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Recommended Citation
DOI: https://doi.org/10.1128/JB.00385-19
Available at: https://irl.umsl.edu/biology-faculty/110

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Multidrug Resistance Regulators MarA, SoxS, Rob, and RamA Repress Flagellar Gene Expression and Motility in Salmonella enterica Serovar Typhimurium

Abstract: Production of flagella is costly and subject to global multilayered regulation, which is reflected in the hierarchical control of flagellar production in many bacterial species. For Salmonella enterica serovar Typhimurium and its relatives, global regulation of flagellar production primarily occurs through the control of flhDC transcription and mRNA translation. In this study, the roles of the homologous multidrug resistance regulators MarA, SoxS, Rob, and RamA (constituting the mar-sox-rob regulon in S. Typhimurium) in regulating flagellar gene expression were explored. Each of these regulators was found to inhibit flagellar gene expression, production of flagella, and motility. To different degrees, repression via these transcription factors occurred through direct interactions with the flhDC promoter, particularly for MarA and Rob. Additionally, SoxS repressed flagellar gene expression via a posttranscriptional pathway, reducing flhDC translation. The roles of these transcription factors in reducing motility in the presence of salicylic acid were also elucidated, adding a genetic regulatory element to the response of S. Typhimurium to this well-characterized chemorepellent. Integration of flagellar gene expression into the mar-sox-rob regulon in S. Typhimurium contrasts with findings for closely related species such as Escherichia coli, providing an example of plasticity in the mar-sox-rob regulon throughout the Enterobacteriaceae family.

IMPORTANCE The mar-sox-rob regulon is a large and highly conserved stress response network in the Enterobacteriaceae family. Although it is well characterized in E. coli, the extent of this regulon in related species is unclear. Here, the control of costly flagellar gene expression is connected to the mar-sox-rob regulon of S. Typhimurium, contrasting with the E. coli regulon model. These findings demonstrate the flexibility of the mar-sox-rob regulon to accommodate novel regulatory targets, and they provide evidence for its broader regulatory role within this family of diverse bacteria.
Motility affords many bacteria the ability to migrate to more favorable environments. Despite its intrinsic benefits, most bacteria selectively engage in or repress motile behavior in response to specific conditions (1, 2). These forms of regulation are particularly true of flagellar motility, for which sizeable energetic costs are associated with synthesis and function (3, 4). Correspondingly, transcriptional and posttranscriptional regulation of flagellar and chemotaxis gene expression is common and requires integration of numerous environmental and nutritional signals. Such integration leads to optimal expression of structural and chemosensory proteins required for flagellar assembly and motility (5, 6).

For *Salmonella enterica* serovar Typhimurium and its close relatives, expression of flagellar and chemotaxis genes is transcriptionally coregulated in a hierarchical manner (Fig. 1A). In this arrangement, the heterohexameric transcription factor FlhD₄C₂ is expressed from the *flhDC* operon (class I genes) and serves as a master regulator that activates a number of downstream flagellar structural genes (class II genes), including an alternative sigma factor, FliA (or σ²₈) (5). FliA subsequently initiates expression of late flagellar and chemotaxis genes (class III genes) (5, 7, 8). Beyond transcriptional regulation, several secretion-dependent feedback mechanisms provide molecular checkpoints during flagellar assembly, often by regulating FliA and FlhD₄C₂ DNA binding or stability (9–13). Based on this regulatory architecture, the majority of known global regulation occurs at the level of *flhDC* transcription or alteration of FlhD₄C₂ activity (1, 2, 5).

Several transcription factors are known to activate and to repress *flhDC* transcription in *S. Typhimurium*. Transcriptional activators include cAMP receptor protein (CRP), Fur, the nucleoid-binding proteins Fis and histone-like nucleoid-structuring (H-NS) protein, and SlyA; autoactivation by FlhD₄C₂ itself also occurs (14–19). HilD, a key regulator of *Salmonella* pathogenicity island 1 (SPI-1), has also been shown to activate *flhDC* transcription, illustrating cross talk between flagellar and pathogenicity-associated gene expression (20). Several regulators, including RtsB, LhrA, OmpR, SsrB, and RcsB, attenuate *flhDC* expression (21–25). RcsB-mediated repression of *flhDC* is coordinated by the FlhD₄C₂-controlled regulator RflM (also known as EcnR) (26, 27). Repression of *flhDC* expression is also mediated through posttranslational regulation of FlhD₄C₂ by FliZ and FliT, YdiV (a nutritional regulator), FimZ (a fimbrial regulator), and others (12, 13, 28, 29). Interactions between these proteins and FlhD₄C₂ result in reduced FlhD₄C₂-dependent activation of *flhDC* expression. Through these varied regulatory systems, numerous environmental and nutritional signals are integrated to control transcription of flagellar and chemotaxis genes.
FIG 1 Repression of motility, flagellin levels, and flagellar gene expression by MarA, SoxS, Rob, and RamA. Each transcription factor was expressed from pBAD30 (pMarA, pSoxS, pRob, or pRamA) in a marRAB soxRS rob ramRA quadruple mutant genetic background (strain LCM2380) unless otherwise noted. (A) Proposed model of MarA, SoxS, Rob, and RamA repression of flagellar gene expression. (B) Effects of MarA, SoxS, Rob, and RamA production on motility, compared to a pBAD30 plasmid control. Motility assays were conducted at room temperature in soft tryptone agar supplemented with 0.2% arabinose to induce expression of marA, soxS, rob, or ramA. (C) Effects of MarA, SoxS, Rob, and RamA production on levels of flagellin (FliC), compared to a pBAD30 plasmid control, as determined by Western blotting. Cell extracts from cultures grown in tryptone broth supplemented with 0.2% arabinose were displayed (100 µg total protein) on 12% acrylamide SDS-PAGE gels prior to transfer to a PVDF membrane and immunoblotting for FliC and DnaK. DnaK levels were used as an internal loading control for each sample. (D) Effects of MarA, SoxS, Rob, and RamA production on levels of flagellin (FliC), compared to a pBAD30 plasmid control, as quantified by an indirect ELISA using FliC-specific primary antibody and horseradish peroxidase-conjugated secondary antibody. Cell extracts (25 µg of total protein) used for ELISA were obtained under the same conditions as for Western blotting. Light emission from each sample was normalized to the pBAD30 plasmid control, and results are presented as a percentage of the FliC level in the pBAD30 plasmid control. Six replicate measurements were made for each plasmid-bearing strain. Letter labels (a to d) represent statistically significant groups (P < 0.05, Tukey’s HSD test). (E) Effects of MarA, SoxS, Rob, and RamA production on flhD, flhB, and fliC transcription, compared to a pBAD30 plasmid control. The marA, soxS, rob, or ramA gene was expressed from pBAD30 in strains in which flhD, flhB, and fliC promoters were transcriptionally fused to yfp in the quadruple mutant genetic background (strains LCM2416, LCM2431, and LCM2446). Fluorescence measurements were made with mid-logarithmic-phase cultures grown in tryptone broth supplemented with 0.2% arabinose, and results were normalized to culture density. Levels of flhD, flhB, and fliC promoter activity in each plasmid-bearing background are presented as a percentage of the expression from each promoter in the pBAD30 plasmid control. Six replicate measurements were made for each plasmid-bearing strain. Letter labels (a to e) represent statistically significant groups (P < 0.05, Tukey’s HSD test) for each transcriptional fusion.

In addition to the transcription-level regulation of flhDC described above, flhDC mRNA is regulated posttranscriptionally. From studies in Escherichia coli and S. Typhimurium, several regulatory small RNAs (sRNAs) with negative effects on flhDC translation, including ArcZ, OmrA, OmrB, and OxyS, have been identified; McaS was observed to positively influence motility through stabilization of the flhDC transcript (30, 31). These sRNAs are conserved in S. Typhimurium and presumably have similar effects on flhDC mRNA translation. Highlighting the importance of sRNA-mediated regulation of flhDC translation, strains with mutations in hfq (encoding the sRNA-binding chaperone Hfq) are severely
impaired in motility (32). Apart from sRNA, *flhDC* mRNA stability is also regulated by direct binding of the carbon storage regulator CsrA (33, 34).

Beyond direct genetic effects, exposure to aromatic acids, phenolic compounds, and other aromatic compounds is known to inhibit the production of flagella and motility in several enteric gammaproteobacteria (35–37). Many aromatic acids, such as salicylic acid, are also well-characterized chemorepellents (38). In the case of membranepermeable aromatic acids, such as benzoic acid, effects on motility have been attributed to disruption of the proton motive force by shuttling of protons across the cytoplasmic membrane (39, 40). Additionally, certain phenols, such as curcumin, have been shown to bind to flagellin monomers, inducing flagellar shedding and loss of motility (41). However, the causes of reductions in flagellar abundance and motility in the presence of aromatic acids have not been rigorously explored.

A common response to many aromatic compounds is increased abundance or activation of MarA, SoxS, RamA, or Rob. The homologous AraC-family transcription factors MarA, SoxS, and Rob are known to coordinately regulate a wide array of genes in *Escherichia coli*, known as the *mar-sox-rob* regulon, resulting in large-scale changes in cellular physiology and metabolism (42–44). Regulation occurs through binding of these transcription factors to a common, degenerate, *marbox* sequence in promoters of *mar-sox-rob* regulon genes (45).

The regulatory targets associated with MarA homologs in related *Enterobacteriaceae* species are largely undefined; however, many common targets in *E. coli* are conserved in related species, such as *S. enterica* (46–48). Beyond *E. coli*, a number of related species, such as *S. enterica* and *Klebsiella pneumoniae*, contain an additional MarA homolog, RamA, which is capable of regulating *mar-sox-rob* regulon genes (49, 50).

Expression of MarA homologs occurs in response to varied chemical cues via disparate mechanisms. For MarA, SoxS, and RamA, expression is controlled by MarR2, SoxR2, and RamR2, respectively, each responding to different compounds. For instance, MarR2-dependent transcriptional repression of the *marRAB* operon is relieved via MarR2 binding to aromatic acids or copper-mediated disulfide bond formation between MarR monomers (51–56). SoxR2-dependent activation of *soxS* transcription occurs through oxidation of an iron-sulfur cluster in SoxR2 by redox-active compounds such as methyl viologen (paraquat) (57–59). Similar to MarR2, RamR2 represses transcription of *ramA* until it is exposed to bile salts or other aromatic compounds, such as indole (60–62). Unlike MarA, SoxS, and RamA, Rob is activated posttranscriptionally via a sequestrationdispersion mechanism in response to aromatic and fatty acids (63, 64). Interestingly, there exists extensive regulatory cross talk between these systems, allowing for the formation of complex feed-forward regulatory loops, depending on the chemical inducers present (65, 66). For instance, exposure to salicylic acid results in Rob activation and *marA* transcription, yielding stronger activation of *mar-sox-rob* targets (65, 67). In this way, species containing *mar-sox-rob* regulatory networks are able to sensitively tune downstream responses to a wide variety of chemical stressors in the environment, based on the intracellular concentrations of MarA, SoxS, Rob, and RamA (68).
Canonically, the downstream effects of MarA homologs have been associated with inducible multidrug resistance (44). Apart from their roles in multidrug resistance, the effects of MarA homologs on prokaryotic physiology are unclear. However, there is growing appreciation of their potential roles in transcriptional regulation of other cellular processes. For instance, RamA has been shown to attenuate expression of virulence traits and efflux pumps in S. Typhimurium (69). The mechanism by which MarA homologs influence traits other than antibiotic resistance in S. Typhimurium or other Enterobacteriaceae species has not been fully explored.

Here, we looked to define the role of the mar-sox-rob regulatory proteins MarA, SoxS, Rob, and RamA in controlling flagellar gene expression and motility in S. Typhimurium. We found that all four of these regulators are repressors of motility, with SoxS and RamA exhibiting the strongest phenotypic effects on swimming and transcriptional repression of the flagellar regulon. Interestingly, the repressive effects of SoxS on motility are due to both transcriptional and posttranscriptional regulation of flhDC expression. Based on these findings, we propose an addition to the flagellar regulatory model. Flagellar repression occurs through coordinated activation of MarA, SoxS, Rob, and RamA in the presence of diverse chemical stressors. This form of repression occurs via transcriptional repression of the flhDC promoter, as well as activation of a posttranscriptional mechanism that inhibits flhDC translation (Fig. 1A). Given that known MarA, SoxS, Rob, and RamA inducers are present in the gastrointestinal environment and these transcription factors are expressed during various stages of S. enterica infections, this mechanism of flagellar gene repression may have implications in the virulence lifestyle of S. enterica and related enterobacterial pathogens.

RESULTS

MarA, SoxS, Rob, and RamA inhibit motility and decrease production of flagellin. Given their broad range of regulatory targets, we examined whether elevated levels of MarA, SoxS, Rob, and RamA have an impact on motility, and we found that all four regulators are capable of inhibiting motility. To test the effect of each regulator on motility, we individually complemented marA, soxS, rob, or ramA expression from an arabinose-inducible vector in a marRAB soxRS rob ramRA quadruple mutant. A genetic background lacking all native loci for these transcription factors was chosen to minimize any possible regulatory cross talk between MarA, SoxS, Rob, and RamA (such as SoxS-dependent activation of marRAB) that might distort the effects on expression of downstream genes. This approach has been used in prior studies examining the mar-sox-rob regulon in E. coli (43, 65). In the quadruple mutant background, we observed that MarA, SoxS, Rob, and RamA repressed swimming motility, with SoxS and RamA having the most pronounced effects (Fig. 1B). Such findings were also observed for swimming and surface-associated swarming motility in a wild-type background with all four native marRAB, soxRS, rob, and ramRA loci intact (see Fig. S1 in the supplemental material).
Expression of each MarA homolog in the quadruple mutant background also resulted in decreased production of flagellin. To delineate whether the effects of MarA, SoxS, Rob, and RamA were the result of reductions in flagellar protein expression or posttranslational effects on flagellar function, we measured the levels of FliC, the flagellar filament protein, by Western blotting (Fig. 1C) and enzyme-linked immunosorbent assay (ELISA) (Fig. 1D), with marA, soxS, rob, or ramA expressed from pBAD30 in the quadruple mutant background. Immunoblots showed that expression of marA, soxS, rob, or ramA repressed FliC production (Fig. 1C). However, quantification by ELISA revealed that, while SoxS and RamA strongly inhibited FliC levels, the effects of MarA and Rob were more modest, as demonstrated by the significantly higher FliC levels, compared to SoxS and RamA levels (all $P \leq 9.5 \times 10^{-3}$, Tukey’s honestly significant difference [HSD] test) (Fig. 1D). These patterns qualitatively correlated with observed effects on motility, with MarA and Rob attenuating motility to a lesser degree than SoxS and RamA (Fig. 1B). Differences between immunoblotting and ELISA measurements likely reflect differences in the detection limits for FliC protein between the assays. Additionally, we found that the phase-variable flagellin system, fljBA, was significantly downregulated under these conditions, suggesting that reduced FliC levels were not a result of increased FljA levels (all $P < 1 \times 10^{-6}$, Student’s $t$ test) (Fig. S2).

All classes of flagellar promoters are downregulated by MarA, SoxS, Rob, and RamA. Based on the effects of MarA, SoxS, Rob, and RamA on motility and expression of FliC, we looked to determine whether these transcription factors repressed specific classes of flagellar genes. To identify which classes of flagellar...
genes are subject to repression by MarA homologs, we constructed single-copy promoter fusions of flhDC (class I), flhB (class II), and fliC (class III) with yfp(Venus), akin to the work of Koirala and coworkers (70, 71). Using these transcriptional fusions in our quadruple mutant background, we found that complementation of marA, soxS, rob, and ramA expression caused significant reductions in expression, compared to a plasmid control (all $P < 1 \times 10^{-7}$, Tukey’s HSD test), from all three classes of flagellar promoters (Fig. 1E). Most importantly, class I was repressed by a range of 29% ± 2.9% to 79.9% ± 0.8%, compared to the plasmid control, bounded by Rob and SoxS, respectively. Concomitantly, these reductions in class I promoter activity were reflected in decreased expression from class II and class III promoters. An exception was MarA, which resulted in ~30% decreases in expression across all classes of flagellar promoters tested. Interestingly, MarA and Rob had nearly identical effects on class I transcription ($P = 0.95$, Tukey’s HSD test), although Rob had stronger negative effects on class II and class III expression (Fig. 1E) but reduced motility less than MarA (Fig. 1B). This finding is likely the result of differences in growth conditions between liquid and motility agar but also may suggest alternative modes of flagellar regulation for these two transcription factors. Considering the architecture of the flagellar regulon, reductions in flhDC expression are likely the principal cause of reductions in flagellar gene expression caused by MarA, SoxS, Rob, and RamA.

MarA and Rob interact directly with the flhDC promoter. To test whether interactions between MarA homologs and the flhDC promoter were direct, we used electrophoretic mobility shift assays (EMSAs) and chromatin immunoprecipitation (ChIP) followed by quantitative PCR (qPCR) to detect binding in vitro and in vivo, respectively. Using purified Rob protein to perform EMSAs, we found that Rob specifically bound the flhDC promoter region (Fig. 2A). When increasing concentrations of Rob protein were incubated with labeled flhDC promoter, a corresponding decrease in gel migration of labeled flhDC promoter DNA was observed. Additionally, incubation with unlabeled flhDC competitor DNA eliminated the binding of Rob to the labeled flhDC promoter DNA, while similar treatment with nonspecific competitor DNA (a gyrA DNA fragment) had no effect on Rob binding. These results showed that Rob specifically bound the flhDC promoter in vitro.

Building on our in vitro observations with Rob, we proceeded to test whether interactions between all four MarA homologs could be detected in vivo using ChIPqPCR with FLAG epitope-tagged derivatives of MarA, SoxS, Rob, and RamA (Fig. 2B). MarA, SoxS, Rob, and RamA are known to bind to similar sites in promoter regions (the marbox) (45). For this reason, we hypothesized that MarA, SoxS, and RamA may bind to flhDC as well. FLAG-tagged MarA, SoxS, Rob, and RamA were independently expressed from an arabinose-inducible vector in a wild-type genetic background, followed by formaldehyde cross-linking, ChIP, and targeted qPCR of the flhDC promoter region. Of the four regulators, MarA, SoxS, and Rob were observed to significantly precipitate flhDC promoter DNA ($P = 0.0035$, $P = 0.014$, and $P = 0.00018$, respectively, Student’s t test), whereas RamA was not ($P = 0.92$, Student’s t test). We note that the fold enrichment from SoxS was modest and might have limited biological significance. There are possible reasons for the discrepancy in binding patterns for MarA and Rob versus SoxS and
RamA. Our findings may reflect different affinities of the homologs for marbox derivatives, with MarA and Rob having higher affinities for flhDC than do SoxS and RamA (72). However, these in vivo ChIP-qPCR data cannot confirm this hypothesis, since the intracellular concentrations and immunoprecipitation efficiency of each transcription factor are not well defined. Alternatively, our cross-linking procedure might have biased detection of SoxS and RamA, compared to MarA and Rob. Other in vivo ChIP studies examining MarA and SoxS binding utilized cross-linking successfully, suggesting that cross-linking is not likely a source of bias (73, 74). Finally, SoxS and RamA are subject to rapid proteolysis, potentially limiting detection by our ChIP-based assay (75). Based on these findings, we conclude that MarA and Rob bind to the flhDC promoter, while SoxS and RamA do not, under the conditions tested.

MarA homologs repress flhDC expression posttranscriptionally. To genetically test MarA, SoxS, Rob, and RamA-dependent control of the flhDC promoter, we replaced the native flhDC promoter (class I) in situ with a tetracycline-inducible promoter cassette in an otherwise wild-type genetic background; this genetic modification has been shown to remove all native transcriptional regulation of the flhDC promoter (11, 13). We induced expression of flhDC from this construct using anhydrotetracycline (ATc), a nontoxic tetracycline derivative, at 1 ng/ml (low ATc concentration) and 100 ng/ml (high ATc concentration). This approach allowed detection of possible posttranscriptional effects on flhDC expression, since reductions in flagellar expression and motility could not occur through transcriptional repression of the native flhDC promoter when MarA, SoxS, Rob, or RamA was ectopically expressed. Using fliC expression as a flagellar regulon readout, induction of flhDC with the low ATc concentration, together with ectopic expression of marA, soxS, rob, or ramA, resulted in all four transcription factors significantly reducing fliC expression levels, to various degrees (all \( P < 1 \times 10^{-7} \), Tukey’s HSD test) (Fig. 3A). When flhDC expression levels were increased by induction with the high ATc concentration, we still found that all MarA homologs significantly reduced fliC expression (all \( P \leq 9.85 \times 10^{-5} \), Tukey’s HSD test), with MarA and Rob having equivalent effects. Expression of fliC was increased by 40.4% \( \pm \) 5.1% and 36.7% \( \pm \) 2.6% under these conditions, compared to treatment with the low ATc concentration, for MarA and Rob, respectively (both \( P \leq 3.9 \times 10^{-7} \), Student’s \( t \) test). Interestingly, fliC levels did not show similar increases with low and high ATc concentrations for SoxS and RamA. These data demonstrate that all four MarA homologs are capable of activating (or repressing) a posttranscriptional regulatory pathway for flhDC. However, MarA and Rob regulate this putative pathway to a lesser degree than SoxS and RamA.

Examining levels of FliC and motility yielded trends similar to those for fliC transcription, particularly at high ATc levels (Fig. 3B and C). Notably, FliC levels were higher at high ATc levels, compared to low ATc levels, for all MarA homologs, matching corresponding increases in motility. An exception was SoxS, for which motility remained completely impaired at low and high ATc levels (Fig. 3C). In the case of MarA at low ATc levels, we observed that FliC levels were higher than SoxS, Rob, and RamA levels but cells were nonmotile (Fig. 3B and C). This increase in FliC levels might be due to MarA-dependent activation of acrAB, which
is capable of removing tetracycline (76), or other downstream targets affecting flagellar expression in motility agar, where oxygen partial pressures are lower than in aerated liquid medium. We also noticed changes in the levels of DnaK, a protein chaperone used as a loading control in immunoblots, in our promoter replacement studies (Fig. 3B). Specifically, DnaK levels decreased between our plasmid control and the MarA homolog vectors at low ATc levels. While DnaK plays a positive role in flagellar assembly, we do not suspect that it plays a role here, as these trends in DnaK levels were not observed at high ATc concentrations (77). Overall, these data suggest that, when flhDC transcript levels are high, posttranscriptional repression can be overcome. This finding is similar to the threshold effects observed for sRNA-based regulation (78, 79).

FIG 3 Production of MarA, SoxS, Rob, and RamA resulting in posttranscriptional repression of flhDC. Expression of flhDC was driven by a tetracycline-inducible promoter at ATc concentrations of 1 ng/ml (low) or 100 ng/ml (high), and marA, soxS, rob, and ramA were ectopically expressed from pBAD30. (A) Effects of MarA, SoxS, Rob, and RamA on fliC transcription in a tetracycline-inducible flhDC genetic background (strain LCM2701) in which native flhDC regulation has been removed. Fluorescence measurements were made with mid-logarithmic-phase cultures grown in tryptone broth supplemented with 0.2% arabinose and the indicated ATc levels, followed by normalization to culture density. Data for each transcriptional fusion are presented as a percentage of fliC expression observed in the pBAD30 plasmid control. Six replicate measurements were made for each plasmid-bearing strain. Letter labels (a to e) indicate statistically significant groups (P < 0.05, Tukey's HSD test) for each ATc treatment. (B) MarA, SoxS, Rob, and RamA effects on FliC levels with low and high ATc levels, as measured by Western blotting. Cell extracts from cultures grown in tryptone broth supplemented with 0.2% arabinose were displayed (100 g total protein) on 12% acrylamide SDS-PAGE gels prior to transfer to a PVDF membrane and immunoblotting for FliC and DnaK. DnaK levels were used as an internal loading control for each sample. (C) Effects of MarA, SoxS, Rob, and RamA on motility with low and high ATc levels. Motility assays were conducted at room temperature in soft tryptone agar supplemented with 0.2% arabinose. Both Western blotting and motility assays were performed using strain LCM2678.

Since SoxS showed the strongest effects on posttranscriptional flagellar regulation with induction of flhDC by low and high ATc concentrations (Fig. 3), we
wanted to understand the effects of native SoxS concentrations on flhDC regulation (Fig. 4). To achieve physiologically relevant levels of SoxS in the absence of toxic inducers, we utilized a constitutive soxR (soxR\textsuperscript{Con}) mutant, which resulted in moderate constitutive levels of soxS expression (Fig. S3) (80). When introduced into the tetracycline-inducible flhDC background, soxR\textsuperscript{Con} resulted in significantly lower levels of class III activation across 2 log units of ATc concentrations, compared to wild-type soxR (soxR\textsuperscript{WT}) (all \(P \leq 0.0018\), Student’s \(t\) test) (Fig. 4A). To test whether translation of flhDC mRNA was inhibited by SoxS expression, we generated a 3 x FLAG epitope-tagged version of FlhC (FlhC-3 x FLAG) expressed by the tetracycline-inducible flhDC construct, similar to the work of Saini and coworkers (13). At high ATc concentrations, moderate levels of SoxS were insufficient to reduce the levels of FlhC-3FLAG. In contrast, induction with low ATc levels resulted in lower FlhC-3FLAG levels. This finding is consistent with the translation of flhDC mRNA being inhibited by a mechanism controlled by SoxS (Fig. 4B). Similarly, the soxR\textsuperscript{Con} mutant had a modest effect on motility at high ATc levels, while it resulted in reduced motility at low ATc levels (Fig. 4C). Taken together, these data indicate that physiological levels of SoxS are capable...
of reducing flagellar expression and repression appears to occur at the level of \textit{flhDC} translation.

Posttranscriptional repression of \textit{flhDC} expression by SoxS is \textit{hfq} independent. Since elevated \textit{soxS} expression resulted in reduced \textit{flhDC} translation, we looked to better understand the mechanism underlying SoxS-dependent posttranscriptional regulation of \textit{flhDC}. Specifically, we explored the possibility of an Hfq-dependent sRNA mediating repression of \textit{flhDC} when SoxS was expressed (Fig. 5). The most common mechanism of posttranscriptional regulation of \textit{flhDC} is via the action of sRNAs, resulting in both positive and negative effects on \textit{flhDC} expression. Exemplifying this fact is the severe motility defect in an \textit{hfq} mutant in \textit{S. Typhimurium} (32). Hfq is a highly conserved sRNA chaperone that is required for the function of many sRNAs (81). If SoxS requires an Hfq-dependent sRNA, then reductions in flagellar expression and motility caused by \textit{soxR}^{Con} (elevated SoxS levels) should be masked by the effects of a \textit{hfq} mutant. Consistent with the observations of Sittka and coworkers (32), our \textit{hfq} mutant had severely reduced \textit{fliC} transcription and motility (Fig. 5). The \textit{soxR}^{Con} and \textit{hfq} mutants both caused significant decreases in \textit{fliC} expression, compared to the wild-type strain (both \(P \leq 2.4 \times 10^{-7}\), Student’s \(t\) test) (Fig. 5A). Notably, the \textit{soxR}^{Con} \textit{hfq} double mutant had significantly lower \textit{fliC} levels than the \textit{soxR}^{Con} mutant (\(P = 8.1 \times 10^{-11}\), Student’s \(t\) test) and the \textit{hfq} mutant (\(P = 1.7 \times 10^{-8}\), Student’s \(t\) test). These data support a regulatory model in which the negative effects of \textit{soxR}^{Con} (i.e., elevated levels of SoxS) on flagellar gene expression are independent of Hfq. We attempted to further validate these findings by measuring FliC protein levels, but we found that levels were too low for quantification of differences with our ELISA method (Fig. S4).
FIG 5 Posttranscriptional regulation of flhDC independent of Hfq. (A) Levels of fliC transcription in wild-type, soxR<sup>Con</sup>, hfq, and soxR<sup>Con</sup> hfq genetic backgrounds (strains LCM2326, LCM2473, LCM2714, and LCM2715). Fluorescence measurements were made with mid-logarithmic-phase cultures grown in tryptone broth. Levels for all mutants were significantly lower than those for the wild-type strain (P ≤ 2.4 x 10<sup>-7</sup>, Student’s t test). The asterisk indicates a significant difference between soxR<sup>Con</sup> and soxR<sup>Con</sup> hfq mutants (P = 8.1 x 10<sup>-11</sup>, Student’s t test). (B) Motility in wild-type, soxR<sup>Con</sup>, hfq, and soxR<sup>Con</sup> hfq backgrounds (strains LCM1930, LCM2449, LCM2597, and LCM2598). Motility assays were conducted at room temperature in soft tryptone agar.

Measuring the effects of soxR<sup>Con</sup> and hfq on motility further demonstrated that soxR<sup>Con</sup> acted independently of hfq in repressing flagellar expression. Consistent with SoxS inhibiting flhDC expression, the soxR<sup>Con</sup> mutant showed reduced motility, compared to the wild-type strain (Fig. 5B). Similar to fliC transcription, combining the soxR<sup>Con</sup> and hfq mutations resulted in complete loss of motility, compared to the decreases observed for either single mutant. We noted an inconsistency between fliC expression and motility for the soxR<sup>Con</sup> mutant that was likely due to different concentrations of oxygen, which is required for oxidation of the Fe-S cluster in SoxR, in the media (58). Nevertheless, both transcription and motility support a model in which SoxS controls expression of an Hfq-independent posttranscriptional pathway to control flhDC expression.

Exposure to chemical inducers of marRAB and soxRS results in reduced flagellar gene expression and motility. To complement our ectopic expression studies performed in a marRAB soxRS rob ramRA mutant background, we explored the effects on flagellar gene expression and motility of two well-characterized chemical inducers of marA and soxS expression in a wild-type genetic background (Fig. 6). Specifically, we examined the effects of salicylic acid and paraquat, inducers of the marRAB and soxRS systems, respectively. Salicylic acid is also a Rob activator and chemorepellent for many bacteria, working through
the chemotactic network, and has been shown to inhibit production of flagella in
diverse species (35, 38, 65). In contrast, the effects of paraquat, a redox-active
electron transfer inhibitor, on bacterial motility are not well characterized.

![Graph](image)

**FIG 6 Repression of flagellar genes, motility, and flagellin production by salicylic acid and paraquat.**
(A) Effects of salicylic acid (Sal) and paraquat (PQ) on transcription of *flhD*, *flhB*, and *fliC* promoters
(strains LCM2324, LCM2325, and LCM2326). Fluorescence measurements were made with mid-
logarithmic-phase cultures grown in tryptone broth supplemented with sodium salicylate (3 mM) or
paraquat (50 M). Fluorescence measurements were normalized to culture density and are
presented as a percentage of the untreated expression level for each promoter fusion. All
decreases in expression were significant (*P* ≤ 1.8 x 10^-5, Student’s *t* test). (B) Levels of FliC protein
determined by Western blotting in the presence of salicylic acid or paraquat in the wild-type strain
(LCM1930). Cell extracts from cultures grown in tryptone broth supplemented with the indicated
concentrations of sodium salicylate or paraquat were displayed (100 μg total protein) on 12%
acrylamide SDS-PAGE gels prior to transfer to a PVDF membrane and immunoblotting for FliC and
DnaK. DnaK levels were used as an internal loading control for each sample. (C) Effects of salicylic
acid and paraquat on motility in the wild-type strain (LCM1930). Motility assays were conducted at
room temperature in soft tryptone agar supplemented with sodium salicylate or paraquat at the
concentrations described above.

Consistent with our ectopic expression data, chemical induction of MarA
expression and activation of Rob with salicylic acid significantly reduced the
expression of all classes of flagellar genes (all *P* ≤ 1.8 x 10^-5, Student’s *t* test), as
did paraquat induction of SoxS (all *P* ≤ 1.5 x 10^-7, Student’s *t* test) (Fig. 6A).
Reductions in class II and class III expression were larger in the presence of
paraquat than salicylic acid (both *P* ≤ 3.0 x 10^-9, Student’s *t* test). This finding is in
agreement with the existence of a posttranscriptional pathway activated
preferentially by SoxS, as characterized above.

Both chemical treatments resulted in reductions in FliC levels and motility.
Commensurate with reductions in *fliC* expression, FliC protein levels were reduced
in the presence of salicylic acid or paraquat, with paraquat resulting in less FliC
than salicylic acid (Fig. 6B). Motility was also reduced in the presence of salicylic
acid or paraquat, with salicylic acid having more pronounced effects (Fig. 6C).
These findings are likely due to salicylic acid functioning as a chemorepellent, in
addition to MarA and Rob-dependent transcriptional repression. Collectively, these
data demonstrate that canonical chemical inducers of the marRAB and soxRS gene systems cause reductions in flagellar gene expression and motility.

MarA and Rob mediate inhibition of motility in the presence of salicylic acid. Having observed salicylic acid-dependent reductions in motility, we looked to test whether MarA and Rob were involved, and we found that they are principal regulators of this response. Both MarA and Rob are known to respond to salicylic acid, forming a coherent feed-forward regulatory loop to control expression of target genes (65, 66). After systematic deletion of marRAB and rob in an otherwise wild-type genetic background, we found that salicylate-induced reductions in all classes of flagellar genes were at least partially restored (Fig. 7A). For flhDC (class I), deletion of marRAB or rob resulted in equivalent, modestly significant increases in class I expression ($P = 0.037$ and $P = 0.041$, respectively, Tukey’s HSD test), while the marRAB rob mutant demonstrated a further increase to $79.2\% \pm 3.7\%$ of untreated levels ($P < 1 \times 10^{-7}$, Tukey’s HSD test). Expression of flhB (class II) yielded similar increases; however, the marRAB rob mutant showed no significant increase, compared to either of the single mutants (both $P = 0.99$, Tukey’s HSD test). Finally, fliC (class III) transcription showed the largest recovery, from $12.5\% \pm 1.7\%$ to $81.8\% \pm 6.0\%$ of untreated levels, comparing the wild-type strain and the marRAB rob mutant during salicylic acid treatment ($P < 1.0 \times 10^{-7}$, Tukey’s HSD test). Correspondingly, the marRAB and rob mutants had nearly equivalent recoveries in fliC expression, although the differences were significant ($P = 0.017$, Tukey’s HSD test). Notably, the differences between the marRAB and rob single mutants and the double mutant, while significant (both $P \leq 3.3 \times 10^{-5}$, Tukey’s HSD test), were not as large as the changes from the wild-type strain and may not be physiologically relevant. Although increases in flagellar expression in the presence of salicylic acid were observed with all permutations of marRAB and rob mutants, expression from all classes of promoters tested remained significantly below untreated wild-type levels (all $P \leq 0.0023$, Student’s $t$ test). These differences may be due to other stress responses to salicylic acid reducing flagellar gene expression.
FIG 7 Recovery of flagellar gene expression and motility in the presence of salicylic acid when MarA and Rob-dependent repression was alleviated. (A) Transcription levels of flhD, flhB, and fliC promoter transcriptional fusions to yfp in wild-type (WT) (strains LCM2324, LCM2325, and LCM2326), marRAB (strains LCM2399, LCM2417, and LCM2432), rob (strains LCM2401, LCM2419, and LCM2434), and marRAB rob (strains LCM2407, LCM2422, and LCM2437) genetic backgrounds. Fluorescence measurements were made with mid-logarithmic-phase cultures grown in tryptone broth supplemented with sodium salicylate (3 mM). Fluorescence measurements were normalized to culture density and are presented as a percentage of the untreated expression level for each promoter fusion. Letter labels (a to d) represent statistically significant groups ($P < 0.05$, Tukey’s HSD test) for each transcriptional fusion. (B) Levels of FliC protein in wild-type (LCM1930), marRAB (LCM2366), rob (LCM2368), and marRAB rob (LCM2371) backgrounds, as measured by Western blotting. Cell extracts from cultures grown in tryptone broth with or without sodium salicylate (SAL) (3 mM) were displayed (100 g total protein) on 12% acrylamide SDS-PAGE gels prior to transfer to a PVDF membrane and immunoblotting for FliC and DnaK. DnaK levels were used as an internal loading control for each sample. (C) Effects of marRAB, rob, and marRAB rob mutations on FliC levels in the presence of salicylic acid, as quantified by ELISA. Cell extracts (25 g total protein) used for ELISAs were obtained under the same conditions as for Western blotting. Light emission from each sample was normalized to wild-type FliC levels, and results are presented as fold increases in FliC abundance. Six replicate measurements were made for each plasmid-bearing strain. Letter labels (a and b) represent statistically significant groups ($P < 0.05$, Tukey’s HSD test) for each genetic background. (D) Effects of marRAB (LCM2366), rob (LCM2368), and marRAB rob (LCM2371) deletions on motility in the presence or absence of salicylic acid, compared with the wild-type strain (LCM1930). Motility assays were conducted at room temperature in soft tryptone agar with or without sodium salicylate (3 mM).

Increases in flagellar gene expression in marRAB and rob mutants during salicylic acid exposure resulted in increased production of FliC. We found that, in marRAB, rob, and marRAB rob mutants, FliC levels were increased in the presence of salicylic acid, with the marRAB rob mutant showing slightly higher levels of FliC (Fig. 7B). Quantification of these differences by ELISA demonstrated no significant differences in FliC levels between the mutants, although all mutants had significantly elevated levels of FliC, compared to the wild-type strain (Fig. 7C). The modest differences between single and double mutants are likely a result of
the interconnected feed-forward loops formed by MarA and Rob, in which loss of either regulator hampers the overall downstream response (65, 66).

Recovery of flagellar gene expression in marRAB and rob mutants in the presence of salicylic acid was also reflected in increases in motility (Fig. 7D). Phenotypically, we observed a larger increase in motility in the marRAB rob mutant, compared to the wild-type strain or either single mutant. Notably, the marRAB mutant did not demonstrate an increase in motility like the rob mutant, in contrast to the gene expression data, in which the marRAB and rob mutants appeared nearly equivalent (Fig. 7A). These differences may reflect differences in liquid medium versus motility agar culturing conditions, under which Rob seemed to have a stronger repressive effect in the presence of salicylic acid. Nevertheless, these data indicate that MarA and Rob-dependent repression of flagellar genes likely works in concert with a known chemosensing pathway to enhance negative chemotaxis away from repellents such as salicylic acid.

DISCUSSION

The expression of genes involved in flagellar biosynthesis and chemotaxis in S. Typhimurium and related flagellated Enterobacteriaceae species is strongly influenced by many environmental signals, through binding of transcription factors to the flhDC promoter and sRNA interactions with flhDC mRNA. Here, we add an additional layer to the flagellar regulatory model. We demonstrated that the homologous transcription factors MarA, SoxS, Rob, and RamA directly inhibit motility by reducing the expression of flagellar genes in Salmonella. In the case of MarA and Rob, this largely occurs through binding repression of the flhDC promoter. SoxS, however, primarily controls flhDC expression through a posttranscriptional pathway, resulting in decreased translation of flhDC. While RamA regulation of flhDC was not explored in detail here, our data indicated that it behaves similarly to SoxS. These findings provide a new role for these regulators, beyond canonical association with multidrug resistance. Importantly, the use of these homologous transcription factors by S. Typhimurium to control the flagellar regulon contrasts with regulatory patterns observed in E. coli. More broadly, these results demonstrate the flexibility of various cellular processes to be integrated into the global mar-sox-rob regulon, a regulon that is conserved throughout the Enterobacteriaceae family.

Regulation of flagellar gene expression by MarA, SoxS, Rob, and RamA occurs in S. Typhimurium but has not been observed in E. coli. MarA, SoxS, and Rob have been well studied in E. coli. In transcriptomic studies by Barbosa and Levy (42) and Martin and Rosner (43), no differential expression of the flagellar regulon was observed under ectopic expression conditions similar to those used in aspects of our study. Transcriptional profiling under salicylate and paraquat inducing conditions found no significant changes in expression of any flagellar or chemotaxis genes shared between S. Typhimurium and E. coli (82). Contrasting these data with our findings suggests that transcriptional repression of flagellar genes by MarA homologs may be unique to S. Typhimurium. Whether these
differences are mediated by differences in MarA homolog promoter discrimination between the two species or divergence in regulatory regions of promoters in the flagellar regulon (namely, \textit{flhDC}) is not known. However, given the nearly complete similarity of MarA, SoxS, and Rob between S. Typhimurium and \textit{E. coli}, we hypothesize that these differences are due to variations in the \textit{flhDC} promoter between these species.

A number of historical differences in flagellar gene expression between \textit{Salmonella} and \textit{E. coli} have been characterized, as reflected in sequence variations in the \textit{flhDC} promoter region between these species (2, 83, 84). Illustrating these differences, several studies have demonstrated the interactions of various regulators of SPI-1 and SPI-2 (not present in \textit{E. coli}) with the \textit{flhDC} promoter in regions not present in the \textit{E. coli flhDC} promoter (20, 21, 24, 84). In fact, variations in interactions of these regulators (specifically, SsrB from SPI-2) with \textit{flhDC} have recently been shown to be part of the evolutionary transition of noninvasive \textit{Salmonella bongori} to \textit{S. enterica}, suggesting that the expression of the flagellar regulon is subject to intense selective pressure during adaptation to different lifestyles or environments, such as within macrophages (24). While we have not yet defined a binding box for MarA and its homologs, it will be interesting to delineate where these interactions occur and whether those regions are conserved in \textit{E. coli}. Further examination of how the flagellar regulon is integrated into the broader \textit{mar-sox-rob} stress response regulon may offer deeper insights into the functional role of \textit{mar-sox-rob} in \textit{Enterobacteriaceae} species.

MarA homolog-dependent posttranscriptional regulation of flagellar gene expression also plays a role in repressing flagellar genes in \textit{Salmonella} in response to chemical stress. Indeed, several sRNA interactions with \textit{flhDC} mRNA have been characterized (30). The MarA homologs in \textit{S. Typhimurium} do not have any known regulatory interactions with these sRNAs. In ongoing transcriptomic research, we have observed increases in the production of OmrA and OmrB, two sRNAs involved in regulating porin and flagellar gene expression (30), during ectopic expression of MarA homologs. However, targeted genetic knockouts of \textit{omrA} and \textit{omrB} yielded no changes in motility phenotypes (data not shown). Therefore, if this posttranscriptional mechanism is sRNA based and the sRNA is activated by MarA homologs similar to MicF (85, 86), then the acting sRNA is likely unknown, as no sRNA-sequencing efforts have been conducted under chemical or ectopic induction of MarA homologs. Adding additional complexity, the posttranscriptional mechanism we observed is Hfq independent. While this finding does not preclude sRNA-mediated repression as a potential mechanism, it excludes the correlation of Hfq RNA immunoprecipitation data to identify possible candidates (87). Finally, we have seen the activation of cryptic MarA-dependent posttranscriptional regulatory pathways affect porin expression in \textit{E. coli} (86), mirroring results seen here. Future efforts in sRNA sequencing under conditions explored in this study will shed light on novel sRNA regulators of flagellar gene expression.

Why might \textit{Salmonella} integrate control of flagellar gene expression into the global \textit{mar-sox-rob} regulon? An answer might be that periods of the \textit{Salmonella} lifestyle in the host are benefited by decreased flagellar synthesis and motility. One
such location might be within macrophages. Notably, within macrophages, \textit{marA}, \textit{soxS}, and \textit{ramA} expression is induced 4- to 25-fold and flagellar gene expression is decreased 20-fold, similar to our \textit{ex vivo} findings (88, 89). The degree to which these events are correlated has not been explored. While it is intriguing to speculate on the importance of these concomitant changes, it is worth noting that Fang and coworkers found that a \textit{soxS} mutation had no apparent effect on virulence in a murine infection model or on survival in murine macrophages (90). Contradicting these findings, Bailey and coworkers more recently demonstrated that mutations in \textit{ramA} did attenuate host survival in a number of infection models, including mice (69). Cast in the light of our current results, it may be that overlapping functional redundancy of MarA homologs masks singular loss of \textit{marA}, \textit{soxS}, \textit{rob}, or \textit{ramA}. Furthermore, each regulator may contribute differently, such as RamA having stronger effects on virulence than MarA, SoxS, or Rob alone, much like flagellar regulation (46, 54, 69, 90). Similarly, MarA homologs may work in conjunction with other regulators, such as SsrB, to enable more robust repression of flagellar expression within macrophages. In this way, \textit{mar-sox-rob} may serve as an additional mechanism of regulatory reinforcement during the transition of \textit{S. Typhimurium} to an intracellular lifestyle.

\textit{Salmonella} uses transcriptional control to enhance negative chemotaxis. Salicylate is a well-documented chemorepellent in \textit{E. coli}, acting through the chemosensing network via the receptor Tsr, as well as alteration of intracellular pH (38, 40). Here, we found that \textit{Salmonella} uses transcriptional and posttranscriptional regulation, in addition to chemotaxis, to avoid toxic aromatic acids. By adding a transcriptional layer of control to negative chemotaxis, \textit{S. Typhimurium} may be able to more finely tune concentrations of chemoreceptors to enhance the sensing of repellent compounds in the midst of attractants. Given that salicylate is sensed by Tsr, which also senses a variety of attractants such as serine, lowering levels of Tsr may allow salicylate to outcompete the binding of attractants, thereby enhancing negative chemotaxis (91, 92).

The regulation of flagellar gene expression in \textit{S. Typhimurium} is complex. Here, we add an additional mechanism of control during chemical stress, namely, repression by the global stress response transcription factors MarA, SoxS, Rob, and RamA. Broadly, this result highlights the evolutionary plasticity of global gene regulation within the \textit{Enterobacteriaceae} family. More specifically, our findings add to a growing understanding of the diverse targets of the \textit{mar-sox-rob} regulatory system throughout this bacterial family. Continued exploration of \textit{mar-sox-rob} regulatory targets will undoubtedly shed light on the role of MarA homologs in regulating the diverse physiology and metabolism of the many relatives of \textit{E. coli}.

**MATERIALS AND METHODS**

Media and growth conditions. Strains were grown in Luria-Bertani (LB) broth at 37°C for plasmid isolation, genetic manipulations, and propagation. When harboring temperature-sensitive plasmids (pKD46, pCP20, or pINT-ts), strains were grown at 30°C. Strains were grown in tryptone medium (1% tryptone, 0.8%
NaCl) for all assays unless otherwise noted. Motility assays were conducted at room temperature in swimming agar (0.3% Difco agar, 1% tryptone, 0.8% NaCl), to enhance resolution. Where indicated, carbenicillin (100 g/ml), kanamycin (50 g/ml), tetracycline (10 g/ml), L-arabinose (0.2% [wt/vol]), or ATc (high concentration, 100 ng/ml; low concentration, 1 ng/ml) was added to the growth medium.

Strain and plasmid construction. Relevant genotypes and properties of strains and plasmids used in this work can be found in Table S1 in the supplemental material. Gene deletions and subsequent marker removal were performed using the Red recombinase method described by Datsenko and Wanner (93), with the oligonucleotides presented in Table S2. Single-copy promoter fusions were integrated into the chromosome using the conditional-replication, integration, and modular (CRIM) plasmid-based system of Haldimann and Wanner (94) or the FLP/FLP recognition target (FRT)-based system of Ellermeier and coworkers (95). All strains were made isogenic to S. Typhimurium LT2, and compound genotypes were constructed by generalized transduction with phage P22 HTint, using standard methods (96).

Ectopic expression vectors and yfp(Venus) transcriptional fusions were constructed using standard molecular cloning procedures (97). Briefly, all genes, promoters, and 3FLAG fusions were amplified by PCR using Phusion DNA polymerase (New England Biolabs), with oligonucleotide primer sets with restriction endonuclease sites containing overhangs (Table S2). PCR products were digested with restriction endonucleases (typically combinations of HindIII, KpnI, and EcoRI) and ligated into the corresponding restriction sites of either pBAD30 (98), pET28a (Novagen), or pVenus (99).

Motility assays. Motility assays were conducted with logarithmic-phase cultures to observe effects on matrix-associated motility. Cultures were inoculated at 1:1,000 from overnight cultures and grown to mid-logarithmic phase (optical density at 600 nm [OD600] of 0.6) in tryptone medium, followed by normalization according to the OD600. Normalized cultures were inoculated (1 l) into the soft agar medium, and plates were incubated overnight at room temperature. Photographs were taken with a charge-coupled device (CCD) camera (Fotodyne).

Transcriptional reporter assays. Fresh cultures were started from overnight cultures and grown to mid-logarithmic phase in tryptone medium. For fluorescence-based reporter assays, 200 l of cultures was transferred into 96-well, black, transparent-bottom plates (96-well Costar assay plate; Corning), followed by fluorescence measurements (excitation wavelength, 500 ± 5 nm; emission wavelength, 520 ± 5 nm). The relative fluorescent units of cultures were corrected for background fluorescence and normalized to the corresponding OD600 values. - Galactosidase assays were performed as described by Thibodeau and coworkers (100), with measurements and analysis as described by Slauch and Silhavy (101). All fluorescence and absorbance measurements were made using a Cytation3 multimode microplate reader (BioTek). All statistical analyses were performed using R. Tukey’s HSD test was used for multiple pairwise comparisons between samples, and Student’s t test was used for direct comparisons. Where both tests were used, the largest P value was reported.
Immunoblotting and ELISAs. For all immunoblotting assays, cell pellets from 2 ml of culture were resuspended and incubated at room temperature in cell lysis buffer (CelLytic B lysis buffer [Sigma-Aldrich], 10 mM Tris-HCl [pH 8.0], 150 mM NaCl). For each sample, a total of 100 g of total protein was separated on morpholineethanesulfonic acid (MES)-Tris-buffered 12% Bis-Tris gels at 150 V for 2.5 h. Proteins were transferred to a polyvinylidene difluoride (PVDF) membrane using Tris–N-cyclohexyl-3aminopropanesulfonic acid (CAPS)-buffered semidy transfer. FliC protein was detected by using an antiflagellin monoclonal antibody (product no. SC-69948; Santa Cruz Biotechnology) at a 1:3,000 dilution. FlhC-3FLAG was detected by using anti-FLAG monoclonal antibody M2 (Sigma) at a 1:3,000 dilution. DnaK protein was used as a loading control for all samples and was detected using E. coli anti-DnaK monoclonal antibody 8E2/2 (Sigma) at a 1:10,000 dilution. Anti-mouse IgG conjugated to horseradish peroxidase (1:30,000 dilution), used as the secondary antibody, was detected with SuperSignal chemiluminescent substrate (Thermo Scientific).

For all ELISAs, strains were started from an overnight seed culture and grown under inducing conditions for 8 h at 37°C. Harvested cells were resuspended in 200 l of Tris-buffered saline (TBS) (10 mM Tris-HCl [pH 8.0], 150 mM NaCl) with protease inhibitors (Roche cOmplete mini, EDTA free) and were sonicated at 30% amplitude with 5-s on/30-s off pulses for four cycles. Lysed cells were centrifuged at 20,000 x g for 10 min to remove debris. Protein concentrations were measured spectroscopically against a bovine serum albumin (BSA) standard (BioTek Cytation3), and lysates were diluted in coating buffer (100 mM sodium bicarbonate-sodium carbonate [pH 9.6]) to a concentration of 500 g/ml. Diluted lysate (50 l) was added to wells of coated 96-well microtiter plates (Costar high affinity) and incubated for 2 h, followed by washing twice with 200 l of phosphate-buffered saline (PBS) (0.137 M NaCl, 2.7 mM KCl, 10 mM Na2HPO4, 1.8 mM KH2PO4). The washed wells were incubated in blocking buffer (PBS buffer with 5% nonfat milk) for 2 h. The wells were washed twice with 200 l PBS, 100 l of antiflagellin monoclonal antibody (1:500 dilution in blocking buffer) was added to each well, and the wells were incubated for 2 h. Two washes with 200 l of PBS were performed to wash the unbound antibody off the wells, 100 l of anti-mouse IgG (Life Technologies) conjugated with horseradish peroxidase (1:1,000 dilution in blocking buffer) was added to the wells, and the wells were incubated for 2 h. This incubation was followed by six washes with 200 l of PBS. Finally, 100 l of SuperSignal chemiluminescence substrate (Thermo Scientific) was added to the wells, and the wells were incubated for 30 s before the luminescence was measured in a microplate reader (BioTek Cytation3).

ChIP-PCR assays. ChIP assays were performed as described by Petrone and coworkers, with minor modifications (102). Briefly, 40-ml fresh cultures were inoculated 1:1,000 into LB broth from overnight cultures and grown at 37°C to mid-logarithmic phase (OD600 of 0.7). Formaldehyde was added at a final concentration of 1% to fix the cells, and the cells were shaken for 5 min at 37°C. Cross-linking was quenched by adding glycine, at a final concentration of 200 mM, to the cultures for 10 min at room temperature, with shaking. Cells were pelleted by
centrifugation at 10,000 x g, washed twice with ice-cold TBS, and frozen at – 80°C until further use.

Cells were then lysed and sonicated, followed by immunoprecipitation. Cell pellets were resuspended in 2 ml of FA cell lysis buffer (50 mM Tris-HCl [pH 7], 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% sodium deoxycholate, 0.1% SDS, 1 tablet of protease inhibitor cocktail [cOmplete mini, EDTA free], 4 mg/ml lysozyme) and incubated at room temperature for 20 min to initiate lysis. The lysate was sonicated for 30 min in a Q800R sonicator (Qsonica) at 100% amplitude with 10-s on/10-s off pulses to shear DNA to 200 to 500-bp fragments, followed by centrifugation at 10,000 x g to remove cell debris. A volume of 100 l of the supernatant was saved as an input control, and the remaining supernatant (1.9 ml) was added to 40 l of preequilibrated protein A/G beads (Pierce) in FA lysis buffer. Following incubation, 10 l of anti-FLAG monoclonal antibody M2 (Sigma) was added to the mixture, and the mixture was incubated at room temperature for 1 h, on a rotator. Protein A/G beads were separated and washed with FA lysis buffer (with 500 mM NaCl), ChIP wash buffer (10 mM Tris-HCl [pH 8], 250 mM LiCl, 1 mM EDTA, 0.5% Nonidet P-40, 0.5% sodium deoxycholate), and finally TE buffer (10 mM Tris-HCl, 1 mM EDTA). Resulting protein-DNA complexes were eluted from the beads in ChIP elution buffer (50 mM Tris-HCl [pH 7.5], 10 mM EDTA, 1% SDS) and incubated at 100°C for 10 min. The eluate and input samples were purified and concentrated using Clean & Concentrator-5 columns (Zymo Research).

Enrichment of flhDC promoter fragments was measured by qPCR. To check whether any of the MarA-3FLAG, SoxS-3FLAG, Rob-3FLAG, or RamA-3FLAG proteins bound the flhDC promoter, the DNA from the ChIP assay was used as the template in a qPCR to check the enrichment of the flhDC promoter region, compared to a control region (gyrA promoter region). Primers specific for flhDC (P-LCM243 and P-LCM242) and gyrA (P-LCM110 and P-LCM111) promoter regions that generated products of approximately 200 bp were used. The enrichment of a target region was calculated according to the 2-ΔΔCt method (103).

EMSAs. A nonradioactive EMSA kit (LightShift; Thermo Scientific) was used to detect protein-DNA binding. Rob protein was purified by nickel affinity chromatography and the tag was removed as described elsewhere (65). Protein concentrations were calculated with the Bradford assay, using a BSA standard. Biotinylated and unmodified 559-bp DNA fragments spanning the promoter region of the flhDC operon were generated by PCR. The binding reaction mixtures, consisting of 10 fmol of probe and different molar amounts of Rob protein in binding buffer [10 mM Tris-HCl, 50 mM KCl, 1 mM dithiothreitol, 2.5% glycerol, 5 mM MgCl2, 1g poly(dl-dC)], were incubated at room temperature for 20 min before being loaded onto a 4% polyacrylamide gel, followed by DNA transfer to a nylon membrane. Biotinylated DNA was detected by blotting with streptavidin-horseradish peroxidase conjugate, followed by chemiluminescence detection (SuperSignal enhanced chemiluminescence [ECL]; Thermo Scientific).
SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/ JB.00385-19.
SUPPLEMENTAL FILE 1, PDF file, 0.6 MB.

ACKNOWLEDGMENTS

We thank J. E. C. Chubiz and B. K. Zolman for critical reading of the manuscript. This work was supported by NIH grant AI137984 to L.M.C.

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