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

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Running title: Regulation of *rcsD* gene by the RcsB regulator

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SUMMARY

The RcsCDB (Rcs) phosphorelay system is involved in the regulation of many envelop genes like those responsible for capsule synthesis, flagella production and O-antigen chain length, as well as in other cellular activities of several enteric bacteria. This 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 issues about this system: a) the *rcsB* gene expression is under the control of P_{rcsDB} and P_{rcsB} promoters, and b) the *rcsD* gene transcription decreased when the bacteria reach high levels of RcsB regulator. In the present work, we demonstrate that RcsB protein represses the *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 was able to modify the bacterial swarming behavior, when it is expressed under the control of the P_{rcsB} promoter.

KEYWORDS

***Salmonella*, autoregulation, Rcs system, RcsB**

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 described, that the flow of phosphoryl groups through the Rcs phosphorelay components occur in the following way: RcsC→RcsD→RcsB (Takeda *et al.*, 2001). The Rcs system appears to be conserved in the *Enterobacteriaceae* family (Huang *et al.*, 2006; Pescaretti *et al.*, 2009) and it is involved in the modulation of many genes expression, such as those controlling the colanic acid biosynthesis (Stout & Gottesman, 1990); the regulator of flagellum synthesis (Francez-Charlot *et al.*, 2003); the cell division (Carballes *et al.*, 1999); the O-antigen chain length determinant (Delgado *et al.*, 2006); the 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 the bacteria growth at low temperature or on solid surface (Ferrieres & Clarke, 2003), the exposition to polymyxin B (Bader *et al.*, 2003; Erickson & Detweiler, 2006), the overproduction of DjlA (Clarke *et al.*, 1997; Chen *et al.*, 2001; Kelley & Georgopoulos, 1997), the *rcsC11* constitutive mutation (Costa & Anton, 2001; Mouslim *et al.*, 2004), *igaA* (Cano *et al.*, 2002) and *mucM* mutants (Costa & Anton, 2001), and the *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* gene decreases the *rcsD* transcription (Pescaretti *et al.*, 2009). The finding of the *rcsD* repression led us to investigate the potential role of RcsB

on the Rcs system regulation mechanism. In the present study, we demonstrated that high levels of RcsB regulator control the *rcsD* expression by direct binding to the P_{rcsDB} promoter, negatively autoregulating the Rcs system. The Rcs negative autoregulation was observed in *rcsC11* mutant or after polymyxin B treatment, indicating their importance in different physiological states. In addition, we also showed a physiological role on swarming behavior repression for the P_{rcsB}, controlling *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 (Davis, 1980). Recombinant DNA techniques and bacteria growth at 37 °C in Luria–Bertani (LB) were performed according to standard protocols (Sambrook, 1989). Kanamycin, ampicillin and chloramphenicol were used at a final concentration of 50 μg ml^{-1}, 50 μg ml^{-1} and 25 μg ml^{-1}, respectively.

Mutations of chromosomal promoters

The promoters P_{rcsDB} or P_{rcsB} were deleted from the chromosome of wild-type *Salmonella enterica* serovar Typhimurium (*S. typimurium*) 14028s strain using the one-step gene-inactivation method (Datsenko & Wanner, 2000). Briefly, a chloramphenicol resistance cassette (Cm) was amplified from pKD3 plasmid, using primers 4894 (5′-CACGGTTATTCACTACACTCCCCTGCTCGACCGTGTAGGCTGGAGCTGCTTC G-3′) and 4504 (5′-CGTTTCACATAACTGCTTGCCGGGTACCAGATTAAGCATG GCCATATGAATATCCTCCTTAG-3′) for P_{rcsDB} deletion; and 2385 (5′-GCGTTGCT

86 TTTACAGGTCGTAAACATAATGTAGGCTGGAGCTGCTTC-3´) and 2386 (5´-
87 GGCAATAATTACGTTCATATTGTTCATATGAATATCCTCCTTAG-3´) for P_{rcsB}
88 deletion. For P_{rcsDB} deletion, the resulting PCR product was introduced into the region
89 from nucleotide -219 upstream of *rcsD* to nucleotide 2541 of *rcsD* coding sequences,
90 leaving intact the P_{rcsB} promoter. On the other hand, for P_{rcsB} deletion, the resulting PCR
91 product was introduced into the complete *rcsD* coding sequence, leaving undamaged
92 P_{rcsDB} promoter (Fig. 2a). These mutations were introduced into strain EG14932
93 containing an Δ*rcsB*::*lacZY* fusion, by P22-mediated transduction. To eliminate some
94 chloramphenicol polar effect the resistance cassette was removed using the plasmid
95 pCP20 as described previously (Datsenko & Wanner, 2000). The proper Cm removal
96 was confirmed by direct nucleotide sequencing. The resulting strains, MDs1032 and
97 MDs1034 respectively, were then transformed with p*rcsB* plasmid.

98 β-*galactosidase assays*

99 The bacteria were grown to an OD_{600}= 0.2 (approximately 2 h) and then supplemented
100 with IPTG (0.35 mM), to overexpress *rcsB* from the P_{lac} promoter of p*rcsB* plasmid, or
101 with polymyxin B (1 μg ml^{-1}) to induce the system. After growing 5 h more, the β-
102 galactosidase activity was measured as previously described (Miller, 1972). Control
103 cultures were grown for 7 h in LB medium at 37 ºC in the absence of IPTG or
104 polymyxin B.

105 *DNase I Footprinting assay*

106 DNase I protection assays were carried out using appropriately labeled primers as
107 described (Delgado *et al.*, 2006). Fragments of DNA used for DNase I footprinting were
108 amplified by PCR using chromosomal DNA from wild-type *S. typhimurium* strain

(14028s) as template. Previously, the primers 4136 (5´-TGCTTCGCATTCGGTTTTTT TTAC-3´) and 4137 (5´-TGATCAGCAATAAGAAGAAACGGGT-3´), which anneal to the coding and non-coding strand of *rcsD* respectively, were labeled with T4 polynucleotide kinase and [γ-^{32}P]-ATP. The *rcsD* promoter region was amplified with the labeled primers 4136 and 4137 for the coding strand or with the labeled 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) were transformed with the plasmid pMS201containing a promoterless *gfp*mut2 gene in which the P_{rcsDB} or P_{rcsB} promoter region were cloned. In this assay, promoter activity was measured as the rate of GFP production divided by the OD_{600} of 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 (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 were deposited onto the surface of 0.4 % LB agar plates; which were incubated for 12 h at 37 °C. To estimate the average speed of migration (mm min^{-1}), the diameter of migrating colonies (mm) were plotted against the incubation times (min). The images in Fig. 4 represent one of three independent experiments; while the data correspond to mean values of these independent experiments.

RESULTS

RcsB overproduction represses the rcsD *transcription*

We previously demonstrated that the *rcsB* overexpression, from p*rcsB* plasmid, 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* was due to the presence of a second promoter, P_{rcsB}, which activated *rcsB* expression independent of *rcsD* (Pescaretti *et al.*, 2009). To determine whether the RcsB repressor effect is also produced in other Rcs-induction condition, the *rcsC11* constitutive allele mutant was used. The *rcsD* expression levels measured as the β-galactosidase activity of the chromosomal Δ*rcsD*::*lacZY* fusion from wild-type *S. typhimurium* (14028s) and *rcsC11* mutant, was determined after 7 h of the growth on LB medium. As shown in Fig. 1(a), the transcription level of *rcsD* in the *rcsC11* mutant was 2-fold lower than the values observed in the wild-type strain. Interestingly, a remarkable decrease (4-fold) was also observed in the mutant containing p*rcsD* plasmid (Fig. 1). This result suggests that the phosphorylated RcsB is more effective than non-phosphorylated form, due to the presence of the intermediary RcsD completing 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 the wild-type strain after 5 hours of exposure to polymyxin B also showed two-fold decreased levels of the *rscD* expression (Fig. 1). These results suggest that the *rcsD* expression is repressed when the bacteria reach high levels of RcsB regulator, under different Rcs system induction conditions.

The rcsB *overexpression represses the* P_{rcsDB} *activity*

To determine whether high levels of RcsB affect the P_{rcsDB} or P_{rcsB} promoter activity, we studied the *rcsB* expression in mutants with deletion in each corresponding promoter region, P_{rcsDB} and P_{rcsB}, transformed with p*rcsB* plasmid. As shown in Fig. 2(b), the β-galactosidase activity decrease 1.6-fold when *rcsB* was overexpressed in the

156 wild-type background compared to their corresponding control without *rcsB*
157 overexpression. Similarly, 1.9-fold decrease was obtained with the P_{rcsB} mutant.
158 Additionally, no essential changes were observed when the overexpression was induced
159 in the P_{rcsDB} mutant. These results clearly suggested that the P_{rcsDB} but not P_{rcsB} promoter
160 is repressed by high levels of RcsB.

161 On the other hand, we expected that the P_{rcsB} promoter activity was off and that the
162 *rcsB* expression was completely abolished by P_{rcsDB} deletion, in the used experimental
163 conditions (Fig. 2b). However, only a 60 % of the *rcsB* expression levels were
164 decreased. This was an unexpected result and could be explained assuming that in the
165 absence (P_{rcsDB} mutant) or repression (wild-type strain overexpressing *rcsB*) of *rcsD,* the
166 P_{rcsB} promoter activity is induced through an unknown mechanism, in order to maintain
167 basal levels of the *rcsB* expression. We investigated this possibility and the decreased
168 levels of *rcsB* expression, obtained when P_{rcsDB} mutant was complemented with the
169 p*rcsD* plasmid, confirmed our assumption (Data not shown).

170 To confirm that only P_{rcsDB} is repressed by high levels of RcsB and simultaneously
171 discard some polar effect, the P_{rcsDB} and P_{rcsB} activities were determined as GFP
172 production. As shown in Fig. 2(c), the *rcsB* overexpression in the wild-type strain
173 decreased the level of GFP when it is under the control of P_{rcsDB} promoter. It is
174 interesting to note that the repressive effect was observed only after four hours of
175 incubation. On the contrary, no effect was detected when the P_{rcsB} controls the
176 expression of GFP (Fig. 2c). The similarity of results obtained with β-galactosidase
177 activity and GFP protein expression assays, let as to conclude that high levels of *rcsB*
178 repress the P_{rcsDB} activity, resulting in lower levels of *rcsD* expression. The P_{rcsDB}
179 promoter activity determined as GFP levels was also measured after polymyxin B
180 treatment. The exposition to polymyxin B of wild-type strain transformed with the

plasmid pMS201 containing the P_{rcsDB} promoter region (Pescaretti *et al.*, 2009), decreased the GFP production compared with control without antibiotic (Fig. 3a). This effect was not observed in *rcsB* mutant background (Fig. 3b), highlighting the role of RcsB regulator. These results confirm our supposition that *rcsD* repression effect occurs at the physiological Rcs system induction levels.

The RcsB protein binds to the P_{rcsDB} *promoter*

With the aim to demonstrate 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 exhibit 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 *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 -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 include the predicted RcsB binding box and overlaps with the P_{rcsDB} promoter -35 box (Fig. 4c).

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

The swarming modulation of *E. coli* and *S. typhimurium* has been previously associated to 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 *rcsB*, P_{rcsDB} and P_{rcsB} mutants. The absence of chromosomal *rcsD* gene in the P_{rcsDB} and P_{rcsB} mutants was complemented with the p*rcsD* plasmid, in order to complete the phosphorylate pathway and produce the RcsB more active form

(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 remark that this assay was carried out in strains harboring the chromosomal *rcsB* gene and under different growth conditions that those used in Fig. 2(b). In agreement with previous observations (Delgado *et al.*, 2006), the wild-type strain and *rcsB* mutant containing p*rcsD* plasmid displayed a very different swarming behavior, migrating at 1.7 x 10^{-2} and 2.3 x 10^{-2} mm min^{-1}, respectively (Fig. 5). Interestingly, the P_{rcsB} mutant migrate appreciably faster (2.1 x 10^{-2} mm min^{-1}) than wild-type strain (1.7 x 10^{-2} mm min^{-1}) and P_{rcsDB} mutant (1.5 x 10^{-2} mm min^{-1}) (Fig. 5). This result could be possible assuming that in the P_{rcsB} mutant the produced RcsB levels exert a negative autoregulation on P_{rcsDB} promoter activity, resulting in a less motility repression. 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.

Taken together, the main conclusion of these results is that the presence of P_{rcsB} was able to keep the swarming repression at similar levels of the wild-type strain; while the P_{rcsDB} promoter did not and its role is 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 presence of P_{rcsB} promoter (Pescaretti *et al.*, 2009). The goal of the present work was go further into the study of the RcsB regulator effect on the control of *rcsD* expression. In addition to the RcsB overproduction reported, the *rcsD* repression occurs also in the *rcsC11* mutant and even more under physiological condition such as polymyxin B treatment. We demonstrated with the β-galactosidase activity and GFP production assays that the *rcsD*

repression induced by high RcsB level is due to a specific effect on the P_{rcsDB} promoter. The P_{rcsDB} promoter activity was repressed only by 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 a direct action of the regulator on the mentioned promoter as established by footprinting assay. No RcsB binding site was found on P_{rcsB} promoter region. Furthermore, the repression effect was strongly supported by the localization of RcsB binding site overlapping the P_{rcsDB} -35 box described previously (Pescaretti *et al.*, 2009).

Cumulatively, these results suggest that Rcs system would have a mechanism of negative autoregulation. Autoregulation has been observed in others two-component regulatory systems, including the *phoPQ* operon of *Salmonella* (Soncini *et al.*, 1995), the *phoBR* operon of *Escherichia coli* (Guan *et al.*, 1983), the *virA* and *virG* genes of *Agrobacterium tumefaciens* (Winans *et al.*, 1994), and the *bvgAS* operon in *Bordetella pertussis* (Stibitz & Miller, 1994). It is important to remark that all of the above cited systems are under a positive autoregulation mechanism. We are describing for 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 full activated due to the 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 phosphorylated and can modulate the expression of those genes that are required for adaptation (Fig. 6 Activation state). After reaching a threshold concentration, RcsB protein represses the *rcsD* transcription by binding to the P_{rcsDB} promoter. This repression breaks off the Rcs phosphorelay pathway, with the consequent dephosphorylation of RcsB by the RcsC phosphatase

activity (Fig. 6 Autoregulation state). At this point, the *rcsB* expression begins to be controlled only by P_{rcsB} promoter and RcsB protein return to the basal levels, which are required to maintain the motility repression.

It was previously 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, accurate correlation between virulence and flagellar regulation has been reported (Ikeda *et al.*, 2001). In this paper we demonstrate that even when the P_{rcsDB} is quantitatively more active than P_{rcsB} promoter the last one is required to maintain the repression of the RcsB-dependent motility phenotype. The presence of two promoters, P_{rcsDB} and P_{rcsB}, acquire an important physiological relevance since allows maintain the bacterial mobility repression even in the negative autoregulation state. On-going experiments are being directed toward identifying the physiological signals capable to activate the Rcs system and determine how the virulence and swarming motility could be influenced.

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Table 1: Bacterial strains and plasmids used in this study.

Strain or plasmid	Description[a]	Reference or source
S. enterica serovar Typhimurium		
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* gene	Pescaretti *et al.* (2009)
p*rcsD*	pUHE2-21 *lacI*^{q} containing *rcsD* gene	This work
pMS201	low copy vector for cloning promoters, pLtet01, derived of pZS21-luc, *gfp*mut2,	Beeston & Surette (2002)
pP_{rcsDB}	Km^r pMS201 containing 122 pb of P_{rcsDB} fused to *gfp*mut2 gene	Pescaretti *et al.* (2009)
pP_{rcsB}	pMS201 containing 131 pb of P_{rcsB} fused to *gfp*mut2 gene	Pescaretti *et al.* (2009)

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

Fig. 1

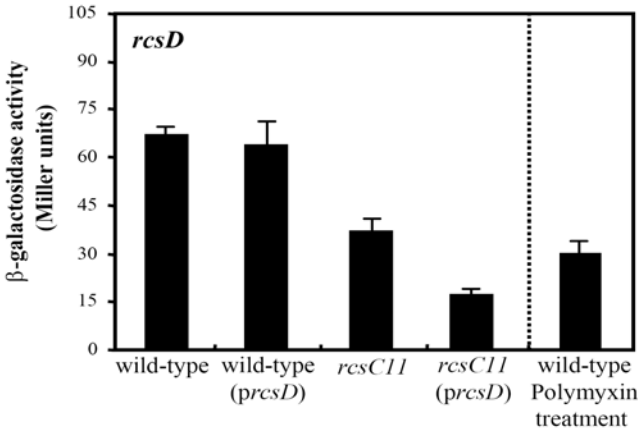


Fig. 1. *rcsB* overexpression represses *rcsD* transcription: The transcriptional activity of Δ*rcsD*::*lacZY* fusion, measured as β-galactosidase activity (Miller units), was investigated in the following genetic backgrounds: wild-type (EG14539) and *rcsC11* (MDs1077) harboring or not the p*rcsD* plasmid,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

Fig. 2

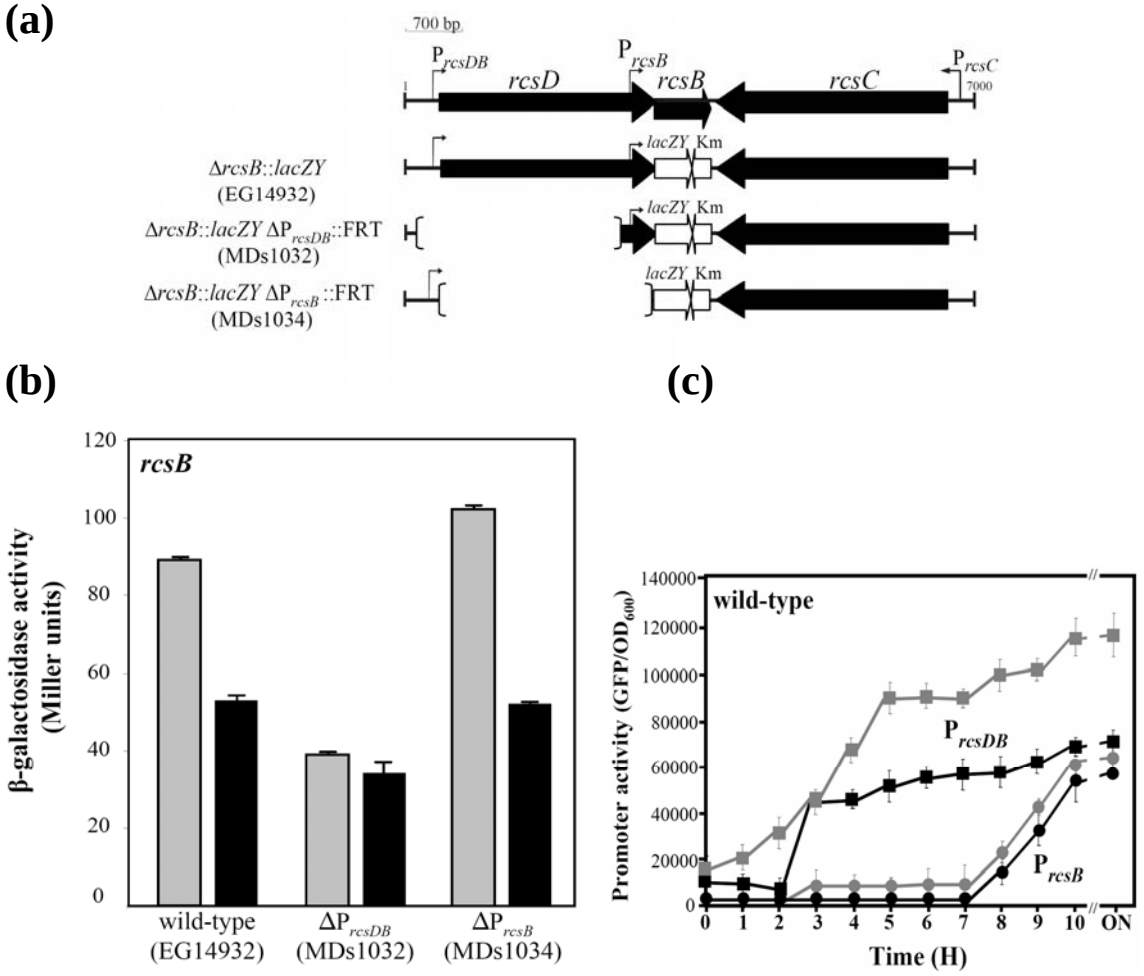


Fig. 2. Contribution of P_{rcsDB} and P_{rcsB} promoters to the *rcsB* expression: (a) Graphic representation of the genetic backgrounds used to determine the transcriptional activity of *rcsB* gene. Spaces between brackets correspond to the deleted promoter regions. (b) The transcriptional activity of 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 p*rcsB* plasmid and grown in the presence (black bars) or in the absence (grey bars) of IPTG. (c) The P_{rcsDB} (squares symbols) and P_{rcsB} (circles symbols) promoters activities, 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 correspond to the cultures grown in the presence of IPTG and grey symbols in the absence. All data correspond to mean values of three independent experiments done in duplicate. Error bars correspond to the standard deviation.

Fig. 3

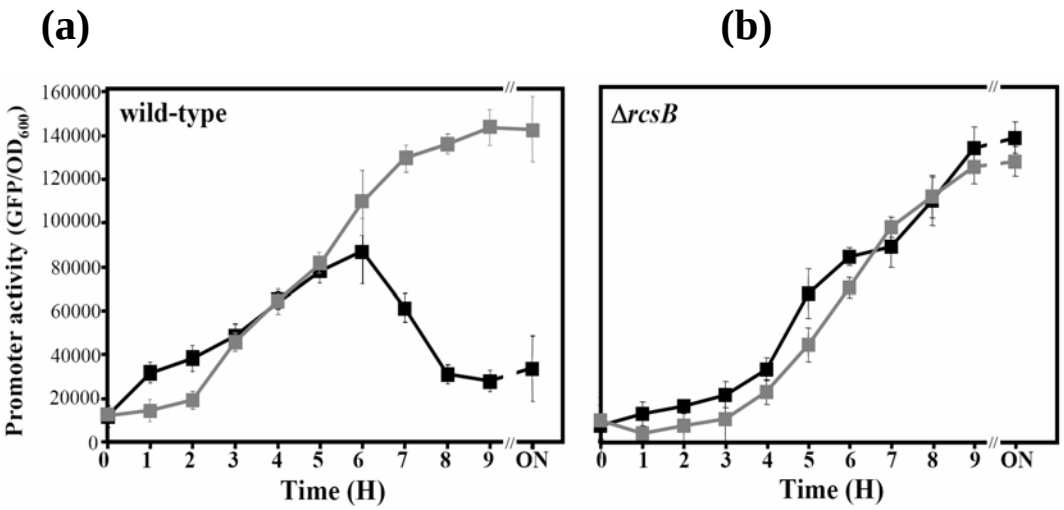
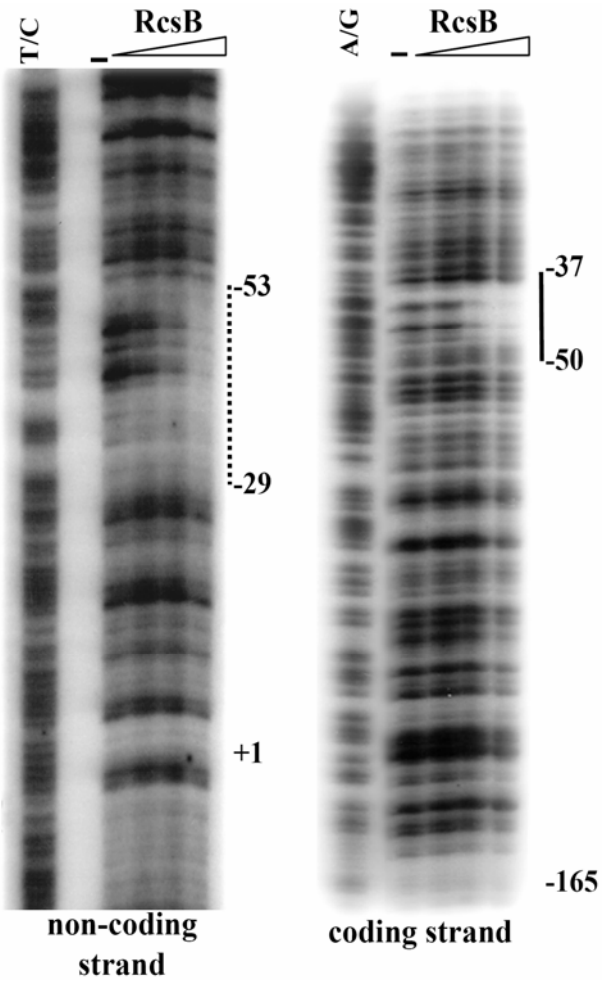


Fig. 3. The P_{rcsDB} activity is repressed by polymyxin B treatment. The P_{rcsDB} promoter activity, measured as GFP production at each time point, was monitored in the: (a) wild-type 14028s strain and (b) *rcsB* mutant (EG12711). Black symbols correspond to the cultures grown in the presence of polymyxin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

Fig. 4

(a)

(b)



(c)

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-248 gttattcactacactcccctgctcgaccgttcgtaagacattagcaaataatttc
-193 ttgatatttagtgctaaacatttataagtagtctttatatttgtaccgttctgcg
-138 aaggttgtacgcttttcccgtcccgccgacggagcgcgtgttaagttgcccggca
 -83 tgacgtaagagtctggaaattcattcattacccttTATACTgcccttcacCttca
                  -35                  -10        +1
 -28 gcgttgcttttacaggtcgtaaacataaATGagtcagtctgacacaacggtc
```

Fig. 4. Interaction of RcsB regulator with 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 *S. enterica* serovar Typhimurium (Se) *ugd*, *Erwinia amylovora*

(Ea) *ams*, and *E. coli* (Ec) *fts* genes. The box indicates conserved sequence of RcsB-binding motif. (b) DNase footprinting analysis of the RcsB-His6 binding to the P_{rcsDB} promoter region. DNA footprinting analysis performed on end-labeled fragments corresponding to the upstream *rcsD* coding and non-coding strands. The RcsB-His6 protein was added at a final concentration of 0, 10, 40, and 80 nM. Solid and dotted black bars represent the RcsB-protected regions. Lanes: A+G and T+C correspond to Maxam and Gilbert sequencing reaction of the labeled fragments. (c) DNA sequence corresponding to the 248-bp region upstream of *rcsD* open reading frame. The sequences underlined by solid and dotted black lines represent the DNA regions footprinted by the RcsB-His6 protein. The conserved sequences corresponding to the putative RcsB -binding motif are boxed.

Fig. 5

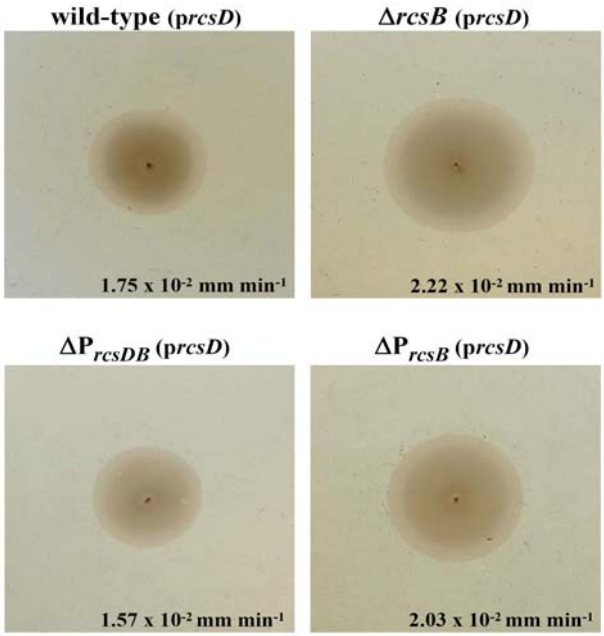

Fig. 5. The *rcsB* gene expression by P_{rcsB} activity controls the swarming phenotype. Swarming behavior of the wild-type (14028s), *rcsB* (EG12711), P_{rcsDB} (MDs1017) and P_{rcsB} (MDs1018) strains carrying the p*rcsD* plasmid was assayed on LB plates containing 0.4 % agar and IPTG. All images were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

Fig. 6

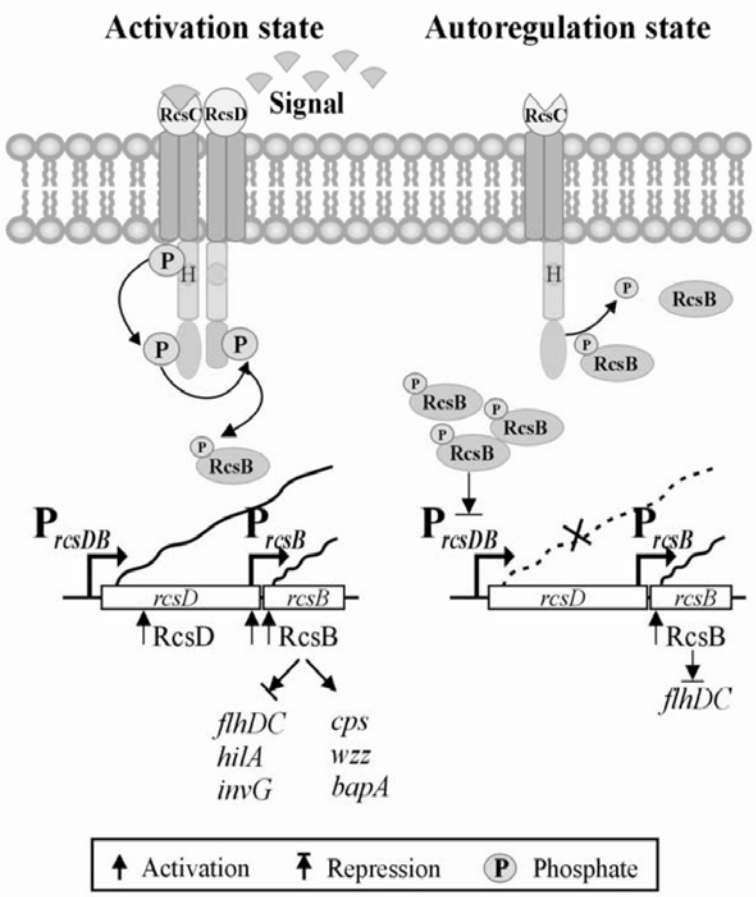

Fig. 6. Negative autoregulation model proposed for the Rcs system: The signal is sensed by RcsC producing full Rcs system activation, which controls the modulation of the indicated genes (Activation state). After reach the threshold concentration of RcsB, the P_{rcsDB} promoter activity is repressed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