

Identification of a new promoter for the response regulator *rcsB* expression in *Salmonella enterica* serovar Typhimurium

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Introduction

The RcsCDB system, first identified as a regulator of the capsular polysaccharide synthesis genes in *Escherichia coli* (Gottesman *et al.*, 1985), consists of the sensor RcsC, the cognate response regulator RcsB and the histidine-containing phosphotransfer RcsD protein. The latter acts as an intermediary in the phosphoryl group transfer from RcsC to RcsB (Takeda *et al.*, 2001; Majdalani & Gottesman, 2005). At present, the Rcs system regulates the transcription of a wide range of genes, including those that encode the master flagellar regulator *flhDC* operon (Francez-Charlot *et al.*, 2003), the cell division genes *ftsA* and *ftsZ* (Carballe *et al.*, 1999), the osmoregulated *osmC* gene (Davalos-García *et al.*, 2001), the O-antigen chain length determinant *wzzB* gene (Delgado *et al.*, 2006), the motility and chemotaxis genes (Cano *et al.*, 2002) and also those involved in the Vi antigen synthesis (Virlogeux *et al.*, 1996) in different enteric species.

Abstract

The RcsCDB (Rcs) phosphorelay system regulates capsule synthesis, flagella production and other cellular activities in several enteric bacteria. This system consists of three proteins: the sensor RcsC, the cognate response regulator RcsB and the histidine-containing phosphotransfer protein RcsD (YojN), which is hypothesized to act as an intermediary in the phosphotransfer from RcsC to RcsB. The *rscC* gene is convergently transcribed toward *rscB*, which follows *rscD* in what appears to be a two-gene operon. Here, it is reported that the overproduction of the *rscB* gene represses *rscD* transcription, but has a weak effect on its own expression. We demonstrated that the differential *rscD* and *rscB* expression is due to the activity of two promoters to transcribe the *rscB* gene: (1) P_{rscDB} located upstream of *rscD* and (2) P_{rscB} located within the *rscD* coding region. In addition, here it was demonstrated that in *Salmonella typhimurium*, P_{rscB} is important to activate the *rscB* expression during the stationary growth phase.

Although the signal activating the Rcs phosphorelay transduction system remains unidentified, many studies have demonstrated that Rcs activation occurs under certain growth conditions, such as overproduction of DjlA (Clarke *et al.*, 1997; Kelley & Georgopoulos, 1997; Chen *et al.*, 2001), growth at a low temperature, osmotic shock, desiccation (Shiba *et al.*, 2004), growth on a solid surface (Ferrieres & Clarke, 2003), mutation of the *tolB* gene (Mouslim *et al.*, 2003), the presence of Fe^{3+} and low Mg^{2+} in a *pmrA* mutant (Mouslim & Groisman, 2003), *igaA* mutation (Cano *et al.*, 2002), *mdo* mutation (Ebel *et al.*, 1997), as well as the *rscC11* constitutive mutation (Costa & Anton, 2001; Mouslim *et al.*, 2004).

While the *rscB* and *rscD* genes are situated in what appears to be an operon controlled by a hypothetical promoter located upstream of the *rscD* coding region (Blattner *et al.*, 1997; Takeda *et al.*, 2001; Detweiler *et al.*, 2003), the *rscC* gene is convergently transcribed to the *rscDB* operon by the

P_{rcsC} promoter (Brill *et al.*, 1988; Mizuno, 1997). In the study reported here, we demonstrated that *rcsB* is transcribed in two different ways: (1) in an *rcsD*-dependent manner, controlled by the operon promoter, P_{rcsDB} , and (2) in an *rcsD*-independent manner, under the control of the P_{rcsB} promoter. In addition, the P_{rcsDB} stimulates the *rcsB* expression during the exponential growth phase, while P_{rcsB} does so during stationary growth at lower levels.

Materials and methods

Bacterial strains, molecular techniques and growth conditions

The bacterial strains and plasmids used in this study are listed in Table 1. Recombinant DNA techniques and bacterial growth at 37 °C in Luria–Bertani (LB) were performed according to standard protocols (Sambrook *et al.*, 1989). Kanamycin was used at a final concentration of 50 µg mL⁻¹, ampicillin at 50 µg mL⁻¹ and chloramphenicol at 25 µg mL⁻¹.

Introduction of gene fusions and mutations in the chromosomal *rcsD*, *rcsB* and *rcsC* loci

The one-step gene-inactivation method (Datsenko & Wanner, 2000) was used to construct the strains containing a deletion of the *rcsD*, *rcsB* or *rcsC* coding sequence, and P_{rcsDB} and P_{rcsB} promoter regions. The chromosomal $\Delta rcsD::lacZY$, $\Delta rcsB::lacZY$ and $\Delta rcsC::lacZY$ gene fusion strains were constructed as described (Ellermeier *et al.*, 2002), with the following modifications. The Cm^R cassette was amplified using pKD3 plasmid DNA as a template and primers 2385 and 2386 for *rcsD*; 1165 and 1166 for *rcsB*; and 2803 and 2804 for *rcsC* (Supporting Information, Table S1). The PCR

products were integrated into the chromosome. The junction region of the *rcsD*, *rcsB* or *rcsC* and the Cm^R cassette was sequenced to confirm the deletion of these genes. After removing the Cm^R cassette, the *lacZY* transcriptional fusion plasmid pCE36 was integrated into the FLP recombination target sequence immediately downstream of the genes by FLP-mediated recombination.

β-Galactosidase assays

The β-galactosidase assays were carried out in duplicate, and the activity was determined as described (Miller, 1972). When the bacteria reached an OD_{600 nm} = 0.2, ≈ 2 h, 0.35 mM of isopropyl-β-D-thiogalactopyranoside (IPTG) was added to the overproduced RcsB regulator from *prcsB*. After 5 h of IPTG addition, the β-galactosidase activity was determined. All data correspond to mean values of three independent experiments performed in duplicate. Error bars correspond to the SD.

S1 mapping of *rcsD* and *rcsB* promoters

The S1 mapping assay was performed as described (Garcia Vescovi *et al.*, 1996). The RNA was harvested from a wild-type *Salmonella typhimurium* strain (14028s) growing in LB media, and isolated using the RNA SV total isolation kit (Promega) according to the manufacturer's specifications. A PCR product was generated with the set of primers 4136/4137 for *rcsD* and 3723/4133 for *rcsB* (Table S1), and chromosomal DNA from wild-type *S. typhimurium* strain (14028s) was the template and used as the probe. This probe was labeled at the 5' end by phosphorylation with [³²P]-ATP using T4 polynucleotide kinase (Gibco BRL) as described (Mousslim *et al.*, 2003).

Table 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Description*	Reference or source
<i>S. enterica</i> serovar Typhimurium		
14028s	Wild-type	Fields <i>et al.</i> (1986)
EG14873	<i>rcsC11</i>	Mousslim <i>et al.</i> (2004)
EG14123	$\Delta rcsC::lacZY$	This work
EG14498	$\Delta rcsC::Cm$	This work
EG14539	$\Delta rcsD::lacZY$	This work
EG14931	$\Delta rcsB$	This work
EG14932	$\Delta rcsB::lacZY$	This work
Plasmids		
pUHE2-2 <i>lacI</i> ^f	rep _{pMB1} Ap ^r <i>lacI</i> ^f	Soncini <i>et al.</i> (1995)
<i>prcsB</i>	pUHE2-21 <i>lacI</i> ^f containing the <i>rcsB</i> gene	This work
<i>prcsBop</i>	pUHE2-21 <i>lacI</i> ^f containing the <i>rcsB</i> gene in opposite direction to the <i>lac</i> promoter	This work
pMS201	Low-copy vector for cloning promoters, pTet01, derived from pZS21-luc, <i>gfpmut2</i> , Km ^r	Beeston & Surette (2002)
pP _{<i>rcsDB</i>}	pMS201 containing 122 pb of P _{<i>rcsDB</i>} fused to the <i>gfpmut2</i> gene	This work
pP _{<i>rcsB</i>}	pMS201 containing 131 pb of P _{<i>rcsB</i>} fused to the <i>gfpmut2</i> gene	This work

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

P_{rcsDB} and P_{rcsB} ::*gfp* fusion construction

The *rcsB* cis-regulatory regions, P_{rcsDB} and P_{rcsB} , were amplified from wild-type *S. typhimurium* (14028s) genomic DNA by PCR using the primers 5325 and 5324, and 5327 and 5326, respectively (Table S1). They were cloned into XhoI and BamHI sites upstream of a promoterless *gfpmut2* gene (Cormack *et al.*, 1996) in a low-copy number plasmid, pMS201, containing pTet01 from pZS21-luc (Ronen *et al.*, 2002; Kalir *et al.*, 2005). These derivative plasmids were used to transform the wild-type *S. typhimurium* strain (14028s).

Determination of promoter activity by the green fluorescent protein (GFP) expression

Cultures (1 mL) inoculated from single colonies were grown for 8 h in LB medium at 37 °C with shaking at 250 r.p.m. The ON cultures were diluted to $OD_{600\text{nm}} = 0.003$ into LB medium, at a final volume of 150 μL per well in a flat-bottom 96-well plate (Sarstedt). The cultures were grown in a Wallac Victor3 multiwell fluorimeter at 37 °C and set with an automatic shaking repeating protocol; $OD_{600\text{nm}}$ and fluorescence readings were determined as described pre-

viously (Ronen *et al.*, 2002; Rosenfeld *et al.*, 2002; Kalir *et al.*, 2005). Measurements were repeated every 6 min, but only the data corresponding to each hour were plotted. Background fluorescence at a given $OD_{600\text{nm}}$ was determined from the fluorescence of cells bearing a promoterless GFP vector at the same $OD_{600\text{nm}}$ as described (Ronen *et al.*, 2002; Rosenfeld *et al.*, 2002; Kalir *et al.*, 2005). All data correspond to mean values of three independent experiments performed in duplicate.

Results

The transcription of *rcsD* is repressed upon *rcsB* gene overexpression

The overexpression of certain response regulator proteins may result in the activation of target genes even in the absence of the cognate sensor or under some inducing condition (Wosten *et al.*, 2000; Oshima *et al.*, 2002; Takaya *et al.*, 2005; Castanie-Cornet *et al.*, 2007). Because the signal and the regulation mechanisms of the RcsCDB system remain unknown, how the *rcsB* gene overexpression may affect the regulation of the Rcs system was investigated. For

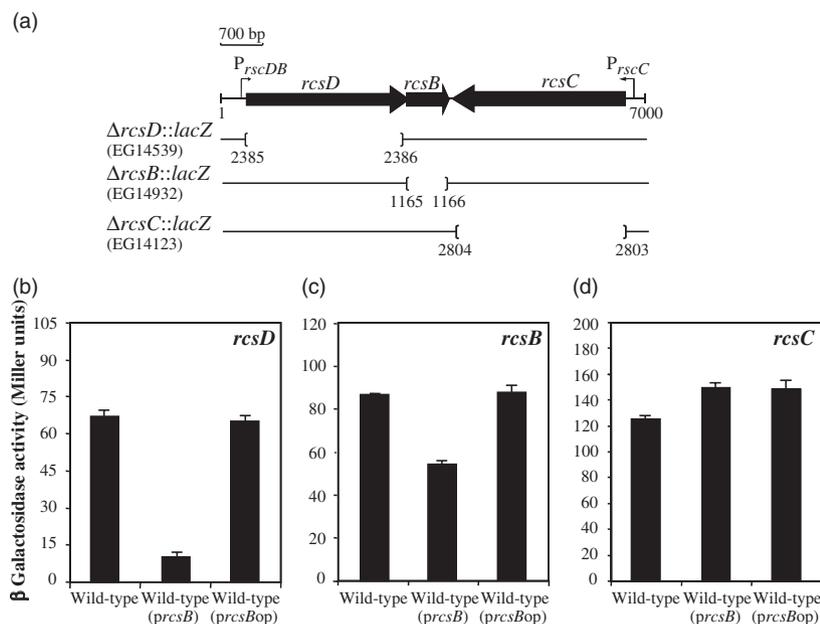
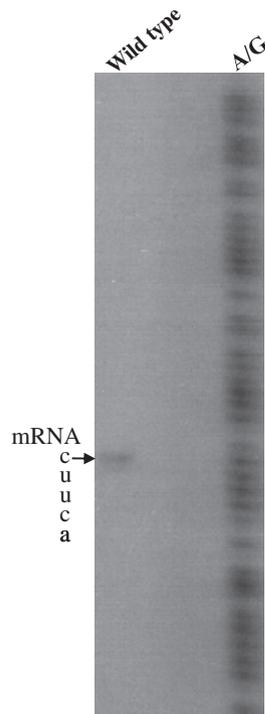


Fig. 1. *rcsB* overexpression represses *rcsD* transcription: (a) Graphic representation of primers (represented by numbers) used to obtain the different deletion fusions in the *rcsD*, *rcsB* or *rcsC* chromosomal genes. The space between brackets represents the region deleted in each mutant, which were replaced by the *lacZ* gene to obtain the transcriptional fusion. (b) β -Galactosidase activity (Miller units) expressed by strains harboring chromosomal *lacZY*-transcriptional fusions to the *rcsD* gene. The transcriptional activity was investigated in three genetic backgrounds: wild-type *Salmonella typhimurium* (EG14539) and wild-type (EG14539) carrying the *prcsB* or *prcsBop* plasmids, growing in LB medium supplemented with 0.35 mM IPTG as described in Materials and methods. (c) β -Galactosidase activity (Miller units) expressed by strains harboring chromosomal *lacZY*-transcriptional fusions to the *rcsB* gene. The transcriptional activity was investigated in three genetic backgrounds: wild-type *S. typhimurium* (EG14932), and wild-type (EG14932) carrying *prcsB* or *prcsBop* following growth as described in (b). (d) β -Galactosidase activity (Miller units) expressed by strains harboring chromosomal *lacZY*-transcriptional fusions to the *rcsC* gene. The transcriptional activity was investigated in three genetic backgrounds: wild-type *S. typhimurium* (EG14123), and wild-type (EG14123) carrying *prcsB* or *prcsBop* following growth as described in (b). Data correspond to mean values of three independent experiments performed in duplicate. Error bars correspond to the SD.

(a)



(b)

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-248 gttattcactacactcccctgctcgaccgttcgtaagacattagcaataatttc
-193 ttgatatttagtgctaaacatttataagtagtctttatattgtaccggtctgcg
-138 aagggtgtacgcttttcccgtcccgccgacggagcgcgtgtaagtggccggca
-83  tgacgtaagagtgcttggaattcatcattaccctttatactgcccttcaccttca
      -35                    -10                    +1
-28  gcggtgcttttacaggtcgtaaacataaATGagtcagtcctgacacaacggtc

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Fig. 2. Molecular analysis of the P_{rcsDB} promoter: (a) S1 mapping of the *rcsD* transcript produced by a wild-type *Salmonella typhimurium* strain (14028s) growing LB medium and harvested at an $OD_{600\text{ nm}}$ of 0.6. The S1 protection assay was performed as described in Materials and methods. Lane T/C corresponds to the Maxam–Gilbert DNA ladder of the target sequence. The transcription start site and the mRNA sequence are marked by an arrow. (b) DNA sequence corresponding to the 248-bp region upstream of the *rcsD* ORF. +1 corresponds to the *rcsD* transcription start site and the bold sequences are the predicted –10 and –35 boxes of the P_{rcsDB} promoter.

this, a derivative pUHE21-2*lacI*^q plasmid containing the *rcsB* gene expressed under the *lac* promoter (*prcsB*) and a negative control plasmid with the *rcsB* gene cloned in the opposite direction of the *lac* promoter (*prcsBop*) were constructed (Table 1). A series of isogenic strains, derivatives of the wild-type *S. typhimurium* strain (14028s) harboring *lacZ* transcriptional fusions to the *rcsD*, *rcsB* or *rcsC* chromosomal genes, were also generated (Fig. 1a, Table 1). The resulting strains were transformed with the plasmids described above. As shown in Fig. 1b, the levels of *rcsD* transcription, measured as β -galactosidase activity, in the wild-type strain harboring *prcsB* were sixfold lower than those observed in the isogenic strain lacking the plasmid or containing *prcsBop*. By contrast, *rcsB* transcription was reduced only 1.5-fold by *rcsB* overexpression (Fig. 1c) even when this and the *rcsD* gene were located in the same operon, and *rcsC* expression was not affected by

this condition (Fig. 1d). These results would indicate that *rcsD* expression is repressed by high levels of RcsB and suggest that an additional promoter could drive *rcsB* expression.

Localization of the *rcsDB* operon promoter

It has been postulated that *rcsD* and *rcsB* genes are transcribed as an operon, under the control of a putative promoter localized upstream of *rcsD* (Detweiler *et al.*, 2003). To probe this possibility, we defined this promoter region, localizing the transcription start site of the *rcsD* gene by S1 nuclease experiments. This assay was carried out using RNA harvested from a wild-type *S. typhimurium* strain (14028s) at an $OD_{600\text{ nm}}$ of 0.6 (Fig. 2a). The wild-type strain yielded an S1 product, located at the *rcsD* transcription start site 33 bp upstream of the *rcsD* ORF (Fig. 2b).

Even when it is not usually observed that the *Salmonella* promoters initiate transcription with a C, we had reported the same +1 for the *wzzB* gene (Delgado *et al.*, 2006). In addition, the +1 site allowed us to locate the -35 and -10 boxes at -32 and -10 bp upstream of the *rcsD* transcription start site, respectively (Fig. 2b). This promoter, controlling the expression of *rcsD* and *rcsB* genes, was designated as P_{rcsDB} .

A new promoter drives *rcsB* expression in addition to P_{rcsDB}

To further explore *rcsB* gene expression, Northern blot analyses were carried out using RNA harvested from the wild-type strain and the isogenic *rscC*, *rscB* and *rscC11* mutants, after 8 h of growth in LB medium (Fig. 3). The *rscC11* mutant, harboring a single nucleotide substitution (C to T) at position 477 of the RcsC protein, within the histidine-kinase domain, expresses RcsB-regulated genes even in the absence of a signal (Costa & Anton, 2001; Mouslim *et al.*, 2004). When the complete *rscB* coding region, amplified by 1106 and 1107 primers (Table S1), was used as a probe, three products were detected: (1) an ≈ 3.5 -

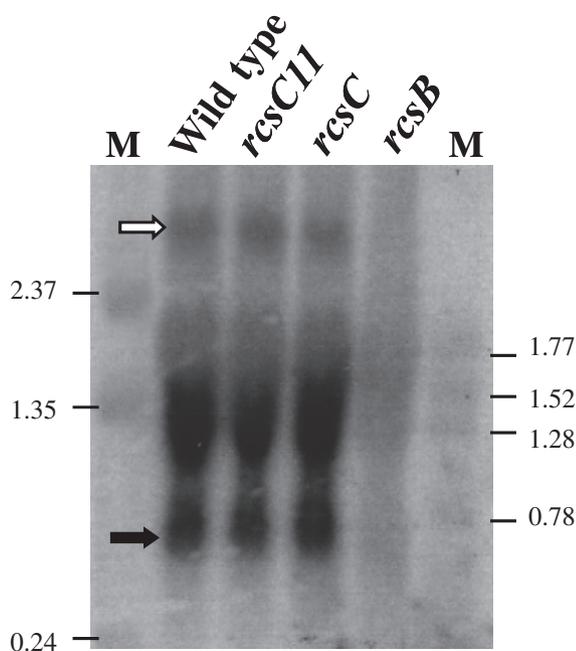


Fig. 3. Analysis of the *rscB* mRNA: Northern blot detection of mRNAs produced in wild-type *Salmonella typhimurium* strain (14028s, lane 2), and in *rscC11* (EG14873, lane 3), *rscC* (EG14498, lane 4) and *rscB* (EG14931, lane 5) mutants extracted after 8 h of growth in LB medium at 37 °C, using a complete *rscB* coding region as a probe. The empty arrow indicates the 3.5-kb band corresponding to the *rscD*–*rscB* cotranscript, while the filled arrow shows the 0.67-kb band corresponding in size to the *rscB* mRNA. M, molecular weight standard (kb); lane 1, 9.49 kb; lane 6, 1.77 kb.

kb product, which likely corresponds to the *rscD*–*rscB* transcript (Fig. 3, empty arrow); (2) an ≈ 1.28 -kb product, which may have originated from degradation of the *rscD*–*rscB* mRNA; and (3) an ≈ 0.67 -kb product, corresponding in size to the *rscB* mRNA transcribed from the 3' end of the *rscD* coding region to the predicted *rscB* ρ -independent terminator (Fig. 3, filled arrow) (Stout & Gottesman, 1990; Aiso & Ohki, 2003). These mRNAs were present in the wild-type strain as well as in the *rscC11* and *rscC* mutants, but were absent in the *rscB* mutant used as a negative control, indicating that they are *rscB*-specific products (Fig. 3).

To verify the possibility that *rscB* would be transcribed from an additional promoter, the 3' end of the *rscD* region was examined using the GEN PROMOTER SCAN (GPS) program (Zwir *et al.*, 2005). A 424-bp region extending from the 2351 nucleotide of the *rscD* coding region to the 61 nucleotide of the *rscB* coding region, including the small intergenic region of 16 bp, was analyzed. The putative promoter was located by this program between the -92 and the -45 nucleotides from the RcsB start codon.

The transcription start site of the putative *rscB* promoter was detected with the S1 mapping assay using the 424-bp region described above and mRNA from the wild-type strain harvested during the exponential and stationary growth phases. An S1 product was detected under both conditions at the -55 position of the RcsB start codon, demonstrating that the 424-bp region also contains the *rscB* +1 start site (Fig. 4a, filled arrow). The S1 product increased when the mRNA from the stationary phase was used (Fig. 4a). Taken together, these results support the presence of a new promoter, which was named here P_{rscB} , that controls *rscB* expression in an *rscD*-independent manner.

P_{rscDB} and P_{rscB} control *rscB* expression at different growth phases

In order to explore the relative contribution of the two promoters driving *rscB* expression, the 122 bp of the P_{rscDB} regulatory region and 131 bp of the 3' end of the *rscD* coding region, containing the P_{rscB} promoter, were separately cloned into the reporter plasmid pMS201 (Table 1, Fig. 5a). The activity of each promoter was determined by the expression levels of GFP as described in Materials and methods. In this assay, promoter activity is measured as the rate of GFP production divided by the $OD_{600\text{ nm}}$ of culture at each time point (Ronen *et al.*, 2002; Rosenfeld *et al.*, 2002; Kalir *et al.*, 2005). The P_{rscDB} activated *rscB* expression after 3 h, during the early exponential growth phase, whereas the P_{rscB} promoted expression after 8 h, when the culture reached the stationary phase (Fig. 5b). Moreover, P_{rscB} displayed a weaker activity than the P_{rscDB} promoter. This assay was also carried out from strains growing at 37 °C in

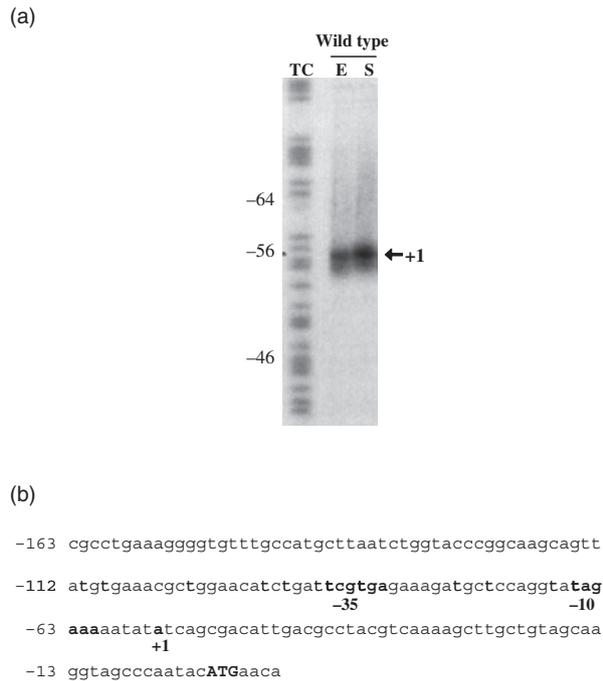


Fig. 4. Molecular analysis of the new *rcsB* promoter: (a) S1 mapping of *rcsB* transcripts produced by wild-type *Salmonella typhimurium* strain (14028s) growing until the exponential (E) or the stationary (S) phase in LB medium. Lane TC corresponds to the Maxam–Gilbert DNA ladder of the target sequence. The transcription start sites are marked with arrows. (b) DNA sequence corresponding to the 163-bp region upstream of the *rcsB* start codon (ATG). +1 corresponds to the transcription start site and the bold sequences are the predicted –10 and –35 boxes of the P_{rcsB} promoter.

50-mL Erlenmeyer flasks at time points of 4, 7 and 10 h, and similar promoter activities as above were observed. These results demonstrate that both promoters are able to support the transcriptional regulation of the *rcsB* gene at different stages of the bacterial growth, as was demonstrated for P_{rcsB} by an S1 nuclease assay (Fig. 4a).

Discussion

It has been reported previously that the expression of the regulon genes of many two-component systems (Soncini *et al.*, 1995; Wosten *et al.*, 2000; Oshima *et al.*, 2002; Takaya *et al.*, 2005) as well as those of the RcsCDB system (Castanie-Cornet *et al.*, 2007) are modulated by high levels of the regulator, even in the absence of the sensor. In this report, for the first time, it is demonstrated that *rcsB* overexpression significantly inhibits *rcsD* transcription, but has a weak effect on *rcsB* expression (Fig. 1), suggesting that the *rcsB* gene may be transcribed in an *rcsD*-independent manner. The identification of the P_{rcsB} promoter driving *rcsB* expression (Figs 4 and 5) as well as a small 0.67-kb

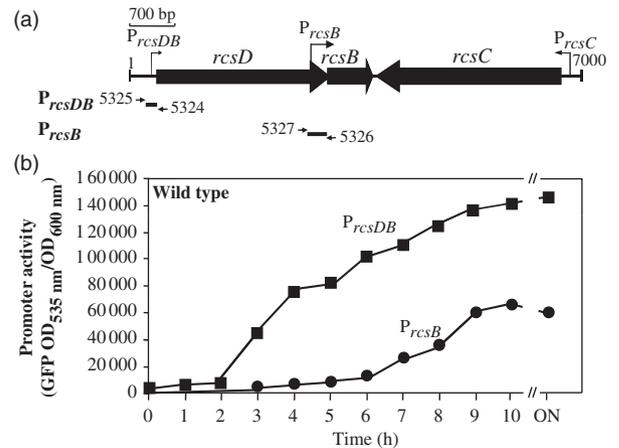


Fig. 5. Analysis of the P_{rcsDB} and P_{rcsB} promoter activities: (a) Graphic representation of the 122- and 131-bp regulatory regions of P_{rcsDB} and P_{rcsB} putative promoters (black lines), respectively, amplified by PCR with appropriate primers (represented by numbers) and cloned into the pMS201 vector to direct transcription of the promoterless *gfp* gene. (b) GFP activity produced by wild-type *Salmonella typhimurium* strain (14028s) harboring the above plasmids, and monitored as the rate of GFP production. Background fluorescence at a given OD_{600 nm} was determined from the fluorescence of cells bearing a promoterless GFP vector at the same OD_{600 nm}. The squares and circle indicate the activity of P_{rcsDB} and P_{rcsB} , respectively. Data correspond to the mean values of three independent experiments performed in duplicate. Error bars correspond to the SD.

transcript corresponding in size to the *rcsB* mRNA strongly support our notion (Fig. 3). However, Detweiler *et al.* (2003) suggested that a region of ≈ 500 nucleotides upstream of *rcsD* was required for the expression of both genes as an operon. Our findings have shown that differential *rcsD* and *rcsB* expression is due to the presence of two promoters controlling the *rcsB* transcription: (1) the P_{rcsDB} , suggested previously by Detweiler and colleagues, and (2) the P_{rcsB} described herein. In this work, the –35 and –10 boxes of the P_{rcsDB} promoter were localized at –32 and –10 bp upstream of the *rcsD* transcription start site, respectively (Fig. 2b).

Furthermore, it was demonstrated that the P_{rcsB} promoter induces *rcsB* transcription when the bacteria reach the stationary growth phase, while the P_{rcsDB} promoter modulates the operon expression during the exponential growth and is maintained to the later growth phase. In addition, we have shown that even when the P_{rcsB} is able to stimulate the *rcsB* expression, a weaker activity than the P_{rcsDB} promoter is shown.

The alignment of P_{rcsDB} and P_{rcsB} promoters with equivalent regions from other enterobacteria demonstrated that both are highly conserved (data not shown). In this alignment, strains of *E. coli*, *Klebsiella*, *Shigella*, *Enterobacter* and

different *Salmonella enterica* serovars were included. The localization of P_{*r*csB} inside the HPT domain-coding sequence suggests that this promoter could be conserved in all the analyzed strains. The presence of the conserved HPT domain allows the RcsD protein to serve as an intermediate in the phosphotransfer from RcsC to RcsB (Takeda *et al.*, 2001; Majdalani & Gottesman, 2005). We observed that the P_{*r*csB} promoter region displays 98% identity between the enterobacteria strains mentioned above (data not shown). This result suggests that the mechanism that controls the RcsB expression could also be conserved.

It was also demonstrated in other two-component regulatory systems that the genes that constitute the system are also transcribed from several promoters. This characteristic has been observed in 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 in *Bordetella pertussis* (Stibitz & Miller, 1994). Perhaps the presence of several promoters in the autoregulation mechanism that control the gene expression constitutes a general feature of these systems. It is important to note here that all of the above-cited systems are under a positive autoregulation mechanism, while the Rcs system is apparently negatively autoregulated. At present, we do not know the pathway by which the RcsB regulator represses *rcsD* expression; however, ongoing experiments are being directed toward elucidating the mechanism.

In summary, these results demonstrate that two promoters control the expression of the *rcsB* gene, which may result in an increase in the level of the RcsB protein. The RcsB regulator overproduction represses the P_{*r*csDB} activity by an unknown mechanism, decreasing the *rcsD* gene expression. Future experiments are being directed to study the expression regulation of Rcs system component genes.

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References

Aiso T & Ohki R (2003) Instability of sensory histidine kinase mRNAs in *Escherichia coli*. *Genes Cells* **8**: 179–187.

- Beeston AL & Surette MG (2002) *pfs*-dependent regulation of autoinducer 2 production in *Salmonella enterica* serovar Typhimurium. *J Bacteriol* **184**: 3450–3456.
- Blattner FR, Plunkett G III, Bloch CA *et al.* (1997) The complete genome sequence of *Escherichia coli* K-12. *Science* **277**: 1453–1474.
- Brill JA, Quinlan-Walsh C & Gottesman S (1988) Fine-structure mapping and identification of two regulators of capsule synthesis in *Escherichia coli* K-12. *J Bacteriol* **170**: 2599–2611.
- Cano DA, Dominguez-Bernal G, Tierrez A, Garcia-Del Portillo F & Casades 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 JP & Cam K (1999) Regulation of *Escherichia coli* cell division genes *ftsA* and *ftsZ* by the two-component system *rscC*–*rscB*. *Mol Microbiol* **34**: 442–450.
- Castanie-Cornet MP, Treffandier H, Francez-Charlot A, Gutierrez C & Cam K (2007) The glutamate-dependent acid resistance system in *Escherichia coli*: essential and dual role of the His-Asp phosphorelay RcsCDB/AE. *Microbiology* **153**: 238–246.
- Chen MH, 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 Biotech Bioch* **65**: 2364–2367.
- Clarke DJ, Holland IB & 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.
- Cormack BP, Valdivia RH & Falkow S (1996) FACS-optimized mutants of the green fluorescent protein (GFP). *Gene* **173**: 33–38.
- Costa CS & Anton DN (2001) Role of the *ftsA1p* 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 KA & Wanner BL (2000) One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. *P Natl Acad Sci USA* **97**: 6640–6645.
- Davalos-Garcia M, Conter A, Toesca I, Gutierrez C & Cam K (2001) Regulation of *osmC* gene expression by the two-component system *rscB*–*rscC* in *Escherichia coli*. *J Bacteriol* **183**: 5870–5876.
- Delgado MA, Mouslim C & Groisman EA (2006) The PmrA/PmrB and RcsC/YojN/RcsB systems control expression of the *Salmonella* O-antigen chain length determinant. *Mol Microbiol* **60**: 39–50.
- Detweiler CS, Monack DM, Brodsky IE, Mathew H & Falkow S (2003) *virK*, *somA* and *rscC* are important for systemic *Salmonella enterica* serovar Typhimurium infection and cationic peptide resistance. *Mol Microbiol* **48**: 385–400.
- Ebel W, Vaughn GJ, Peters HK III & Trempy JE (1997) Inactivation of *mdoH* leads to increased expression of colanic acid capsular polysaccharide in *Escherichia coli*. *J Bacteriol* **179**: 6858–6861.

- Ellermeier CD, Janakiraman A & Slauch JM (2002) Construction of targeted single copy *lac* fusions using lambda Red and FLP-mediated site-specific recombination in bacteria. *Gene* **290**: 153–161.
- Ferrieres L & Clarke DJ (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 PI, Swanson RV, Haidaris CG & Heffron F (1986) Mutants of *Salmonella typhimurium* that cannot survive within the macrophage are avirulent. *P Natl Acad Sci USA* **83**: 5189–5193.
- Francez-Charlot A, Laugel B, Van Gemert A, Dubarry N, Wiorowski F, Castanie-Cornet MP, Gutierrez C & Cam K (2003) RcsCDB His-Asp phosphorelay system negatively regulates the *flhDC* operon in *Escherichia coli*. *Mol Microbiol* **49**: 823–832.
- Garcia Vescovi E, Soncini FC & Groisman EA (1996) Mg²⁺ as an extracellular signal: environmental regulation of *Salmonella* virulence. *Cell* **84**: 165–174.
- Gottesman S, Trisler P & Torres-Cabassa A (1985) Regulation of capsular polysaccharide synthesis in *Escherichia coli* K-12: characterization of three regulatory genes. *J Bacteriol* **162**: 1111–1119.
- Guan CD, Wanner B & Inouye H (1983) Analysis of regulation of *phoB* expression using a *phoB-cat* fusion. *J Bacteriol* **156**: 710–717.
- 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**: 1–6.
- Kelley WL & 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.
- Majdalani N & Gottesman S (2005) The Rcs phosphorelay: a complex signal transduction system. *Annu Rev Microbiol* **59**: 379–405.
- Miller JH (1972) *Experiments in Molecular Genetics*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
- Mizuno T (1997) Compilation of all genes encoding two-component phosphotransfer signal transducers in the genome of *Escherichia coli*. *DNA Res* **4**: 161–168.
- Mousslim C & Groisman EA (2003) Control of the *Salmonella* *ugd* gene by three two-component regulatory systems. *Mol Microbiol* **47**: 335–344.
- Mousslim C, Latifi T & Groisman EA (2003) Signal-dependent requirement for the co-activator protein RcsA in transcription of the RcsB-regulated *ugd* gene. *J Biol Chem* **278**: 50588–50595.
- Mousslim C, Delgado M & Groisman EA (2004) Activation of the RcsC/YojN/RcsB phosphorelay system attenuates *Salmonella* virulence. *Mol Microbiol* **54**: 386–395.
- Oshima T, Aiba H, Masuda Y, Kanaya S, Sugiura M, Wanner BL, Mori H & Mizuno T (2002) Transcriptome analysis of all two-component regulatory system mutants of *Escherichia coli* K-12. *Mol Microbiol* **46**: 281–291.
- Ronen M, Rosenberg R, Shraiman BI & Alon U (2002) Assigning numbers to the arrows: parameterizing a gene regulation network by using accurate expression kinetics. *P Natl Acad Sci USA* **99**: 10555–10560.
- Rosenfeld N, Elowitz MB & Alon U (2002) Negative autoregulation speeds the response times of transcription networks. *J Mol Biol* **323**: 785–793.
- Sambrook J, Fritsch EF & Maniatis T (1989) *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
- Sanderson KE, Hessel A & Rudd KE (1995) Genetic map of *Salmonella typhimurium*, edition VIII. *Microbiol Rev* **59**: 241–303.
- Shiba Y, Yokoyama Y, Aono Y, Kiuchi T, Kusaka J, Matsumoto K & Hara H (2004) Activation of the Rcs signal transduction system is responsible for the thermosensitive growth defect of an *Escherichia coli* mutant lacking phosphatidylglycerol and cardiolipin. *J Bacteriol* **186**: 6526–6535.
- Soncini FC, Vescovi EG & Groisman EA (1995) Transcriptional autoregulation of the *Salmonella typhimurium* *phoPQ* operon. *J Bacteriol* **177**: 4364–4371.
- Stibitz S & Miller JF (1994) Coordinate regulation of virulence in *Bordetella pertussis* mediated by the *vir* (*byg*) locus. *Molecular Genetics of Bacterial Pathogenesis* (Miller VL, Kaper JB, Portney DA & Isberg RR, eds), pp. 407–422. American Society of Microbiology, Washington, DC.
- Stout V & Gottesman S (1990) RcsB and RcsC: a two-component regulator of capsule synthesis in *Escherichia coli*. *J Bacteriol* **172**: 659–669.
- Takaya A, Suzuki A, Kikuchi Y, Eguchi M, Isogai E, Tomoyasu T & Yamamoto T (2005) Derepression of *Salmonella* pathogenicity island 1 genes within macrophages leads to rapid apoptosis via caspase-1- and caspase-3-dependent pathways. *Cell Microbiol* **7**: 79–90.
- 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.
- Virlogeux I, Waxin H, Ecobichon C, Lee JO & Popoff MY (1996) Characterization of the *rcaA* and *rcaB* genes from *Salmonella typhi*: *rcaB* through *tviA* is involved in regulation of Vi antigen synthesis. *J Bacteriol* **178**: 1691–1698.
- Winans SC, Mantis NJ, Chen CY, Chang CH & Han DC (1994) Host recognition by the VirA, VirG two-component regulatory proteins of *Agrobacterium tumefaciens*. *Res Microbiol* **145**: 461–473.
- Wosten MM, Kox LF, Chamnongpol S, Soncini FC & Groisman EA (2000) A signal transduction system that responds to extracellular iron. *Cell* **103**: 113–125.

Zwir I, Shin D, Kato A, Nishino K, Latifi T, Solomon F, Hare JM, Huang H & Groisman EA (2005) Dissecting the PhoP regulatory network of *Escherichia coli* and *Salmonella enterica*. *P Natl Acad Sci USA* **102**: 2862–2867.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Primers used in this work.

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