulons of most transcription factors have not been mapped, even for *E. coli*, suggesting that most intragenic binding sites remain to be identified. Indeed, a study of 51 transcription factors in *Mycobacterium tuberculosis* suggests that a typical bacterial genome contains >10,000 intragenic binding sites (J. E. Galagan *et al.*, 2013). The transcriptional activities of most intragenic transcription/σ factor binding sites have not been extensively studied, but many are likely to be functional (J. E. Galagan *et al.*, 2013). Although transcription regulatory networks evolve rapidly, individual regulatory interactions are often maintained by purifying selection (Lozada-Chávez *et al.*, 2006; Perez and Groisman, 2009; Stringer *et al.*, 2014). Hence, many intragenic transcription/σ factor binding sites may be functional, and thus are likely to be conserved. A previous study suggested that purifying selection on intragenic transcription/σ factor binding sites in human cells constrains the evolution of overlapping protein-coding genes (Stergachis *et al.*, 2013). The impact of bacterial intragenic binding sites on overlapping protein-coding genes has not been assessed.

FliA (σ^{28}) is an alternative σ factor involved in transcription of genes associated with flagellar motility and chemotaxis (reviewed (Paget, 2015)). FliA also initiates transcription of some non-flagellar genes in *E. coli* (Fitzgerald *et al.*, 2014), and is encoded by some non-motile bacteria, such as *Chlamydia* (Yu and Tan, 2003), suggesting additional non-flagellar

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roles. Recently, we reported that over half of *E. coli* FliA binding sites are located inside genes, often far from gene starts (Fitzgerald *et al.*, 2014). These intragenic sites were split approximately evenly between those occurring in the sense and antisense orientations, with respect to the overlapping gene. Most intragenic FliA binding sites were not associated with detectable FliA-dependent RNAs, so it is unclear whether they represent functional promoters. Notably, FliA is the most highly and broadly conserved alternative σ factor (Feklístov *et al.*, 2014; Paget, 2015). The interactions between FliA, RNA polymerase, and promoter DNA are so highly conserved that the *Bacillus subtilis* homolog, σ^D, can complement an *E. coli fliA* strain (Chen and Helmann, 1992). Like many alternative σ factors, FliA has a decreased ability to melt DNA as compared to housekeeping σ factors (Koo, Rhodius, Nonaka, *et al.*, 2009; Feklístov *et al.*, 2014). Thus, FliA-dependent transcription initiation requires a stringent match to its consensus promoter sequence (Koo, Rhodius, Campbell, *et al.*, 2009). Together, the high conservation and readily identifiable motif make FliA a good model for evolutionary analysis of intragenic σ factor binding.

In this study, we evaluate the promoter activity of intragenic FliA binding sites in *E. coli*. We also assess the conservation of intragenic FliA promoters and map the *Salmonella* FliA regulon. We conclude that most intragenic FliA binding sites represent bona fide promoters that transcribe unstable intragenic RNAs. We show that extensive intragenic transcription by FliA is likely to be a conserved phenomenon, but the genetic locations of intragenic FliA promoters are generally not conserved. Nonetheless, we show that a single intragenic FliA promoter is under strong selective pressure that constrains the evolution of the FlhC protein. This is the first documented example of intragenic regulatory sequence impacting evolution of the overlapping protein-coding gene in a bacterium, and suggests that selective pressure on intragenic binding sites for σ factors and transcription factors is an overlooked factor in protein evolution in compact bacterial genomes.

RESULTS

Most intragenic FliA binding sites represent transcriptionally active promoters

To test whether FliA binding sites previously identified by ChIP-seq (Fitzgerald *et al.*, 2014) represent active promoters, we generated transcriptional fusions of potential promoters to the *lacZ* reporter gene. For each of the 52 putative FliA promoters, the region from approximately −200 to +10 was cloned upstream of *lacZ* on a single-copy plasmid (Figure 1A). We chose to include 200 bp upstream sequence because at least one FliA promoter is regulated by a transcription factor binding upstream (Hollands *et al.*, 2010). Plasmids were transformed into a motile strain of *E. coli* MG1655 (i.e. expressing FliA), or an isogenic *fliA* derivative, and assayed for β-galactosidase activity. Of the 20 intergenic promoters, 15 displayed significant FliA-dependent activity (*t*-test, *p* 0.05; Figure 1B). Of the 30 intragenic promoters, 10 out of 16 sense- and 7 out of 14 antisense-orientation putative intragenic promoters showed significant FliA-dependent activity (*t*-test, *p* 0.05; Figure 1C). These intragenic FliA-dependent promoters include all five that have been previously associated with transcription of stable RNAs ((*flhC*)*motAB-cheAW*, (*yafY*)*ykfB*, (*yjdA*)*yjcZ*, (*uhpT*), and antisense (*hypD*), where genes in parentheses indicate those with an internal FliA promoter. One of the two putative promoters located in convergent intergenic

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regions also showed significant FliA-dependent activity (*t*-test, $p \leq 0.05$; Figure 1C). It should be noted that some fusions had very high levels of background activity, which may have prevented the detection of lower levels of FliA-dependent transcription from these promoter fusions. Of note, no FliA-dependent activity was detected for the well-characterized promoters upstream of *fliA*, *fliD*, and *fliL*, likely due to overwhelming transcriptional activity from the strong, σ^{70}-dependent, FlhDC-activated promoters known to be immediately upstream (Liu and Matsumura, 1996; Stafford *et al.*, 2005; Fitzgerald *et al.*, 2014). High β-galactosidase activity associated with the *lacZ* fusions for *pntA*, *cvrA*, *glyA*, *proK*, and *insB-4*/*cspH* suggest they are also likely to include σ^{70} promoters that may preclude identification of FliA-dependent transcription. Consistent with this, we previously detected σ^{70} binding sites <200 bp upstream of all of these putative FliA promoters (Singh *et al.*, 2014).

We previously identified FliA-regulated transcripts using RNA-seq, although most intragenic FliA sites were not associated with a detectable RNA (Fitzgerald *et al.*, 2014). However, this method often fails to detect unstable RNAs. To independently assess whether intragenic FliA binding sites act as promoters, we analyzed two published datasets generated from motile *E. coli* strains: (i) genome-wide transcription start site (TSS) mapping by differential RNA-seq (dRNA-seq) (Thomason *et al.*, 2015), and (ii) Nascent Elongating Transcript sequencing (NET-seq) (Larson *et al.*, 2014). dRNA-seq identifies TSSs by selectively degrading processed transcripts bearing a 5′ monophosphate, and then preparing a library from the remaining 5′ triphosphate-bearing primary transcripts (Sharma and Vogel, 2014). By focusing reads to the 5′ ends of transcripts, this technique is more sensitive than standard RNA-seq, and can distinguish intragenic RNAs from overlapping mRNAs. NET-seq isolates nascent RNA still bound to RNAP, facilitating detection of unstable transcripts prior to degradation (Churchman and Weissman, 2011).

To compare FliA binding site location to TSS mapping data, we determined the distance from the predicted FliA promoter sequence associated with each FliA binding site (Fitzgerald *et al.*, 2014) to all downstream TSSs within 500 bp (Figure 2A). For most well-characterized FliA-dependent promoters for flagellar genes, the distance between the center of the promoter sequence and TSS was between 18 and 22 bp. For other FliA binding sites, we observed a strong enrichment for TSSs between 18 and 23 bp downstream of FliA motif centers. In total, 38 of the 52 FliA binding sites have a TSS located 18–23 bp downstream of the center of their predicted promoter. This positional enrichment is highly significant when compared to the same analysis performed with a randomized TSS dataset; only one random TSS was between 18–23 bp downstream of a FliA motif center (Fisher's exact test, $p<0.0001$).

To systematically assess whether FliA binding sites are associated with signal in the NET-seq dataset, the sequence read coverage upstream and downstream of FliA binding sites was determined. For FliA binding sites associated with a TSS, the read coverage at each position from −100 to +100 was determined relative to the TSS. For all other FliA binding sites, a TSS was predicted at 20 bp downstream of the predicted promoter sequence center (average position of other TSSs), and coverage was determined from −100 to +100 relative to this position. The coverage profile for each binding site was normalized to the minimum and

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maximum coverage in the region and plotted as a heatmap (Figure 2B). There is a clear trend of higher NET-seq read coverage downstream of FliA binding sites, compared to the regions immediately upstream. To quantify this trend, the ratio of NET-seq read coverage upstream and downstream of the TSS was calculated for each putative FliA-dependent promoter. In total, 44 out of the 52 putative promoters showed at least 2-fold higher coverage in the region 100 bp downstream of the TSS than in the region 100 bp upstream of the TSS. These 44 putative promoters included 19 that are intragenic (Table 1). As expected, there is a high degree of overlap between the FliA binding sites with transcriptional activity detected by NET-seq and those detected by TSS association (Table 1).

In total, 26 of the 30 intragenic FliA binding sites, and one of the two FliA sites in a convergent intergenic region, show evidence of promoter activity from at least one assay. Table 1 summarizes the existing evidence for these sites. It should be noted that neither the TSS nor NET-seq datasets have matched *fliA* controls, so it is formally possible that TSSs/transcripts are associated with FliA-independent promoters. However, this is highly unlikely given the position of putative TSSs and the position of NET-seq signal with respect to the predicted FliA promoter sequences. Overall, there is substantial overlap between the sets of putative intragenic promoters that display FliA-dependent activity in promoter fusion assays, those with appropriately positioned TSSs, and those that have high NET-seq read coverage ratios (downstream:upstream).

Most intragenic FliA promoters are not conserved across species

To assess whether intragenic FliA promoters and binding sites are likely to be functionally important, we determined conservation of these sites bioinformatically. The sequence surrounding each of the 52 FliA binding sites previously identified by ChIP-seq (Fitzgerald *et al.*, 2014) was extracted and used as a BLAST query to search genomes from 24 γ-proteobacterial genera (Table S1). All genomes queried encode FliA, except for those of *Klebsiella* and *Raoultella*, which were included as controls. If a homologous region was identified, it was scored against the previously determined *E. coli* FliA position-weight matrix (Fitzgerald *et al.*, 2014). These scores are depicted as a heatmap in Figure 3A, where yellow represents the highest-scoring sites and blue the lowest-scoring. Sites are categorized by location and orientation, and then ranked by total degree of conservation within each category, from left to right. The well-characterized FliA-dependent promoter inside *flhC*, which drives transcription of the downstream *motABcheAW* operon, was the most highly conserved. All other well-characterized, flagellar-related FliA promoters were well-conserved at the sequence level, with the exception of the promoter upstream of the *fliLMNOPQR* operon, which is also transcribed by σ^{70} in *E. coli*. Most novel intergenic and intragenic FliA binding sites showed no evidence of conservation, even in close relatives such as *Salmonella*. It should be noted that a few intragenic FliA binding sites, such as those inside *hslU*, *glyA*, and *ybhK*, appear conserved, but score equally well in species that lack *fliA* (*Klebsiella* and *Raoultella*), suggesting they are maintained for reasons independent of their ability to bind FliA, most likely because of high levels of conservation for these protein-coding genes. A few other intragenic promoters, such as those inside *uhpC*, *hypD*, *metF*, and *speA*, show possible sequence conservation in *Salmonella*, but not in more distantly related genera.

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Intragenic FliA promoters are not conserved across *E. coli* strains

Previous studies suggest that while intragenic promoters may not be conserved between species (Raghavan *et al.*, 2012), they may be conserved within strains of the same species (Shao *et al.*, 2014). Hence, we bioinformatically determined the conservation of all FliA sites across 9,432 *E. coli* strains for which a genome sequence is available (Table S2). The sequence surrounding each of the 52 FliA binding sites previously identified by ChIP-seq (Fitzgerald *et al.*, 2014) was extracted and used as a BLAST query to search genomes from each *E. coli* genome contig. If a homologous region was identified, we determined whether each position in each *E. coli* K-12 FliA site is conserved. We then determined the proportion of strains with a homologous region in which each position of each FliA site is conserved. Figure 3B shows the level of conservation of each position of FliA sites divided into two classes: (i) sites that represent promoters of mRNAs (based on our previous RNA-seq data and other studies of the FliA regulon (Fitzgerald *et al.*, 2014)), and (ii) all other sites. The second class includes most of the intragenic FliA sites. FliA sites that represent promoters of mRNAs are highly conserved in the −10 and −35 regions, but less well conserved in the spacer region (Figure 3B), consistent with the lack of sequence requirements in the spacer region for FliA binding. By contrast, FliA sites that do not represent promoters for mRNAs are less well conserved in the −10 and −35 regions, and show no difference in conservation between these regions and the spacer. We conclude that, as a group, FliA binding sites that do not represent mRNA promoters are not under purifying selection.

Genome-wide mapping of the *Salmonella* Typhimurium FliA regulon

Salmonella enterica and *E. coli* diverged approximately 100 million years ago and exhibit substantial drift at wobble positions (Gordienko *et al.*, 2013). As an independent, empirical test of FliA binding site conservation, we determined the genome-wide binding profile of *S. enterica* serovar Typhimurium FliA using ChIP-seq of a C-terminally tagged derivative expressed from its native locus. To facilitate comparison with *E. coli* ChIP-seq data, we grew cells under similar conditions as those used in our previous study of *E. coli* FliA (Fitzgerald *et al.*, 2014). A total of 23 high-confidence FliA binding sites were identified (Table 2, Figure 4A). Of these 23 sites, three are inside genes but within 300 bp of a gene start (13%; Figure 4B), and five are inside genes and far from a gene start (22%). No equivalent ChIP-seq peaks were identified using a control, untagged strain of *S.* Typhimurium. All 23 *S.* Typhimurium FliA binding sites are associated with a match to the consensus FliA motif (Figure 4C; MEME, E-value = $7.4e^{-49}$), and motif positions were enriched in the region ~25 bp upstream of peak centers, as previously described for FliA binding sites in *E. coli* (Fitzgerald *et al.*, 2014). As predicted by the sequence conservation analysis (Figure 3A), FliA-dependent promoters upstream of key flagellar operons were conserved in *S.* Typhimurium. However, with the exception of the *motA* promoter that is located inside *flhC*, no intragenic FliA binding sites were found to be conserved between *E. coli* and *S.* Typhimurium.

RNA-seq was used to assess FliA-dependent changes in gene expression by comparing wild-type and *fliA* strains of *S.* Typhimurium (Figure 5). As for the ChIP-seq experiment, cells were grown under similar conditions as those used in our previous study of *E. coli* FliA (Fitzgerald *et al.*, 2014). Overall, 344 genes were significantly differentially expressed

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between the two strains (*q*-value 0.01, fold-change 2), of which 36 were downstream of FliA binding sites identified by ChIP-seq (Table 2). The intragenic FliA binding sites within *flhC*, *STM14_3340*, and *STM14_3895* were associated with FliA-dependent regulation of the downstream genes, all of which are known flagellar genes. The other intragenic binding sites were not associated with detectable transcripts.

The *motA* promoter within *flhC* constrains evolution of the FlhC protein

Although most intragenic FliA promoters in *E. coli* are not well conserved in other species, the *motA* promoter, located inside *flhC*, is highly conserved (Figure 3A). However, it is unclear whether this conservation is due to selective pressure on the promoter or on the amino acid sequence of FlhC, which is encoded by the same DNA. As expected given the conservation of the *motA* promoter inside *flhC*, the two FlhC amino acids, Ala177-Asp178, that are encoded by sequence overlapping the −10 region, are highly conserved among γ-proteobacteria (Figure 6A; Table S3). Strikingly, the amino acids flanking the Ala-Asp sequence are poorly conserved (Figure 6A), leading us to hypothesize that the Ala-Asp motif is conserved due to selective pressure on the *motA* promoter, rather than on the amino acids themselves. To test this hypothesis, we determined whether Asp178 is required for FlhC function. We created a strain of motile *E. coli* MG1655 in which the *flhDC* promoter is transcriptionally active, but *flhC* is replaced with a cassette containing *thyA* under the control of a constitutive σ^{70} promoter. Thus, this strain lacks the *motA* promoter, but we reasoned that *motA* would be co-transcribed with *thyA* (Figure 6B). We then introduced either wild-type FlhC or D178A FlhC from a plasmid, or an empty vector control. Cells containing the empty vector control were non-motile, as expected given that they lack FlhC (Figure 6B). By contrast, cells expressing wild-type FlhC from the plasmid were fully motile. Strikingly, cells expressing D178A FlhC were also fully motile (mean motility level relative to wild-type FlhC of 0.97 ± s.d. 0.09, n = 3; Figure 6B). We conclude that the conserved Asp178 is likely not required for FlhC function.

To further investigate the conservation of the Ala-Asp motif in FlhC, we aligned the sequences of FlhC homologues from 98 different proteobacterial species, each from a different genus in which *motA* is positioned immediately downstream of *flhC* (Table S4). Although Ala177 and Asp178 are well conserved across these species (conserved in 70% and 56% of species, respectively), we identified 44 species in which Asp178 is not conserved (Table S4). We reasoned that if Asp178 is broadly conserved due to selective pressure on the overlapping *motA* promoter, species in which Asp178 is not conserved are likely to have repositioned the *motA* promoter. To test this hypothesis, we extracted the intergenic sequences between *flhC* and *motA* for each of the 43 species where Asp178 is not conserved (Figure S1). Consistent with our hypothesis, we identified a strongly enriched sequence motif in 19 species (MEME E-value = $1.5e^{-32}$) corresponding to a consensus FliA promoter (Figure 6C). By contrast, when we repeated this analysis for the 55 species where Asp178 is conserved (Figure S1), we did not observe enrichment of a FliA promoter motif in the *flhC-motA* intergenic region. Having a FliA promoter for *motA* within *flhC* is likely to be the ancestral state, since the position of FliA promoters in *flhC-motA* intergenic regions differs extensively between species, as do the sequences flanking these promoters. We also compared the length of the *flhC-motA* intergenic region in (i) the 19 species where FlhC

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Asp178 is not conserved and for which we identified a likely intergenic FliA promoter, and (ii) the 55 species where FlhC Asp178 is conserved. Intergenic distances in group (i) are significantly higher (median length 207 bp) than those in group (ii) (median length 131 bp; Mann-Whitney U Test $p = 4.0e^{-7}$). We conclude that the selective pressure on Asp178 is lost in species that reposition the *motA* promoter to the *flhC*-*motA* intergenic region, and that this repositioning likely occurs by sequence insertion.

DISCUSSION

Most FliA Binding Sites are Active Promoters for Unstable RNAs

Most FliA binding sites identified by ChIP-seq display FliA-dependent promoter activity when fused upstream of the *lacZ* reporter gene (Figure 1). Many of these FliA binding sites, and some additional sites that had inactive *lacZ* fusions, are associated with correctly positioned TSSs and NET-seq signal from published studies (Larson *et al.*, 2014; Thomason *et al.*, 2015). Together, these data suggest that almost all FliA binding sites represent transcriptionally active FliA-dependent promoters, regardless of their location relative to protein-coding genes. The small subset of FliA binding sites that appear to be transcriptionally inert were amongst the most weakly bound sites detected by ChIP-seq (Fitzgerald *et al.*, 2014). Three of these sites have at least one mismatch to key −10 region residues (Koo, Rhodius, Campbell, *et al.*, 2009), suggesting that the sites are unlikely to be active promoters, or are so weakly transcribed that their activity is undetectable using standard assays.

Although most intragenic FliA binding sites are likely to represent active promoters, they are not associated with the transcription of stable RNAs, since we previously detected very few such RNAs using standard RNA-seq (Fitzgerald *et al.*, 2014). We conclude that most intragenic FliA promoters drive transcription of unstable RNAs. This is consistent with the previously described phenomenon of “pervasive transcription” that generates large numbers of short, unstable transcripts, primarily from promoters within genes (Lybecker *et al.*, 2014; Wade and Grainger, 2014). Intragenic promoters typically drive transcription of non-coding RNAs. Transcription of these RNAs is rapidly terminated by Rho (Peters *et al.*, 2012), and the transcripts are rapidly degraded by RNases (Lybecker *et al.*, 2014; Wade and Grainger, 2014).

Limited conservation of the FliA regulon outside of core flagellar genes

Evolutionary conservation of DNA sequences is due to purifying selection, and suggests that the sequence has beneficial function.. As expected, most flagella-associated FliA promoters are highly conserved at the sequence level (Figure 3). Of the intragenic FliA binding sites, only those that drive transcription of an mRNA for a downstream gene appear to be at all functionally conserved. A few intragenic promoters, such as those within *hslU*, *glyA*, and *ybhK*, are conserved at the sequence level between *E. coli* and many species (Figure 3A). However, the fact that these sites are also conserved in two genera not encoding *fliA* – *Klebsiella* and *Raoultella* – suggests that the DNA sequences are maintained for reasons independent of FliA, most likely purifying selection on the codons for the overlapping protein-coding genes.

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To experimentally validate the sequence-based conservation predictions, we performed ChIP-seq on *S.* Typhimurium FliA. As predicted based on sequence conservation, all key flagellar promoters were functionally conserved, except the one upstream of *fliLMNOPQR*. In *E. coli*, this operon is primarily transcribed from a σ^{70} promoter that is activated by FlhDC (Liu and Matsumura, 1996; Stafford *et al.*, 2005; Fitzgerald *et al.*, 2014). Conservation of the σ^{70} promoter and FlhDC regulation would ensure that these genes are coordinately regulated with other flagellar genes in *S.* Typhimurium, potentially relieving the selective pressure to maintain the FliA promoter. Our ChIP-seq data indicate the only intragenic FliA promoter functionally conserved between *E. coli* and *S.* Typhimurium is that within *flhC*. While specific intragenic FliA binding sites were not conserved, *S.* Typhimurium FliA binds multiple intragenic sites. This suggests that the factors affecting FliA specificity, or lack thereof, are similar between *E. coli* and *S.* Typhimurium, and that the phenomenon of intragenic FliA promoters is conserved, even if the specific promoters are not. Note that we identified fewer intragenic FliA sites in *S.* Typhimurium than we previously identified in *E. coli* (Fitzgerald *et al.*, 2014), but this is likely due to the data for *S.* Typhimurium having slightly lower signal-to-noise ratios (compare ChIP-seq enrichment (“FAT”) scores in Table 2 to those in our previous study (Fitzgerald *et al.*, 2014)).

It should be noted that lack of conservation of specific promoters does not necessarily indicate a lack of functional importance, but could instead reflect lineage-specific evolution. Indeed, regulatory small RNAs are often poorly conserved, even between closely related species (Toffano-Nioche *et al.*, 2012; Beauregard *et al.*, 2013; Patenge *et al.*, 2015). However, our analysis of conservation within *E. coli* suggests that most intragenic FliA promoters are not conserved even within the species, although this multi-promoter analysis does not rule out the possibility that a small proportion of the intragenic promoters are functional. Indeed, one of the two stable, FliA-transcribed non-coding RNAs – that transcribed from within *uhpT* – is likely a functional regulator. A recent study detected numerous Hfq-mediated interactions between mRNAs and RNA originating from the 3′ end of *uhpT* (Melamed *et al.*, 2016). Although the *uhpT* sequences from these interactions map to locations downstream of the sRNA predicted by RNA-seq (Fitzgerald *et al.*, 2014), an earlier microarray study and NET-seq data suggest that the FliA-transcribed sRNA extends further downstream (Reppas *et al.*, 2006; Larson *et al.*, 2014). The other stable, FliA-transcribed non-coding RNA – that transcribed from within *hypD* – was not detected in any sRNA:mRNA interactions (Melamed *et al.*, 2016), suggesting that it is not functional. Unstable FliA-transcribed non-coding RNAs are also unlikely to be functional, given their transient nature, and the lack of promoter conservation.

Intragenic FliA promoters likely arise as a result of sequence drift during evolution, although the likelihood of creating a FliA promoter as a result of a base substitution is lower than for some other σ factors, since FliA promoters require a more stringent match to the consensus sequence. Nonetheless, we estimate that there are 474 possible single base substitutions in the *E. coli* genome that would create a new FliA promoter (see Methods). Strikingly, this number is similar to the number of single base substitutions that we predict would destroy an existing FliA site, based on the number of actual FliA sites and the information content of the binding motif. We propose that the number of intragenic FliA sites in *E. coli* is in equilibrium, but that non-functional sites turn over relatively frequently. The prevalence of

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intragenic FliA promoters in *E. coli* and *S.* Typhimurium suggests that they do not substantially impact expression of the overlapping genes. Consistent with this, we detected significant FliA-dependent regulation of only three *S.* Typhimurium genes that have an internal FliA site (Figure 5; Table 2); one of these genes (*STM14_3340*) is immediately upstream of a FliA-transcribed flagellar gene, and another (*motB*) is a downstream gene in a FliA-transcribed operon. While most intragenic FliA promoters are unlikely to be individually functional, the phenomenon of widespread intragenic FliA sites may be functional. For example, intragenic FliA sites could titrate cellular FliA, thereby sensitizing other FliA promoters to the level of FliA expression (Brewster *et al.*, 2014). Alternatively, titration of FliA could reduce stochasticity in effective FliA levels, by requiring that FliA levels be maintained at higher levels. These functions would be independent of the specific locations of FliA promoters, and more dependent on the number and strength of promoters. Spontaneous creation of FliA binding sites by genetic drift may also provide a source of novel, functional FliA promoters, e.g. if there is a selective advantage of coordinately regulating the downstream gene with flagellar genes.

The *motA* promoter inside *flhC* constrains the evolution of FlhC

Although most intragenic FliA promoters are not conserved, the promoter within *flhC* is the most highly conserved of all FliA promoters. This promoter has been described previously, and drives transcription of the *motAB-cheAW* operon mRNA (Ide *et al.*, 1999; Park *et al.*, 2001; Fitzgerald *et al.*, 2014). FliA promoters require a stringent match to the consensus promoter sequence (Koo, Rhodius, Campbell, *et al.*, 2009), and this is reflected by the high information content in the sequence motif associated with FliA binding, especially in the −10 region (Figure 4C) (Fitzgerald *et al.*, 2014). Hence, conservation of an intragenic FliA promoter is likely to result in conservation of the amino acid sequence for the overlapping codons. The −10 region of the FliA promoter in *flhC* corresponds to an Ala-Asp motif in the FlhC protein. This motif is broadly conserved. Multiple independent lines of evidence support the idea that the Ala-Asp sequence motif is conserved due to selective pressure on the intragenic FliA promoter and not on the amino acids themselves: (i) amino acids close to the Ala-Asp motif that are not associated with FliA promoter elements are poorly conserved (Figure 6A); (ii) the Ala-Asp motif is not present in the X-ray crystal structure of FlhDC (Wang *et al.*, 2006), suggesting that it is in a disordered region; (iii) Asp178 does not detectably contribute to FlhC function (Figure 6B); and (iv) in proteobacterial species where *flhC* and *motA* are adjacent genes but FlhC Asp178 is not conserved, an alternative FliA promoter is often located in the intergenic region between *flhC* and *motA* (Figure 6C). Thus, even in cases where the specific FliA promoter inside *flhC* is not conserved, the presence of a FliA promoter upstream of *motA* is conserved. If the FliA promoter inside *flhC* were conserved because of selective pressure on the Ala-Asp motif, we would expect that (i) surrounding amino acids would also be conserved, regardless of whether they are encoded in sequence overlapping key FliA promoter elements, (ii) the Ala-Asp motif would be part of an important structural motif, (iii) Asp178 would be required for motility, and (iv) in species where Asp178 is not conserved, there would be no selective pressure to acquire an alternative FliA promoter for *motA*. We therefore conclude that the amino acid sequence of FlhC is constrained by the internal promoter for *motA*. Thus, the evolution of FlhC protein sequence is directly impacted by the function of the downstream gene.

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The potential for an abundance of bacterial regulatory sequences that constrain protein evolution

A recent study reported large numbers of putative transcription factor binding sites in the coding sequences of the human genome, and suggested that these sequences are under selective pressure for both their regulatory and coding functions (Stergachis *et al.*, 2013). While the specific findings of that study have been questioned (Xing and He, 2015), the FliA promoter inside *flhC* is clearly analogous. We propose that conservation of intragenic sequences due to selective pressure on their regulatory function is likely to occur far more frequently in bacteria than in eukaryotes. The compact nature of bacterial genomes causes them to be gene-dense, greatly limiting the non-coding sequence space; in *E. coli*, ~90% of the genome is protein-coding, in stark contrast to the human genome, which is <2% protein-coding. Consistent with the paucity of non-coding sequence in bacterial genomes, numerous intragenic binding sites have been identified for transcription factors and σ factors (Wade *et al.*, 2006; Shimada *et al.*, 2008; Hartkoorn *et al.*, 2012; J. Galagan *et al.*, 2013; J. E. Galagan *et al.*, 2013; Bonocora *et al.*, 2013; Wade and Grainger, 2014; Bonocora *et al.*, 2015; Grainger, 2016). In some cases, low stringency in the DNA sequence requirements for binding may allow for sequence changes that change encoded amino acids while maintaining regulatory function. For example, there are many intragenic σ^{70} promoters in *E. coli* (Singh *et al.*, 2014), but σ^{70} promoters can still be active with multiple mismatches to the consensus (Singh *et al.*, 2014). Hence, even if an intragenic σ^{70} promoter is under selective pressure, it could acquire mutations that alter the overlapping coding potential without affecting promoter strength. However, bacterial transcription factors and some alternative σ factors tend to have high information content binding sites, especially compared to their eukaryotic equivalents (Wade *et al.*, 2005; Wunderlich and Mirny, 2009). This suggests that functional conservation of intragenic transcription/σ factor binding sites in bacteria will often constrain evolution of the overlapping gene.

Identification of regulatory sequences that constrain protein evolution requires further investigation of intragenic regulatory sites. Although numerous intragenic binding sites have been identified, their regulatory capacity is often unclear, and their conservation has not been extensively analyzed. Intragenic promoters have been reported in numerous bacterial species (Lybecker *et al.*, 2014; Wade and Grainger, 2014). Limited evolutionary analysis suggests that most promoters for antisense RNAs are not conserved (Raghavan *et al.*, 2012), although there is evidence for lineage-specific conservation (Shao *et al.*, 2014). Importantly, there are specific examples of intragenic σ factor binding that likely constrain evolution of the amino acid sequence encoded by the overlapping protein-coding gene. First, an intragenic promoter for the alternative σ factor, σ^{24}, is conserved both at the sequence level and functionally (Guo *et al.*, 2014; Li *et al.*, 2015). This promoter drives transcription of a non-coding, regulatory RNA, MicL, that is also conserved (Guo *et al.*, 2014). Hence, both the promoter and non-coding RNA might represent dual-usage sequence. Second, an alternative σ factor, σ^{54}, binds many intragenic sites in *E. coli* and *S.* Typhimurium that are conserved both at the sequence level and functionally (Bonocora *et al.*, 2015; Bono *et al.*, 2017), suggesting that they may constrain protein evolution. Since conserved intragenic σ^{54} binding sites are likely to be promoters for downstream genes (Bonocora *et al.*, 2015), evolution of the amino acid

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sequence of proteins encoded by genes containing σ^{54} promoters may often be constrained by the function of the downstream gene.

Extrapolating from our data for FliA, the majority of intragenic transcription/σ factor binding sites are likely to be non-functional, and hence not under selective pressure. These sites would therefore not impact protein evolution. Even though the complete regulons of most *E. coli* transcription/σ factors remain to be mapped, thousands of intragenic sites have already been identified, implying that there are thousands more sites yet to be discovered. Even if only a small fraction of intragenic sites are under selection, this would indicate the existence of many such sequences that constrain protein evolution. Hence, our data suggest that the evolutionary impact of intragenic regulatory sequences should be considered more broadly, as it is likely to be an important factor shaping bacterial genome evolution.

MATERIALS AND METHODS

Strains, plasmids, and growth conditions

All bacterial strains and plasmids used in this study are listed in Table 3. All oligonucleotides used in this study are listed in Table S5. All *E. coli* strains are derivatives of the motile MG1655 strain (DMF36) described previously (Fitzgerald *et al.*, 2014). To construct strains used for β-galactosidase assays, the native *lacZ* gene of DMF36, or the isogenic *fliA* strain (DMF40) (Fitzgerald *et al.*, 2014) was replaced by *thyA* using FRUIT recombineering (Stringer *et al.*, 2012) with oligonucleotides JW5397 and JW5398, generating strains DMF122 and DMF123, respectively. *flhC* and 106 bp downstream sequence was replaced with *thyA* in DMF36 using FRUIT recombineering (Stringer *et al.*, 2012) to generate strain CDS105. *Salmonella* strains are derivatives of *S. enterica* serovar Typhimurium 14028s (Jarvik *et al.*, 2010). *S.* Typhimurium FliA was N-terminally epitope tagged with a 3x-FLAG tag at the native chromosomal locus using FRUIT recombineering (Stringer *et al.*, 2012), generating strain DMF087. The *S.* Typhimurium *fliA* strain, DMF088, was constructed using FRUIT recombineering (Stringer *et al.*, 2012).

Wild-type *flhC* was PCR-amplified using oligonucleotides JW8879 and JW8880, and cloned into the *Sac*I and *Sal*I restriction sites of pBAD30 (Guzman *et al.*, 1995) using the In-Fusion method (Clontech) to generate pCDS043. D178A mutant *flhC* was PCR-amplified using oligonucleotides JW8879 and JW8881, and cloned as described for wild-type *fhlC*, to generate pCDS044. Transcriptional fusions of putative FliA promoters to *lacZ* were constructed in plasmid pAMD-BA-lacZ (Stringer *et al.*, 2014). Putative promoter regions (nucleotide positions −200 to +10, relative to the predicted TSS) were PCR-amplified from MG1655 cells. PCR products were cloned into pAMD-BA-lacZ cut with *Sph*I and *Nhe*I using the In-Fusion method (Clontech). Oligonucleotides used for the plasmid cloning are listed in Table 3.

For all experiments involving liquid growth, subcultures were grown in LB at 37 °C, with aeration, to OD_{600} 0.5–0.7.

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β-galactosidase assays

Transcriptional *lacZ* promoter fusion plasmids were transformed into *lacZ* strains with (DMF122) or without *fliA* (DMF123). Promoter activity was assessed by β-galactosidase assay, as previously described (Stringer *et al.*, 2014).

Analysis of published TSS data

To determine whether FliA binding sites were associated with TSSs, a published list of TSS locations derived from dRNA-seq was used (Thomason *et al.*, 2015). Orientation of putative FliA promoters was determined based on associated motifs. For each putative FliA promoter, the distance from the motif center to each downstream TSS on the correct strand was calculated. All pairwise distances <500 bp are plotted in Figure 2A. As a control, a randomized TSS dataset was generated with the same total number and distribution (with respect to strand and being intragenic/intergenic) as the experimental dataset. The analysis was repeated with this dataset.

Analysis of published NET-seq data

Raw sequencing data files from NET-seq experiments (Larson *et al.*, 2014) were obtained and mapped to the *E. coli* MG1655 genome using CLC Genomics Workbench. Sequence read depths at positions surrounding putative FliA promoters were calculated using a custom Python script. For FliA binding sites associated with a TSS, the NET-seq read coverage was calculated at every position from −100 to +100 relative to the TSS. For FliA binding sites not associated with a TSS, a TSS was predicted to be located 20 bp downstream of the motif center, and NET-seq read coverage was calculated from −100 to +100 relative to this position. For each region, NET-seq read coverage was normalized to local minimum and maximum values. Normalized read coverage was plotted as a heat map in Figure 3B.

FliA binding site conservation analysis across species

The locations of all *E. coli* FliA binding sites described previously (Fitzgerald *et al.*, 2014) were used to identify homologous sequences in 24 other species (Table S1). A Position Specific Scoring Matrix (PSSM) was derived from the identified FliA binding sites in *E. coli* (Fitzgerald *et al.*, 2014), as described previously (Bonocora *et al.*, 2015). We then took a 300 bp sequence surrounding each FliA site in *E. coli* MG1655. For sites within ORFs we used BLASTX (Altschul *et al.*, 1990) to search for homologous protein sequences in the selected bacterial species (BLAST E-value cut-off of $1e^{-04}$, low-complexity filter turned off). Using the PSSM, we scored the top-scoring BLAST hit for each species, searching within 100 bp of the position corresponding to the binding site in *E. coli*. For sites within intergenic regions, we used BLASTN to search for regions homologous to each of the 300 bp sequences in each of the selected species (BLAST E-value cut-off of $1e^{-04}$, low-complexity filter turned off), and extracted 100 bp on either side of the position corresponding to the position of the site in *E. coli*. If no hits were found, we took the sequence of the downstream gene in *E. coli* and used BLASTX to search for homologues in the selected species (BLAST E-value cut-off of $1e^{-04}$, low-complexity filter turned off). For each top BLAST hit, we used the position of the binding site in *E. coli* relative to the downstream gene to determine the predicted site of binding, and extracted 100 bp on either side. We calculated PSSM scores

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for all sequences in each of the selected regions. The best score for each region tested was selected for plotting in Figure 3A.

FliA binding site conservation in *E. coli* strains

All complete or partial genome sequences for *E. coli* (9432 genomes or contigs; Table S2) were downloaded directly from NCBI and individually scored for the presence FliA sites using the method described above for comparison to other species.

ChIP-seq of *S.* Typhimurium FliA

ChIP-seq was performed with strains DMF087 (FliA-FLAG$_3$) or 14028s (untagged control) as previously described (Stringer *et al.*, 2014). Sequence reads were mapped to the *S.* Typhimurium 14028s genome using CLC Genomics Workbench (Version 8). Peaks were called using a previously described analysis pipeline (Fitzgerald *et al.*, 2014). Three peaks with a FAT score of 1 were identified in the control dataset; these peaks were all >30 kbp from any putative FliA binding site.

RNA-seq

RNA-seq was performed with strains 14028s and DMF088, as previously described (Stringer *et al.*, 2014). Read mapping and differential expression analysis were performed using Rockhopper (McClure *et al.*, 2013). The normalized expression values and indicators of statistical significance in Table 2 were generated using Rockhopper.

Analysis of FlhC sequence conservation

We used the RSAT "Comparative Genomics/Get Orthologs" tool (default parameters, except we required 50% amino acid sequence identity; (Medina-Rivera *et al.*, 2015)) to identify 52 FlhC homologues from γ-proteobacterial species, each from a different genus. We aligned protein sequences using MUSCLE (v3.8, default parameters; (Edgar, 2004); Table S3), and for each FlhC homologue we counted matches at each amino acid position to the aligned *E. coli* FlhC sequence.

Identification of enriched sequence motifs in *flhC*-*motA* intergenic regions

We used the RSAT "Comparative Genomics/Get Orthologs" tool (default parameters, except we required 40% amino acid sequence identity; (Medina-Rivera *et al.*, 2015)) to identify 130 FlhC homologues from proteobacterial species, each from a different genus. We aligned these protein sequences using MUSCLE (v3.8, default parameters; (Edgar, 2004); Table S4). To determine whether the *flhC* and *motA* genes are adjacent in each of the 131 species selected, we first used the RSAT "Comparative Genomics/Get Orthologs" tool (default parameters except required 40% amino acid sequence identity; (Medina-Rivera *et al.*, 2015)) to extract 100 bp of sequence immediately downstream of the end of the intergenic region following *flhC* for each species. We then searched for open reading frames similar to that of *E. coli* K-12 *motA* using BLASTX (v2.2.3, hosted on EcoGene 3.0, default parameters, searching against the *E. coli* annotated proteome; (Altschul *et al.*, 1997; Zhou and Rudd, 2013)). We discarded 32 FlhC sequences for which there was no BLASTX match to MotA with the corresponding sequence downstream of *flhC*. For each of the 98 remaining FlhC

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homologues, using the MUSCLE alignment described above (Table S4), we determined whether *E. coli* K-12 Asp178 is conserved.

We used the RSAT "Comparative Genomics/Get Orthologs" tool (Medina-Rivera *et al.*, 2015) to extract intergenic sequence downstream of *flhC* for the 98 FlhC homologues from genomes where *flhC* and *motA* are adjacent genes. We discarded intergenic sequences <50 bp. We used MEME (v4.12.0, default settings, except we selected the "look on given strand only" option; (Bailey and Elkan, 1994)) to identify enriched sequence motifs in intergenic regions from species where FlhC Asp178 is conserved (n = 55) or is not conserved (n = 43), respectively.

Motility assays

Motility assays were performed as previously described (Fitzgerald *et al.*, 2014).

Estimating the number of single base substitutions that would create a new FliA site in *E. coli*

We used the *E. coli* FliA PSSM (Fitzgerald *et al.*, 2014) to calculate motif scores for all 27mer sequences in the *E. coli* MG1655 genome. For each score window between integer values (e.g. scores between 10 and 11, scores between 11 and 12, etc.), we determined the frequency of sequences that represent actual FliA binding sites, as determined previously by ChIP-seq (Fitzgerald *et al.*, 2014). We then calculated motif scores for every 27mer in the genome with every possible single base substitution (i.e. 81 scores for each sequence). We binned scores in whole integer windows (e.g. a bin for scores between 10 and 11, a bin for scores between 11 and 12, etc.) and used the frequencies calculated for actual sites to estimate the number of mutated 27mers that would represent real FliA sites.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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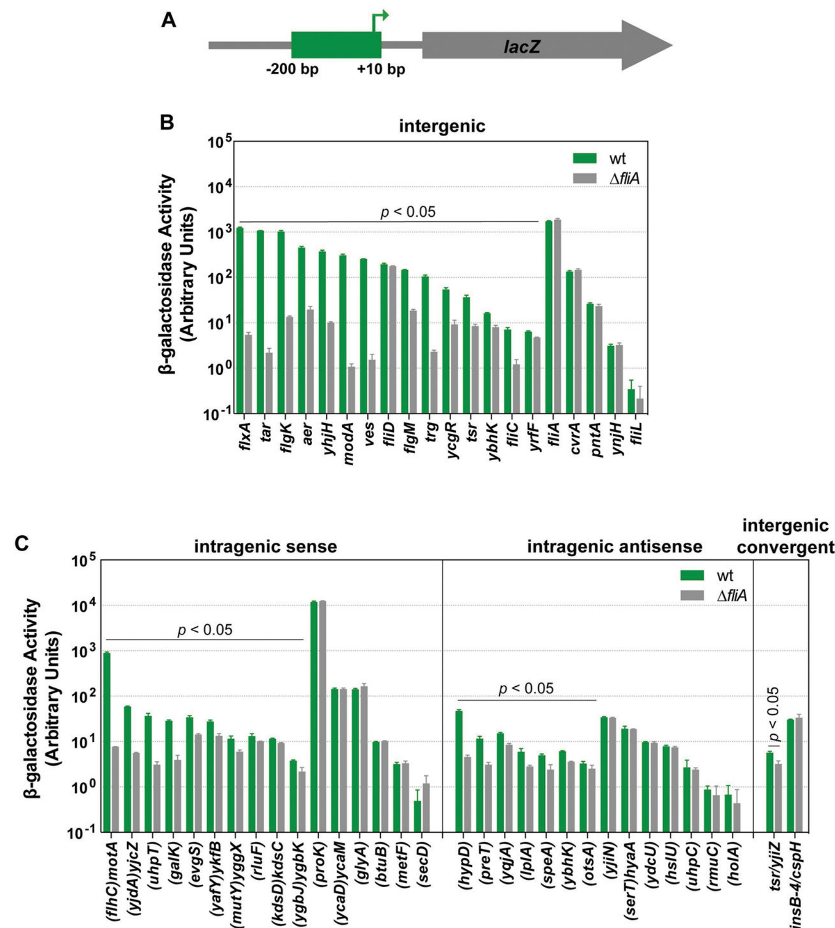

Figure 1. Identification of transcriptionally active FliA binding sites using reporter gene fusions

(A) Schematic of transcriptional fusions of potential FliA promoters to the *lacZ* reporter gene. For all FliA binding sites identified in a previous study, transcriptional fusions to *lacZ* were constructed using positions −200 to +10 relative to the predicted TSS based on the previously identified FliA binding motif (Fitzgerald *et al.*, 2014). **(B)** β-galactosidase activity for transcriptional fusions for FliA binding sites in intergenic regions upstream of genes, for wild-type (wt; DMF122; green bars) and *fliA* (DMF123; gray bars) cells. Reporter fusions that showed significantly lower β-galactosidase activity in *fliA* cells than wild-type cells (*t*-test $p < 0.05$) are indicated. The genes downstream of the FliA binding sites are listed on the x-axis. **(C)** As above, but for FliA binding sites within genes or between convergently transcribed genes. Genes containing FliA binding sites are listed on the x-axis in parentheses. Genes not in parentheses are downstream of the corresponding FliA binding site. Error bars indicate one standard deviation from the mean (n = 3).

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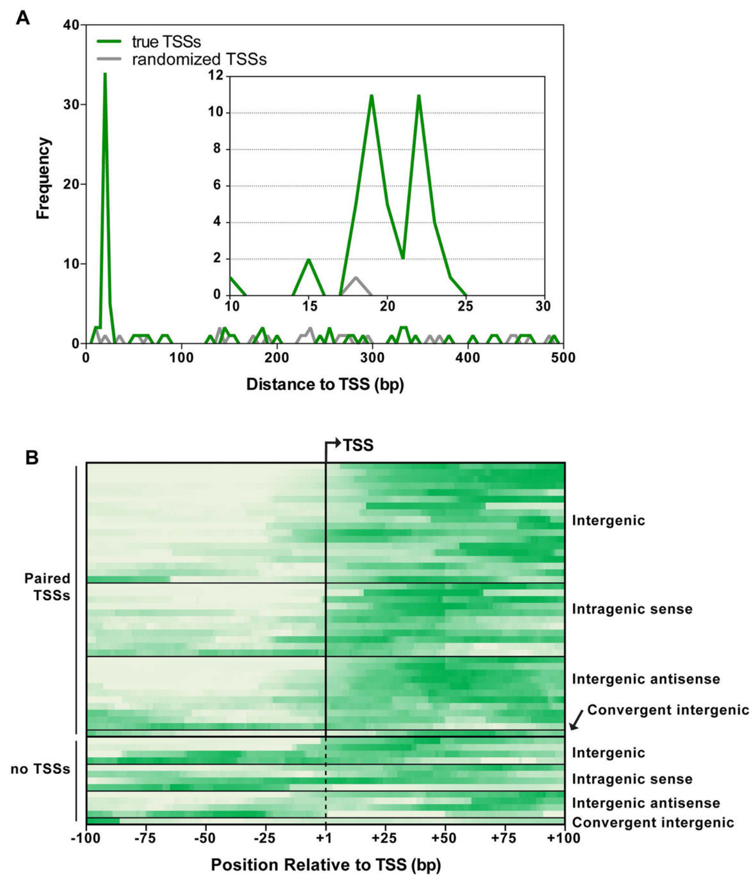

Figure 2. Identification of transcriptionally active FliA binding sites by mining genome-scale transcriptome datasets

(A) For each FliA binding site identified previously (Fitzgerald *et al.*, 2014), we determined the distance to each downstream TSS identified previously (Thomason *et al.*, 2015) within a 500 bp range. The frequencies of these distances are plotted in 10 bp bins (green line), with the inset showing the frequency of binding sites 10–30 bp upstream of TSSs with a bin size of 1 bp. The gray line shows the frequency of distances from FliA binding sites to a control, randomized TSS dataset (see Methods). **(B)** Normalized sequence read coverage from published NET-seq data (Larson *et al.*, 2014) (see Methods) for each previously identified FliA binding site (Fitzgerald *et al.*, 2014), plotted 100 bp upstream and downstream of the known/predicted TSS. Predicted TSSs are indicated by the dashed vertical line. Darker green indicates higher sequence read density.

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Figure 3. Sequence conservation of FliA binding sites between *E. coli* and related bacterial species

(A) Heat-map depicting the match to the FliA consensus binding site for regions in the genomes of a range of bacterial species, where the region analyzed is homologous to a region surrounding a FliA binding site in *E. coli*. Genera are listed on the left. *E. coli* genes associated with the binding sites are listed across the top of the heat-map. FliA binding sites are grouped by location/orientation category, as indicated by category labels across the bottom of the heat-map. Genes containing FliA binding sites are listed in parentheses. Genes not in parentheses are downstream of the corresponding FliA binding site. The color scale indicating the strength of the sequence match is shown next to the heat-map. Empty squares in the heat-map indicate that the corresponding genomic region in *E. coli* is not sufficiently conserved in the species being analyzed. **(B)** Conservation of FliA sites across 9,432 *E. coli* strains. For each site from *E. coli* K-12, conservation was determined at each position within the site for all strains of *E. coli* where the surrounding sequence is conserved. Thus, the fraction of genomes in which each base is conserved was calculated. Values plotted represent the average (mean) level of conservation for (i) 18 FliA sites that represent promoters for mRNAs (filled circles; Table 1), and (ii) the remaining 34 FliA sites (empty circles). The FliA binding motif is shown above the graph as a reference point for each of the site positions.

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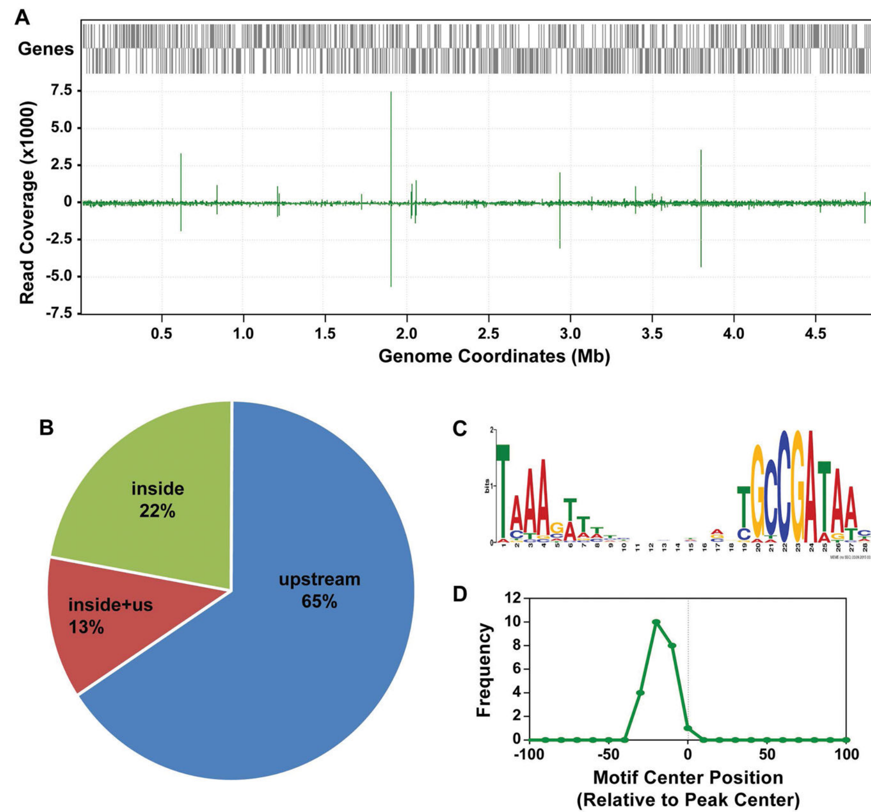

Figure 4. Identification of FliA binding sites in *Salmonella* Typhimurium using ChIP-seq

(A) Sequence read coverage across the *S.* Typhiumurium genome for a FliA ChIP-seq dataset. Annotated genes are indicated by gray bars. The green graph shows relative sequence read coverage, with "spikes" corresponding to sites of FliA association. **(B)** Pie-chart showing the distribution of identified FliA binding sites relative to genes. "Inside" = FliA binding within a gene. "Upstream" = FliA binding upstream of a gene. "Inside + us" = FliA binding within a gene but within 300 bp of a downstream gene start. **(C)** Enriched sequence motif associated with FliA binding sites identified by ChIP-seq. **(D)** Distribution of motifs relative to ChIP-seq peak centers for all FliA binding sites identified by ChIP-seq. Motifs are enriched in the region ~25 bp upstream of the peak center, relative to the motif orientation.

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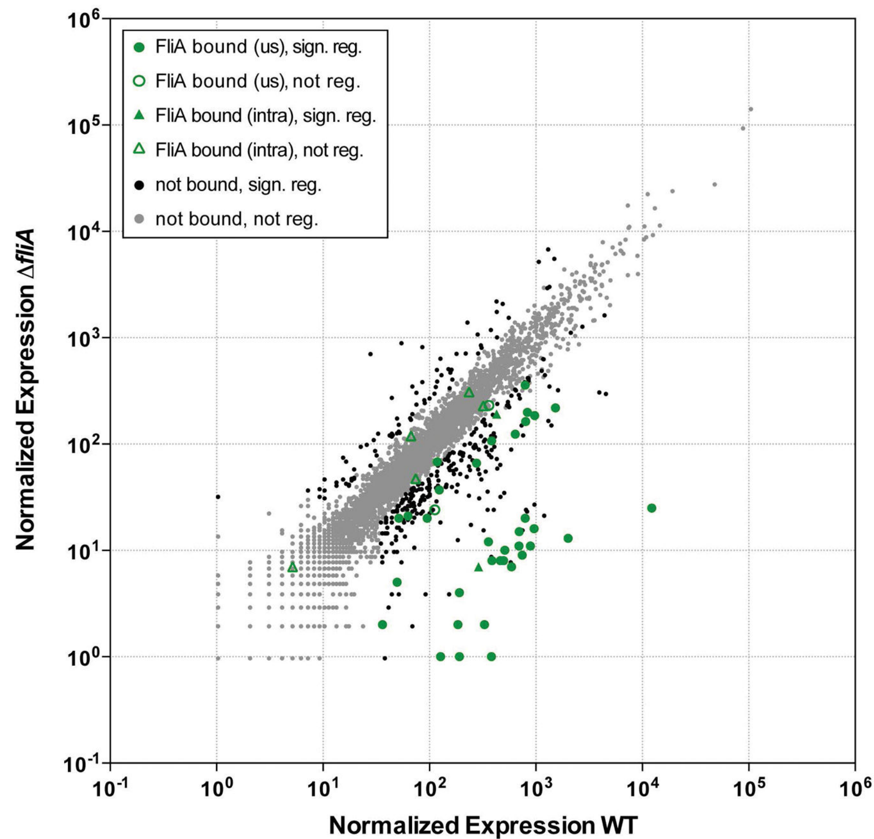

Figure 5. Transcriptome analysis of the FliA regulon in *Salmonella* Typhimurium

The scatter-plot shows normalized expression (see Methods) for each gene in *S.* Typhimurium for wild-type cells (14028s; x-axis) or *fliA* cells (DMF088; y-axis). Gray dots represent genes that are not associated with a FliA binding site and are not significantly differentially expressed between wild-type and *fliA* cells. Black dots represent genes that are not associated with a FliA binding site and are significantly differentially expressed between wild-type and *fliA* cells. Green circles represent genes that are associated with an upstream FliA binding site. Green triangles represent genes that are associated with an internal FliA binding site. Filled green circles/triangles indicate genes that are significantly differentially expressed between wild-type and *fliA* cells. Empty green circles/triangles represent genes that are not differentially expressed between wild-type and *fliA* cells.

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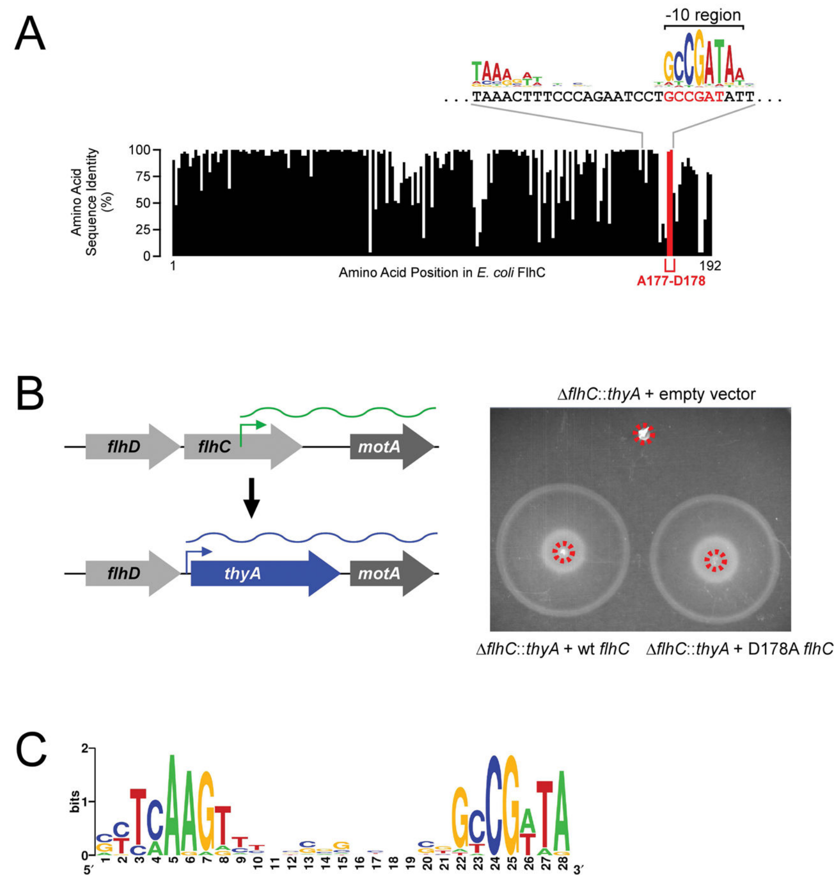

Figure 6. The FliA promoter within *flhC* constrains evolution of FlhC amino acid sequence

(A) Sequence conservation of FlhC amino acid sequence between *E. coli* and 51 other γ-proteobacterial species. The graph indicates the level of identity across all species analyzed for each amino acid in FlhC; data for Ala177 and Asp178 are highlighted in red. The nucleotide sequence of *flhC* in the *motA* promoter region is indicated, aligned with the previously reported FliA binding motif logo (Fitzgerald *et al*., 2014). Codons 177 and 178 are shown in red. **(B)** Motility assay for *flhC::thyA E. coli* (CDS105) containing either empty vector (pBAD30), or plasmid expressing wild-type FlhC (pCDS043) or D178A mutant FlhC (pCDS044). Dashed red circles indicate the inoculation sites. Plates were incubated for 7 hours. The schematic to the left of the plate image shows how the strain was constructed. **(C)** Enriched sequence motif found in the *flhC*-*motA* intergenic regions of species in which FlhC Asp178 is not conserved. This motif is a close match to the known FliA binding site consensus.

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Table 1

Intragenic FliA binding sites show evidence of transcriptional activity.

Putative Promoter[1]	β-gal[2]	TSS[3]	NET-seq[4]	RNA-seq[5]
Intergenic				
*aer**	✓	✓	✓	✓
*fliC**	✓	✓	✓	✓
*flxA**	✓	✓	✓	✓
*modA**	✓	✓	✓	✓
*tar**	✓	✓	✓	✓
*trg**	✓	✓	✓	✓
*ycgR**	✓	✓	✓	✓
*yhjH**	✓	✓	✓	✓
*flgK**	✓	✓	✓	-
*flgM**	✓	✓	✓	-
*fliA**	-	✓	✓	✓
*tsr**	✓	-	✓	✓
*ves**	✓	✓	✓	-
ybhK	✓	✓	✓	-
cvrA	-	✓	✓	-
*fliD**	-	✓	✓	-
*fliL**	-	-	✓	-
pntA	-	-	✓	-
*ynjH**	-	-	-	✓
yrfF	✓	-	-	-
Intragenic Sense				
*(flhC)motA**	✓	✓	✓	✓
(uhpT)	✓	✓	✓	✓
*(yafY)ykfB**	✓	✓	✓	✓
*(yjdA)yjcZ**	✓	✓	✓	✓
(evgS)	✓	✓	✓	-
(galK)	✓	✓	✓	-
(kdsD)kdsC	✓	✓	✓	-
(mutY)yggX	✓	✓	✓	-
(ygbJ)ygbK	✓	✓	✓	-
(metF)	-	✓	✓	-
(rluF)	✓	✓	-	-
(btuB)	-	✓	-	-
(secD)	-	-	✓	-
(glyA)	-	-	-	-

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Putative Promoter[1]	β-gal[2]	TSS[3]	NET-seq[4]	RNA-seq[5]
(proK)	-	-	-	-
(ycaD)ycaM	-	-	-	-
Intragenic Antisense				
(hypD)	✓	✓	✓	✓
(lplA)	✓	✓	✓	-
(preT)	✓	✓	✓	-
(speA)	✓	✓	✓	-
(ybhK)	✓	✓	✓	-
(yqjA)	✓	✓	✓	-
(holA)	-	✓	✓	-
(otsA)	-	✓	✓	-
(rmuC)	-	✓	✓	-
(hslU)	-	✓	-	-
(uhpC)	-	-	✓	-
(ydcU)	-	-	✓	-
(yjiN)	-	-	✓	-
(serT)hyaA	-	-	-	-
Intergenic (between convergent genes)				
tsr/yjiZ	-	✓	✓	-
insB-4/cspH	-	-	-	-

[1]Genes associated with FliA binding sites. Genes in parentheses have an internal FliA binding site; genes not in parentheses start <300 bp downstream of a FliA binding site and are orientated in the same direction as the putative promoter. Asterisks indicate FliA binding sites previously reported to be associated with transcription of an mRNA (Fitzgerald *et al.*, 2014).

[2]Check marks indicate a significant difference in β-galactosidase activity between *fliA*$^+$ and *fliA* cells for the corresponding *lacZ* transcriptional fusion (Figure 1).

[3]Check marks indicate association with a nearby TSS.

[4]Check marks indicate a downstream:upstream (relative to the putative TSS) coverage ratio 2.

[5]Check marks indicate regulation of the corresponding gene(s), as determined using RNA-seq (Fitzgerald *et al.*, 2014).

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Table 2

FliA regulon of *Salmonella* Typhimurium 14028s.

Peak Center[1]	FAT[2]	Motif Center[3]	Motif Strand[4]	Motif[5]	Gene(s)[6]	Expression (wt)[7]	Expression (*fliA*)[7,8]
624724	29	624746	–	TAAAAAGAAAAGAGTGCGCGCCGATATA	STM14_0662	115	68*
845911	8	845901	+	TCAAGATCCTGCTTTTCCTGCCGATATT	*modA*	120	37*
1215557	9	1215575	–	TAAAGATTTTGTCGCGGCTGCCGATGAG	*flgM*	776	163*
1223602	5	1223586	+	TCAAGTCCACGTAGTCGCTGCCGATAAC	*flgK*	943	185*
1727514	4	1727546	–	TAAGCAAACGTCACGAAGTGCCGATGAC	*trg*	185	1*
1907932	58	1907909	+	TTAAGTTCTTTCTACCGATGCCGATAAC	*ycgR*	179	2*
2027840	8	2027866	–	TAAAGTTATCACCGCAGGTGCCGATAAC	*cheM*	370	1*
2031328	1	2031360	–	TAAAGAATTAATTCAGATTGCCGAATAT	*(motB)*	(572)	(7)*
2032570	8	2032596	–	TAAACTTTCCCGAGATGCTGCCGATATT	*(flhC)motA*	(311)483	(228)8*
2056900	7	2056910	–	TGTAGAAACGGATAATCATGCCGATAAC	*fliA*	–	–
2060603	10	2060633	–	TAAAGTTCGAAATTCAGGTGCCGATACA	*fliC*	11938	25*
2060764	6	2060755	+	TAAACTTTGCCTCCAGATTGCCGATAAC	*fliD*	1478	219*
2472155	1	2472147	+	TCCATTTTTTGTCCTCATTGCCGATAAC	STM14_2852	932	16*
2728195	1	2728218	–	AAAACTGTTCCTGTGCTGCGCCGATAAT	*(pepB)*	(65)	(119)
2935946	23	2935935	+	TAAAGTTTATGCCTCAAGTGTCGATAAC	(STM14_3340) *fljBA*	(280)1954	(7)*13*
3334267	2	3334287	–	TATATATTCTCCGCGCCTTGCCGATAAC	STM14_3817	48	5*
3400660	9	3400673	–	TAAAGTTAGTAACATTATTGCCGATAAA	STM14_3893	863	11*
3402562	2	3402592	–	TAAAGATAAATAGATTAGCGCCGAAATA	(STM14_3895) *aer*	(5)185	(7)4*
3504766	2	3504795	–	TAAAAAATTCTGGGATGCCACCGATAAA	*(arcB)*	(228)	(308)
3559655	4	3559683	–	TAACAAAATGCTCGATCGTGACGATATG	*(yhdA)*	(72)	(47)
3801092	34	3801114	–	TAAAGTTCTCTGCCTGAACGCCGATAAC	*yhjH*	318	2*
4531425	5	4531410	+	TCAACCCGAACAATAAATTGCCGATAAC	*(nrfB)*	(414)	(191)*
4802894	10	4802872	+	TAAAGTTTTCCCTTTCCAGGCCGAAAAT	*tsr*	450	8*

[1]Genome coordinate of the ChIP-seq peak center. Coordinates are relative to the 14028s chromosomal reference sequence (NC_003198.1).

[2]Fold Above Threshold (FAT) score, a measure of relative ChIP-seq enrichment.

[3]Genome coordinate of the sequence motif identified using MEME. Coordinates are relative to the 14028s chromosomal reference sequence (NC_003198.1).

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[4]Genomic strand of the sequence motif identified using MEME.

[5]Sequence of the motif identified using MEME.

[6]For intergenic FliA binding sites, the downstream gene is listed. Genes containing intragenic FliA binding sites are listed in parentheses. Underlining indicates that the putative promoter is in the antisense orientation relative to the overlapping gene. If a gene start is located within 300 bp of a putative intragenic FliA promoter, that gene name is listed as well.

[7]Normalized expression values for the indicated genes, as determined by RNA-seq.

[8]Asterisks indicate significant differential expression between wild-type and *fliA* cells ($q < 0.01$).

Table 3

Strains and Plasmids used in this study.

Strains		
Name	**Description**	**Source**
DMF36	Motile *Escherichia coli* MG1655	(Fitzgerald *et al.*, 2014)
DMF122	DMF36 *lacZ*	This study
DMF123	DMF123 *fliA*	This study
CDS105	DMF36 *thyA flhC::thyA*	This study
14028s	Wild-type *Salmonella* Typhimurium	(Jarvik *et al.*, 2010)
DMF087	14028s $FLAG_3$-*fliA*	This study
DMF088	14028s *fliA*	This study

Plasmids			
Name	**Description**	**Oligonucleotides used for Cloning**	**Source**
pAMD-BA-lacZ	Single-copy lacZ expression vector (cam^R)	n/a	(Stringer *et al.*, 2014)
pDMF35	pAMD-BA-lacZ with FliA promoter (*yafY*) *ykfB*	JW5523/JW5567	This study
pDMF36	pAMD-BA-lacZ with FliA promoter (*secD*)	JW5356/JW5357	This study
pDMF37	pAMD-BA-lacZ with FliA promoter (*holA*)	JW5352/JW6127	This study
pDMF38	pAMD-BA-lacZ with FliA promoter (*galK*)	JW5524/JW5568	This study
pDMF39	pAMD-BA-lacZ with FliA promoter *modA*	JW5525/JW5569	This study
pDMF40	pAMD-BA-lacZ with FliA promoter (*ybhK*)	JW5526/JW6130	This study
pDMF41	pAMD-BA-lacZ with FliA promoter *ybhK*	JW5527/JW5571	This study
pDMF42	pAMD-BA-lacZ with FliA promoter (*ycaD*) *ycaM*	JW5528/JW6132	This study
pDMF43	pAMD-BA-lacZ with FliA promoter (*serT*) *hyaA*	JW5529/JW5573	This study
pDMF44	pAMD-BA-lacZ with FliA promoter *insB-4*/*cspH*	JW5530/JW5574	This study
pDMF45	pAMD-BA-lacZ with FliA promoter *flgM*	JW5531/JW6135	This study
pDMF46	pAMD-BA-lacZ with FliA promoter *flgK*	JW5532/JW6136	This study
pDMF47	pAMD-BA-lacZ with FliA promoter *cvrA*	JW5533/JW5577	This study
pDMF48	pAMD-BA-lacZ with FliA promoter *ycgR*	JW5534/JW6138	This study
pDMF49	pAMD-BA-lacZ with FliA promoter *trg*	JW5535/JW5579	This study
pDMF50	pAMD-BA-lacZ with FliA promoter (*ydcU*)	JW5536/JW5580	This study
pDMF51	pAMD-BA-lacZ with FliA promoter *flxA*	JW5537/JW5581	This study
pDMF52	pAMD-BA-lacZ with FliA promoter *pntA*	JW5538/JW5582	This study
pDMF53	pAMD-BA-lacZ with FliA promoter *ves*	JW5539/JW6143	This study
pDMF54	pAMD-BA-lacZ with FliA promoter *ynjH*	JW5540/JW6144	This study
pDMF55	pAMD-BA-lacZ with FliA promoter *tar*	JW5541/JW6145	This study
pDMF56	pAMD-BA-lacZ with FliA promoter (*flhC*) *motA*	JW5542/JW5586	This study
pDMF57	pAMD-BA-lacZ with FliA promoter (*otsA*)	JW5543/JW6147	This study
pDMF58	pAMD-BA-lacZ with FliA promoter *fliA*	JW5544/JW5588	This study
pDMF59	pAMD-BA-lacZ with FliA promoter *fliC*	JW5342/JW5343	This study

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Plasmids			
Name	**Description**	**Oligonucleotides used for Cloning**	**Source**
pDMF60	pAMD-BA-lacZ with FliA promoter *fliD*	JW5545/JW6150	This study
pDMF61	pAMD-BA-lacZ with FliA promoter *fliL*	JW5546/JW6151	This study
pDMF62	pAMD-BA-lacZ with FliA promoter (*preT*)	JW5547/JW6152	This study
pDMF63	pAMD-BA-lacZ with FliA promoter (*evgS*)	JW5548/JW5592	This study
pDMF64	pAMD-BA-lacZ with FliA promoter (*glyA*)	JW5549/JW6154	This study
pDMF65	pAMD-BA-lacZ with FliA promoter (*hypD*)	JW5348/JW5349	This study
pDMF66	pAMD-BA-lacZ with FliA promoter (*ygbJ*)*ygbK*	JW5550/JW5594	This study
pDMF67	pAMD-BA-lacZ with FliA promoter (*speA*)	JW5350/JW5351	This study
pDMF68	pAMD-BA-lacZ with FliA promoter (*mutY*)*yggX*	JW5551/JW6158	This study
pDMF69	pAMD-BA-lacZ with FliA promoter *aer*	JW5552/JW5596	This study
pDMF70	pAMD-BA-lacZ with FliA promoter (*yqjA*)	JW5553/JW5597	This study
pDMF71	pAMD-BA-lacZ with FliA promoter (*kdsD*)*kdsC*	JW5554/JW5598	This study
pDMF72	pAMD-BA-lacZ with FliA promoter *yrfF*	JW5344/JW5345	This study
pDMF73	pAMD-BA-lacZ with FliA promoter *yhjH*	JW5555/JW5599	This study
pDMF74	pAMD-BA-lacZ with FliA promoter (*proK*)	JW5556/JW6164	This study
pDMF75	pAMD-BA-lacZ with FliA promoter (*uhpT*)	JW5346/JW5347	This study
pDMF76	pAMD-BA-lacZ with FliA promoter (*uhpC*)	JW5557/JW5601	This study
pDMF77	pAMD-BA-lacZ with FliA promoter (*rmuC*)	JW5558/JW5602	This study
pDMF78	pAMD-BA-lacZ with FliA promoter (*hslU*)	JW5559/JW6168	This study
pDMF79	pAMD-BA-lacZ with FliA promoter (*metF*)	JW5560/JW6169	This study
pDMF80	pAMD-BA-lacZ with FliA promoter *btuB*	JW5561/JW5605	This study
pDMF81	pAMD-BA-lacZ with FliA promoter (*rluF*)	JW5354/JW5355	This study
pDMF82	pAMD-BA-lacZ with FliA promoter (*yjdA*)*yjcZ*	JW5562/JW5606	This study
pDMF83	pAMD-BA-lacZ with FliA promoter (*yjiN*)	JW5563/JW5607	This study
pDMF84	pAMD-BA-lacZ with FliA promoter *tsr*	JW5564/JW6174	This study
pDMF85	pAMD-BA-lacZ with FliA promoter *tsr*/*yjiZ* convergent	JW5565/JW6175	This study
pDMF86	pAMD-BA-lacZ with FliA promoter (*lplA*)	JW5566/JW6176	This study
pBAD30	Empty pBAD30	n/a	(Guzman *et al.*, 1995)
pCDS043	pBAD30-*flhC*	JW8879/8880	This study
pCDS044	pBAD30–D178A *flhC*	JW8879/8881	This study

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