Supplementary Information

RflM mediates target specificity of the RcsCDB phosphorelay system for transcriptional repression of flagellar synthesis in *Salmonella enterica*

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Tab. S1. List of bacterial strains used in this study.

Strain	Genotype	Source or Reference
Salmonella enterica serovar Typhimurium LT2 strains		
TH437	WT	J. Roth
TH3466	F'128 (pro-lac) *zzf382*::Tn10*d*Tc[*del-20*] proAB47	Lab collection
TH9386	Δ*araBAD921*::*rflM*$^+$	J. Karlinsey
TH12883	Δ*araBAD996*::*rcsB*$^+$	Lab collection
TH12949	Δ*araBAD921*::*rflM*$^+$ *rcsB*::Mu*dJ*	Lab collection
TH13069	Δ*araBAD921*::*rflM*$^+$ *flhC5213*::Mu*dJ*	Lab collection
TH13654	Δ*araBAD1005*::FRT *rcsB*::Mu*dJ*	Lab collection
EM162	Δ*araBAD996*::*rcsB*$^+$ *rflM*::Mu*dJ*	This study
EM163	Δ*araBAD1005*::FRT *rflM*::Mu*dJ*	This study
EM169	Δ*araBAD1005*::FRT *rflM*::Mu*dJ* Δ*rcsDBC*::FCF	This study
EM221	Δ*araBAD1005*::FCF *flhC5213*::Mu*dJ rcsB*::T-POP	This study
EM222	Δ*araBAD996*::*rcsB*$^+$ *flhC5213*::Mu*dJ rflM*2::T-POP	This study
EM229	Δ*araBAD921*::*rflM*$^+$ *flhC5213*::Mu*dJ rcsB*::T-POP	This study
EM423	pNK2880 Δ*araBAD921*::*rflM*$^+$ *flhC5213*::Mu*dJ*	This study
EM477	Δ*araBAD1170*::*rflM*(*rcsB*-HTH) *flhC5213*::Mu*dJ*	This study
EM490	Δ*araBAD1170*::*rflM*(*rcsB*-HTH) *flhC5213*::Mu*dJ rcsB*::T-POP	This study
EM504	Δ*araBAD1005*::FRT *flhC5213*::Mu*dJ rflM*2::T-POP	This study
EM506	Δ*araBAD1171*::*rcsB*(*rflM*-HTH) *flhC5213*::Mu*dJ rflM*2::T-POP	This study
EM515	Δ*araBAD1005*::FRT *flhC5213*::Mu*dJ rflM*2::T-POP Δ*rcsDBC*::FCF	This study
EM517	Δ*araBAD1005*::FRT *flhC5213*::Mu*dJ*	Singer *et al*. 2014
EM521	Δ*araBAD996*::*rcsB*$^+$ *flhC5213*::Mu*dJ rflM*2::T-POP Δ*rcsDBC*::FCF	This study
EM541	Δ*araBAD1171*::*rcsB*(*rflM*-HTH) *flhC5213*::Mu*dJ rflM*2::T-POP Δ*rcsDBC*::FCF	This study

EM656	Δ*araBAD1165*::*rflM*(*sdiA*-HTH) *flhC5213*::Mu*dJ*	This study
EM677	Δ*araBAD1180*::*rcsB*(*sdiA*-HTH) *flhC5213*::Mu*dJ rflM*2::T-POP	This study
EM676	Δ*araBAD1181*::*rflM*(*sdiA*-HTH) *flhC5213*::Mu*dJ rcsB*::T-POP	This study
EM704	Δ*araBAD1005*::FRT *flhC5213*::Mu*dJ rcsB*::T-POP	This study
EM705	Δ*araBAD1180*::*rcsB*(*sdiA*-HTH) *flhC5213*::Mu*dJ rflM*2::T-POP Δ*rcsDBC*::FCF	This study
EM706	P_{flhDC}8093 (P_{flhDC}-*luxCDBAE*-Km-P*flhDC*$^{+}$) Δ*araBAD1005*::FRT	Singer *et al*. 2014
EM707	P_{flhDC}8124 (P_{flhDC} P1+ (-10 of P2,P3,P4,P5,P6 changed to GTTGGT)-*luxCDBAE*-Km-P_{flhDC}^{+}) Δ*araBAD1005*::FRT	Singer *et al*. 2014
EM711	P_{flhDC}8128 (P_{flhDC} P5+ (-10 of P1,P2,P3,P4,P6 changed to GTTGGT)-*luxCDBAE*-Km-P_{flhDC}^{+}) Δ*araBAD1005*::FRT	Singer *et al*. 2014
EM808	Δ*araBAD1005*::FRT	Lab collection
EM1055	P_{flhDC}8093 (P_{flhDC}-*luxCDBAE*-Km-P_{flhDC}^{+}) Δ*araBAD921*::*rflM*$^{+}$	This study
EM1056	P_{flhDC}8124 (P_{flhDC} P1+ (-10 of P2,P3,P4,P5,P6 changed to GTTGGT)-*luxCDBAE*-Km-P_{flhDC}^{+}) Δ*araBAD921*::*rflM*$^{+}$	This study
EM1060	P_{flhDC}8128 (P_{flhDC} P5+ (-10 of P1,P2,P3,P4,P6 changed to GTTGGT)-*luxCDBAE*-Km-P_{flhDC}^{+}) Δ*araBAD921*::*rflM*$^{+}$	This study
EM1062	P_{flhDC}8093 (P_{flhDC}-*luxCDBAE*-Km-P_{flhDC}^{+}) Δ*araBAD996*::*rcsB*$^{+}$	This study
EM1063	P_{flhDC}8124 (P_{flhDC} P1+ (-10 of P2,P3,P4,P5,P6 changed to GTTGGT)-*luxCDBAE*-Km-P_{flhDC}^{+}) Δ*araBAD996*::*rcsB*$^{+}$	This study
EM1067	P_{flhDC}8128 (P_{flhDC} P5+ (-10 of P1,P2,P3,P4,P6 changed to GTTGGT)-*luxCDBAE*-Km-P_{flhDC}^{+}) Δ*araBAD996*::*rcsB*$^{+}$	This study
EM1325	Δ*rcsB*	This study
EM1432	Δ*araBAD1005*::FRT *flhC5213*::Mu*dJ* Δ*rcsB*	This study
EM1434	Δ*araBAD1005*::FRT *flhC5213*::Mu*dJ rflM*2::T-POP Δ*rcsB*	This study
EM1435	Δ*araBAD996*::*rcsB*$^{+}$ *flhC5213*::Mu*dJ rflM*2::T-POP Δ*rcsB*	This study
EM1740	*rflM33*::HA-FRT	This study
EM1766	Δ*araBAD1201*::*rflM*$^{+}$-HA-FRT	This study
EM2004	$RcsB_{D56E}$	This study
EM2005	$RcsB_{D56N}$	This study

EM2423	*ΔaraBAD1005*::FRT *flhC5213*::Mu*d*J Δ*rflM* $RcsB_{D56E}$	This study
EM2424	*ΔaraBAD1005*::FRT *flhC5213*::Mu*d*J Δ*rflM* $RcsB_{D56N}$	This study
EM2426	*ΔaraBAD921*::*rflM*$^{+}$ *flhC5213*::Mu*d*J Δ*rflM* $RcsB_{D56E}$	This study
EM2427	*ΔaraBAD921*::*rflM*$^{+}$ *flhC5213*::Mu*d*J Δ*rflM* $RcsB_{D56N}$	This study
EM2486	*ΔaraBAD921*::*rflM*$^{+}$ *flhC5213*::Mu*d*J Δ*rcsB*	This study
EM2504	Δ*araBAD1201*::*rflM*$^{+}$-HA-FRT Δ*rcsB*	This study
EM4358	*trp*::(SpcR T7-RNAP *lacO lacP lacI*) *flhC5213*::Mu*d*A	This study
EM4365	Δ*araBAD921*::*rflM*$^{+}$ Δ*rcsB*	This study
EM4366	Δ*araBAD996*::*rcsB*$^{+}$ Δ*rflM33*	This study
EM4369	Δ*araBAD921*::*rflM*$^{+}$ Δ*lrhA*	This study
EM4370	Δ*araBAD921*::*rflM*$^{+}$ Δ*slyA*	This study
EM4372	Δ*araBAD921*::*rflM*$^{+}$ Δ*rtsB*	This study
EM4373	Δ*araBAD921*::*rflM*$^{+}$ Δ*fliZ*	This study
EM4420	*rflM33*::HA-FRT Δ*clpXP*::FRT	This study
EM4421	*rflM33*::HA-FRT Δ*clpX*	This study
EM4422	*rflM33*::HA-FRT Δ*clpA*	This study
EM4451	*rflM33*::HA-FRT Δ*clpXP-lon*::FRT	This study
EM4454	Δ*araBAD1211*::$rcsB_{D56N}$ *flhC5213*::Mu*d*J *rflM*2::T-POP Δ*rcsB*	This study
EM4470	Δ*araBAD921*::*rflM*$^{+}$ Δ*prgH-hilD-hilA7791*	This study
EM4528	*trp*::(SpcR T7-RNAP *lacO lacP lacI*) *flhC5213*::Mu*d*A pSUMO-His_6-RflM	This study
EM4529	*trp*::(SpcR T7-RNAP *lacO lacP lacI*) *flhC5213*::Mu*d*A pSUMO-His_6-RcsB	This study
EM4530	*trp*::(SpcR T7-RNAP *lacO lacP lacI*) *flhC5213*::Mu*d*A pSUMO-His_6-RflM-RBS-RcsB	This study
EM4570	*trp*::(SpcR T7-RNAP *lacO lacP lacI*) *flhC5213*::Mu*d*A pSUMO(-)	This study
EM4586	Δ*araBAD1201*::*rflM*$^{+}$-HA-FRT *rcsB*::T-POP	This study
EM4598	*trp*::(SpcR T7-RNAP *lacO lacP lacI*) *flhC5213*::Mu*d*A Δ*rcsB*::*tetRA* pSUMO-His_6-RflM	This study

EM4599	*trp*::(SpcR T7-RNAP *lacO lacP lacI*) *flhC5213*::Mu*d*A Δ*rcsB*::*tetRA* pSUMO-His_6-RcsB	This study
EM4600	*trp*::(SpcR T7-RNAP *lacO lacP lacI*) *flhC5213*::Mu*d*A Δ*rcsB*::*tetRA* pSUMO-His_6-RflM-RBS-RcsB	This study
EM4601	*trp*::(SpcR T7-RNAP *lacO lacP lacI*) *flhC5213*::Mu*d*A Δ*rcsB*::*tetRA* pSUMO(-)	This study
EM4655	*rflM33*::HA-FRT Δ*lon*	This study
EM4665	Δ*araBAD1170*::*rflM*(*rcsB*-HTH) *flhC5213*::Mu*d*J Δ*rcsB*	This study
EM4666	Δ*araBAD1181*::*rflM*(*sdiA*-HTH) *flhC5213*::Mu*d*J Δ*rcsB*	This study
EM4697	Δ*araBAD1213*::*rflM*(ΔHTH) *flhC5213*::Mu*d*J	This study
EM4711	Δ*araBAD1213*::*rflM*(ΔHTH) *flhC5213*::Mu*d*J *rcsB*::T-POP	This study
EM4727	Δ*araBAD1213*::*rflM*(ΔHTH) *flhC5213*::Mu*d*J Δ*rcsB*	This study
Escherichia coli K12 strains		
TH5314	BacterioMatch reporter strain, F' P_{lac}OR2-62 P_{lac}-*bla-lacZ*	Stratagene
EM567	BacterioMatch pBT pTRG	This study
EM571	BacterioMatch pBT-RflM pTRG-RflM	This study
EM572	BacterioMatch pBT-RflM pTRG-RcsB	This study
EM574	BacterioMatch pBT-RcsB pTRG-RcsB	This study
EM575	BacterioMatch pBT-RcsB pTRG-RflM	This study
EM576	BacterioMatch pBT-LGF2 pTRG-Gal11P	This study
EM577	BacterioMatch pBT-RcsB pTRG-Gal11P	This study
EM578	BacterioMatch pBT-RflM pTRG-Gal11P	This study
EM579	BacterioMatch pBT-LGF2 pTRG-RcsB	This study
EM580	BacterioMatch pBT-LGF2 pTRG-RflM	This study
EM765	BL21(λDE3) pSUMO-His_6-RcsB	This study
EM1020	BL21(λDE3) pSUMO-His_6-RflM-RBS-RcsB	This study
EM3064	BL21(λDE3) pSUMO-His_6-SUMO-$RcsB_{D56E}$	This study
EM4413	BL21(λDE3) pSUMO-His_6-SUMO-RflM(ΔHTH)-RBS-RcsB	This study

Supplemental Fig. S1.

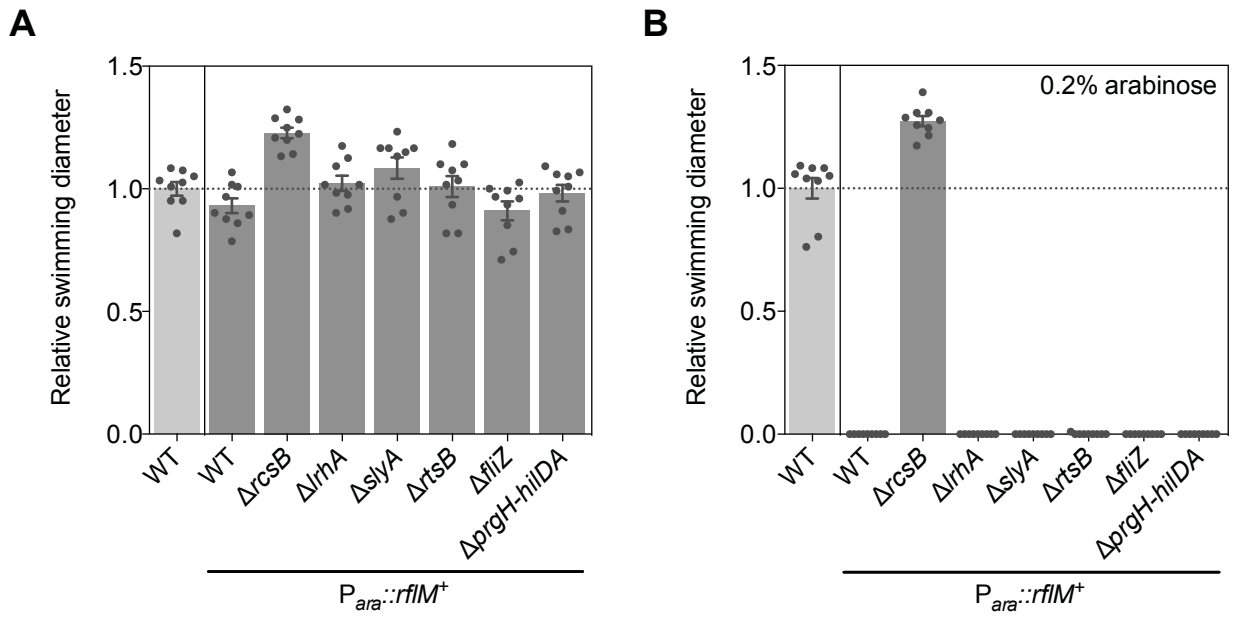

Fig. S1. RflM-mediated repression of motility does depend on the presence of RcsB, but not other *flhDC*-specific regulators.

Relative swimming diameters of deletion mutants of *flhDC*-specific regulators (Δ*rcsB*, Δ*lrhA*, Δ*slyA*, Δ*rtsB*, Δ*fliZ*, Δ*prgH-hilDA*) that harbored *rflM* under control of an arabinose-inducible promoter (P$_{araBAD}$::*rflM*$^{+}$) were analyzed under non-inducing (A) and inducing conditions (B). Swimming diameters were measured after incubation on 0.3% motility agar plates and normalized to the P$_{araBAD}$::FRT wildtype control (WT). Bars represent mean values of 12 biological replicates and error bars represent the standard errors of the mean.

Supplemental Fig. S2.

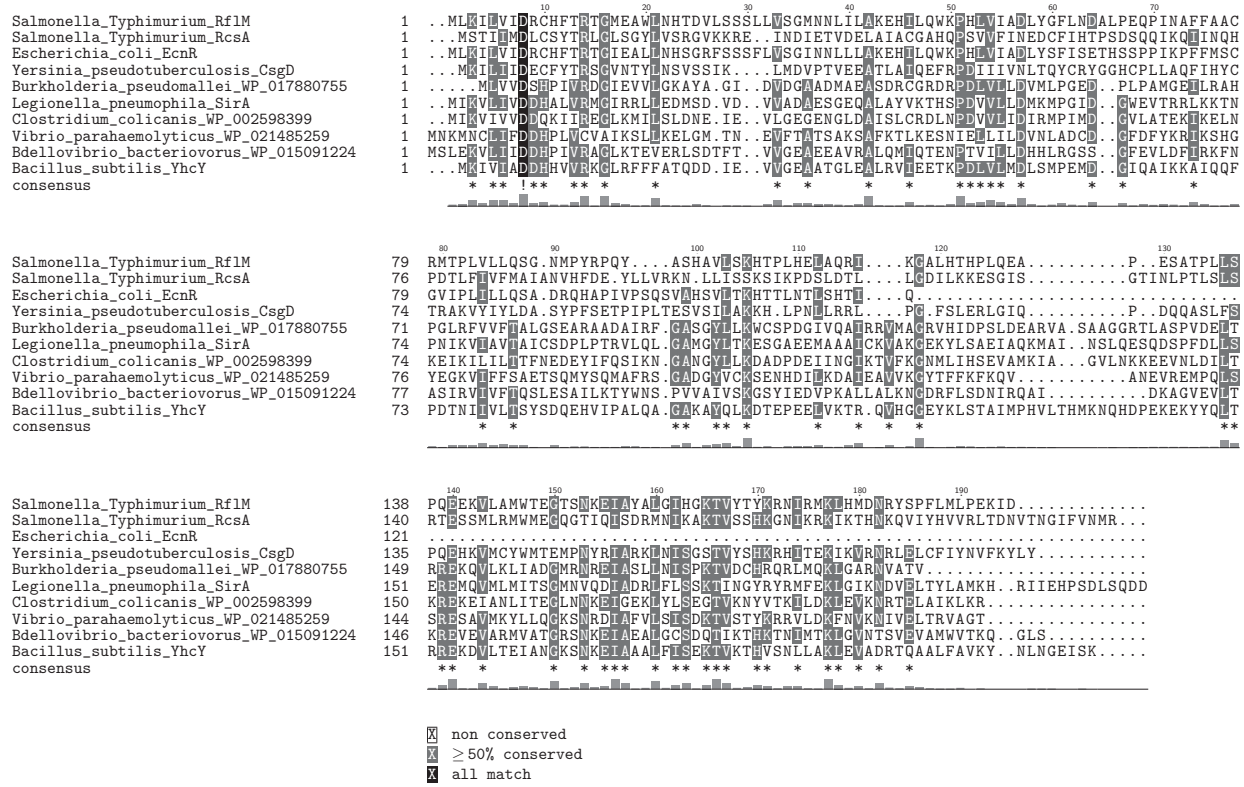

Fig. S2. Comparison of RflM homologs by multiple sequence alignment.

Basic Local Alignment Search Tool (BLAST) was used to search for RflM homologous sequences. The amino acid consensus sequence is shown at the bottom. Identical amino acids are shown with black shading marked with an exclamation mark (all match), whereas conserved amino acids are shown with grey shading marked with an asterisk (≥ 50% conserved). Bar heights below the consensus sequence indicate the degree of conservation.

Supplemental Fig. S3.

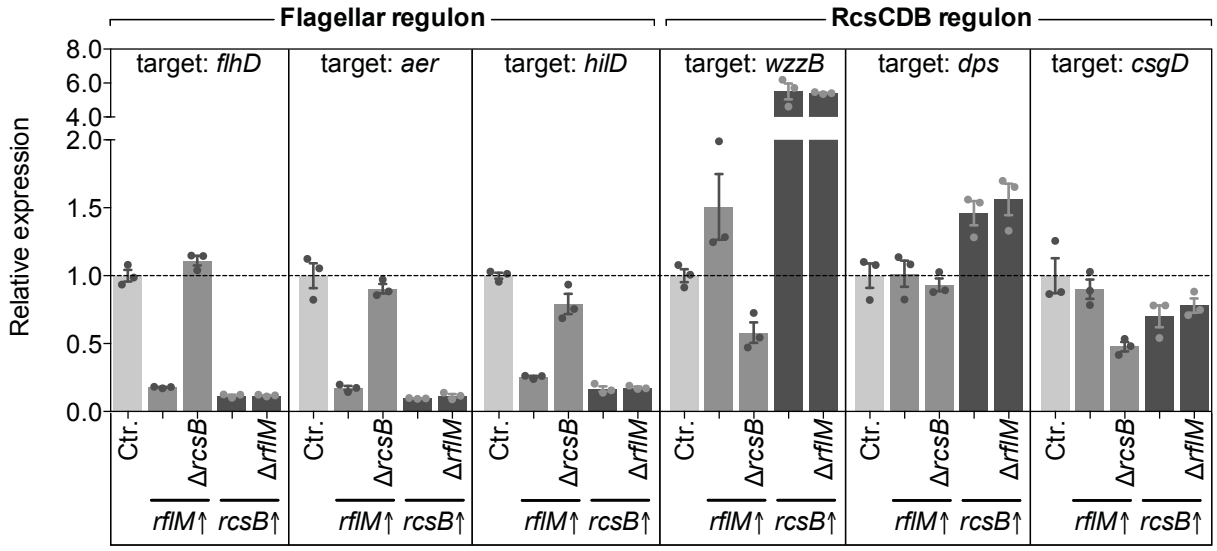

Fig. S3. Relative transcript levels of flagellar and RcsCDB regulated genes.

Transcript levels of target genes belonging to the flagellar regulon (*flhD*, *aer* and *hilD*) and the RcsCDB regulon (*wzzB*, *dps*, *csgD*) were determined by qRT-PCR analysis. Analyzed strains include wildtype (Ctr.; EM808), *rflM* overexpressed from the P_{araBAD} promoter (*rflM*↑) in presence (TH9386) or absence of *rcsB* (Δ*rcsB;* EM4365) and *rcsB* overexpressed from the P_{araBAD} promoter (*rcsB*↑) in presence (TH12883) or absence of *rflM* (Δ*rflM*; EM4366). Transcript levels were normalized against the reference genes *rpoB*, *gyrB* and *gmk*, and reported as relative expression compared to the wildtype control. Bars represent mean values of three independent biological replicates and error bars represent the standard errors of the mean.

Supplemental Fig. S4.

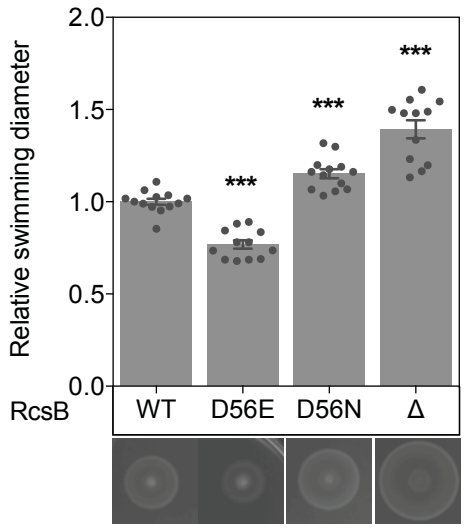

Fig. S4. RcsB phosphorylation mutants are functional.

Relative swimming diameters of RcsB phosphorylation mutants $RcsB_{D56E}$ (mimics phosphorylation; EM2004) and $RcsB_{D56N}$ (cannot be phosphorylated; EM2005) compared to the wildtype control ($RcsB_{WT}$; TH437) and a *rcsB* deletion mutant (Δ*rcsB*; EM1325). Strains were incubated on motility plates containing 0.3% agar, swimming halo diameters were measured and normalized to the wildtype control. Bars represent mean values of eight biological replicates and error bars represent the standard errors of the mean. Exemplary images of the motility phenotypes are shown. Asterisks indicate significant difference to the WT control according to Student's t-test (*** $P<0.0001$).

Supplemental Fig. S5.

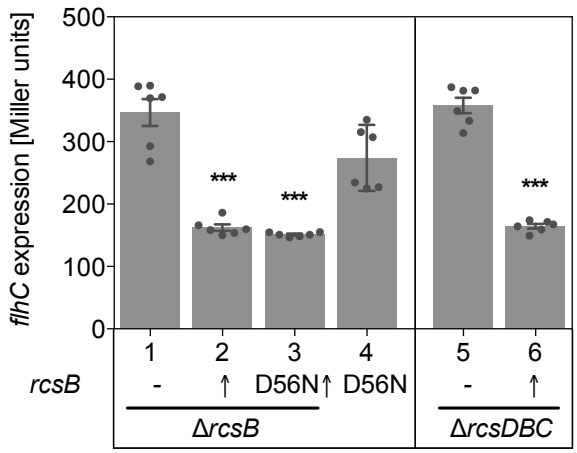

Fig. S5. Overexpression of *rcsB* bypasses the requirement of RcsB phosphorylation for repression of *flhDC* operon expression.

Expression of a transcriptional *flhC-lac* fusion was analyzed upon P_{araBAD}-dependent *rcsB* overexpression (↑) and compared to a control strain (-). The $rcsB_{D56N}$ mutation prevented RcsB phosphorylation. Strain backgrounds were either deleted for *rcsB* (Δ*rcsB*) or for the RcsCDB phosphorelay cascade (Δ*rcsDBC*) preventing phosphoryl group transfer to RcsB. Strains analyzed were EM1434 (1), EM1435 (2), EM4454 (3), EM4453 (4), EM515 (5) and EM521 (6). Bars represent mean values of six independent biological replicates and error bars represent the standard errors of the mean. Asterisks indicate significant difference to the corresponding control according to Student's t-test (*** $P<0.0001$).

Supplemental Fig. S6.

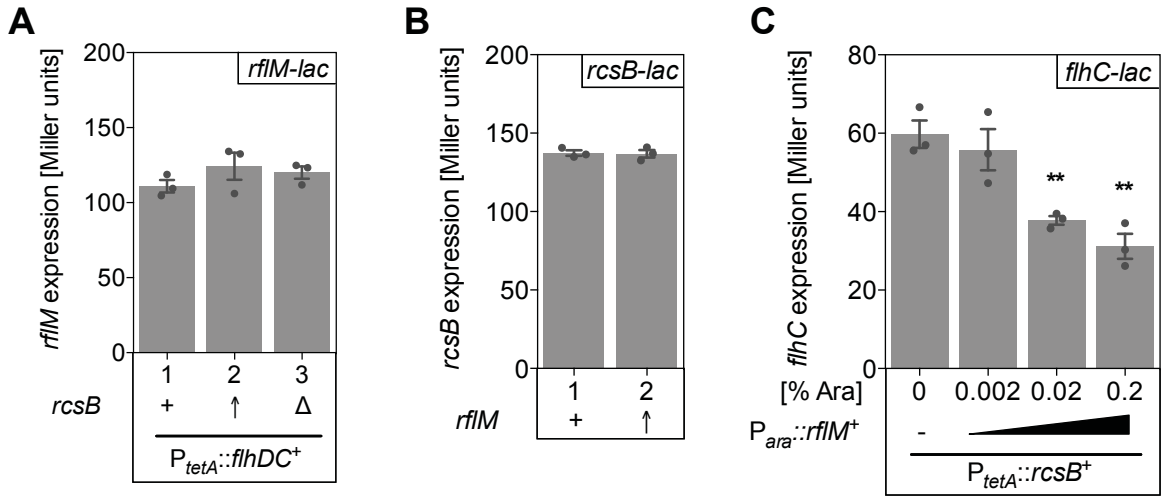

Fig. S6. RcsB and RflM do not regulate each other, but cooperatively repress *flhDC* expression.

A. Expression analysis of a transcriptional *rflM-lac* fusion under physiological *rcsB* expression level (+; EM163), P_{araBAD}-induced *rcsB* overexpression (↑; EM162) or in a RcsCDB deletion strain (Δ; EM169). All strains constitutively expressed *flhDC* from an ATc–inducible promoter (P_{tetA}::*flhD*$^+$*C*$^+$) to ensure $FlhD_4C_2$-dependent *rflM* expression.

B. Expression analysis of a transcriptional *rcsB-lac* fusion under physiological expression level of *rflM* (+; TH13654) in comparison to P_{araBAD}-induced *rflM* overexpression (↑; TH12949).

C. Cooperativity of RcsB and RflM in repression of *flhDC*. *flhC-lac* levels were monitored under conditions of constant ATc-induced *rcsB* expression (P_{tetA}-*rcsB*) and additional titration of arabinose-induced P_{araBAD}-*rflM* (EM229) at the indicated arabinose concentrations [% Ara].

A-C. Bars represent mean values of three biological replicates. Individual data points are shown and error bars represent the standard errors of the mean. Asterisks indicate significant difference to the control according to Student's t-test (** P<0.005; *** P<0.0001).

Supplemental Fig. S7.

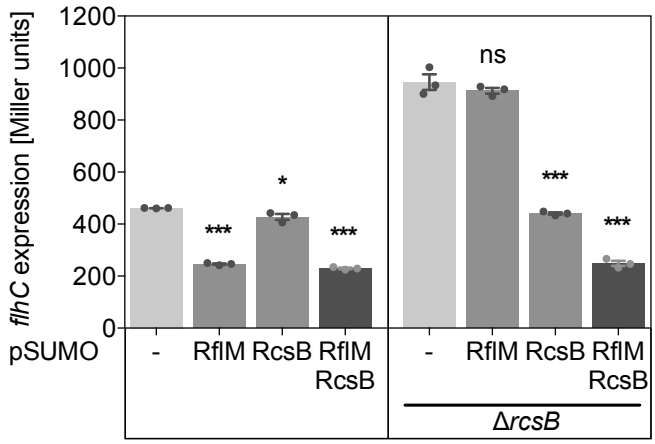

Fig. S7. His_6-SUMO-RcsB and His_6-SUMO-RflM fusions used for protein purification are functional in repression of *flhC-lac* in *Salmonella enterica* strain LT2.

The activity of pSUMO-RflM, pSUMO-RcsB and pSUMO-RflM/RcsB fusion constructs in repression of *flhDC* was analyzed *in vivo* using a transcriptional *flhC-lac* fusion upon induction of *rcsB* or *rflM* overexpression with 0.2 mM IPTG. Induction of the pSUMO-RflM/RcsB construct resulted in co-expression of *rcsB* and *rflM*. The pSUMO empty plasmid (-) served as control. The *Salmonella enterica* reporter strains in the LT2 background constitutively expressed T7 RNA polymerase for expression of the RflM/RcsB constructs from the T7 promoter of the pSUMO plasmid and harbored a wildtype copy of *rcsB* or a deletion of *rcsB* (Δ*rcsB*). Expression of *rflM* is $FlhD_4C_2$-dependent and the strains do not express *rflM* due to the transcriptional *flhC-lac* fusion. Bars represent mean values of three independent biological replicates and error bars represent the standard errors of the mean. Asterisks indicate significant difference to the corresponding control according to Student's t-test (* $P<0.05$, *** $P<0.0001$).

Supplemental Fig. S8.

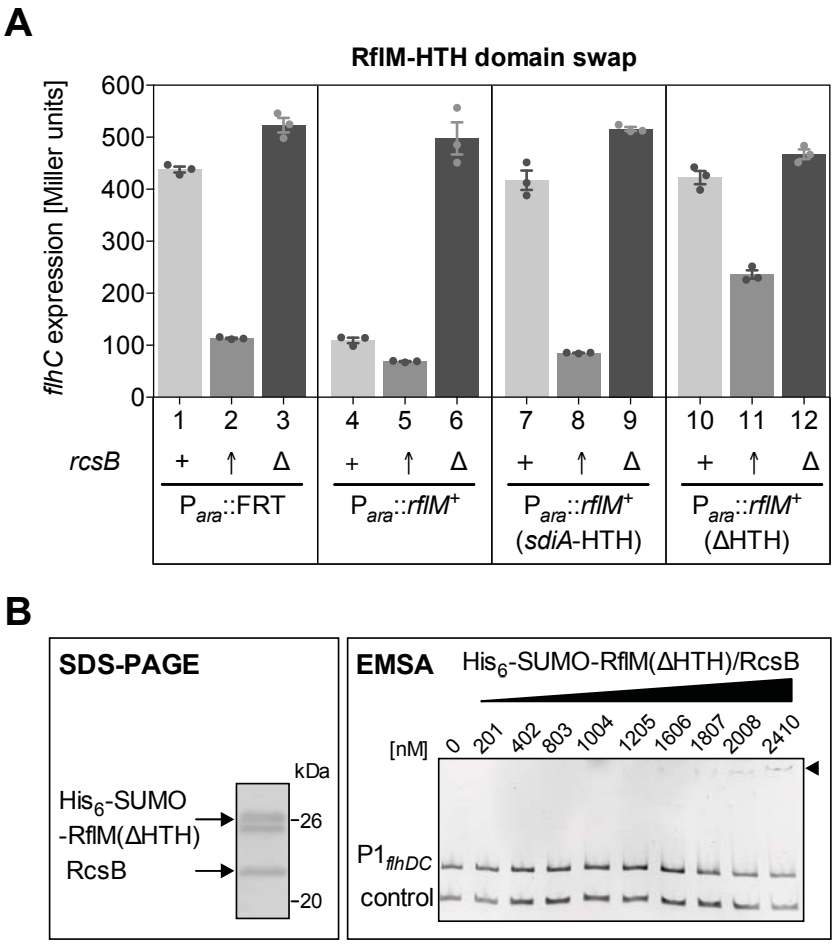

Fig. S8. The DNA-binding domain of RflM is indispensable for repression of *flhDC*.

A. The helix-turn-helix (HTH) DNA-binding domain of RflM was exchanged with the HTH domain of the LuxR-homolog SdiA of *Salmonella* (P_{araBAD}::*rflM*(*sdiA*-HTH)) or deleted (P_{araBAD}::*rflM*(ΔHTH)) and the hybrid constructs were analyzed for their ability to repress *flhDC*. The wildtype *rflM* control and the hybrid constructs were expressed from an arabinose-inducible promoter and the transcript levels of a *flhC-lac* fusion were determined in strains with physiological *rcsB* level (*rcsB*+), ATc-induced *rcsB* overexpression (*rcsB*↑) or *rcsB* deletion (Δ*rcsB*). Strains analyzed were EM517 (1), EM704 (2), EM1432 (3), TH13069 (4), EM229 (5), EM2486 (6), EM656 (7), EM676 (8), EM4666 (9), EM4697 (10), EM4711 (11) and EM4727 (12).

B. Expression analysis and functionality test of purified RflM protein deleted for its HTH domain in complex with RcsB (His_6-SUMO-RflM(ΔHTH)/RcsB). Left: SDS-polyacrylamide gel electrophoresis of purified His_6-SUMO-RflM(ΔHTH)/RcsB complex. Arrows indicate His_6-SUMO-RflM(ΔHTH) (26 kDa) and presumably a degradation product of His_6-SUMO-RflM(ΔHTH), as well as RcsB (24 kDa). Right: Electrophoretic mobility shift assay of the $P1_{flhDC}$ promoter fragment (-271 to -71 relative to *flhD* ATG start site) with increasing amounts of purified His_6-SUMO-RflM(ΔHTH)/RcsB complex as indicated. *gyrA* served as negative control DNA. The arrowhead indicates protein-DNA complexes.