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 (Ferrie`res & 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 & Grosman, 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

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Description*</th>
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<tr>
<td>S. Typhimurium strains</td>
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<tr>
<td>14028s</td>
<td>Wild-type</td>
<td>Fields et al. (1986)</td>
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<tr>
<td>EG12711</td>
<td>ΔrcsB::Cm</td>
<td>This work</td>
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<tr>
<td>EG14873</td>
<td>rcsC11</td>
<td>Mousslim et al. (2004)</td>
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<td>EG14539</td>
<td>ΔrcsD::lacZY</td>
<td>Pescaretti et al. (2009)</td>
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<td>ΔrcsB::lacZY</td>
<td>Pescaretti et al. (2009)</td>
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<td>ΔrcsD::lacZY rcsC11</td>
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<td>Plasmids</td>
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<tr>
<td>pUHE2-2lacPf</td>
<td>repMB Ap&lt;sup&gt;+&lt;/sup&gt; lacPlac0&lt;sup&gt;+&lt;/sup&gt;</td>
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<td>pUHE2-21 lacP&lt;sup&gt;+&lt;/sup&gt; containing rcsB</td>
<td>Pescaretti et al. (2009)</td>
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<td>prcsD</td>
<td>pUHE2-21 lacP&lt;sup&gt;+&lt;/sup&gt; containing rcsD</td>
<td>This work</td>
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<td>pMS201</td>
<td>Low copy vector for cloning promoters, pLtet01, derived from pZS21-luc,</td>
<td>Beeston &amp; Surette (2002)</td>
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<td>pPrcsB</td>
<td>KM&lt;sup&gt;+&lt;/sup&gt; pMS201 containing 122 bp of P&lt;sub&gt;rcsB&lt;/sub&gt; fused to gfpmut2</td>
<td>Pescaretti et al. (2009)</td>
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<td>pPrcsDB</td>
<td>Km&lt;sup&gt;+&lt;/sup&gt; pMS201 containing 131 bp of P&lt;sub&gt;rcsDB&lt;/sub&gt; fused to gfpmut2</td>
<td>Pescaretti et al. (2009)</td>
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*Gene designations are summarized by Sanderson et al. (1995).

**Mutation of chromosomal promoters.** The promoters P<sub>rcsB</sub> or P<sub>rcsDB</sub> 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'-TACACTCCCCTGCTCGACCGTGTAGGCTGGAGCTGCTTCG-3') and 4504 (5'-CGTTCACATAAATGCTGCCGGGTACCAAGATT-AAGCATG GACATATGAAATTCCTCCTCTAG-3') from plasmid pKD3, using primers 4894 (5'-TACACTCCCCTGCTCGACCGTGTAGGCTGGAGCTGCTTCG-3') and 4504 (5'-CGTTCACATAAATGCTGCCGGGTACCAAGATT-AAGCATG GACATATGAAATTCCTCCTCTAG-3'). Amplification by PCR using chromosomal DNA from a wild-type S. Typhimurium strain (14028s) as template. Primers 4136 (5'-TGATCTCATGAGTAGTTTTCACACGTCC-3') and 4504 (5'-C GGTTCATGAGTAGTTTTCACACGTCC-3') were used to amplify DNA fragments containing promoters P<sub>rcsB</sub> and P<sub>rcsDB</sub>, respectively, were then transformed with plasmid pCP20, as described by Beeston & Surette (2002). Proper Cm removal was confirmed by direct nucleotide sequencing. The resulting strains, MDx1032 and MDx1034, respectively, were then transformed with plasmid pprcsB.

**β-Galactosidase assays.** Bacteria were grown to OD<sub>600</sub> 0.2 (about 2 h) and then supplemented with IPTG (0.35 mM), to overexpress rcsB from the P<sub>lac</sub> promoter of plasmid pBAD33, or with polymyxin B (1 µg ml<sup>-1</sup>), 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'-TGATCTCATGAGTAGTTTTCACACGTCC-3') and 4137 (5'-TGATCTCA GAAATAAGAAGAAACGGGT-3'), which anneal to the coding and

Fig. 1. rcsB overexpression represses rcsD transcription. The transcriptional activity of a ΔrcsD::lacZY fusion, measured as β-galactosidase activity (Miller units), was investigated in the following genetic backgrounds: wild-type (EG14539) and ccsC11 (MDs1077), harbouring or not harbouring plasmid pprcsB, 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 \( P_{\text{rcsDB}} \) and \( P_{\text{rcsB}} \) 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 \( \Delta rcsB::\text{lacZY} \) fusion, measured as \( \beta \)-galactosidase activity (Miller units), was investigated in the following genetic backgrounds: wild-type (EG14932), and \( P_{\text{rcsDB}} \) (MDs1032) and \( P_{\text{rcsB}} \) (MDs1034) mutants, all carrying the \( \text{prcsB} \) plasmid and grown in the presence (black bars) or absence (grey bars) of IPTG. (c) \( P_{\text{rcsDB}} \) (squares) and \( P_{\text{rcsB}} \) (circles) promoter activity, measured as GFP production at each time point, was monitored in the wild-type 14028s strain co-transformed with \( \text{prcsB} \) and \( pP_{\text{rcsDB}} \) or \( pP_{\text{rcsB}} \) 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 $[^{32}P]ATP$. 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 gfpmut2 gene in which the PrcsDB or PrcsB 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 pRSB 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, PrcsDb 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 ΔrcsD::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 pRSB (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 rcsD 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 PrcsDB activity**

To determine whether high levels of RcsB affect PrcsDB or PrcsB promoter activity, we studied rcsB expression in mutants with a deletion in each corresponding promoter region, PrcsDB and PrcsB transformed with pRSB. 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 observed with the PrcsB mutant. Additionally, essentially no changes were observed when the overexpression was induced in the PrcsDB mutant. These results clearly suggested that the PrcsDB but not the PrcsB promoter is repressed by high levels of RcsB.
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.

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**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.
investigated this possibility, and the decreased levels of rcsB expression, obtained when the P_{rcsDB} mutant was complemented with the prcsD 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 phosphor-ey 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 prcsD 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 prcsD. 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 prcsD 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 prcsD 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 \( rcsC^{11} \) mutant and even more so under a physiological condition such as polymyxin B treatment. With the \( \beta \)-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.

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 \( \text{phoPQ} \) operon of *Salmonella* (Soncini et al., 1995), the \( \text{phoBR} \) operon of *E. coli* (Guan et al., 1983), the \( \text{virA} \) and \( \text{virG} \) genes of *Agrobacterium tumefaciens* (Winans et al., 1994), and the \( \text{bvgAS} \) 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\( \text{rcsD} \) transcription by binding to the \( P_{\text{rcsDB}} \) 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, \( \text{rcsB} \) expression begins to be controlled only by the \( P_{\text{rcsB}} \) 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 \textit{Salmonella} Rcs regulatory system interferes with the ability of \textit{Salmonella} to cause a lethal infection in mice (Mouslim \textit{et al.}, 2004). Furthermore, a close correlation between virulence and flagellar regulation has been reported (Ikeda \textit{et al.}, 2001). In this paper we demonstrate that even when \( P_{\text{rcsDB}} \) is quantitatively more active than the \( P_{\text{rcsB}} \) promoter, the latter is required to maintain the repression of the RcsB-dependent motility phenotype. The presence of two promoters, \( P_{\text{rcsDB}} \) and \( P_{\text{rcsB}} \), acquires an important physiological relevance, since it allows the maintenance of bacterial mobility repression even in the negative auto-regulation 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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