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RflM Functions as a Transcriptional Repressor in the Autogenous Control of the *Salmonella* Flagellar Master Operon *flhDC*

Hanna M. Singer,[a] Marc Erhardt,[a,b] Kelly T. Hughes[c]

Microbiologie, Département de Médecine, Université de Fribourg, Fribourg, Switzerland[a]; Helmholtz Centre for Infection Research, Braunschweig, Germany[b]; Department of Biology, University of Utah, Salt Lake City, Utah, USA[c]

Motility of bacteria like *Salmonella enterica* is a highly regulated process that responds to a variety of internal and external stimuli. A hierarchy of three promoter classes characterizes the *Salmonella* flagellar system, and the onset of flagellar gene expression depends on the oligomeric regulatory complex and class 1 gene product $FlhD_4C_2$. The *flhDC* promoter is a target for a broad range of transcriptional regulators that bind within the *flhDC* promoter region and either negatively or positively regulate *flhDC* operon transcription. In this work, we demonstrate that the RflM protein is a key component of *flhDC* regulation. Transposon mutagenesis was performed to investigate a previously described autoinhibitory effect of the flagellar master regulatory complex $FlhD_4C_2$. RflM is a LuxR homolog that functions as a flagellar class 1 transcriptional repressor. RflM was found to be the negative regulator of *flhDC* expression that is responsible for the formerly described autoinhibitory effect of the $FlhD_4C_2$ complex on *flhDC* operon transcription (K. Kutsukake, Mol. Gen. Genet. 254:440–448, 1997). We conclude that upon commencement of flagellar gene expression, the $FlhD_4C_2$ complex initiates a regulatory feedback loop by activating *rflM* gene expression. *rflM* encodes a transcriptional repressor, RflM, which fine-tunes *flhDC* expression levels.

The biosynthesis, assembly, and rotation of flagella require a significant amount of biosynthetic resources and energy (1, 2). The expression of flagellar genes is regulated in response to various environmental conditions, which can determine the onset of flagellar biosynthesis and the overall degree of flagellation (3, 4). One difference between flagellar gene expression in *Escherichia coli* and *Salmonella* is that under low-nutrient conditions, the flagellar regulon can be either induced (*E. coli*) or repressed (*Salmonella*) (5–8). In all systems examined to date, flagellar synthesis is repressed during biofilm formation and upon entry into host cells (9–12).

The more than 60 genes of the flagellar regulon are organized into a transcriptional hierarchy of three promoter classes. The flagellar master operon, *flhDC* (under the control of the flagellar class 1 promoter), is transcribed to produce the $FlhD_4C_2$ transcriptional activator complex that is needed for the activation of genes downstream in the flagellar transcriptional hierarchy, which are transcribed from flagellar class 2 promoters. $FlhD_4C_2$-dependent genes are required for the structure and assembly of the flagellar hook-basal body (HBB), which functions as the motor of the flagellum. Also transcribed from a class 2 flagellar promoter is the *fliA* gene, which encodes the flagellum-specific transcription factor σ^{28}, required to transcribe flagellar class 3 promoters. Class 3 flagellar genes encode proteins required after HBB completion, including filament (*fliC* and *fljB*), motor force generators (*motAB*), and components of the chemosensory system (*che*).

A broad range of factors positively and negatively regulate expression of the *flhDC* operon. Transcription of *flhDC* from the class 1 promoter is dependent on the binding of the cyclic AMP-catabolite gene activator protein complex and can also be activated by the iron-regulatory protein Fur and by the nucleoid proteins Fis and H-NS (13–17). Those regulatory factors bind directly to the *flhDC* promoter region, as shown for Fis in *Salmonella* and for Fur and H-NS in *E. coli* (15, 17, 18). There are numerous negative regulators of flagellar biosynthesis, including SlyA, a transcriptional regulator of *flhDC* (19) that is also required for *Salmonella* virulence (20), RtsB, a *Salmonella* pathogenicity island 1 (SPI-1)-encoded repressor of flagellar class 1 transcription (21), LrhA (22), and RcsB. RcsB is reported to positively regulate SPI-2 expression and favor *Salmonella* growth in macrophages (23). RcsB also binds an RcsB box in the *flhDC* promoter region to repress the flagellar master operon (23, 24). Finally, *flhDC* transcription is reported to be under autogenous control (13). $FlhD_4C_2$ is also under posttranscriptional regulation. The flagellar protein FliZ is a product of the *fliAZ* operon, which is transcribed from flagellar class 2 and 3 promoters. FliZ positively regulates additional flagellar class 2 gene expression (25). According to Saini et al., FliZ posttranslationally alters the concentration of $FlhD_4C_2$ (26). Data provided by Wada et al. suggest that FliZ has a repressing effect on YdiV, another posttranscriptional anti-$FlhD_4C_2$ factor (27). YdiV binds to FlhD and prevents the $FlhD_4C_2$ complex from binding to class 2 promoters, either by physically keeping the FlhDC complex away from free promoter DNA or by releasing FlhDC from the DNA-bound state (7, 28).

In an earlier study, we identified RflM, previously known as EcnR (see Materials and Methods), as a repressor of *flhDC* transcription. RflM is encoded in close proximity to the antidote/toxin gene pair *ecnAB* and exhibits the typical characteristics of an OmpR-like response regulator (29). It was therefore originally named EcnR (entericidin gene R), but experimental analyses showed *ecnAB* to be positively regulated by σ^S and negatively regulated by EnvZ/OmpR (29). There is no evidence for regulation of *ecnAB* by RflM (EcnR). In our previous work, we isolated *flhDC* promoter mutants that suppressed RflM inhibition (30). The inhibitory effect of RflM on motility has

Address correspondence to Kelly T. Hughes, hughes@biology.utah.edu.

TABLE 1 *Salmonella enterica* serovar Typhimurium LT2 and ATCC 14028s (ST14028) strains used in this study

Strain or plasmid	Relevant characteristics	Reference or source
EM57	LT2 Δ*araBAD1007*::*flhD*$^+$*C*$^+$ Δ*rflM32 flhC5213*::Mu*dJ*	This study
EM59	LT2 Δ*araBAD1007*::*flhD*$^+$*C*$^+$ *flhC5213*::Mu*dJ*	This study
EM71	LT2 Δ*araBAD925*::*tetRA flhC5213*::Mu*dJ* Δ*rflM32*	This study
EM153	LT2 Δ*araBAD921*::*rflM*$^+$ *rflM3*::Mu*dJ* P(*flhDC*)*5451*::Tn*10d*Tc[*del-25*]	This study
EM154	LT2 Δ*araBAD1005*::FCF P(*flhDC*)*5451*::Tn*10d*Tc[*del-25*] *rflM3*::Mu*dJ*	This study
EM635	14028s Δ*rcsB*::*tetRA luxCDABE* (Km^r)::*ybaJ*	This study
EM636	14028s Δ*rflM5*::FCF *luxCDABE* (Km^r)::*ybaJ*	This study
EM642	14028s Δ*araBAD925*::*rcsB*$^+$ *luxCDABE* (Km^r)::*ybaJ*	This study
EM643	14028s Δ*araBAD921*::*rflM*$^+$ *luxCDABE* (Km^r)::*ybaJ*	This study
EM666	14028s Δ*araBAD1005*::FRT *luxCDABE* (Km^r)::*ybaJ*	This study
EM672	14028s Δ*araBAD1007*::*flhD*$^+$*C*$^+$ *rflM3*::Mu*dJ*	This study
EM673	14028s Δ*araBAD1005*::FRT *rflM3*::Mu*dJ*	This study
EM700	14028s Δ*araBAD1007*::*flhD*$^+$*C*$^+$ *rflM3*::Mu*dJ* Δ*invH-sprB*::FCF	This study
EM701	14028s Δ*araBAD1005*::FRT *rflM3*::Mu*dJ* Δ*invH-sprB*::FCF	This study
TH437	LT2	J. Roth
TH3923	pJS28 (Ap^r P22-9$^+$) F′114(Ts) Lac$^+$ *zzf-20*::Tn*10*[*tetA*::Mu*dP*] (Tc^s) *zzf-3823*::Tn*10d*Tc[*del-25*] *leuA414 hsdSB* Fels2$^-$	Lab collection
TH5971	Δ*hin-5717*::FCF	
TH6701	LT2 Δ*araBAD925*::*tetRA*	Lab collection
TH8972	LT2 Δ*araBAD925*::*tetRA flhC5213*::Mu*dJ*	P. Aldridge
TH9386	LT2 Δ*araBAD921*::*rflM*$^+$	J. Karlinsey
TH10068	LT2 *rflM3*::Mu*dJ*	Wozniak et al., 2009 (30)
TH13067	LT2 Δ*araBAD996*::*rcsB*$^+$ *flhC5213*::Mu*dJ*	Lab collection
TH13069	LT2 *flhC5213*::Mu*dJ* Δ*araBAD921*::*rflM*$^+$	Lab collection
TH14156	LT2 Δ*araBAD1007*:: *flhD*$^+$*C*$^+$	Erhardt and Hughes, 2010 (19)
TH15941	LT2 Δ*araBAD1007*::*flhD*$^+$*C*$^+$ *flhC5213*::Mu*dJ fliA5886* (R91C L207P)	This study
TH16205	LT2 Δ*araBAD1007*::*flhD*$^+$*C*$^+$ *rflM3*::Mu*dJ*	This study
TH16952	Δ*rflM4*::FKF Δ*hin-5717*::FCF	This study
TH16964	Δ*rcsB*::*tetRA* Δ*hin-5717*::FCF	This study

been shown to be dependent on the RcsCDB system (30). An additional deletion of *rcsDBC* or an insertion in *rcsB* prevented the reported loss of motility of P$_{ara}$*rflM*. Here, we characterized the inhibitory effect of RflM on *flhDC* transcription. FlhD_4C_2 activates transcription of *rflM*, and in turn, RflM represses *flhDC* transcription. The RflM-FlhD_4C_2 feedback loop thereby accounts for the formerly described autoregulatory effect of FlhD_4C_2 on *flhDC* operon transcription.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. All bacterial strains used in this study are listed in Table 1. Cells were cultured in lysogeny broth (LB) medium, supplemented with tetracycline (15 μg ml^{-1}) or anhydrotetracycline (AnTc) (1 μg ml^{-1}) if needed. Gene expression from the arabinose promoter was achieved by addition of 0.2% L-arabinose to the medium. The generalized transducing phage of *Salmonella enterica* serovar Typhimurium P22 *HT105/1 int-201* was used in all crosses (31). Experiments using the virulent *Salmonella enterica* serovar Typhimurium ATCC 14028s background were performed under SPI-1-inducing conditions (24, 32, 33). Cultures were grown under high-osmolarity (1% NaCl, final) and low-oxygen (without agitation) conditions and if necessary supplemented with anhydrotetracycline and arabinose as described above. Phenotypic Lac activity was observed using MacConkey lactose (MacLac) agar supplemented with 0.2% arabinose if required.

Nomenclature. The gene *rflM* was originally named *ecnR* since it was a putative regulatory protein linked to the toxin-antitoxin genes *ecnAB* (29). The change in nomenclature thereby displays the newly characterized function of RflM as a modulator of the flagellar master operon *flhDC* (*r*egulator of *fl*agellar *m*aster operon).

Isolation of random T-POP insertions. Strain TH15941 [Δ*araBAD1007*::*flhD*$^+$*C*$^+$ *flhC5213*::Mu*dJ fliA5886*(R91C L207P)] carries the *flhD*$^+$*C*$^+$ operon expressed from the chromosomal *araBAD* promoter (P$_{araBAD}$) (19). It also carries a *lac* operon (via insertion of the Mu*dJ* transposon into the *flhC* gene) transcriptional reporter fusion to the chromosomal *flhDC* promoter (*flhC5213*::Mu*dJ*) and a *fliA* null allele that is defective in binding DNA (34). The introduction of the *fliA* null allele was to prevent any potential effects of σ^{28} activity on the *flhDC* autoregulatory control. Strain TH15941 is Lac$^+$ but becomes Lac$^-$ in the presence of arabinose (Ara-Lac$^-$) due to induction of *flhD*$^+$*C*$^+$ transcription from P$_{araBAD}$, resulting in autorepression of *flhC-lac* reporter transcription by FlhD_4C_2. P22 phage prepared from T-POP donor strain TH3923 {pJS28 (Ap^r P22-9$^+$) F′114(Ts) Lac$^+$ *zzf-20*::Tn*10*[*tetA*::Mu*dP*] (Tc^s) *zzf-3823*::Tn*10d*Tc[*del-25*] *leuA414 hsdSB* Fels2$^-$} was used to introduce T-POP into strain TH15941 carrying plasmid pNK2881, which constitutively expresses the Tn*10* transposase gene carrying the *ats-1* and *ats-2* (*a*ltered *t*arget *s*pecificity mutations) alleles, which result in the random insertion of Tn*10* derivatives into the chromosome (35). About 30,000 T-POP insertions in TH15941 were screened for loss of FlhD_4C_2 autorepression, an Ara-Lac$^+$ phenotype, in the presence of tetracycline (Tc-Ara-Lac$^+$). Thirty T-POP insertions that had an initial Ara-Lac$^+$ phenotype in the presence of Tc were isolated and were further characterized as described in Results.

β-Galactosidase assays. β-Galactosidase activity was measured according to method of Zhang and Bremer (36) with minor modifications as described in the following. For each strain, a minimum of three independent biological replicates were picked from individual colonies. For experiments performed with LT2, cells were grown overnight in general LB medium. Strain 14028s was grown under SPI-1 inducing conditions in LB medium supplemented with 1% NaCl (final) and without shaking. LT2 samples were diluted 1:100 in LB medium supplemented with 0.2% L-arabinose and 1 μg ml^{-1} anhydrotetracycline if needed. Cultures were grown until mid-log phase at 37°C before permeabilization of the cells

using 100 mM Na_2HPO_4, 20 mM KCl, 2 mM $MgSO_4$, 0.08% CTAB (hexadecyl-trimethyl ammonium bromide), 0.04% sodium deoxycholate, and 5.4 μl ml^{-1} β-mercaptoethanol.

To start the reaction, substrate solution containing 60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 1 mg ml^{-1} *o*-nitrophenyl-β-D-galactoside (ONPG) and 2.7 μg ml^{-1} β-mercaptoethanol was added, and the time was measured until sufficient color had been developed. The enzyme reaction was then stopped by addition of 1 M sodium carbonate (Na_2CO_3), and remaining cell fragments were pelleted by centrifugation at full speed. The optical density of the supernatant at 405 nm was measured, and Miller units were calculated according to the method of Miller (37).

All samples were normalized against their corresponding arabinose-induced wild-type control in the presence or absence of anhydrotetracycline.

For statistical analysis, an unpaired *t* test was performed using the software program GraphPad Prism version 5.0d for Mac (GraphPad Software, San Diego, CA, USA). The *P* value summaries are displayed above the respective bars.

RNA isolation and quantitative real-time PCR. RNA isolation was performed for three independent biological replicates using the RNeasy minikit (Qiagen). For removal of genomic DNA, RNA was treated with DNase I for 30 min at 37°C using a DNA-free RNA kit (Zymo Research) or on-column treatment was performed using kit 79254 (Qiagen). Subsequently, RNA samples were reverse transcribed according to the RevertAid first-strand cDNA synthesis kit (Fermentas). Quantitative real-time PCRs were carried out using the EvaGreen quantitative real-time PCR (qPCR) master mix (Bio-Rad) and primers 5′-TCTCAACGATGCCTTACCCGAACA plus 5′-GCAAGCTCATGTAAAGGCGTGTGT (*rflM*), 5′-CTGCTCAAAGAGCTGGTGTATCA plus 5′-AGCGCGTTACAGTCTGCTCAT (*gyrB*), 5′-CAACCTGTTCGTACGTATCGAC plus 5′-CAGCTCCATCTGCAGTTTGTTG (*rpoB*), 5′-CAACAGTATGCGCGTGATGAT plus 5′-CGACGCAGAGCTTCATGATC (*rpoD*), 5′-TTGCAGAAATGAGCCATTACGCCG plus 5′-GACGTTCAGC GCGAATGATGGTTT (*gmk*), 5′-GTAGGCAGCTTTGCGTGTAG plus 5′-TCCAGCAGTTGTGGAATAATATCG (*flhDC*), 5′-AACGTCTATTTTGTGAAAACCAAAG plus 5′-AGACTCCAGAATCCCGTTTTC (*flgE*), and 5′-AACGACGGTATCTCCATTGC plus 5′-ATTTCAGCCTGGATGGAGTC (flagellin, conserved region of *fliC* and *fljB*). Experiments were performed on a CFX96 real-time PCR instrument (Bio-Rad), with the exception of the experiment leading to Fig. 5. Here we used a Rotor-Gene Q 2plex real-time PCR system (Qiagen). Relative changes in mRNA levels were analyzed according to the Pfaffl method (38) and normalized against the transcript levels of the reference genes *rpoB*, *rpoD*, *gyrB*, and *gmk*.

Motility plates. Motility plates were prepared as described previously (30, 39). Strains were picked from fresh individual colonies grown on LB and pocked into 0.2% arabinose-containing (+Ara) and arabinose-free (−Ara) motility agar. Plates were kept at 30°C and grown for 5.5 h (−Ara) and 6.5 h (+Ara), respectively. The diameter of the motility swarm was measured using the software program NIH ImageJ 1.44g. In order to increase the contrast between motility and background, contrast levels were equally adjusted using iWork Pages software.

Flagellar staining. For immunostaining of flagellar filaments, *fliC*-ON phase-locked (Δ*hin-5717*) *Salmonella* bacteria containing a deletion of either *rflM* or *rcsB* were grown to mid-log phase and immobilized on poly-L-lysine-treated coverslips. The bacteria were fixed by addition of 5% formaldehyde and 0.5% glutaraldehyde. Membrane staining was performed using FM-64 (0.5 mg ml^{-1}). Flagella were stained using polyclonal anti-FliC antibodies (rabbit) and anti-rabbit-Alexa 488 secondary antibodies (Invitrogen). Images were collected using an inverted Axio Observer.Z1 fluorescence microscope (Zeiss).

RESULTS

$FlhD_4C_2$ activates its own repressor, RflM. The master regulatory operon of flagellar gene expression, *flhDC*, is under autogenous repression (13). It was reported that increased levels of active $FlhD_4C_2$ complex resulted in decreased *flhDC* transcription in cases where σ^{28} (FliA) was absent or where σ^{28} was in the presence of its cognate anti-σ^{28} factor FlgM (13). We confirmed the autoregulatory effect of $FlhD_4C_2$ on *flhDC* operon transcription in the presence of both σ^{28} and FlgM as shown in Fig. 1. When $FlhD_4C_2$ was overexpressed from the arabinose promoter upon addition of 0.2% arabinose, a chromosomal *flhC-lac* transcriptional fusion under the control of the *flhDC* promoter reported a drop in *flhC-lac* expression of about 2-fold (Fig. 1A). It was shown previously that tuning expression of $FlhD_4C_2$ from the arabinose promoter by addition of 0.05% to 0.6% arabinose resulted in a steady 60-fold increase in *flhDC* mRNA levels compared to *flhDC* expressed from the native P_{flhDC} promoter (19). In addition, the $FlhD_4C_2$ levels needed for maximal activation of downstream flagellar genes appear to be rapidly saturated, since class 2 and class 3 gene expression increased only 3- to 5-fold in the tested range of P_{ara}*flhDC* overexpression (19).

To address whether $FlhD_4C_2$ could directly repress its own transcription or if $FlhD_4C_2$ mediated autorepression indirectly by activating the transcription of an unknown *flhDC* operon repressor, we performed T-POP transposon mutagenesis and screened for potential $FlhD_4C_2$-dependent repressors of *flhDC* gene transcription. T-POP transposons are derivatives of transposon Tn*10* that can transcribe chromosomal genes adjacent to the site of insertion from tetracycline-inducible promoters within the T-POP element (40). T-POP lacks transposition functions, which can be provided in *trans* by a Tn*10* transposase expression plasmid present in recipient cells (40). After transposon insertions have been isolated and moved into a strain lacking Tn*10* transposase, the T-POP transposon is no longer capable of further transposition. Strain TH15941 (Δ*araBAD1007*::*flhD*$^+$*C*$^+$ *flhC5213*::Mu*dJ fliA*5886) carries an *flhC-lac* transcriptional fusion and the *flhD*$^+$*C*$^+$ operon expressed from an arabinose-inducible promoter (P_{ara}*flhD*$^+$*C*$^+$). This strain is Lac$^-$ in the presence of arabinose due to $FlhD_4C_2$ autorepression. The phenotype on lactose MacConkey (MacLac) agar is shown in Fig. 2. MacLac indicator medium shows the optimal sensitivity in the intermediate *lac* operon expression range (41). It thus represents the indicator medium of choice to visualize repression of *flhDC*, such as *flhDC* autorepression (P_{ara}*flhD*$^+$*C*$^+$) or repression of *flhDC* by the known transcriptional regulator RcsB (P_{ara}*rcsB*$^+$). Using MacLac indicator medium, a transition from white (Lac$^-$) to dark-pink (Lac$^+$) colonies can be readily observed (Fig. 2). Strain TH15941 was mutagenized with the T-POP transposon Tn*10d*Tc[*del-25*] and screened for insertions that were Lac$^+$ (dark pink) in the presence of arabinose due to loss of $FlhD_4C_2$ autorepression. The T-POP transposon Tn*10d*Tc[*del-25*] is deleted for the terminator of the *tetA* tetracycline resistance gene transcript, allowing transcription from the *tetA* promoter (P_{tetA}) within the T-POP transposon into adjacent chromosomal DNA (40). Four groups of T-POP transposon insertion mutants that resulted in the apparent loss of $FlhD_4C_2$ autorepression (i.e., Lac$^+$ in the presence of arabinose [Ara-Lac$^+$]) were obtained. One group included T-POP insertions linked to the arabinose region and are presumed to have disrupted the *flhDC* operon. A second group included T-POP insertions linked to the *flhC-lac* operon fusion and are presumed to be polar insertions between the *flhDC* promoter and the *lac* operon inserted in *flhC*, thereby putting *lac* directly under P_{tetA} control. These two groups of Ara-Lac$^+$ T-POP insertions in strain TH15941 were not further charac-

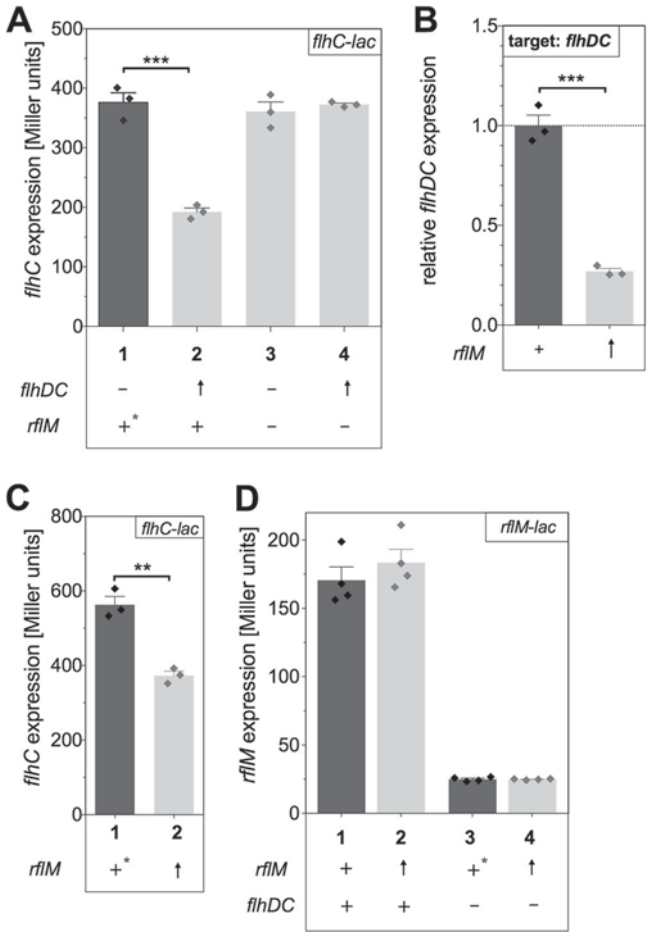

FIG 1 RflM inhibits its own activator (*flhDC*). (A) Expression levels of an *flhC-lac* transcriptional fusion under P_{araBAD}::*flhD*$^+$*C*$^+$ inducing conditions (0.2% arabinose), measured by β-galactosidase activity as described in Materials and Methods. Three independent biological replicates of the P_{araBAD}::*flhD*$^+$*C*$^+$ *flhC*::Mu*dJ* (EM59; lane 2) and P_{araBAD}::*flhD*$^+$*C*$^+$ Δ*rflM flhC*::Mu*dJ* (EM57; lane 4) strains were measured and compared to Δ*araBAD*::*tetRA flhC*::Mu*dJ* (TH8972; lane 1) and Δ*araBAD*::*tetRA* Δ*rflM flhC*::Mu*dJ* (EM71; lane 3) control strains. (B) Relative *flhDC* expression compared to wild-type mRNA levels of strain TH6701 Δ*araBAD*::*tetRA* or TH437 LT2, respectively. Transcript levels were compared by quantitative real-time PCR as described in the text. The effect of arabinose-induced overexpression of RflM (TH9386; P_{araBAD}::*rflM*$^+$) on *flhDC* expression was monitored in independent experiments using total mRNA of three independent biological samples grown in arabinose-containing medium. (C) β-Galactosidase activity showing transcription levels in strain expressing RflM from the arabinose promoter (TH13069 P_{araBAD}::*rflM*$^+$ *flhC*::Mu*dJ*, labeled ↑), compared to results for the wild-type control (TH8972; Δ*araBAD*::*tetRA flhC*::Mu*dJ*). TH8972 contains chromosomal *rflM* but lacks *rflM* expression due to the absence of functional *flhDC* (labeled as +*). (D) *rflM* expression levels in a β-galactosidase assay showing repression of *rflM* transcription in strains lacking *flhDC*. For each strain, four independent replicates were analyzed. EM154 (Δ*araBAD*::FCF P_{flhDC}::Tn10*d*Tc[*del-25*] *rflM*::Mu*dJ*; lanes 1 and 3) and EM153 (P_{araBAD}::*rflM*$^+$ P_{flhDC}::Tn10*d*Tc[*del-25*] *rflM*::Mu*dJ*; lanes 2 and 4) were grown until mid-log phase in arabinose-supplemented medium in the presence and absence of 1 μg ml^{-1} anhydrotetracycline (AnTc). The relevant genotype is labeled as followed: a chromosomal wild-type copy of the gene is present (+), the gene is chromosomally overexpressed from an arabinose-inducible promoter (↑), or the strain is deleted for the respective gene (−). It is important to note that *rflM* requires the presence of FlhDC in order to be expressed. The asterisk (*) indicates conditions where the wild-type *rflM* gene is present but not transcribed due to the absence of *flhDC*. Error bars represent the standard deviations of the means. Data were analyzed by the Student *t* test. Gene expression levels that differed significantly are shown (**, $P < 0.01$; ***, $P < 0.001$).

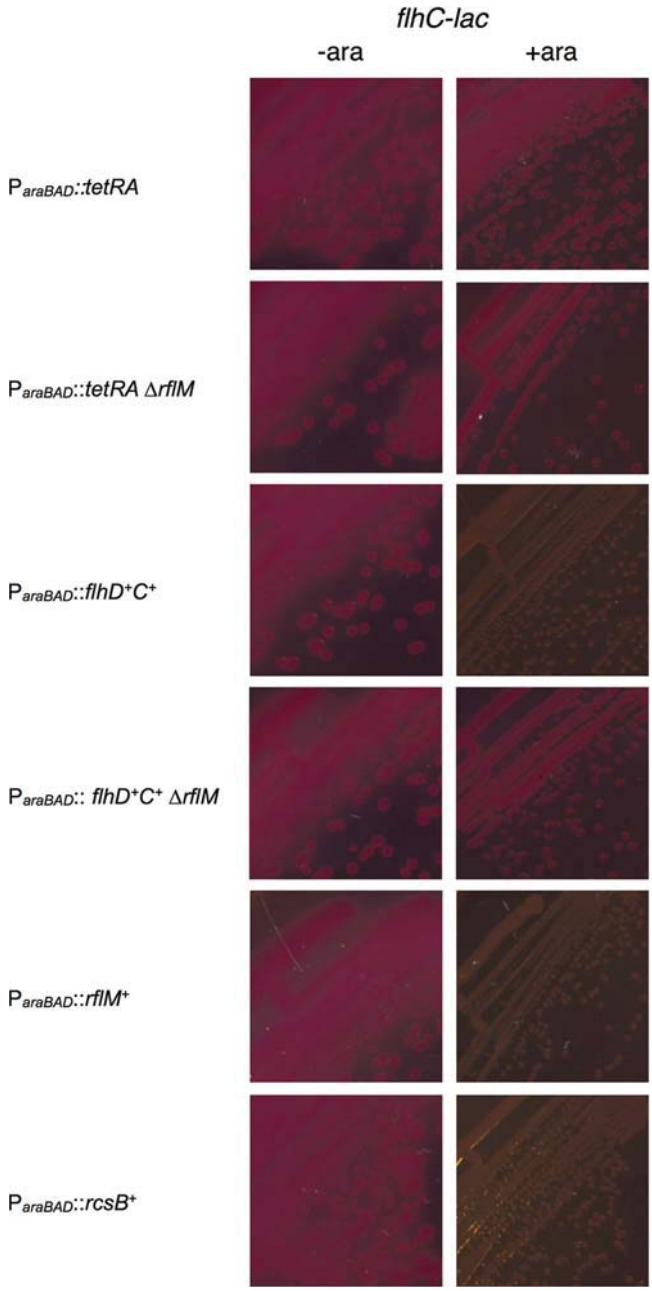

FIG 2 Phenotypes of different P_{ara} constructs on MacLac indicator plates in the presence (+ara) and absence (−ara) of arabinose. Plates were incubated at 37°C overnight. TH8972 P_{araBAD}::*tetRA*, EM71 P_{araBAD}::*tetRA* Δ*rflM*, EM59 P_{araBAD}::*flhDC*, EM57 P_{araBAD}::*flhDC* Δ*rflM*, TH13069 P_{araBAD}::*rflM*$^+$, and TH13067 P_{araBAD}::*rcsB* are shown.

terized. The remaining two groups of Ara-Lac$^+$ T-POP insertions were unlinked to both *ara* and *flhC-lac* fusion chromosomal positions and represented candidates for insertions in a gene or genes responsible for $FlhD_4C_2$-dependent repression of *flhDC* operon transcription. One group included two insertions that exhibited an Ara-Lac$^+$ phenotype only in the presence of tetracycline (Tc). These were presumed to turn on an activator of *flhDC* gene expression from P_{tetA} that is currently being subjected to further characterization. The final group of

TABLE 2 Locations of tetracycline-independent Ara-Lac$^+$ T-POP insertions

T-POP no.	Location of insertion (bp downstream of *rflM* start site)	DNA strand
4	157	Minus strand
5 and 9	39	Minus strand
6 and 24	200	Minus strand
10	157	Minus strand
11	191	Reference (plus) strand
17	542	Reference (plus) strand
22	473	Minus strand
25	149	Reference (plus) strand

insertion mutants was Ara-Lac$^+$ in either the presence or absence of added Tc, and their Ara-Lac$^+$ phenotype was presumed to be due to the loss of function of the gene into which the T-POP was inserted. T-POP insertions of this group that were linked to known negative regulators of *flhDC* expression (e.g., *lrhA*, *rtsB*, and *rcsB*) were not characterized further. Of the remaining unlinked T-POP insertions, we sequenced 10 Tc-independent Ara-Lac$^+$ insertion mutants, and DNA sequence analysis revealed transposition of the T-POP element into the *rflM* locus (Table 2). This indicated that *rflM* encodes an $FlhD_4C_2$-induced repressor of *flhDC* transcription (the "autorepressor").

RflM inhibits *flhC* gene transcription. The results of the random T-POP transposon mutagenesis suggested that RflM functions as an $FlhD_4C_2$-dependent inhibitor of *flhDC* operon transcription. Accordingly, we tested autoinhibition of *flhDC* transcription upon induction of P_{ara}*flhD*$^+$*C*$^+$ in the absence of the putative regulator RflM (Fig. 1A). The inhibitory effect of P_{ara}*flhD*$^+$*C*$^+$ induction on *flhDC* gene transcription was lost in the *rflM* deletion strain, confirming that RflM was responsible for the $FlhD_4C_2$ autoregulation effect observed by Kutsukake (13). In order to confirm that RflM represses *flhDC* operon transcription, we analyzed *flhDC* mRNA levels in a strain that expresses excess *rflM* from an arabinose-inducible promoter (P_{ara}*rflM*$^+$). As shown in Fig. 1B, under P_{ara}*rflM*$^+$-inducing conditions, *flhDC* expression decreased about 4-fold in a reverse transcriptase qPCR (RT-qPCR) assay. Reduction in *flhDC* transcription was also observed under P_{ara}*rflM*$^+$-inducing conditions using an *flhC-lac* operon fusion reporter shown in Fig. 1C.

Effect of RflM on *rflM* gene transcription. We investigated whether RflM was under autogenous control. To test if RflM autoregulates its own transcription, we utilized a previously isolated *rflM-lac* operon transcriptional fusion (30). A strain overexpressing RflM from the arabinose promoter (P_{ara}*rflM*$^+$) was analyzed in comparison to the wild-type control, where the arabinose genes *araBAD* were deleted by inserting an Flp recombination target (FRT)-chloramphenicol acetyltransferase-FRT (FCF) resistance cassette (Δ*araBAD*::FCF). Both strains carried the above-mentioned *rflM-lac* reporter and a T-POP insertion in the promoter region of *flhDC*. The latter allows the activation of *flhDC* transcription upon addition of anhydrotetracycline (AnTc), which activates T-POP-encoded *tetA* and *tetR* gene transcription, and prevents *flhDC* autorepression. As shown in Fig. 1D, the absence of $FlhD_4C_2$ (e.g., in the absence of the inducer AnTc, the *flhDC* operon in these strains is not transcribed) resulted in a reduction of *rflM-lac* transcription, presumably because the *rflM* gene required $FlhD_4C_2$ for its transcription. However, induction of *rflM* from the arabinose locus had no effect on the remaining *rflM-lac* transcription, suggesting that RflM does not play an additional role in its own transcriptional regulation except through regulation of *flhDC*.

$FlhD_4C_2$ activates its own repressor, RflM. The results described above indicate that $FlhD_4C_2$ activates its own repressor, RflM. To analyze the effects of P_{ara}*flhD*$^+$*C*$^+$ induction on *rflM* gene expression, we analyzed β-galactosidase activity of a transcriptional *rflM-lac* reporter in *Salmonella enterica* LT2 and the virulent strain *Salmonella enterica* ATCC 14028s to exclude strain-specific effects and in addition analyze a potential cross talk between the interconnected virulence and flagellar gene regulation networks. The attenuated strain, LT2, was grown under regular LB conditions, whereas the virulent strain, 14028s, was grown under high-salt and low-oxygen conditions, known to induce the virulence-associated *Salmonella* pathogenicity island 1 (SPI-1) (24, 32, 33). In both strain backgrounds, a 3-fold induction of *rflM* was observed under P_{ara}*flhD*$^+$*C*$^+$-inducing conditions (Fig. 3A). We

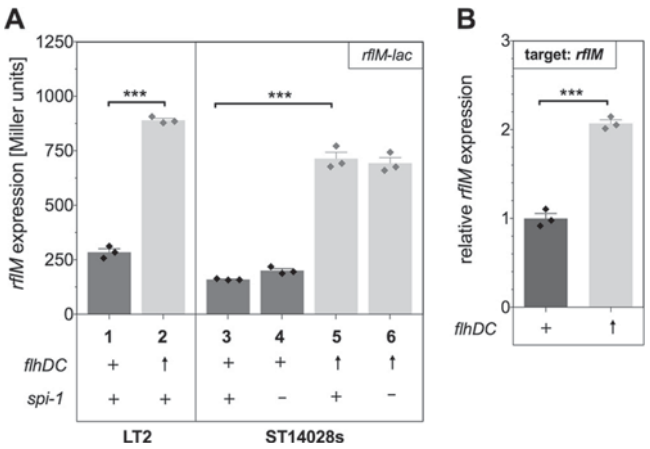

FIG 3 $FlhD_4C_2$ activates its own repressor, RflM. (A) Expression of an *rflM-lac* transcriptional fusion under *flhDC* overexpression conditions compared to wild-type *flhDC* expression conditions. Transcription of *rflM-lac* as determined by β-galactosidase assays for *Salmonella enterica* serovar Typhimurium LT2 and ATCC 14028s shows activation of *rflM* gene expression in a strain expressing excess $FlhD_4C_2$. Three independent biological replicates were grown in LB medium containing 0.2% arabinose. TH10068 (LT2 *rflM*::Mu*dJ*; lane 1) and TH16205 (LT2 P_{araBAD}::*flhD*$^+$*C*$^+$ *rflM*::Mu*dJ*; lane 2) were grown shaking in normal LB medium. For ST14028s, strains EM673 (Δ*araBAD*::FRT *rflM*::Mu*dJ*; lane 3), EM701 (Δ*araBAD*::FRT *rflM*::Mu*dJ* Δ*invH-sprB*::FCF; lane 4), EM672 (P_{araBAD}::*flhD*$^+$*C*$^+$ *rflM*::Mu*dJ*; lane 5), and EM700 (P_{araBAD}::*flhD*$^+$*C*$^+$ *rflM*::Mu*dJ* Δ*invH-sprB*::FCF; lane 6) were grown under SPI-1-inducing conditions as further described in Materials and Methods. (B) Effects of excess $FlhD_4C_2$ on *rflM* gene expression. Strain TH14156 P_{araBAD}::*flhD*$^+$*C*$^+$ (↑) was grown to mid-log phase in LB medium containing 0.2% arabinose. Total RNA of three biological replicates was isolated, and *rflM* transcript levels were analyzed in independent experiments by real-time qPCR as described in the text. Relative gene expression was determined using the Pfaffl method. Individual mRNA levels were normalized against those for multiple reference genes (*gmk*, *gyrB*, *rpoB*, and *rpoD*) and are presented as fold change relative to those for the wild-type control, TH6701 Δ*araBAD*::*tetRA* or TH437 LT2. Data were analyzed by the Student *t* test. Gene expression levels that differed significantly are indicated (***, $P < 0.001$). Error bars represent the standard deviations of the means. The relevant genotype is labeled as followed: a chromosomal wild-type copy of the gene is present (+), the gene is chromosomally overexpressed from an arabinose-inducible promoter (↑), or the strain is deleted for the respective gene (−).

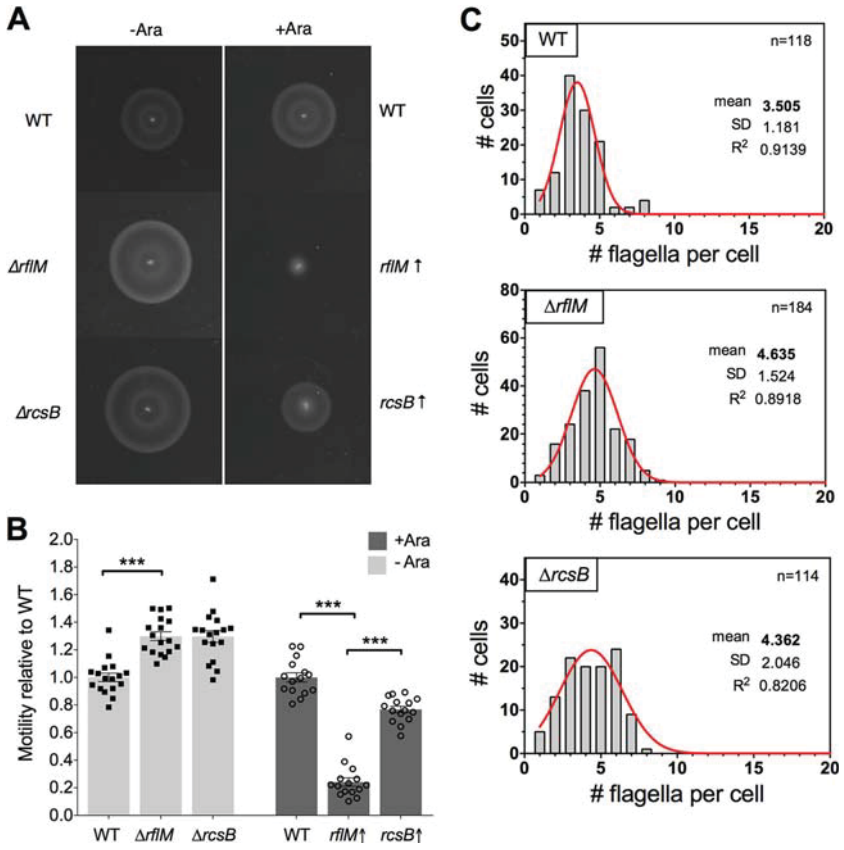

FIG 4 Motility and flagellation of *rflM* and *rcsB* mutants. (A) Motility of *rflM* and *rcsB* mutants with or without induction by arabinose. Fresh colonies were poked into motility plates and grown at 30°C for 5.5 h (−Ara) or 6.5 h (+Ara). (B) Motility diameter was measured using ImageJ, and values for EM636 (Δ*rflM*::FCF), EM635 (Δ*rcsB*::*tetRA*), EM643 (Δ*araBAD*::*rflM*$^+$), and EM642 (Δ*araBAD*::*rcsB*$^+$) are shown relative to those for the wild type (WT) EM666 (Δ*araBAD*::FRT). Relevant genotypes are listed. For details, see the list of strains (Table 1). (C) Numbers of flagella per cell in a *fliC*-ON (Δ*hin-5717*) phase-locked background. Cell bodies were stained using FM-64, and flagella were labeled using antibodies against the expressed filament subunit *fliC* as described in Materials and Methods. The number of flagella per cell body was manually counted using ImageJ. TH5971 (Δ*hin-5717*), TH16952 (Δ*ecnR* Δ*hin-5717*), and TH16964 (Δ*rcsB* Δ*hin-5717*) were analyzed.

also performed complementary quantitative real-time PCR assays. Upon P_{ara}*flhD*$^+$*C*$^+$-inducing conditions, the *rflM* mRNA level was significantly increased, as shown in Fig. 3B.

Effect of RflM on motility and flagellation. We have shown above that overexpression of *rflM* resulted in a substantial repression of the flagellar master regulatory operon, *flhDC*. We therefore analyzed the effect of a deletion of the *rflM* gene and *rflM* overexpression on motility and flagellar assembly. A deletion in *rflM* resulted in an increase in motility compared to the wild type (Fig. 4A and B) and on average one additional flagellum per cell body (Fig. 4C). Under *rflM*-overexpressing conditions (labeled *rflM*↑), a substantial loss of motility was observed and motility was significantly impaired compared to that with the known repressor RcsB (Fig. 4B).

Additionally, we analyzed gene expression of the three flagellar promoter classes by quantitative real-time PCR. Expression of all the classes was upregulated in the Δ*rflM* background, with a decreasing effect along the transcriptional flagellar hierarchy (Fig. 5). While no significant difference could be observed between the two regulators RflM and RcsB in terms of class 1 (*flhDC*) expression, the RflM effect is clearly visible at later class 2 (*flgE*) and class 3 (*fliC* and *fljB*) transcription. This effect supports the observation that the RflM effect on motility and flagellation was more pronounced than that of RcsB.

In summary, our results support a regulatory feedback loop for the autogenous control of *flhDC* operon transcription. Expression of *flhDC* leads to production of a functional $FlhD_4C_2$ activator complex, which, in addition to activating flagellar class 2 pro-

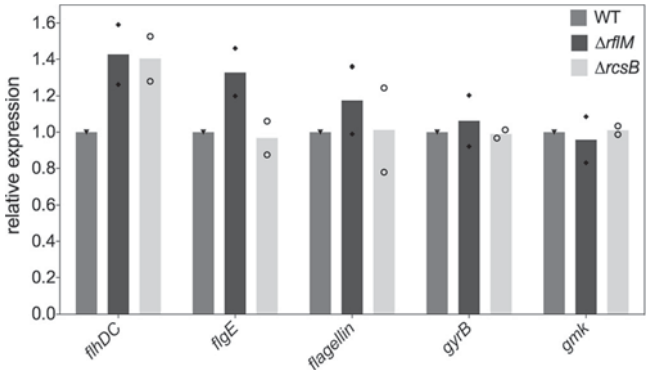

FIG 5 Flagellar class 1, 2, and 3 gene expression of *rflM* and *rcsB* mutants. Relative expression of the different flagellar promoter classes under wild-type, Δ*rflM*, and Δ*rcsB* conditions is shown. Quantitative real-time analysis was performed with two independent mRNA purifications. Expression levels are shown for class 1 (*flhDC*), class 2 (hook subunit *flgE*), and class 3 (flagellin subunits *fliC* and *fljB*) gene products. *gyrB*, *rpoB*, and *gmk* served as reference genes for normalization.

moter transcription for hook-basal body assembly, activates *rflM* gene transcription. RflM, when produced, acts to inhibit *flhDC* transcription. We have thereby demonstrated that this feedback loop is responsible for the formerly observed autoregulatory effect of $FlhD_4C_2$.

DISCUSSION

In this study, we show that the formerly observed autoregulatory effect of the flagellar master regulator $FlhD_4C_2$ is caused by $FlhD_4C_2$-dependent production of a negative regulator, RflM. $FlhD_4C_2$ and RflM thereby form a regulatory feedback loop, which regulates the rate of flagellar gene expression with respect to the level of already-initiated flagellar biosynthesis. Expression of *rflM* is directly coupled to the presence of functional $FlhD_4C_2$, which could be concluded from the fact that the amount of *rflM* mRNA was significantly increased under $FlhD_4C_2$ overexpression conditions. Conclusively, *flhDC* expression decreased in the presence of excess RflM, and $FlhD_4C_2$ autoregulation was completely abolished in an *rflM* deletion background.

Flagellar synthesis is dependent on a hierarchy of three transcriptional promoter classes, with the master regulator, $FlhD_4C_2$, at the top. $FlhD_4C_2$ is a known DNA binding complex that has been shown to bind to the −40 to −80 regions of multiple flagellar operons (42, 43) and together with σ^{70} targets RNA polymerase to transcribe class 2 flagellar promoters (44). $FlhD_4C_2$ allows expression of the subsequent flagellar genes (under the control of the $FlhD_4C_2$-dependent class 2 promoters) needed for hook-basal-body assembly. The presence of functional $FlhD_4C_2$ thereby plays the main role in determining whether or not flagella are synthesized. Accordingly, negative feedback between the $FlhD_4C_2$ protein and *flhDC* operon expression would prevent the cell from synthesizing an excess of flagella. Overexpression of *flhDC* has previously been described to turn off *flhDC* operon transcription and was attributed to a direct inhibition of the *flhDC* operon by the $FlhD_4C_2$ complex (13). It was unclear, however, whether this inhibitory function was direct or indirect. Thus, we designed a genetic selection and screen to differentiate between two possibilities: in addition to the known role of $FlhD_4C_2$ as a transcriptional activator of flagellar class 2 promoters, $FlhD_4C_2$ could either act as a direct repressor of its own (*flhDC* operon) transcription or as an activator of an unknown repressor of *flhDC* operon transcription. Our results support the latter possibility.

The identification of an $FlhD_4C_2$-dependent *flhDC* regulator allows us to postulate a novel regulatory feedback loop that accounts for the previously described autoregulation of $FlhD_4C_2$. The loop consists of two mechanisms: (i) $FlhD_4C_2$ activates *rflM* and (ii) RflM represses *flhDC* transcription. With this study, we added a new component to the complex regulatory mechanisms that controls expression and activity of the flagellar master regulator $FlhD_4C_2$. Many environmental and regulatory stimuli are integrated at the level of σ^{70}-dependent transcription of the *flhDC* operon. Expression of $FlhD_4C_2$ therefore forms the prerequisite for the initiation of flagellar synthesis by activation of flagellar class 2 and 3 gene expression. Regulation at the level of *flhDC* operon transcription is thus the main target by which flagellar synthesis is regulated. In this study, we further characterized the *flhDC* master operon regulation that is placed on top of the flagellar gene expression cascade. In addition to environmental (external) stimuli that control expression of *flhDC*, an endogenous (internal) regulation mechanism that directly provides feedback of

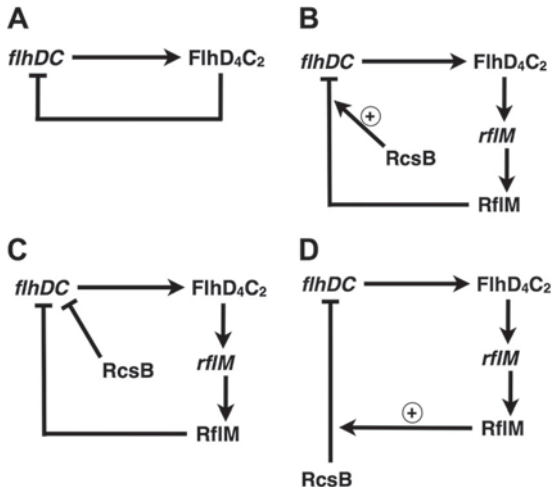

FIG 6 Possible models of the regulatory *flhDC* feedback loop. Panel A shows the formerly described autoregulatory effect of *flhDC*, where $FlhD_4C_2$ directly represses *flhDC* operon transcription. In contrast to this previous model, $FlhD_4C_2$ induces expression of *rflM*, and the RflM protein acts as a repressor of *flhDC* transcription. Repression of RflM might be direct but facilitated by RcsB (B). Alternatively, RflM might repress *flhDC* independently of RcsB (C) or act as a corepressor of RcsB (D).

$FlhD_4C_2$ protein levels exists. An increase in *flhDC* expression and the subsequent flagellar synthesis requires a mechanism of counterbalance. Such an effect was previously referred to as $FlhD_4C_2$ autorepression (Fig. 6A), which our results show to be a regulatory feedback loop via RflM. A previous study indicated an additional role of the RcsCDB system in the RflM-mediated repression of *flhDC* (30). The response regulator RcsB has been shown to directly bind to the *flhDC* promoter region (23, 24). Accordingly, different scenarios of RflM-dependent repression of *flhDC* can be envisioned, and follow-up experiments are currently in progress to test the role RcsB in this regulation network. RflM could directly repress *flhDC*, while RcsB takes over a supporting role (Fig. 6B). Alternatively, RcsB could directly repress *flhDC* independently of RflM, and both proteins might compete for binding to the *flhDC* promoter region (Fig. 6C). Finally, RflM could act as a corepressor of RcsB (Fig. 6D).

The complexity and the hierarchical structure of the flagellar system explain the frequency by which endogenous regulation mechanisms occur. Feedback regulation in the flagellar system targets multiple levels throughout biosynthesis of the flagellum: (i) on the level of *flhDC* gene expression via $FlhD_4C_2$-dependent activation of the LuxR-type repressor RflM, (ii) on the level of FlhDC protein complex via posttranscriptional repression (FliT and YdiV) and activation (FliZ) of FlhDC-dependent class 2 gene expression, and (iii) on the level of flagellar class 3 gene expression by secretion of the anti-σ^{28} factor FlgM after HBB completion and positive regulation of *flgM* translation by FlgN (45–47). In summary, the following scenario could be envisioned to couple the assembly process of the flagellum to flagellar gene expression. σ^{70}-dependent class 1 transcription of *flhDC* leads to formation of the flagellar master regulatory complex $FlhD_4C_2$. $FlhD_4C_2$ activates class 2 gene transcription, as well as that of its own repressor, *rflM*. While class 2 products build up the HBB, FliZ activates FlhDC complex and RflM acts as a repressor of *flhDC* transcription. Upon completion of the HBB, the secretion specificity switch re-

sults in secretion of FlgM and FliD, and class 3 gene expression takes place. Secretion of FliD upon HBB completion allows FliT to bind FlhDC complex and inhibit $FlhD_4C_2$ activity. Reduced activity of FlhDC decreases expression of *rflM* and allows a new cycle of flagellar gene expression and assembly starting with derepression of the *flhDC* promoter.

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REFERENCES

1. **Macnab RM.** 1996. Flagella and motility, p 123–145. *In* Neidhardt FC, Curtiss R, III, Ingraham JL, Lin ECC, Low KB, Magasanik B, Reznikoff WS, Riley M, Schaechter M, Umbarger HE (ed), *Escherichia coli* and *Salmonella*: cellular and molecular biology, 2nd ed, vol 1. American Society for Microbiology, Washington, DC.
2. **Fontaine F, Stewart EJ, Lindner AB, Taddei F.** 2008. Mutations in two global regulators lower individual mortality in *Escherichia coli*. Mol. Microbiol. **67:**2–14.
3. **Aldridge C, Pooncharoen K, Saini S, Ewen T, Soloyva A, Rao CV, Imada K, Minamino T, Aldridge PD.** 2010. The interaction dynamics of a negative feedback loop regulates flagellar number in *Salmonella enterica* serovar Typhimurium. Mol. Microbiol. **78:**1416–1430.
4. **Soutourina OA, Bertin PN.** 2003. Regulation cascade of flagellar expression in Gram-negative bacteria. FEMS Microbiol. Rev. **27:**505–523.
5. **Yokota T, Gots JS.** 1970. Requirement of adenosine 3′,5′-cyclic phosphate for flagella formation in *Escherichia coli* and *Salmonella typhimurium*. J. Bacteriol. **103:**513–516.
6. **Zhao K, Liu M, Burgess RR.** 2007. Adaptation in bacterial flagellar and motility systems: from regulon members to 'foraging'-like behavior in *E. coli*. Nucleic Acids Res. **35:**4441–4452.
7. **Wada T, Morizane T, Abo T, Tominaga A, Inoue-Tanaka K, Kutsukake K.** 2011. EAL domain protein YdiV acts as an anti-$FlhD_4C_2$ factor responsible for nutritional control of the flagellar regulon in *Salmonella enterica* serovar Typhimurium. J. Bacteriol. **193:**1600–1611.
8. **Wada T, Hatamoto Y, Kutsukake K.** 2012. Functional and expressional analyses of the anti-$FlhD_4C_2$ factor gene *ydiV* in *Escherichia coli*. Microbiology **158:**1533–1542.
9. **Prigent-Combaret C, Vidal O, Dorel C, Lejeune P.** 1999. Abiotic surface sensing and biofilm-dependent regulation of gene expression in *Escherichia coli*. J. Bacteriol. **181:**5993–6002.
10. **Beloin C, Ghigo JM.** 2005. Finding gene-expression patterns in bacterial biofilms. Trends Microbiol. **13:**16–19.
11. **Eriksson S, Lucchini S, Thompson A, Rhen M, Hinton JC.** 2003. Unravelling the biology of macrophage infection by gene expression profiling of intracellular *Salmonella enterica*. Mol. Microbiol. **47:**103–118.
12. **Cummings LA, Wilkerson WD, Bergsbaken T, Cookson BT.** 2006. *In vivo*, *fliC* expression by *Salmonella enterica* serovar Typhimurium is heterogeneous, regulated by ClpX, and anatomically restricted. Mol. Microbiol. **61:**795–809.
13. **Kutsukake K.** 1997. Autogenous and global control of the flagellar master operon, *flhD*, in *Salmonella typhimurium*. Mol. Gen. Genet. **254:**440–448.
14. **Komeda Y, Suzuki H, Ishidsu JI, Iino T.** 1976. The role of cAMP in flagellation of *Salmonella typhimurium*. Mol. Gen. Genet. **142:**289–298.
15. **Soutourina O, Kolb A, Krin E, Laurent-Winter C, Rimsky S, Danchin A, Bertin P.** 1999. Multiple control of flagellum biosynthesis in *Escherichia coli*: role of H-NS protein and the cyclic AMP-catabolite activator protein complex in transcription of the *flhDC* master operon. J. Bacteriol. **181:**7500–7508.
16. **Campoy S, Jara M, Busquets N, de Rozas AM, Badiola I, Barbe J.** 2002. Intracellular cyclic AMP concentration is decreased in *Salmonella typhimurium fur* mutants. Microbiology **148:**1039–1048.
17. **Kelly A, Goldberg MD, Carroll RK, Danino V, Hinton JC, Dorman CJ.** 2004. A global role for Fis in the transcriptional control of metabolism and type III secretion in *Salmonella enterica* serovar Typhimurium. Microbiology **150:**2037–2053.
18. **Stojiljkovic I, Baumler AJ, Hantke K.** 1994. Fur regulon in gram-negative bacteria. Identification and characterization of new iron-regulated *Escherichia coli* genes by a Fur titration assay. J. Mol. Biol. **236:**531–545.
19. **Erhardt M, Hughes KT.** 2010. C-ring requirement in flagellar type III secretion is bypassed by FlhDC upregulation. Mol. Microbiol. **75:**376–393.
20. **Libby SJ, Goebel W, Ludwig A, Buchmeier N, Bowe F, Fang FC, Guiney DG, Songer JG, Heffron F.** 1994. A cytolysin encoded by *Salmonella* is required for survival within macrophages. Proc. Natl. Acad. Sci. U. S. A. **91:**489–493.
21. **Ellermeier CD, Slauch JM.** 2003. RtsA and RtsB coordinately regulate expression of the invasion and flagellar genes in *Salmonella enterica* serovar Typhimurium. J. Bacteriol. **185:**5096–5108.
22. **Lehnen D, Blumer C, Polen T, Wackwitz B, Wendisch VF, Unden G.** 2002. LrhA as a new transcriptional key regulator of flagella, motility and chemotaxis genes in *Escherichia coli*. Mol. Microbiol. **45:**521–532.
23. **Wang Q, Zhao Y, McClelland M, Harshey RM.** 2007. The RcsCDB signaling system and swarming motility in *Salmonella enterica* serovar typhimurium: dual regulation of flagellar and SPI-2 virulence genes. J. Bacteriol. **189:**8447–8457.
24. **Francez-Charlot A, Laugel B, Van Gemert A, Dubarry N, Wiorowski F, Castanie-Cornet MP, Gutierrez C, Cam K.** 2003. RcsCDB His-Asp phosphorelay system negatively regulates the flhDC operon in Escherichia coli. Mol. Microbiol. **49:**823–832.
25. **Saini S, Koirala S, Floess E, Mears PJ, Chemla YR, Golding I, Aldridge C, Aldridge PD, Rao CV.** 2010. FliZ induces a kinetic switch in flagellar gene expression. J. Bacteriol. **192:**6477–6481.
26. **Saini S, Brown JD, Aldridge PD, Rao CV.** 2008. FliZ is a posttranslational activator of $FlhD_4C_2$-dependent flagellar gene expression. J. Bacteriol. **190:**4979–4988.
27. **Wada T, Tanabe Y, Kutsukake K.** 2011. FliZ acts as a repressor of the *ydiV* gene, which encodes an anti-$FlhD_4C_2$ factor of the flagellar regulon in *Salmonella enterica* serovar typhimurium. J. Bacteriol. **193:**5191–5198.
28. **Takaya A, Erhardt M, Karata K, Winterberg K, Yamamoto T, Hughes KT.** 2012. YdiV: a dual function protein that targets FlhDC for ClpXP-dependent degradation by promoting release of DNA-bound FlhDC complex. Mol. Microbiol. **83:**1268–1284.
29. **Bishop RE, Leskiw BK, Hodges RS, Kay CM, Weiner JH.** 1998. The entericidin locus of *Escherichia coli* and its implications for programmed bacterial cell death. J. Mol. Biol. **280:**583–596.
30. **Wozniak C, Lee C, Hughes K.** 2009. T-POP array identifies EcnR and PefI-SrgD as novel regulators of flagellar gene expression. J. Bacteriol. **191:**1498–1508.
31. **Sanderson KE, Roth JR.** 1983. Linkage map of *Salmonella typhimurium*, edition VI. Microbiol. Rev. **47:**410–453.
32. **Saini S, Ellermeier JR, Slauch JM, Rao CV.** 2010. The role of coupled positive feedback in the expression of the SPI1 type three secretion system in *Salmonella*. PLoS Pathog. **6:**e1001025. doi:10.1371/journal.ppat.1001025.
33. **Bajaj V, Lucas RL, Hwang C, Lee CA.** 1996. Co-ordinate regulation of *Salmonella typhimurium* invasion genes by environmental and regulatory factors is mediated by control of *hilA* expression. Mol. Microbiol. **22:**703–714.
34. **Aldridge PD, Karlinsey JE, Aldridge C, Birchall C, Thompson D, Yagasaki J, Hughes KT.** 2006. The flagellar-specific transcription factor, σ^{28}, is the type III secretion chaperone for the flagellar-specific anti- σ^{28} factor FlgM. Genes Dev. **20:**2315–2326.
35. **Bender J, Kleckner N.** 1992. IS10 transposase mutations that specifically alter target site recognition. EMBO J. **11:**741–750.
36. **Zhang X, Bremer H.** 1995. Control of the *Escherichia coli rrnB* P1 promoter strength by ppGpp. J. Biol. Chem. **270:**11181–11189.
37. **Miller J.** 1972. Assay of beta-galactosidase, p 352–355. *In* Experiments in molecular genetics.. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
38. **Pfaffl MW.** 2001. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res. **29:**e45. doi:10.1093/nar/29.9.e45.
39. **Gillen KL, Hughes KT.** 1991. Molecular characterization of *flgM*, a gene

encoding a negative regulator of flagellin synthesis in *Salmonella typhimurium*. J. Bacteriol. **173:**6453–6459.
40. **Rappleye CA, Roth JR.** 1997. A Tn*10* derivative (T-POP) for isolation of insertions with conditional (tetracycline-dependent) phenotypes. J. Bacteriol. **179:**5827–5834.
41. **Shuman HA, Silhavy TJ.** 2003. The art and design of genetic screens: *Escherichia coli*. Nat. Rev. Genet. **4:**419–431.
42. **Liu X, Matsumura P.** 1994. The FlhD/FlhC complex, a transcriptional activator of the *Escherichia coli* flagellar class II operons. J. Bacteriol. **176:**7345–7351.
43. **Wang S, Fleming RT, Westbrook EM, Matsumura P, McKay DB.** 2006. Structure of the *Escherichia coli* FlhDC complex, a prokaryotic heteromeric regulator of transcription. J. Mol. Biol. **355:**798–808.
44. **Liu X, Fujita N, Ishihama A, Matsumura P.** 1995. The C-terminal region of the α subunit of *Escherichia coli* RNA polymerase is required for transcriptional activation of the flagellar level II operons by the FlhD/FlhC complex. J. Bacteriol. **177:**5186–5188.
45. **Fraser GM, Bennett JC, Hughes C.** 1999. Substrate-specific binding of hook-associated proteins by FlgN and FliT, putative chaperones for flagellum assembly. Mol. Microbiol. **32:**569–580.
46. **Karlinsey JE, Lonner J, Brown KL, Hughes KT.** 2000. Translation/secretion coupling by type III secretion systems. Cell **102:**487–497.
47. **Aldridge P, Karlinsey J, Hughes KT.** 2003. The type III secretion chaperone FlgN regulates flagellar assembly via a negative feedback loop containing its chaperone substrates FlgK and FlgL. Mol. Microbiol. **49:**1333–1345.