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Transcriptional regulation of the *Salmonella enterica std* fimbrial operon by the RcsCDB system

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Abstract

In *Salmonella enterica* serovar Typhimurium, the RcsCDB regulatory system controls the expression of genes involved in synthesis of colanic acid, formation of flagella and virulence. Here, we show that activation of the RcsCDB system downregulates expression of *std,* an operon that encodes fimbriae involved in *Salmonella* attachment to the mucus layer in the large intestine. Bioinformatic analysis predicts the existence of an RcsB-binding site located 180 bp upstream to the +1 transcription start site of the *std* promoter, and electrophoretic mobility shift assays confirm that RcsB binds the *std* promoter region *in vitro*. This study adds RcsB to the list of regulators of *std* transcription and provides an example of modulation of fimbriae synthesis by a signal transduction system.

INTRODUCTION

Salmonella enterica, a facultative intracellular pathogen, is a ubiquitous bacterium that can respond to changes in the environment including the animal host [1–3]. *Salmonella* survival within the host depends on coordinated expression of multiple genes, and signal transduction systems are relevant to detect specific signals and modulate the expression of genes required for adaptation [4, 5]. One such system is RcsCDB, which comprises the sensors RcsC and RcsD, the response regulator RcsB, and additional ancillary proteins [6–8]. These proteins are (i) the co-regulator RcsA; (ii) an outer membrane lipoprotein RcsF; and (iii) an integral inner membrane protein IgaA [8]. Constitutive activation of the RcsCDB system attenuates virulence in *S.* Typhimurium [9, 10]. The attenuated phenotype of Rcs constitutive mutants is partly produced by capsule overproduction and by repression of the *flhDC* operon and pathogenicity island 1 (SPI-1) [10–12]. In addition, the Rcs system protects *Salmonella* from oxidative stress [13] and controls the acid resistance response [14].

Adhesion of *Salmonella enterica* to host tissues is mediated by fimbrial and non-fimbrial adhesins that recognize and bind-specific receptor moieties of host cells, often distinguishing between different epithelial cell types [15]. Fimbriae are classified in six types: α, *β*, γ, κ, π and σ [16]. π-type fimbriae are formed by a major fimbrial subunit, an usher protein and a chaperone [16]. Members of this group are well-characterized virulence factors such as the pyelonephritis-associated (P) fimbriae in *E. coli* and the mannose-resistant/*Proteus*-like (MR/P) fimbriae of *Proteus mirabilis* [16]. *Salmonella* Std fimbriae belong also to the π-type [16, 17]. Std fimbriae contribute to cecal colonization by binding to α- [1, 2] fucose residues, which are abundant in the host cecal mucosa [18]. Expression of *std* is bistable, resulting in the formation of Std^{OFF} and Std^{ON} subpopulations [19]. The difficulty to detect *std* expression under laboratory conditions [20, 21] is caused by the small size of the Std^{ON} subpopulation outside the animal host [19]. However, *std* expression increases in the intestine of infected animals, with concomitant production of large Std^{ON} subpopulations [22].

The *std* operon contains six genes, named *stdABCDEF*, which are co-transcribed from a single promoter located upstream of *stdA* [23] (Fig. 1a). The downstream genes *stdE* and *stdF* encode transcription factors that control expression of multiple genes including the *std* operon itself [19]. Transcriptional control of *std* expression is complex, and involves

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Abbreviations: EDTA, Ethylenediaminetetraacetic acid; EMSA, Electrophoretic Mobility Shift Assay; MR/P, Mannose-Resistant/*Proteus*; PAGE, Polyacrylamide Gel Electrophoresis; PBS, Phosphate Buffered Saline; PCR, Polymerase Chain Reaction; SPI-1, Salmonella Pathogenicity Island 1; UAS, Upstream Activating Sequence.

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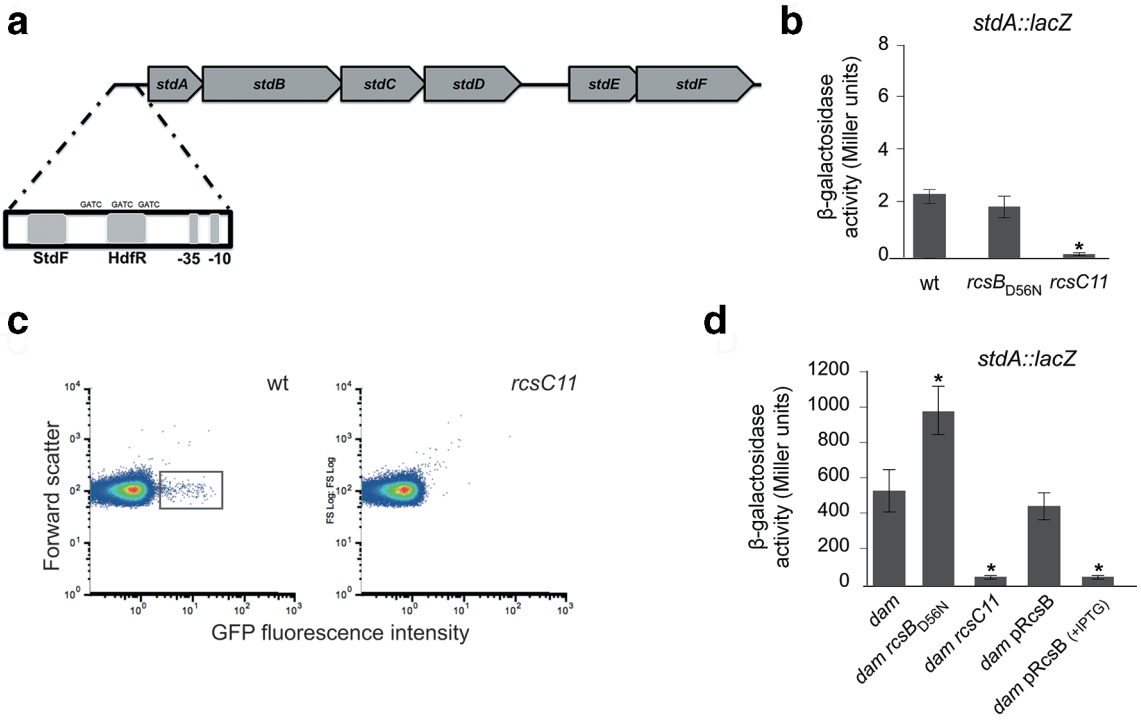

Fig. 1. Regulation of *std* transcription by the RcsCDB system. (a) Diagram of the *std* operon with amplification of the promoter and the upstream regulatory region. Binding sites for StdF and HdfR are shown. The region harbours three GATC sites, and two such sites are located within the HdfR-binding site. (b) *β*-galactosidase activity of strains harbouring an *stdA::lacZ* fusion. Strains were SV5206 (*stdA::lacZ*, in a wild-type background), MDs1696 (*stdA::lacZ rcsB*$_{D56N}$) and MDs1695 (*stdA::lacZ rcsC11*). Asterisk represents statistically different *β*-galactosidase activity values compared to the wild-type strain (Tukey test, $P<0.05$). (c) Flow cytometry analysis of *stdA::gfp* expression in the wild-type and *rcsC11* backgrounds (SV9597 and SV8528, respectively). The box shows Std^{ON} cells. (d) *β*-galactosidase activity of the *stdA::lacZ* fusion in *dam* (MDs1697), *dam rcsB*$_{D56N}$ (MDs1699), *dam rcsC11* (MDs1698) and *dam* pRcsB (SV9318) backgrounds. Asterisk represents statistically different *β*-galactosidase activity values compared to *dam* background (Tukey test, $P<0.05$). Data in (b) and (d) are averages and standard deviations from three independent experiments, each carried out in duplicate.

a double feedback loop: StdE activates transcription of *hdfR*, a gene located outside the *std* operon, and HdfR activates *std* transcription together with StdF [24]. HdfR binding to the *std* upstream activating sequence (UAS) is hindered by methylation of GATC sites located at the *std* UAS, and formation of Std^{ON} cells requires GATC methylation hindrance, which is caused by HdfR binding [21, 24]. This study shows that the RcsCDB system is an additional regulator of *std* transcription, thus adding a fimbrial operon to the list of loci under RcsCDB control.

METHODS

Bacterial strains, molecular techniques and growth conditions

The *S.* Typhimurium strains used in this work derive from ATCC 14028 s (Table 1). Mutations were introduced into different genetic backgrounds by P22-mediated transduction following the protocol described by Davis *et al.* [25]. The pRcsB plasmid is derivative of the pUHE2-21 *lacI*^{q} expression vector harbouring the *rcsB* gene under the control of P$_{lac}$ [26]. Bacterial growth and recombinant DNA techniques followed standard protocols [27]. Kanamycin (Km), ampicilin (Ap) and chloramphenicol (Cm) were used at final concentrations of 50, 50 and 25 μg ml^{-1}, respectively.

β-galactosidase assays

Levels of *β*-galactosidase activity were assayed using the $CHCl_3$-sodium dodecyl sulfate permeabilization procedure [28]. *β*-galactosidase activity data are the averages and standard deviations from three independent experiments.

Protein extracts and Western blot analysis

Total protein extracts were obtained from bacterial cultures grown for 5 h at 37 °C in Luria broth (LB) medium. Bacterial cells were collected by centrifugation (16000 ***g***, 2 min) and suspended in 100 ml of Laemmli sample buffer [1.3% SDS, 10% (v/v) glycerol, 50 mM Tris-HCl, 1.8% *β*-mercaptoethanol, 0.02% bromophenol blue, pH 6.8]. Proteins were resolved by Tris-Tricine-PAGE (12%). The Western blot assay was performed using conditions for protein transfer described elsewhere [23]. Optimal primary antibody dilutions were used as follows: anti-FLAG M2

monoclonal antibody (Sigma Chemical), 1:5000; and anti-DnaK polyclonal antibody (Sigma Chemical), 1:20000. The Goat anti-mouse horseradish peroxidase-conjugated antibody (1:5000, BioRad, Hercules, CA, USA) or Goat anti-rabbit horseradish peroxidase conjugated antibody (1:20000, Santa Cruz Biotechnology, Heidelberg, Germany) were used as secondary antibodies. Proteins recognized by the antibodies were visualized by chemoluminescence using luciferin-luminol reagents in a LAS 3000 Mini Imaging System (Fujifilm, Tokyo, Japan). DnaK was used as the loading control of each extraction.

Electrophoretic mobility shift assay (EMSA)

EMSA was carried out according to the protocol previously described [29]. DNA sequences containing the putative RcsB-binding site were amplified by PCR using wild-type ATCC 14028 s chromosomal DNA as a template. The PCR product of the *std* promoter (735 bp), containing the predicted RcsB-binding site, was obtained using primers no. 9053 (5′-CGAT TCTAGACGCATTAATATCCCCCAGCC-3′) and no. 9055 (5′-ATTACGCATAGATAATATGTC-3′). The 234 bp PCR product of the *dps* promoter, where a predicted RcsB-binding site was not present, was amplified using primers no. 8032 (5′-GCGCTATTACTTCGTC-3′) and no. 8033 (5′-CGGG ATCCCTCATATCCTCTTGATGTTTGTGT-3′) and used as a negative control. In this assay, 2 pmol of each PCR product were incubated together at room temperature with 0, 5, 10, 40 or 80 nM of RcsB-His_6 protein previously purified as described [12]. The samples were analysed on a 6% non-denaturing Tris-borate-EDTA polyacrylamide gel and it was run at 8 mA at 4 °C. The polyacrylamide gel was stained with ethidium bromide, and migration of PCR fragments was visualized under UV irradiation.

Table 1. *Salmonella enterica* serovar Typhimurium strains used in this study

Strain	Description	Reference[a]
MDs1695	*rcsC11 stdA::lacZ*	
MDs1696	$rcsB_{D56N}$ *stdA::lacZ*	
MDs1697	*dam*-201::Tn*10d*Tc *stdA::lacZ*	
MDs1698	*rcsC11dam*-201::Tn*10d*Tc *stdA::lacZ*	
MDs1699	$rcsB_{D56N}$*dam*-201::Tn*10d*Tc *stdA::lacZ*	
SV9318	*dam*-201::Tn*10d*Tc/pRcsB	
SV5206	*stdA::lacZ*	[21]
SV6502	*dam*-201::Tn*10d*Tc *stdF*::3xFLAG	[23]
SV6533	*dam*-201::Tn*10d*Tc *stdF::lacZ*	[23]
SV8783	*rcsC11 dam*-201::Tn*10d*Tc *stdF::lacZ*	
SV8784	*dam*-201::Tn*10d*Tc *stdF*::3xFLAG *rcsC11*	
SV9597	*stdA::gfp*	[19]
SV8528	*stdA::gfp rcsC11*	

a, Omitted for strains described in this study.

Flow cytometry

Bacterial cultures were grown at 37 °C in LB medium until stationary phase (OD_{600}≅2). Cells were then diluted in PBS to a final concentration of ~10^7 cells ml^{-1}. Data acquisition was performed using a Cytomics FC500-MPL cytometer (Beckman Coulter, Brea, CA, USA). Data were collected for 100000 events per sample and were analysed with CXP and FlowJo 8.7 softwares. Data are shown by dot plots representing

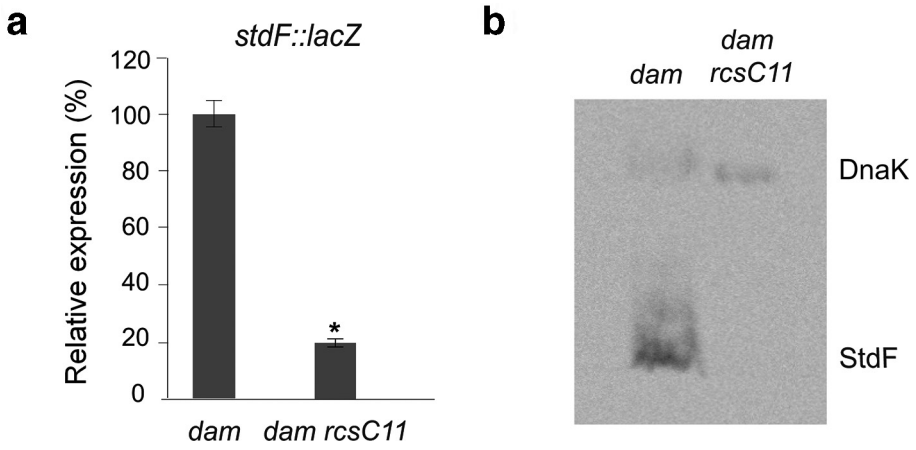

Fig. 2. Regulation of *stdF* expression by RcsCDB. (a) β-galactosidase activity of the *stdF::lacZ* fusion measured in *dam* (SV6533) and *dam rcsC11* (SV8783) mutants. Data are averages and standard deviations of three independent experiments, carried out in duplicate. Asterisk represents statistically different β-galactosidase activity values (Tukey test, $P<0.05$). (b). Western blot analysis using the *stdF*::3xFLAG fusion expressed in *dam* (SV6502) and *dam rcsC11* (SV8784) backgrounds.

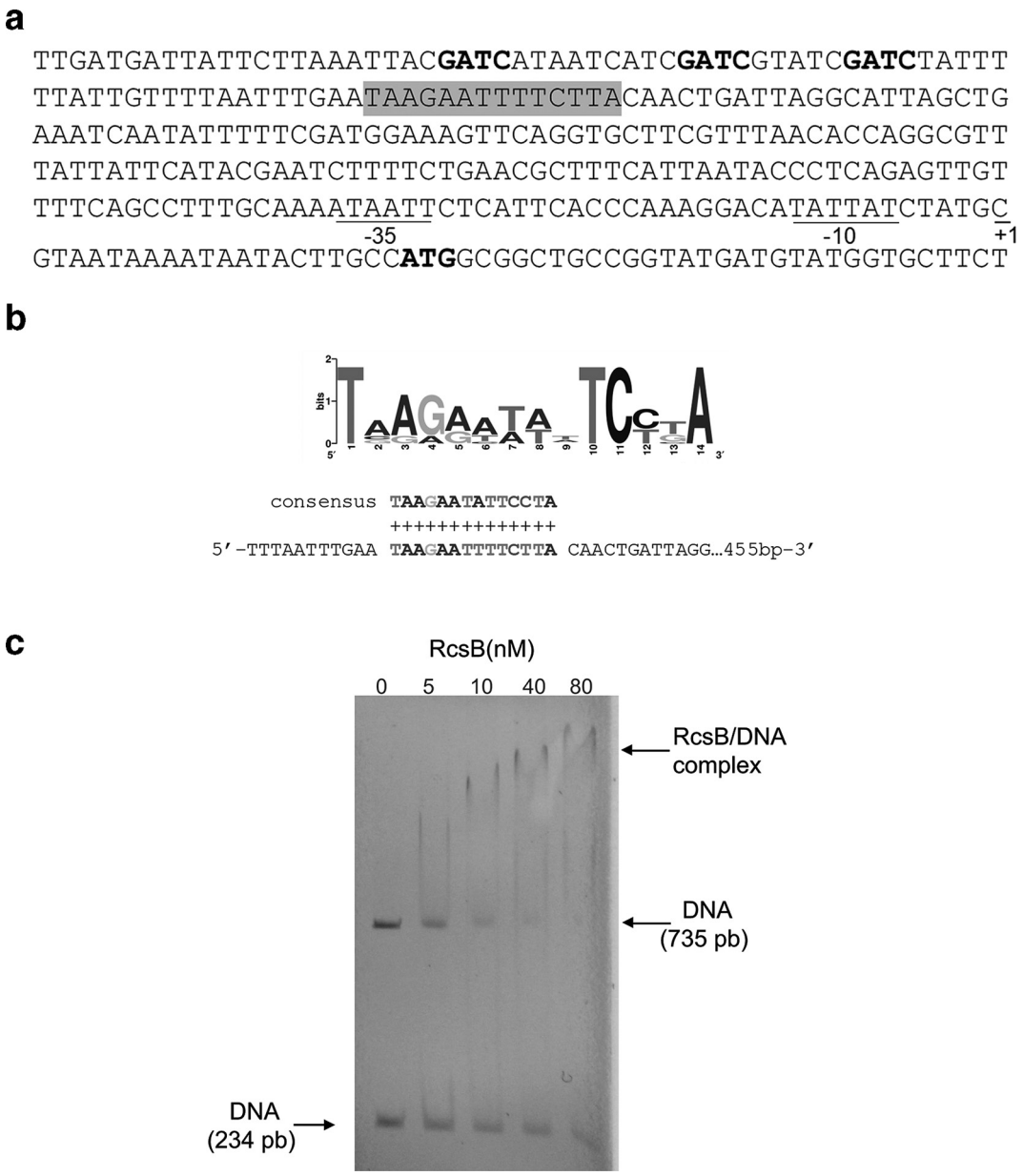

Fig. 3. Binding of RcsB to the *std* promoter region. (a) Nucleotide sequence upstream of the *std* start codon (bold sequence) harbouring the +1 transcription start site and −10 and −35 conserved boxes. The RcsB-binding site is highlighted. The GATC Dam-dependent methylation sites are in bold. (b) Sequence alignment of the *std* promoter region with RcsB-binding sites identified in the promoters of other RcsB-dependent genes, using the MEME/MAST motif detection program**.** (c) EMSA of a 735 bp PCR product that contains the putative RcsB-binding site in the *std* promoter in the presence of RcsB-His6 protein. A 234 bp PCR product amplified from the *dps* promoter without the RcsB-binding site was used as a negative control.

forward scatter (cell size) in the *y* axis vs fluorescence intensity in the *x* axis.

RESULTS

Regulation of *std* transcription by the RcsCDB system

To investigate whether the RcsCDB system controls the *std* operon expression, a *stdA::lacZ* transcriptional fusion harboured in the *Salmonella* chromosome (SV5206) was transduced to strains carrying *rcsC11* and $rcsB_{D56N}$ mutations. The *rcsC11* allele contains a point mutation that constitutively activates the RcsCDB system [30]. In turn, the $rcsB_{D56N}$ allele contains a point mutation in the receiver domain of the *rcsB* gene that prevents RcsB phosphorylation [31]. The expression levels of *stdA* were low in both backgrounds as well as in the wild-type strain (Fig. 1b), in accordance with previous reports indicating that *std* operon expression is difficult to detect under

laboratory conditions [20, 21]. However, we observed that the *rcsC11* mutant (MDs1695) produced a very low expression level, suggesting that activation of the RcsCDB phosphorelay might repress *std* transcription (Fig. 1b). Further evidence for RcsB-mediated repression was provided by flow cytometry analysis using strains carrying a *stdA::gfp* fusion (Fig. 1c). The wild-type strain (SV9597) showed bistable expression as previously described [19], with formation of a small Std^{ON} subpopulation (approx. 0.3% cells). RcsCDB activation by the *rcsC11* mutation (SV8528) abolished bistability, and Std^{ON} cells were not detected (Fig. 1c).

Because lack of Dam methylation activates *std* transcription [21], we tested the effects of *rcsC11* and $rcsB_{D56N}$ mutations in a *dam* background. As expected, the *dam* mutation increased *std* expression compared with the wild-type strain [Fig. 1(b) and (d)]. In turn, the β-galactosidase activity of the *stdA::lacZ* fusion increased in the *dam* $rcsB_{D56N}$ background (MDs1699) and decreased in the *dam rcsC11* background (MDs1698) (Fig. 1d). Repression was likewise observed upon RcsB overexpression from a plasmid (SV9318) (Fig. 1d). Altogether, these observations strengthened the evidence that activation of the RcsCDB system represses *std* expression.

To confirm that RcsB-dependent modulation of *std* expression affects the entire *std* operon, we monitored the expression of downstream gene of this operon [23]. For this purpose, we determined the activity of an *stdF::lacZ* transcriptional fusion in *dam* and *dam rcsC11* genetic backgrounds (SV6533 and SV8783, respectively). The β-galactosidase activity of the *stdF::lacZ* fusion decreased fivefold in the presence of the *rcsC11* mutation (Fig. 2). In addition, the StdF protein levels in *dam* and *dam rcsC11* backgrounds (SV6502 and SV8784, respectively) were analysed by Western blot using an anti-3xFLAG antibody. StdF protein was not detected in the *dam rcsC11* double mutant, while a 32 kDa band corresponding to StdF was observed in the *dam* mutant (Fig. 2b). The overall conclusion from the above experiments was that activation of the RcsCDB system does repress *std* operon expression.

Binding of RcsB to the *std* promoter region

To determine whether *std* downregulation upon RcsCDB activation involves specific binding of RcsB to the *std* promoter region, we carried out an *in silico* search to identify a potential RcsB-binding motif in the *std* promoter region. Using the MEME/MAST motif detection program [32, 33], we detected a sequence homologous to RcsB-binding sites present in other RcsB-regulated genes [34–36] (Fig. 3a, b). The location of this putative RcsB-binding site is 180 bp upstream to the +1 transcription start site, near but not overlapping the HdfR-binding site or the Dam methylation sites [24] (Figs 3a and 1a).

A direct test of RcsB binding was performed by EMSA. For this purpose, we amplified a DNA fragment of 735 bp harbouring the *std* promoter and upstream regulatory sequences including the putative RcsB-binding site. As a negative control, we used a 234 bp PCR product containing a variant of the RcsB-dependent *dps* promoter, which lacked the RcsB-binding sequence [13]. Retardation of the 735 bp PCR product migration was detected while the electrophoretic mobility of the 234 bp PCR product (negative control) was not modified (Fig. 3c). These observations indicate that RcsB binds the *std* promoter region.

DISCUSSION

We show that activation of the RcsCDB system represses transcription of the *std* fimbrial operon, and provide evidence that repression may be caused by binding of RcsB upstream of the *std* promoter. The RcsB-binding site does not overlap with the StdF- and HdfR-binding sites nor with the GATC sites present in the region (Figs 1a and 3a). Detection of RcsCDB-mediated repression in both dam^+ and *dam* backgrounds (Fig. 1d, c) suggests that RcsB binding may counter transcriptional activation by the StdE-StdF-HdfR regulatory loop [24]. RcsB binding may thus repress *std* transcription in a manner that is independent of previously known mechanisms of *std* control.

The physiological relevance of RcsCDB-mediated repression of *std* is difficult to understand because the environmental cues that activate the RcsCDB signal transduction cascade remain largely unknown [6–8]. A tentative speculation, however, is that RcsCDB might permit environmental control of *std* switching as described in other Dam-dependent bistable switches. For instance, phase variation of the *pap* operon of uropathogenic *E. coli* is controlled by the CpxAR stress response [37] and by the global regulator CRP [38]. In *std*, environmental control might prevent production of Std fimbriae under circumstances that are not appropriate for attachment, either in the environment or inside the animal host. The possibility of environmental control may be tentatively supported by the observation that the sizes of Std^{OFF} and Std^{ON} subpopulations in the large intestine are different from those observed in the laboratory [19, 22]. Control of *std* expression by RcsCDB might thus be part of the regulatory networks that adjust *Salmonella* gene expression to distinct host environments [2, 3, 5].

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Conflicts of interest
The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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