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Transcriptional autoregulation of the RcsCDB phosphorelay system in *Salmonella enterica* serovar Typhimurium

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The RcsCDB (Rcs) phosphorelay system is involved in the regulation of many envelope genes, such as those responsible for capsule synthesis, flagella production and O-antigen chain length, as well as in other cellular activities of several enteric bacteria. The system is composed of three proteins: the sensor RcsC, the response regulator RcsB, and the phospho-transfer intermediary protein RcsD. Previously, we reported two important aspects of this system: (a) *rcsB* gene expression is under the control of P_{rcsDB} and P_{rcsB} promoters, and (b) *rcsD* gene transcription decreases when the bacteria reach high levels of the RcsB regulator. In the present work, we demonstrate that the RcsB protein represses *rcsD* gene expression by binding directly to the P_{rcsDB} promoter, negatively autoregulating the Rcs system. Furthermore, we report the physiological role of the RcsB regulator, which is able to modify bacterial swarming behaviour when expressed under the control of the P_{rcsB} promoter.

INTRODUCTION

The Rcs phosphorelay is an uncommon adaptive response system, composed of three proteins: the sensor RcsC, the cognate response regulator RcsB, and the intermediary in the phosphoryl transfer RcsD (Majdalani & Gottesman, 2005). It has been determined that the flow of phosphoryl groups through the Rcs phosphorelay components occurs as follows: RcsC→RcsD→RcsB (Takeda *et al.*, 2001). The Rcs system appears to be conserved in the family *Enterobacteriaceae* (Huang *et al.*, 2006; Pescaretti *et al.*, 2009), and it is involved in the modulation of the expression of many genes, such as those controlling colanic acid biosynthesis (Stout & Gottesman, 1990), regulation of flagellum synthesis (Francez-Charlot *et al.*, 2003), cell division (Carballes *et al.*, 1999), O-antigen chain length determination (Delgado *et al.*, 2006), motility (Cano *et al.*, 2002) and Vi antigen synthesis (Virlogeux *et al.*, 1996). The signals leading to induction of the Rcs system remain unknown, even though a wide range of activation conditions has been described, such as bacterial growth at low temperature or on solid surfaces (Ferrières & Clarke, 2003), exposure to polymyxin B (Bader *et al.*, 2003; Erickson & Detweiler, 2006), overproduction of DjlA (Clarke *et al.*, 1997; Chen *et al.*, 2001; Kelley & Georgopoulos, 1997), *rcsC11* constitutive mutation (Costa & Anton, 2001; Mouslim *et al.*, 2004), *igaA* (Cano *et al.*, 2002) and *mucM* mutants (Costa & Anton, 2001), and *tolB* and *pmrA* mutants affecting the cell envelope (Mouslim & Groisman, 2003).

Previously, we reported that the *rcsB* gene is transcribed from two promoters: (i) P_{rcsDB}, located upstream of *rcsD*, and (ii) P_{rcsB}, located within the *rcsD* coding region, and that the overexpression of *rcsB* decreases *rcsD* transcription (Pescaretti *et al.*, 2009). The discovery of *rcsD* repression led us to investigate the potential role of RcsB in the mechanism of Rcs system regulation. In the present study, we demonstrated that high levels of the RcsB regulator control *rcsD* expression by direct binding to the P_{rcsDB} promoter, negatively autoregulating the Rcs system. Rcs negative autoregulation was observed in an *rcsC11* mutant or after polymyxin B treatment, indicating the importance of the promoters in different physiological states. In addition, we also showed a physiological role in swarming behaviour repression for P_{rcsB}, via the control of *rcsB* expression.

METHODS

Bacterial strains, molecular techniques and growth conditions. Bacterial strains and plasmids used in this study are listed in Table 1. Phage P22-mediated transductions were used to introduce mutations into different genetic backgrounds, as described by Davis *et al.* (1980). Recombinant DNA techniques and bacterial growth at 37 °C in Luria–Bertani (LB) medium were performed according to standard protocols (Sambrook *et al.*, 1989). Kanamycin, ampicillin and chloramphenicol were used at final concentrations of 50 μg ml^{-1}, 50 μg ml^{-1} and 25 μg ml^{-1}, respectively.

Table 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Description*	Reference or source
***S.* Typhimurium strains**		
14028s	Wild-type	Fields *et al.* (1986)
EG12711	Δ*rcsB*::Cm	This work
EG14873	*rcsC11*	Mouslim *et al.* (2004)
EG14539	Δ*rcsD*::*lacZY*	Pescaretti *et al.* (2009)
EG14932	Δ*rcsB*::*lacZY*	Pescaretti *et al.* (2009)
MDs1077	Δ*rcsD*::*lacZY rcsC11*	This work
MDs1017	ΔP_{rcsDB}::Cm	This work
MDs1018	ΔP_{rcsB}::Cm	This work
MDs1026	Δ*rcsB*::*lacZY* ΔP_{rcsDB}::Cm	This work
MDs1032	Δ*rcsB*::*lacZY* ΔP_{rcsDB}::FRT	This work
MDs1027	Δ*rcsB*::*lacZY* ΔP_{rcsB}::Cm	This work
MDs1034	Δ*rcsB*::*lacZY* ΔP_{rcsB}::FRT	This work
Plasmids		
pUHE2-2*lacI*^{q}	rep_{pMB1} Ap^r *lacI*^{q}	Soncini *et al.* (1995)
p*rcsB*	pUHE2-21 *lacI*^{q} containing *rcsB*	Pescaretti *et al.* (2009)
p*rcsD*	pUHE2-21 *lacI*^{q} containing *rcsD*	This work
pMS201	Low copy vector for cloning promoters, pLtet01, derived from pZS21-luc, *gfp*mut2	Beeston & Surette (2002)
pP_{rcsDB}	Km^r pMS201 containing 122 bp of P_{rcsDB} fused to *gfp*mut2	Pescaretti *et al.* (2009)
pP_{rcsB}	pMS201 containing 131 bp of P_{rcsB} fused to *gfp*mut2	Pescaretti *et al.* (2009)

*Gene designations are summarized by Sanderson *et al.* (1995).

Mutation of chromosomal promoters. The promoters P_{rcsDB} or P_{rcsB} were deleted from the chromosome of wild-type *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium) strain 14028s using the one-step gene-inactivation method (Datsenko & Wanner, 2000). Briefly, a chloramphenicol-resistance cassette (Cm) was amplified from plasmid pKD3, using primers 4894 (5′-CACGGTTATTCACTACACTCCCCTGCTCGACCGTGTAGGCTGGAGCTGCTTCG-3′) and 4504 (5′-CGTTTCACATAACTGCTTGCCGGGTACCAGATTAAGCATGGCCATATGAATATCCTCCTTAG-3′) for P_{rcsDB} deletion, and 2385 (5′-GCGTTGCTTTTACAGGTCGTAAACATAATGTAGGCTGGAGCTGCTTC-3′) and 2386 (5′-GGCAATAATTACGTTCATATTGTTCATATGAATATCCTCCTTAG-3′) for P_{rcsB} deletion. For P_{rcsDB} deletion, the resulting PCR product was introduced into the region from nucleotide −219 upstream of *rcsD* to nucleotide 2541 of the *rcsD* coding sequence, leaving the P_{rcsB} promoter intact. On the other hand, for P_{rcsB} deletion, the resulting PCR product was introduced into the complete *rcsD* coding sequence, leaving an undamaged P_{rcsDB} promoter (Fig. 2a). These mutations were introduced into strain EG14932, which contains an Δ*rcsB*::*lacZY* fusion, by P22-mediated transduction. To eliminate any polar effect, the chloramphenicol resistance cassette was removed using plasmid pCP20, as described by Datsenko & Wanner (2000). Proper Cm removal was confirmed by direct nucleotide sequencing. The resulting strains, MDs1032 and MDs1034, respectively, were then transformed with plasmid p*rcsB*.

β-Galactosidase assays. Bacteria were grown to OD_{600} 0.2 (about 2 h) and then supplemented with IPTG (0.35 mM), to overexpress *rcsB* from the P_{lac} promoter of plasmid p*rcsB*, or with polymyxin B (1 μg ml^{-1}), to induce the Rcs system. After growth for an additional 5 h, the β-galactosidase activity was measured as described by Miller (1972). Control cultures were grown for 7 h in LB medium at 37 °C in the absence of IPTG or polymyxin B.

DNase I footprinting assay. DNase I protection assays were carried out using appropriately labelled primers, as described by Delgado *et al.* (2006). Fragments of DNA used for DNase I footprinting were amplified by PCR using chromosomal DNA from a wild-type *S.* Typhimurium strain (14028s) as template. Previously, primers 4136 (5′-TGCTTCGCATTCGGTTTTTTTTAC-3′) and 4137 (5′-TGATCAGCAATAAGAAGAAACGGGT-3′), which anneal to the coding and

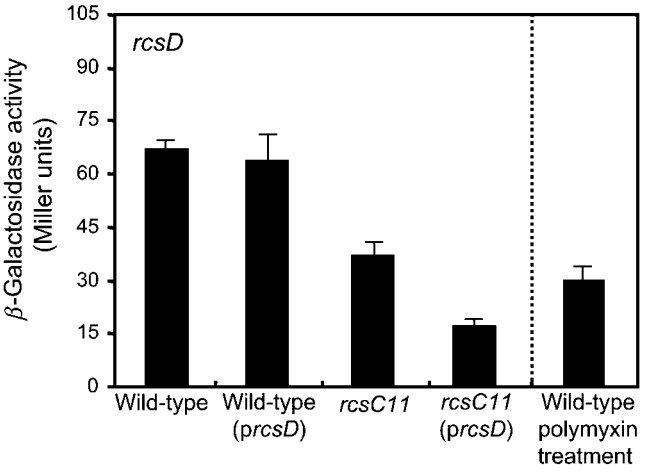

Fig. 1. *rcsB* overexpression represses *rcsD* transcription. The transcriptional activity of a Δ*rcsD*::*lacZY* fusion, measured as β-galactosidase activity (Miller units), was investigated in the following genetic backgrounds: wild-type (EG14539) and *rcsC11* (MDs1077), harbouring or not harbouring plasmid p*rcsD*, and the wild-type (EG14539) strain in the presence of polymyxin B, as described in Methods. All data correspond to mean values of three independent experiments done in duplicate; error bars, SD.

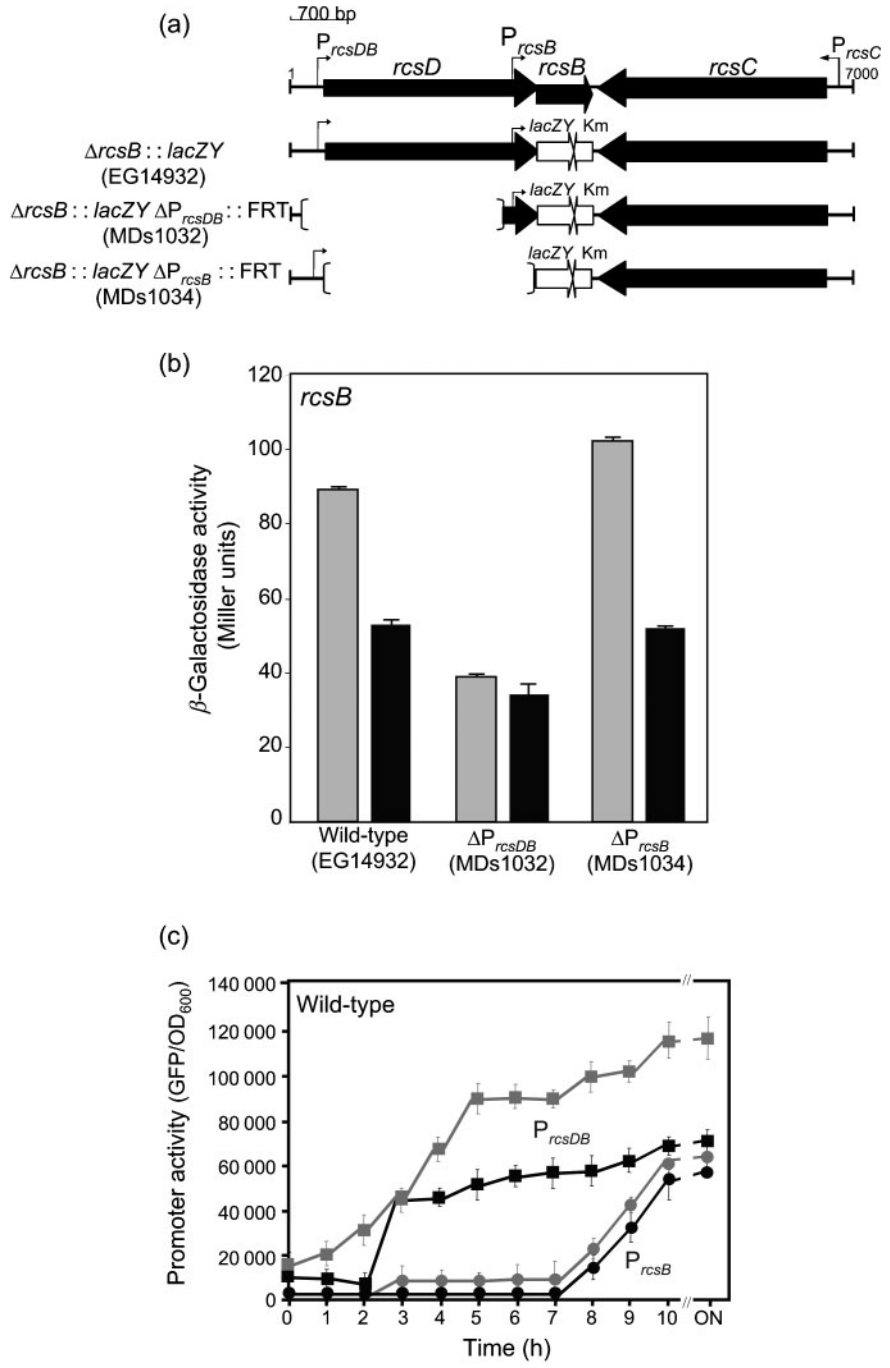

Fig. 2. Contribution of P_{rcsDB} and P_{rcsB} promoters to *rcsB* expression. (a) Graphic representation of the genetic backgrounds used to determine the transcriptional activity of the *rcsB* gene. Spaces within brackets correspond to the deleted promoter regions. (b) Transcriptional activity of the Δ*rcsB*::*lacZY* fusion, measured as β-galactosidase activity (Miller units), was investigated in the following genetic backgrounds: wild-type (EG14932), and P_{rcsDB} (MDs1032) and P_{rcsB} (MDs1034) mutants, all carrying the p*rcsB* plasmid and grown in the presence (black bars) or absence (grey bars) of IPTG. (c) P_{rcsDB} (squares) and P_{rcsB} (circles) promoter activity, measured as GFP production at each time point, was monitored in the wild-type 14028s strain co-transformed with p*rcsB* and pP_{rcsDB} or pP_{rcsB} plasmids, respectively. Black symbols, cultures grown in the presence of IPTG; grey symbols, cultures grown in the absence of IPTG. All data correspond to mean values of three independent experiments done in duplicate; error bars, SD.

non-coding strands of *rcsD*, respectively, had been labelled with T4 polynucleotide kinase and [γ-^{32}P]ATP. The *rcsD* promoter region was amplified with labelled primers 4136 and 4137 for the coding strand, or with labelled primers 4137 and 4136 for the non-coding strand. The histidine-tagged RcsB protein used in this work was purified as previously described (Delgado *et al.*, 2006).

Determination of promoter activity by GFP production. As previously described (Pescaretti *et al.*, 2009), wild-type *S.* Typhimurium (14028s) was transformed with plasmid pMS201, which contains a promoterless *gfp*mut2 gene in which the P_{rcsDB} or P_{rcsB} promoter region has been cloned. In this assay, promoter activity was measured as the rate of GFP production divided by the OD_{600} of the culture at each time point (Kalir *et al.*, 2005; Ronen *et al.*, 2002; Rosenfeld *et al.*, 2002).

Swarming motility assay. Swarming assays were carried out as described by Kim & Surette (2004). Briefly, the overnight LB cultures of tested strains were adjusted to OD_{600} 1.0. Then, 5 µl of these normalized cultures was deposited onto the surface of 0.4 % LB agar plates, which were incubated for 12 h at 37 °C. To estimate the mean speed of migration (mm min^{-1}), the diameter of migrating colonies (mm) was plotted against the incubation time (min). The images in Fig. 4 represent one of three independent experiments, while the data correspond to mean values for these independent experiments.

RESULTS

RcsB overproduction represses *rcsD* transcription

We have previously demonstrated that *rcsB* overexpression from plasmid p*rcsB* results in strong repression of *rcsD* gene expression, while its own expression is not affected (Pescaretti *et al.*, 2009). The differential expression of *rcsD* and *rcsB* is due to the presence of a second promoter, P_{rcsB}, which activates *rcsB* expression independently of *rcsD* (Pescaretti *et al.*, 2009). To determine whether the RcsB repressor effect is also produced under other Rcs-induction conditions, the *rcsC11* constitutive allele mutant was used. *rcsD* expression levels, measured as the β-galactosidase activity of the chromosomal Δ*rcsD*::*lacZY* fusion from wild-type *S.* Typhimurium (14028s) and the *rcsC11* mutant, were determined after 7 h of growth on LB medium. As shown in Fig. 1, the transcription level of *rcsD* in the *rcsC11* mutant was twofold lower than that observed in the wild-type strain. Interestingly, a remarkable decrease (fourfold) was also observed in the mutant containing plasmid p*rcsD* (Fig. 1). This result suggests that the phosphorylated RcsB is more effective than the non-phosphorylated form, due to the presence of the intermediary RcsD, which completes the Rcs phosphorelay pathway (Takeda *et al.*, 2001). In a second approach, polymyxin B was used to induce the Rcs system. The data indicate that after 5 h of exposure to polymyxin B, the wild-type strain also showed twofold decreased levels of *rscD* expression (Fig. 1). These results suggest that *rcsD* expression is repressed when the bacteria reach high levels of the RcsB regulator, under different Rcs system induction conditions.

rcsB overexpression represses P_{rcsDB} activity

To determine whether high levels of RcsB affect P_{rcsDB} or P_{rcsB} promoter activity, we studied *rcsB* expression in mutants with a deletion in each corresponding promoter region, P_{rcsDB} and P_{rcsB}, transformed with p*rcsB*. As shown in Fig. 2(b), the β-galactosidase activity decreased 1.6-fold when *rcsB* was overexpressed in the wild-type background compared with the corresponding control without *rcsB* overexpression. Similarly, a 1.9-fold decrease was obtained with the P_{rcsB} mutant. Additionally, essentially no changes were observed when the overexpression was induced in the P_{rcsDB} mutant. These results clearly suggested that the P_{rcsDB} but not the P_{rcsB} promoter is repressed by high levels of RcsB.

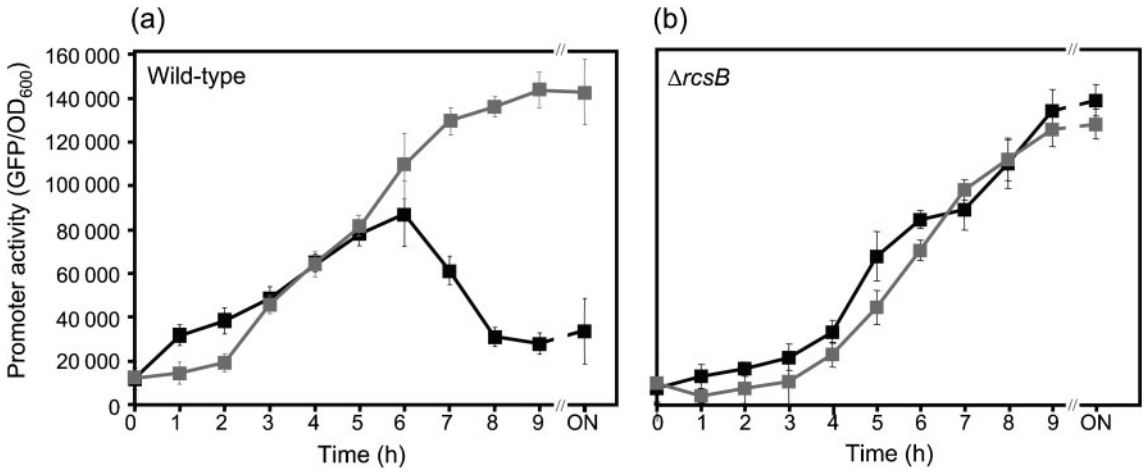

Fig. 3. P_{rcsDB} activity is repressed by polymyxin B treatment. P_{rcsDB} promoter activity, measured as GFP production at each time point, was monitored in (a) wild-type strain 14028s and (b) the *rcsB* mutant (EG12711). Black symbols, cultures grown in the presence of polymyxin B; grey symbols, cultures grown in the absence of polymyxin B. All data correspond to mean values of three independent experiments done in duplicate; error bars, SD.

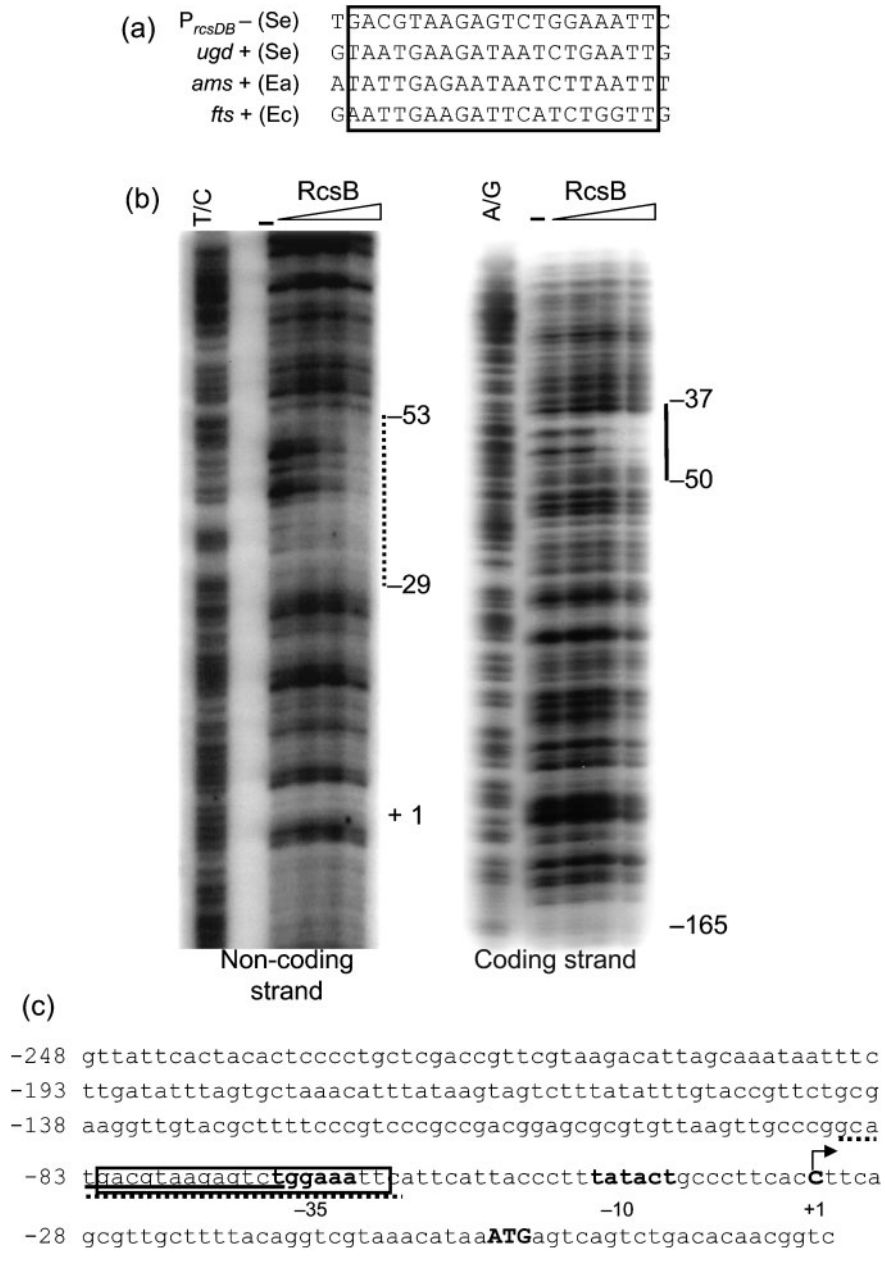

Fig. 4. Interaction of the RcsB regulator with the P_{rcsDB} promoter region. (a) Alignment of the RscB boxes conserved in the P_{rcsDB} promoter in comparison with the reported RcsB-binding sequences of the *S.* Typhimurium (Se) *ugd*, *Erwinia amylovora* (Ea) *ams*, and *E. coli* (Ec) *fts* genes. The box indicates the conserved sequence of the RcsB-binding motif. (b) DNase footprinting analysis of RcsB–His6 binding to the P_{rcsDB} promoter region. DNA footprinting analysis was performed on end-labelled fragments corresponding to the upstream *rcsD* coding and non-coding strands. The RcsB–His6 protein was added at final concentrations of 0, 10, 40 and 80 nM. Solid and dotted black bars represent the RcsB-protected regions. Lanes: A/G and T/C, Maxam and Gilbert sequencing reaction of the labelled fragments. (c) DNA sequence corresponding to the 248 bp region upstream of the *rcsD* ORF. The sequences underlined by solid and dotted black lines represent the DNA regions footprinted by the RcsB–His6 protein. Conserved sequences corresponding to the putative RcsB-binding motif are boxed.

On the other hand, we expected that P_{rcsB} promoter activity would be absent and that *rcsB* expression would be completely abolished by P_{rcsDB} deletion, under the experimental conditions employed (Fig. 2b). However, the *rcsB* expression levels decreased by only 60 %. This was an unexpected result and could be explained by assuming that in the absence (P_{rcsDB} mutant) or repression (wild-type strain overexpressing *rcsB*) of *rcsD*, the P_{rcsB} promoter activity is induced through an unknown mechanism, in order to maintain basal levels of *rcsB* expression. We

investigated this possibility, and the decreased levels of *rcsB* expression, obtained when the P_{rcsDB} mutant was complemented with the p*rcsD* plasmid, confirmed our assumption (data not shown).

To confirm that P_{rcsDB} alone is repressed by high levels of RcsB and to simultaneously discount any polar effect, the P_{rcsDB} and P_{rcsB} activities were determined as GFP production. As shown in Fig. 2(c), *rcsB* overexpression in the wild-type strain decreased the level of GFP when it was under the control of the P_{rcsDB} promoter. It is interesting to note that the repressive effect was observed only after 4 h of incubation. In contrast, no effect was detected when P_{rcsB} controlled the expression of GFP (Fig. 2c). The similarity of the results obtained with β-galactosidase activity and GFP expression assays let us conclude that high levels of *rcsB* repress P_{rcsDB} activity, resulting in lower levels of *rcsD* expression. The P_{rcsDB} promoter activity determined as GFP level was also measured after polymyxin B treatment. The exposure to polymyxin B of the wild-type strain transformed with plasmid pMS201 containing the P_{rcsDB} promoter region (Pescaretti *et al.*, 2009) decreased GFP production compared with the control without antibiotic (Fig. 3a). This effect was not observed in the *rcsB* mutant background (Fig. 3b), highlighting the role of the RcsB regulator. These results confirm our supposition that the effect of *rcsD* repression occurs at the physiological levels of Rcs system induction.

RcsB protein binds to the P_{rcsDB} promoter

With the aim of demonstrating a direct repression effect of RcsB, we searched by bioinformatics analysis a putative RcsB-binding site on the P_{rcsDB} promoter region sequence. This analysis revealed the presence of a DNA sequence that exhibits homology with the previously predicted RcsB-binding box (Fig. 4a) (Carballes *et al.*, 1999; Mouslim *et al.*, 2003; Wehland & Bernhard, 2000). DNase I footprinting assay of the 248 bp region upstream of the *rcsD* coding sequence established that the RcsB protein binds to the characterized P_{rcsDB} promoter (Pescaretti *et al.*, 2009). Specifically, the region from position -50 to -37 on the coding strand and from -53 to -29 on the non-coding strand relative to the transcription start site was protected by the RcsB regulator (Fig. 4b). The protected sequences included the predicted RcsB-binding box and overlapped with the P_{rcsDB} promoter -35 box (Fig. 4c).

Swarming is controlled by the *rcsB* gene expressed under the P_{rcsB} promoter

The swarming modulation of *Escherichia coli* and *S.* Typhimurium has been associated with the Rcs phosphorelay system (Harshey, 2003; Takeda *et al.*, 2001; Toguchi *et al.*, 2000). Here, we studied the motility phenotype of the wild-type *S.* Typhimurium strain, and of *rcsB*, P_{rcsDB} and P_{rcsB} mutants. The absence of a chromosomal *rcsD* gene in the P_{rcsDB} and P_{rcsB} mutants was complemented with the p*rcsD* plasmid, in order to complete the phosphorylation pathway and produce the more active form of RcsB (phospho-RcsB) (Mariscotti & Garcia-del Portillo, 2009). In order to unify the genetic background, the wild-type *S.* Typhimurium strain and *rcsB* mutant were also transformed with p*rcsD*. It is important to note that this assay was carried out in strains harbouring the chromosomal *rcsB* gene and under growth conditions different from those used in Fig. 2(b). In agreement with previous observations (Delgado *et al.*, 2006), the wild-type strain and the *rcsB* mutant containing plasmid p*rcsD* displayed very different swarming behaviours, migrating at 1.8×10^{-2} and 2.2×10^{-2} mm min^{-1}, respectively (Fig. 5). Interestingly, the P_{rcsB} mutant migrated appreciably faster (2.0×10^{-2} mm min^{-1}) than the wild-type strain (1.7×10^{-2} mm min^{-1}) and P_{rcsDB} mutant (1.6×10^{-2} mm min^{-1}) (Fig. 5). This result could be explained by assuming that in the P_{rcsB} mutant, the RcsB levels produced exert a negative autoregulation on P_{rcsDB} promoter activity, resulting in less repression of motility. In contrast, the negative autoregulation effect on the P_{rcsDB} promoter was not observed in the results obtained in Fig. 2(b) due to the absence of the *rcsB* gene.

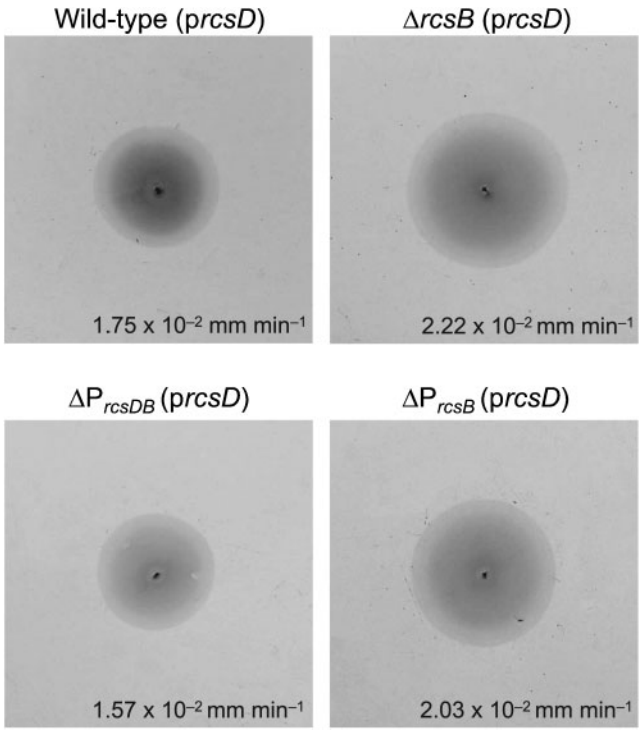

Fig. 5. *rcsB* gene expression determines the swarming phenotype under the control of P_{rcsB}. The swarming behaviour of the wild-type (14028s), *rcsB* (EG12711), P_{rcsDB} (MDs1017) and P_{rcsB} (MDs1018) strains carrying the p*rcsD* plasmid was assayed on LB plates containing 0.4 % agar and IPTG. All images were captured after 12 h incubation at 37 °C. The images represent one of three independent experiments; the values shown at the foot of each panel correspond to mean values of the independent experiments.

Taken together, the main conclusion of these results is that the presence of P_{rcsB} is able to maintain swarming repression at levels similar to those of the wild-type strain, while the P_{rcsDB} promoter does not, and its role is currently under investigation.

DISCUSSION

We previously reported that a high level of RcsB regulator inhibits *rcsD* gene transcription, showing a weak effect on *rcsB* expression due to the presence of the P_{rcsB} promoter (Pescaretti *et al.*, 2009). The goal of the present work was to study further the effect of the RcsB regulator on the control of *rcsD* expression. In addition to the RcsB overproduction reported, *rcsD* repression also occurs in the *rcsC11* mutant and even more so under a physiological condition such as polymyxin B treatment. With the *β*-galactosidase activity and GFP production assays we demonstrated that the *rcsD* repression induced by a high RcsB level is due to a specific effect on the P_{rcsDB} promoter. The P_{rcsDB} promoter activity was repressed only by the RcsB protein, since no effect was observed in the *rcsB* mutant, indicating the RcsB-dependence of this effect. Additionally, the identification of a conserved RcsB-binding site on the P_{rcsDB} promoter confirmed the direct action of the regulator on this promoter, as established by the footprinting assay. No RcsB-binding site was found in the P_{rcsB} promoter region. Furthermore, the repression effect was strongly supported by the localization of the RcsB-binding site, overlapping the P_{rcsDB} −35 box described previously (Pescaretti *et al.*, 2009).

Cumulatively, these results suggest that the Rcs system has a mechanism of negative autoregulation. Autoregulation has been observed in other two-component regulatory systems, including the *phoPQ* operon of *Salmonella* (Soncini *et al.*, 1995), the *phoBR* operon of *E. coli* (Guan *et al.*, 1983), the *virA* and *virG* genes of *Agrobacterium tumefaciens* (Winans *et al.*, 1994), and the *bvgAS* operon of *Bordetella pertussis* (Stibitz & Miller, 1994). It is important to note that all of the above systems are under a positive autoregulation mechanism. We are describing for what is believed to be the first time that the Rcs system could be negatively autoregulated.

On the basis of our results, a negative autoregulation model for the *S.* Typhimurium Rcs regulatory system is proposed (Fig. 6). In the presence of the signal, the Rcs phosphorelay system is fully activated due to phosphate transfer from RcsC to RcsB, mediated by RcsD. The *rcsB* gene is expressed from both promoters, P_{rcsDB} and P_{rcsB}, producing high levels of RcsB, which is then phosphorylated and can modulate the expression of those genes that

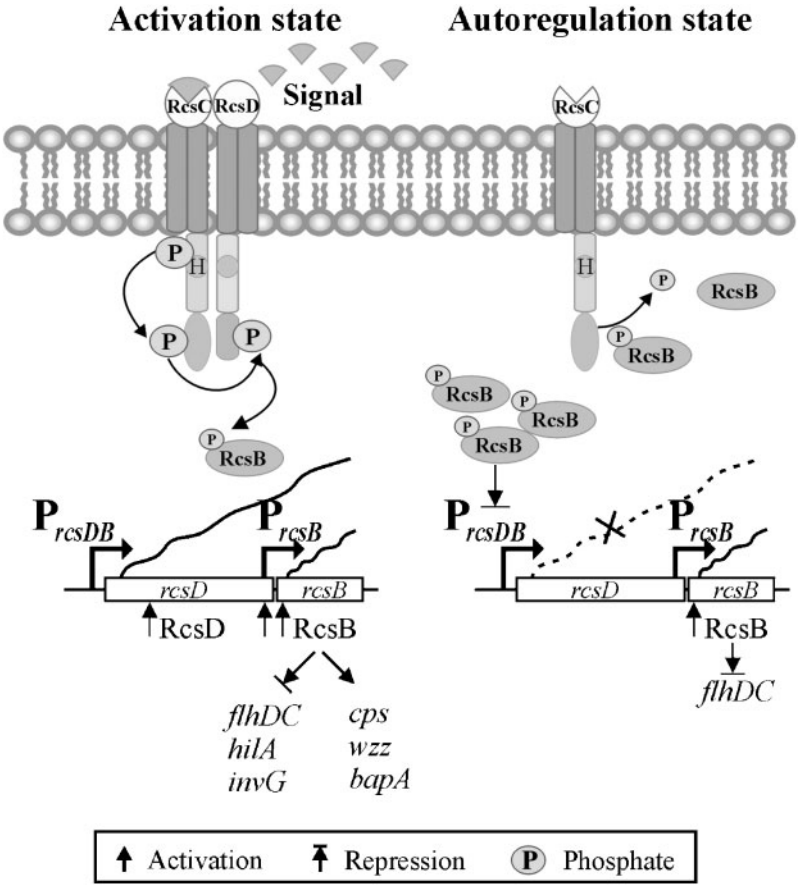

Fig. 6. Negative autoregulation model proposed for the Rcs system. The signal is sensed by RcsC, producing full Rcs system activation, which in turn controls the modulation of the indicated genes (Activation state). After reaching the threshold concentration of RcsB, the P_{rcsDB} promoter activity is repressed and *rcsB* expression is maintained at low levels by the P_{rcsB} promoter activity (Autoregulation state).

are required for adaptation (Fig. 6, activation state). After reaching a threshold concentration, RcsB protein represses *rcsD* transcription by binding to the P_{rcsDB} promoter. This repression breaks off the Rcs phosphorelay pathway, with the consequent dephosphorylation of RcsB by RcsC phosphatase activity (Fig. 6, autoregulation state). At this point, *rcsB* expression begins to be controlled only by the P_{rcsB} promoter, and the RcsB protein returns to basal levels, which are required to maintain the repression of motility.

It has previously been established that activation of the *Salmonella* Rcs regulatory system interferes with the ability of *Salmonella* to cause a lethal infection in mice (Mouslim *et al.*, 2004). Furthermore, a close correlation between virulence and flagellar regulation has been reported (Ikeda *et al.*, 2001). In this paper we demonstrate that even when P_{rcsDB} is quantitatively more active than the P_{rcsB} promoter, the latter is required to maintain the repression of the RcsB-dependent motility phenotype. The presence of two promoters, P_{rcsDB} and P_{rcsB}, acquires an important physiological relevance, since it allows the maintenance of bacterial mobility repression even in the negative autoregulation state. Ongoing experiments are being directed toward identifying the physiological signals able to activate the Rcs system and determining how virulence and swarming motility could be influenced.

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REFERENCES

Bader, M. W., Navarre, W. W., Shiau, W., Nikaido, H., Frye, J. G., McClelland, M., Fang, F. C. & Miller, S. I. (2003). Regulation of *Salmonella typhimurium* virulence gene expression by cationic antimicrobial peptides. *Mol Microbiol* **50**, 219–230.

Beeston, A. L. & Surette, M. G. (2002). *pfs*-dependent regulation of autoinducer 2 production in *Salmonella enterica* serovar Typhimurium. *J Bacteriol* **184**, 3450–3456.

Cano, D. A., Dominguez-Bernal, G., Tierrez, A., Garcia-Del Portillo, F. & Casadesus, J. (2002). Regulation of capsule synthesis and cell motility in *Salmonella enterica* by the essential gene *igaA*. *Genetics* **162**, 1513–1523.

Carballes, F., Bertrand, C., Bouche, J. P. & Cam, K. (1999). Regulation of *Escherichia coli* cell division genes *ftsA* and *ftsZ* by the two-component system *rcsC-rcsB*. *Mol Microbiol* **34**, 442–450.

Chen, M. H., Takeda, S., Yamada, H., Ishii, Y., Yamashino, T. & Mizuno, T. (2001). Characterization of the RcsC→YojN→RcsB phosphorelay signaling pathway involved in capsular synthesis in *Escherichia coli*. *Biosci Biotechnol Biochem* **65**, 2364–2367.

Clarke, D. J., Holland, I. B. & Jacq, A. (1997). Point mutations in the transmembrane domain of DjlA, a membrane-linked DnaJ-like protein, abolish its function in promoting colanic acid production via the Rcs signal transduction pathway. *Mol Microbiol* **25**, 933–944.

Costa, C. S. & Anton, D. N. (2001). Role of the *ftsA*1p promoter in the resistance of mucoid mutants of *Salmonella enterica* to mecillinam: characterization of a new type of mucoid mutant. *FEMS Microbiol Lett* **200**, 201–205.

Datsenko, K. A. & Wanner, B. L. (2000). One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. *Proc Natl Acad Sci U S A* **97**, 6640–6645.

Davis, R. W., Bolstein, D. & Roth, J. R. (1980). *Advanced Bacterial Genetics*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Delgado, M. A., Mouslim, C. & Groisman, E. A. (2006). The PmrA/PmrB and RcsC/YojN/RcsB systems control expression of the *Salmonella* O-antigen chain length determinant. *Mol Microbiol* **60**, 39–50.

Erickson, K. D. & Detweiler, C. S. (2006). The Rcs phosphorelay system is specific to enteric pathogens/commensals and activates *ydeI*, a gene important for persistent *Salmonella* infection of mice. *Mol Microbiol* **62**, 883–894.

Ferrières, L. & Clarke, D. J. (2003). The RcsC sensor kinase is required for normal biofilm formation in *Escherichia coli* K-12 and controls the expression of a regulon in response to growth on a solid surface. *Mol Microbiol* **50**, 1665–1682.

Fields, P. I., Swanson, R. V., Haidaris, C. G. & Heffron, F. (1986). Mutants of *Salmonella typhimurium* that cannot survive within the macrophage are avirulent. *Proc Natl Acad Sci U S A* **83**, 5189–5193.

Francez-Charlot, A., Laugel, B., Van Gemert, A., Dubarry, N., Wiorowski, F., Castanie-Cornet, M. P., Gutierrez, C. & Cam, K. (2003). RcsCDB His-Asp phosphorelay system negatively regulates the *flhDC* operon in *Escherichia coli*. *Mol Microbiol* **49**, 823–832.

Guan, C. D., Wanner, B. & Inouye, H. (1983). Analysis of regulation of *phoB* expression using a *phoB-cat* fusion. *J Bacteriol* **156**, 710–717.

Harshey, R. M. (2003). Bacterial motility on a surface: many ways to a common goal. *Annu Rev Microbiol* **57**, 249–273.

Huang, Y. H., Ferrières, L. & Clarke, D. J. (2006). The role of the Rcs phosphorelay in *Enterobacteriaceae*. *Res Microbiol* **157**, 206–212.

Ikeda, J. S., Schmitt, C. K., Darnell, S. C., Watson, P. R., Bispham, J., Wallis, T. S., Weinstein, D. L., Metcalf, E. S., Adams, P. & other authors (2001). Flagellar phase variation of *Salmonella enterica* serovar Typhimurium contributes to virulence in the murine typhoid infection model but does not influence *Salmonella*-induced enteropathogenesis. *Infect Immun* **69**, 3021–3030.

Kalir, S., Mangan, S. & Alon, U. (2005). A coherent feed-forward loop with a SUM input function prolongs flagella expression in *Escherichia coli*. *Mol Syst Biol* **1**, 2005.0006.

Kelley, W. L. & Georgopoulos, C. (1997). Positive control of the two-component RcsC/B signal transduction network by DjlA: a member of the DnaJ family of molecular chaperones in *Escherichia coli*. *Mol Microbiol* **25**, 913–931.

Kim, W. & Surette, M. G. (2004). Metabolic differentiation in actively swarming *Salmonella*. *Mol Microbiol* **54**, 702–714.

Majdalani, N. & Gottesman, S. (2005). The Rcs phosphorelay: a complex signal transduction system. *Annu Rev Microbiol* **59**, 379–405.

Mariscotti, J. F. & Garcia-del Portillo, F. (2009). Genome expression analyses revealing the modulation of the *Salmonella* Rcs regulon by the attenuator IgaA. *J Bacteriol* **191**, 1855–1867.

Miller, J. H. (1972). *Experiments in Molecular Genetics*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Mouslim, C. & Groisman, E. A. (2003). Control of the *Salmonella ugd* gene by three two-component regulatory systems. *Mol Microbiol* **47**, 335–344.

Mouslim, C., Latifi, T. & Groisman, E. A. (2003). Signal-dependent requirement for the co-activator protein RcsA in transcription of the RcsB-regulated *ugd* gene. *J Biol Chem* **278**, 50588–50595.

Mouslim, C., Delgado, M. & Groisman, E. A. (2004). Activation of the RcsC/YojN/RcsB phosphorelay system attenuates *Salmonella* virulence. *Mol Microbiol* **54**, 386–395.

Pescaretti, M. M., Morero, R. & Delgado, M. A. (2009). Identification of a new promoter for the response regulator *rcsB* expression in *Salmonella enterica* serovar Typhimurium. *FEMS Microbiol Lett* **300**, 165–173.

Ronen, M., Rosenberg, R., Shraiman, B. I. & Alon, U. (2002). Assigning numbers to the arrows: parameterizing a gene regulation network by using accurate expression kinetics. *Proc Natl Acad Sci U S A* **99**, 10555–10560.

Rosenfeld, N., Elowitz, M. B. & Alon, U. (2002). Negative autoregulation speeds the response times of transcription networks. *J Mol Biol* **323**, 785–793.

Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989). *Molecular Cloning: a Laboratory Manual*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Sanderson, K. E., Hessel, A. & Rudd, K. E. (1995). Genetic map of *Salmonella typhimurium*, VIII edition. *Microbiol Rev* **59**, 241–303.

Soncini, F. C., Vescovi, E. G. & Groisman, E. A. (1995). Transcriptional autoregulation of the *Salmonella typhimurium phoPQ* operon. *J Bacteriol* **177**, 4364–4371.

Stibitz, S. & Miller, J. F. (1994). Coordinate regulation of virulence in *Bordetella pertussis* mediated by the *vir* (*bvg*) locus. In *Molecular Genetics of Bacterial Pathogenesis*, pp. 407–422. Edited by V. L. Miller, J. B. Kaper, D. A. Portney & R. R. Isberg. Washington, DC: American Society for Microbiology.

Stout, V. & Gottesman, S. (1990). RcsB and RcsC: a two-component regulator of capsule synthesis in *Escherichia coli*. *J Bacteriol* **172**, 659–669.

Takeda, S., Fujisawa, Y., Matsubara, M., Aiba, H. & Mizuno, T. (2001). A novel feature of the multistep phosphorelay in *Escherichia coli*: a revised model of the RcsC → YojN → RcsB signalling pathway implicated in capsular synthesis and swarming behaviour. *Mol Microbiol* **40**, 440–450.

Toguchi, A., Siano, M., Burkart, M. & Harshey, R. M. (2000). Genetics of swarming motility in *Salmonella enterica* serovar Typhimurium: critical role for lipopolysaccharide. *J Bacteriol* **182**, 6308–6321.

Virlogeux, I., Waxin, H., Ecobichon, C., Lee, J. O. & Popoff, M. Y. (1996). Characterization of the *rcsA* and *rcsB* genes from *Salmonella typhi*: *rcsB* through *tviA* is involved in regulation of Vi antigen synthesis. *J Bacteriol* **178**, 1691–1698.

Wehland, M. & Bernhard, F. (2000). The RcsAB box. Characterization of a new operator essential for the regulation of exopolysaccharide biosynthesis in enteric bacteria. *J Biol Chem* **275**, 7013–7020.

Winans, S. C., Mantis, N. J., Chen, C. Y., Chang, C. H. & Han, D. C. (1994). Host recognition by the VirA, VirG two-component regulatory proteins of *Agrobacterium tumefaciens*. *Res Microbiol* **145**, 461–473.

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