Regulatory effect of SlyA on *rcsB* expression in *Salmonella* Typhimurium

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Running title: *rcsB* gene regulation by SlyA

Abstract

The *Salmonella* Typhimurium RcsCDB system regulates the synthesis of colanic acid and flagellum as well as the expression of virulence genes. We previously demonstrated that the *rcsC11* mutant, which constitutively activates the RcsB regulator, attenuates *Salmonella* virulence in an animal model. This attenuated phenotype could be also produced by deletion of the *slyA* gene. In this work, we investigated if this antagonistic behavior is produced by the modulation of the expression of both regulator-encoding genes. We demonstrated that SlyA overproduction negatively regulates *rcsB* transcription. A bioinformatic analysis allowed us to identify a putative SlyA binding site on each of both promoters, P_{rcsDB} and P_{rcsB}, which control *rcsB* transcriptional levels. We also determined that SlyA is able to recognize and bind to these predicted sites to modulate the activity of both *rcsB* promoters. According to these results, SlyA represses *rcsB* transcription by direct binding to specific sites located on the *rcsB* promoters, thus accounting for the attenuate/virulence antagonistic behaviors. Moreover, we showed that the opposite effect between both regulators also physiologically affects the *Salmonella* motility phenotype. In this sense, we observed that under SlyA overproduction P_{rcsB} is repressed and consequently bacterial motility is increased. On the basis of these results, we suggest that during infection the different RcsB levels produced act as a switch between the *Salmonella* virulent or attenuated form. Thereby, we propose that higher concentrations of RcsB tilt the balance towards the attenuated form, while absence or low concentrations resulting from SlyA overproduction does it towards the virulent form.

Importance

47 The antagonistic behavior of RcsB and SlyA on virulence genes expression led us to hypothesize
48 that both regulators interplay in a regulatory network and could be considered coordinators of
49 this process. We here report that the SlyA virulence factor influence the motility behavior by
50 controlling the *rcsB* transcription from the P_{rcsB} promoter. We also demonstrate that SlyA
51 negatively affect the expression of *rcsB* gene by a direct binding to P_{rcsDB} and P_{rcsB} promoters.
52 We here suggest that different levels of RcsB act as a switch between the virulent and attenuated
53 forms of *Salmonella*, where high concentrations of the regulator tend to tilt the balance towards
54 the attenuated form, while low concentrations or its absence do this towards the virulent form.
55
56 **Introduction**
57
58 *S.* Typhimurium virulence is directly related to the presence of pathogenicity islands (SPI),
59 containing virulence genes. *Salmonella* virulence is controlled by a complex interplay between
60 different transcriptional regulators that modulate the expression of these genes (1). Among the
61 major regulators involved in this process we can mention to RcsB, PhoP, PmrA, SlyA and OmpR
62 (1-6). Several reports demonstrate that the above interactions coordinately control the expression
63 of the genes involved in pathogen adhesion, invasion, replication or survival within
64 macrophages, in a specific time and space (7).
65 The SlyA transcriptional factor belongs to the MarR (Multiple antibiotic resistance Regulator)
66 regulator family, which are distributed in both Archaea and Bacteria. The MarR family members
67 regulate a large number of cellular processes including resistance to antibiotics, organic solvents,
68 disinfectants and oxidative stress-generating agents (8, 9). These kinds of regulators are linked to
69 the induction of virulence in pathogenic bacteria in humans and plants (8). The *slyA* gene was

70 isolated from *S.* Typhimurium and characterized to confer a hemolytic phenotype on *Escherichia*
71 *coli* K-12 (10, 11). Further studies reveal that the *slyA* gene of *S.* Typhimurium is required to
72 control the expression of the SPI-2 virulence genes (12). Furthermore, it is reported that this
73 transcriptional factor is also involved in the regulation of genes required for adhesion and
74 survival within macrophages as well for resistance to H_2O_2, magainin 2 and polymyxin B (6, 10,
75 13-16). Notably, Libby *et al.* (1994) reported that the *slyA* mutant show a virulence attenuation
76 phenotype in mouse infection assays.

77 The RcsB protein is the transcriptional regulator that belongs to the RcsCDB phosphorelay
78 system (17). This is an unusual two component system because consists of three proteins: RcsC
79 and RcsD, which acts as sensor proteins, and RcsB, which is the response regulator (18, 19).
80 Other components of the system are the upstream regulators RcsF and IgaA (17). RcsF is a
81 lipoprotein anchored to the outer membrane that can transduce stress signal to RcsC, but is not
82 fully required for RcsCDB system activation (20). We demonstrated that the *Salmonella rcsB*
83 gene is controlled by two promoters, P_{rcsDB} and P_{rcsB}, whose activities depend on the bacteria
84 growth phase, causing that the *rcsB* regulator be expressed with *rcsD* in a bicistronic transcript
85 and/or as a monocistronic mRNA (21). In addition, we showed that high levels of the RcsB-
86 active form control the *rcsDB* transcript, since RcsB is able to bind to the P_{rcsDB} promoter, thus
87 inhibit its activity, but not to the P_{rcsB} promoter (22).
88 Interestingly, it was reported that the activation of the RcsB regulator affects both invasion and
89 replication inside of eukaryotic cells, repressing SPI-2 genes (23-25). Therefore, the presence of
90 the RcsB factor causes *Salmonella* attenuation, while deletion of the *rcsB* gene increases the
91 virulence phenotype (23, 26). In contrast, an opposite effect was reported for SlyA, whose
92 overproduction or activation increases virulence through a positive control exerted on SPI-2

93 genes (12), being the *slyA* gene mutant attenuated in virulence in a mouse infection model (10).
94 On the basis of the antagonistic behavior of RcsB and SlyA on virulence, we decided to
95 investigate if these transcriptional factors interplay in a regulatory network that could account for
96 the attenuated/virulent phenotype. Since both proteins are transcriptional factors, we
97 hypothesized that such effect would be based on a cross modulation of their encoding genes.
98 Here, we reported that the SlyA transcriptional factor exerts a negative and direct control on
99 *rcsB* transcription. Our results revealed a putative SlyA binding sequence upstream of the P_{rcsDB}
100 and P_{rcsB} promoters. We demonstrated that this regulator binds to DNA fragments containing
101 these promoters. We also found that the repression of *rcsB* transcription, produced by SlyA
102 overproduction, affected *Salmonella* motility behavior. Our results allow us to suggest that
103 during the *Salmonella* infection steps, bacteria reach different RcsB protein levels, controlled by
104 SlyA, which acts as a switch between the virulent or the attenuated bacterial state. The virulent
105 state is also favored during the stationary phase by increased SlyA concentration, explaining the
106 antagonist effects of the RcsB and SlyA regulators on *Salmonella* pathogenesis.
107
108 **Results**
109
110 **SlyA downregulates the *rcsB* expression**
111 The hypothesis that RcsB and SlyA affect each other's activity, led us to investigate the role of
112 SlyA on *rcsB* expression. To test this assumption, we measured the β-galactosidase activity
113 produced by the chromosomal transcriptional *rcsB*::*lacZY* fusion in wild type and *slyA* genetic
114 backgrounds. As shown in Fig. 1, β-galactosidase levels in the *slyA* mutant were 1.6-fold higher
115 than those observed in the wild type strain, suggesting that SlyA regulator is able to repress

116 transcription of *rcsB* gene. In order to confirm that the effect displayed in the *slyA* mutant was
117 not due to a polar effect produced by the gene deletion, we complemented *in trans* the *slyA*
118 mutation. Here, we observed that the *rcsB* expression was repressed by *slyA* overexpression,
119 since the β-galactosidase levels were 3.2-fold lower in the wild type strain in the presence of the
120 p*slyA* plasmid and IPTG. Moreover, in the *slyA* mutant harboring p*slyA*, β-galactosidase levels
121 were also repressed by IPTG addition, while maximal repression levels was observed in the wild
122 type strain overexpressing the *slyA* and *rcsB* plasmidic genes (Fig. 1A and B). We here suggest
123 that the SlyA negative effect was independent of the RcsB-autoregulatory mechanism exerted on
124 its own transcription, since the levels of *rcsB* expression in wild type and in *slyA* mutant both
125 harboring p*rcsB** were similar under arabinose induction (Fig. 1B). These results demonstrate
126 that SlyA represses *rcsB* transcription, suggesting that SlyA could act on one or both *rcsB*
127 promoters.
128
129 **Bioinformatics analysis of the *rcsB* promoters**
130 In order to determine whether the *rcsB* promoters were affected by SlyA, we decided to
131 analyze both *rcsB* promoter sequences. For this purpose, we performed an alignment between the
132 P_{rcsDB} or P_{rcsB} promoter regions with the previously described SlyA consensus box (15), using the
133 Multiple Expectation Maximization for motif Elicitation (MEME) tools (27). First, we studied
134 the -248 bp region upstream of the *rcsDB* operon coding region, corresponding to the P_{rcsDB}
135 promoter, and we observed the presence of a putative SlyA box located at -200 bp of the *rcsD*
136 start codon and -127 bp of the conserved -35 box (Fig. 2A). It is important to mention that *rcsD*
137 is the first gene in the *rcsDB* operon controlled by the P_{rcsDB} promoter. The results obtained
138 showed that this putative binding site displays 66.67% identity with the SlyA consensus box,

keeping intact the first half of the palindrome (Fig. 2, lower panels). On the other hand, when we aligned the SlyA consensus box with the P_{rcsB} promoter region, we found another sequence that could serve as a SlyA binding site. This new site was located within the *rcsD* encoding sequence, at 8 bp upstream of the *rcsB* translational start codon (Fig. 2B). The location of this putative SlyA binding box suggests that it could function as a negative regulation site for *rcsB* transcription (Fig. 2B). Furthermore, this putative SlyA binding site showed a 75% DNA sequence identity with the palindromic consensus sequence reported by Stapleton *et al*. (2002) (Fig. 2B). These results indicate that both transcript *rcsDB* and *rcsB* could be controlled by the SlyA virulence transcriptional factor.

SlyA overproduction affects the activity of P_{rcsDB} and P_{rcsB} promoters

In order to examine the role of SlyA on the activity of each of the *rcsB* promoters, we decided to study *rcsB* expression under SlyA overproduction, using single promoter mutants (Fig. 3A). These assays were carried out using the *rcsB*::*lacZY* chromosomal transcriptional fusion, in which the *rcsB* genes was eliminated in order to avoid any effect of this regulator on its own expression (Fig. 3A). When the P_{rcsDB} mutant was analyzed, where *rcsB* is expressed by P_{rcsB} activity, we observed that the *slyA* overexpression produces a strong decrease in β-galactosidase levels with respect to those obtained without IPTG or with the empty vector (Fig. 3B). Interestingly, when *rcsB* was transcribed from P_{rcsDB} promoter, in the P_{rcsB} mutant, the β-galactosidase levels were decreased by *slyA* overexpression compared to the control without IPTG or to the empty vector (Fig. 3B). Moreover, the *rcsB* repression levels of each mutant by SlyA increased amount were lower than those determined in the wild type background grown

under the same condition (Fig. 3B). These data demonstrate that SlyA modulates the activity of both promoters, P_{rcsDB} and P_{rcsB}, downregulating not only *rcsB* but also *rcsD* transcription.

SlyA protein binds to the *rcsB* promoters *in vitro*

Based on the results described above, we investigated whether SlyA could bind to the P_{rcsDB} and P_{rcsB} promoters and modulate *rcsB* and *rcsD* expression. To this end, we performed EMSAs using PCR products containing the P_{rcsDB} or P_{rcsB} promoter regions, both harboring the putative SlyA binding sites previously identified (467 bp and 126 bp, respectively). In addition, we used a 122 bp deleted PCR product of the P_{rcsDB} and a 102 bp deleted PCR product of the P_{rcsB} promoter regions lacking the putative SlyA regulatory box, as negative control.

As shown in Fig. 4A and B, the SlyA protein specifically bound to both P_{rcsDB} and P_{rcsB} promoters. We observed the appearance of a slower migration band when each PCR product was incubated with the SlyA-His6 protein. These bands were more intense at higher SlyA-His6 concentrations, while staining intensity decreased in the band of PCR product alone. These observations suggested that the lower migration bands corresponded to the DNA/SlyA-His6 complex, since these bands were not observed when the PCR products lacking the SlyA binding box was analyzed (Fig. 4A and B). Remarkably, we noticed that higher concentrations of SlyA-His6 were required to observe the P_{rcsB} promoter shift, compared to the optimal concentration to shift the P_{rcsDB} promoter (Fig. 4A and B). Taken together, these results demonstrate that SlyA could binds to the sequences identified by bioinformatics analysis on the P_{rcsDB} and P_{rcsB} promoter regions.

Physiological role of *rcsB* repression mediated by SlyA

We hypothesized that SlyA repression of *rcsB* transcription could affect the motility behavior of *Salmonella*, since both regulators also participate in the regulation of flagellar gene expression (6, 28-30). In order to investigate this assumption, we studied the swimming phenotype of the *S.* Typhimurium wild type strain, *rcsB* and *rcsC11* mutants; and of the wild type, *rcsB*, P_{rcsDB} and P_{rcsB} strains harboring the p*slyA* plasmid. The *rcsB* and *rcsC11* mutants were used as motility-phenotype controls of the RcsCDB system activation effect. As shown in Fig. 5, we observed that the *rcsB* mutant displayed a migration rate 1.43-fold greater than the wild type strain, while the *rcsC11* mutant did not swim, which is in agreement with previous results (28, 31). In this assay we observed that the wild type strain overexpressing *slyA* displayed a 1.25-fold lower migration rate than without *slyA* induction (Fig. 5, + and – IPTG, respectively). We also detected that the motility of the P_{rcsB} mutant harboring p*slyA* decreased 1.34-fold in the presence of IPTG compared to the same mutant growing in its absence (Fig. 5). Interestingly, an opposite motility phenotype was observed by the P_{rcsDB} mutant when the *slyA* gene was overexpressed, displaying a 1.3-fold increase in the migration rate compared to the same strain without IPTG addition (Fig. 5). Moreover, the increased migration levels were similar to those observed in the *rcsB* mutant overexpressing *slyA* (Fig. 5). These data would indicate that the increased motility displayed by the P_{rcsDB} mutant could be due to the SlyA repression effect on P_{rcsB}, a weak activity promoter. Taken together, these results demonstrated that SlyA activation would significantly affect the motility behavior only at low RcsB levels.

To confirm the SlyA repression effect on *rcsB*, we used other RcsB-dependent gene like *dps* (46). When the levels of *dps*::*lacZY* transcription were determined we observed that *slyA* overexpression decreased such *dps* expression in wild type but not in *rcsB* strain (Fig. 5C).

206 We here demonstrate that the repression of *rcsB* by SlyA regulator results in a modulating
207 effect of the RcsB dependent genes.
208
209 **Discussion**
210 It is known that the *Salmonella* infection process is controlled by a network of transcriptional
211 regulators acting to modulate many virulence genes. Numerous interplays are described: i-
212 PhoPQ and PmrAB systems interact to remodel the lipid A in order to resist the bactericidal
213 action of cationic peptides and to replicate within macrophages (32, 33); ii- PhoPQ and RcsCDB
214 interact to coordinately control the expression of *pag* and *ugd* genes required for lipid A
215 modification, colanic acid synthesis, and several virulence genes (34, 35); iii- PhoPQ and SpiR-
216 SsrB interact to control the genes located in SPI-2, which are necessary to allow the maturation
217 of *Salmonella*-containing vacuoles (SCV) within the host cell (2); iv- SpiR-SsrB and
218 OmpR/EnvZ interact to control genes encoding the SPI-2-type III secretion system, required for
219 bacterial replication inside macrophages and to establish systemic infection (5, 36, 37); v-
220 SirA/BarA interacts with CsrAB to induce the *hilA* expression, whose product acts as a master
221 regulator of the SPI-1 genes required to establish eukaryotic cell invasion (38). However, at the
222 moment no RcsB/SlyA interaction has been reported.
223 Libby *et al.* (1994) demonstrated that SlyA induces the SPI-2 virulence genes; the *slyA*
224 deletion attenuates the *Salmonella* virulence (10). However, an opposite effect is reported for
225 RcsB, whose activation represses both invasion and intracellular replication genes, attenuating
226 *Salmonella* virulence (23-25). Therefore, *rcsB* gene deletion from the bacterial chromosome
227 displays a greater infective capacity. On the basis of this observation, we hypothesized that the
228 opposite effect of SlyA and RcsB on virulence genes may be due to a cross regulation

mechanism exerted by one of these factors on the gene that codes for the opposite factor, and vice versa.

In this work we report for the first time that an overlapping regulatory effect exists between SlyA and RcsB regulators on *Salmonella* motility behavior. Previously, we demonstrated that activation of the P_{rcsDB} promoter starts in early exponential growth face and maintains its activity along with growth; while the P_{rcsB} promoter becomes active in stationary phase (21). On the other hand, it was reported that the physiological *slyA* gene expression was induced during the stationary growth phase (13). Considering these data we assumed that SlyA affects *rcsB* expression. We found that P_{rcsDB} and P_{rcsB} promoter sequences harbor a SlyA regulatory box as imperfect palindrome sequences, with 66.67% and 75% of identity with the consensus. Accordingly, other authors also reported an imperfect but functional palindrome for the SlyA binding on the *ugtL* and *slyA* promoter regions (15, 39), indicating that those motifs identified on the P_{rcsDB} and P_{rcsB} sequence could function as SlyA regulatory sites. We confirmed these results by EMSAs, where the SlyA was able to shift the mobility of P_{rcsDB} and P_{rcsB} PCR products. Meanwhile, deletion of SlyA binding nucleotides on the P_{rcsDB} and P_{rcsB} PCR products prevented SlyA binding to these products. These results indicate that the predicted sequences are effectively the site of SlyA regulation on the *rcsB* promoters. It is important to note that the formation of P_{rcsB}/SlyA-His6 complex required a higher protein levels than that required for P_{rcsDB}/SlyA complex. This result is consistent with a higher affinity of SlyA for the P_{rcsDB} promoter site. This possibility is supported by the different levels of identity displayed by each SlyA binding box found when they were aligned with the SlyA consensus site described by Stapleton *et al*. (2002).

Based in the results described above, we studied which of *rcsB* promoters was modulated by SlyA. We demonstrated that overexpression of *slyA* resulted in decreased *rcsB* expression levels from both promoter mutants, suggesting that SlyA represses *rcsB* transcription mainly during stationary phase. Moreover, when the *slyA* gene was remove from the *Salmonella* chromosome, the *rcsB* expression level increased 1.6-fold. Similarly, *rcsD* expression is consequently repressed by high amounts of SlyA, only when the P_{rcsDB} activity is affected as expected. In agreement with Dolan *et al*. (2011), who reported that the SlyA/DNA complex produces a conformational change on DNA topology and its bending, we propose that this effect could prevent the recruitment of RNA polymerase to *rcsB* promoters and prevent their transcription.

In order to evaluate the physiological importance of SlyA on the RcsCDB system components, we analyzed motility since SlyA and RcsB are involved in the control of this process. Our results led us to conclude that SlyA increases bacterial motility by repressing *rcsB* transcription from the P_{rcsB} promoter. Interestingly, Mouslim and Hughes (2014) observed that SlyA had no effect on the motility phenotype. In fact, they reported that this regulator represses the activity of the $P5_{flhDC}$ flagellar promoter, which in turn controls the *flhDC* operon expression during the stationary phase (30). These results may differ from ours due to the fact that Mouslim and Hughes only investigate the effect on motility of SlyA absence. In previous studies, we demonstrated that *rcsB* is constitutively expressed at very low levels by P_{rcsB} activity. However, these basal levels are not enough to maintain certain motility in the wild type strain. Meanwhile, the complete deletion of the *rcsB* gene produces an exacerbated motility phenotype (22). Taking into account that: i- the activity of $P1_{flhDC}$ ensures the synthesis of flagellum positively controlling motility; ii- $P1_{flhDC}$ is negatively affected by RcsB, even at basal levels; and iii- high levels of SlyA repress the expression of *rcsB* from its two promoters, hence no repression of

274 P1$_{flhDC}$ take place; we here propose that SlyA activation indirectly modulates *Salmonella* motility
275 in a RcsB-dependent pathway. Similar effect was observed using other RcsB-dependent gene
276 like *dps*, the *slyA* overexpression decreased the levels of *dps* in wild type but not in *rcsB* strain.
277 Therefore, our results complement Mouslim and Hughes (2014) findings demonstrating that
278 there is a negative interaction of SlyA and RcsB in the control of numerous virulence factors
279 depending on the environment, exemplified by the regulation of flagella and motility behavior.
280 The results obtained in this work represent an important advance in the knowledge of the role
281 of the RcsCDB system in *Salmonella* pathogenesis, and of the role that SlyA plays in the control
282 of the RcsCDB system components in this bacterial. Thus, we suggest that different levels of
283 RcsB act as a switch between the virulent and attenuated forms of *Salmonella*, represented in the
284 model of Fig. 6: (A) high concentrations of the regulator tilts the balance towards the attenuated
285 form, (B) while low concentrations or their absence do this towards the virulent form. Based on
286 our results, SlyA represents one additional mechanism by which *Salmonella* controls this
287 balance, through regulation of the *rcsB* transcription.
288
289
290 **Materials and Methods**
291
292 **Bacterial strains, molecular techniques and growth conditions**
293 The bacterial strains used in this work are listed in Table 1. Bacteria were routinely grown in
294 Luria-Bertani (LB) broth or agar plates. The double mutant strains construction was conducted
295 by P22 phage-mediated transduction (40). Standard methods for DNA recombination and
296 bacterial growth were used as previously described (41). β-galactosidase assay were performed

following the protocol developed by Miller (1972) (42). Kanamycin (50 μg/ml), ampicilin (50 μg/ml) and chloramphenicol (25 μg/ml) were added to the medium for antibiotic selection.

Plasmid construction

Plasmid p*slyA*-His6 was constructed by amplifying the *slyA* gene from wild type 14028s chromosomal DNA with primers #8081 (CGGATCCTTGGAATCGCCACTAGGTTC) and #8082 (CCCAAGCTTAATCGTGAGAGTGCAATT). Then, the PCR product was digested with *Bam*HI and *Hin*dIII and subsequently cloned into the corresponding sites of pACYC-Duet1 vector (Novagen), which encodes the six-histidine tag sequence. The correct *slyA*-His6 DNA sequence was confirmed by sequencing.

SlyA purification

E. coli BL21 (DE3) harboring plasmid p*slyA*-His6 was grown at 37°C at OD_{600}=0.2 and then 0.5 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG) was added and the culture was incubated for another 3 h at 30°C. Cells were harvested by centrifugation and resuspended in 5 ml of purification buffer (300 mM NaCl; 30 mM NaH_2PO_4 pH 8.3) and then were disrupted with a French Press. The cell lysate was mixed with HIS-Select Nickel Affinity Gel (SIGMA) following the manufacturer instructions, and incubated overnight at 4°C. Then, the resin containing proteins was washed 3 times with lysis buffer including 15 and 30 mM of imidazole by centrifugation at 3,000 X *g*. Finally, the SlyA-H6 protein was eluted from the Ni-NTA resin using 100 mM imidazole, which was stored with glycerol (50%, v/v) and at -70°C.

Electrophoretic mobility shift assay (EMSA)

EMSAs were carried out as previously described (43). Briefly, two fragments containing the putative SlyA binding site of P_{rcsDB} and P_{rcsB} promoters (467 bp and 126 bp, respectively) were generated by PCR using primers Fwd P_{rcsDB} (CCGCTCGAGCGGGTTAAATTGATGAAATTC) /Rev P_{rcsDB} (CGCGGATCCTTATGTTTACGACCTGTAAAAG) and Fwd P_{rcsB} (CCGCTCGAG GGTACCCGGCAAGCAGTTATGTG)/ Rev P_{rcsB} (CGCGGATCCGTATTGGGCTACCTTGC TACAG) respectively, and using wild type 14028s chromosomal DNA as template. In addition, two smaller fragments of the P_{rcsDB} and P_{rcsB} promoters were used as control (122 bp and 102 bp, respectively) and generated by PCR, lacking the SlyA binding site, using the set of primers Fwd P_{rcsDB} control (CTCGAGCCCGTCCCGCCGACGGAGCGCG)/Rev P_{rcsDB} and Fwd P_{rcsB}/Rev P_{rcsB} control (TTGACGTAGGCGTCAATGTCGC), respectively. These DNA fragments were incubated with 100, 200 or 300 nM of SlyA-H6 protein in binding buffer (25 mM Tris/HCl pH 8, 50 mM NaCl, 5 mM $MgCl_2$, 5 mM DTT and 10% glycerol) at room temperature for 20 min. The samples were run on a 6% polyacrylamide non-denaturing gel using 0.38× Tris-borate-EDTA buffer, at 8 mA for 3 hours. Gels were stained with ethidium bromide and visualized under UV irradiation.

Motility assays

Swimming plates were performed following the protocol previously described (44). Briefly, single colonies from overnight streak plates were placed into swimming agar using toothpicks (10 g/l tryptone, 5 g/l NaCl and 0.35% agar) and incubated at 37°C for 4 h. An average of at least eight independent colonies was performed for each strain assayed.

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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	(45)
EG14932	*rcsB*::*lacZY*	(21)
MDs1026	*rcsB*::*lacZY* P_{rcsDB}::Cm	(22)
MDs1027	*rcsB*::*lacZY* P_{rcsB}::Cm	(22)
EG14078	*slyA*::Cm	(39)
MDs1138	*rcsB*::*lacZY slyA*::Cm	This work
EG12711	*rcsB*::Cm	(22)
EG14873	*rcsC11*	(23)
MDs1017	P_{rcsDB}::Cm	(22)
MDs1018	P_{rcsB}::Cm	(22)
MDs1568	*dps*::*lacZY*	(46)
MDs1569	*dps*::*lacZY rcsB*::Cm	(46)
Plasmids		
pUHE2-2*lacI*^{q}	rep_{pMB1} Ap^r *lacI*^{q}	(47)
p*rcsB*	pUHE2-21 *lacI*^{q} containing *rcsB* gene	(21)
pBAD33	pBR322 *ori*, arabinose induction, Cm	(48)
p*rcsB**	pBAD33 containing *rcsB* gene	This work
p*slyA*	pUHE2-21 *lacI*^{q} containing *slyA* gene	(39)
pACYCDuet-1	P15A *ori lacI* T7*lac* Cm	Novagen
p*slyA*-His6	pACYCDuet-1 containing *slyA* gene	This work

Gene designations are summarized by Sanderson *et al* (1995). (49)

547 **Figure Legends**

548

549 **Fig. 1. The *rcsB* gene transcription is modulated by the SlyA regulator.** (**A**) The ß-
550 galactosidase activity (Miller units) of the *rcsB*::*lacZY* chromosomal transcriptional fusion was
551 investigated in the wild type (EG14932) and *slyA* (MDs1138) strains, harboring the p*slyA*
552 plasmid and grown at 37 ºC in LB medium in absence (-, white bars) or presence (+, black bars)
553 of 0.5 mM IPTG. (**B**) The *rcsB*::*lacZY* expression was measured in the wild type (EG14932)
554 strains harboring p*slyA*, p*rcsB** or both plasmids, as well in *slyA* (MDs1138) mutant containing
555 p*rcsB** plasmid, to analyzed the effect of RcsB autoregulation. These strains were grown in same
556 above condition and under induction (+) or not (-) of *slyA* and *rcsB* plasmidics gene with IPTG
557 or arabinose, respectively. In these assays the empty vectors were used as control, under IPTG or
558 arabinose treatment, respectively. The data correspond to the average of three independent
559 experiments, performed in duplicate. The error bars correspond to standard deviation. The
560 asterisk represent statistically different values (Tukey test, p=0.05).

561 **Fig. 2. Sequence analysis of the *rcsB* promoter regions.** DNA sequence (upper panel) and
562 alignment (lower panel) of the SlyA consensus box with the *rcsDB* (**A**) and *rcsB* (**B**) promoter
563 regions (248 bp and 163 bp, respectively). The start codon of the *rcsDB* operon or *rcsB* gene, -35
564 and -10 boxes are indicated with bold letters. The SlyA binding sequences found in this work are
565 boxed. The numbering is referred from the start codon of the *rcsDB* operon (**A**) and *rcsB* gene
566 (**B**). In the sequence alignments, the conserved nucleotides positions of putative SlyA binding
567 sites are highlighted in bold.

568 **Fig. 3. Effect of *slyA* overexpression on the P_{rcsDB} or P_{rcsB} promoter activities. A)** Graphic
569 representation of the mutant backgrounds in which the *rcsB* gene transcriptional expression was

570 determined. The empty spaces between brackets correspond to each promoter deletion. **B)** The ß-
571 galactosidase activity (Miller units) of the *rcsB*::*lacZY* chromosomal transcriptional fusion was
572 investigated in the wild type (EG14932) strain and P_{rcsDB} (MDs1026) and P_{rcsB} (MDs1027)
573 promoter mutants carrying the p*slyA* plasmid and grown to stationary phase at 37 ºC in LB
574 medium, in absence (-, white bars) or presence (+, black bars) of 0.5 mM IPTG. The empty
575 vector was used as control, under IPTG treatment. The data correspond to the average of three
576 independent experiments, performed in duplicate. The error bars correspond to standard
577 deviation. The asterisk represent statistically different values (Tukey test, p=0.05).

578 **Fig. 4. The SlyA protein binds to the P_{rcsDB} and P_{rcsB} promoters.** The electrophoretic mobility
579 shift assay (EMSA) was performed using 2 pmol of P_{rcsDB} (**A**), P_{rcsB} (**B**) or control P_{rcsDB} (**C**)
580 PCR products and different SlyA-His6 protein concentrations (100, 200 or 300 nM, from left to
581 right) where a control was performed without protein (-). The DNA fragments were separated on
582 6% polyacrylamide gel and visualized with ethidium bromide to detect the SlyA/DNA complex.

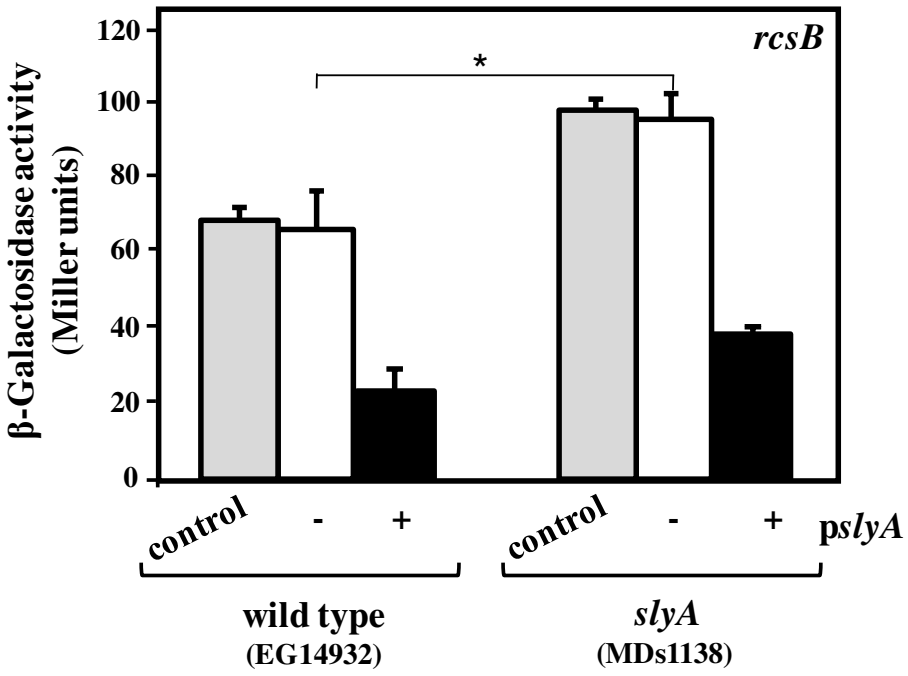
583 **Fig. 5. *Salmonella* RcsB-dependent motility phenotype.** (**A**) Motility rate was determined
584 in the wild type *S.* Typhimurium 14028s strain and *rcsB* (EG12711), *rcsC11* (EG14873) mutants
585 (gray bars), and in wild type, *rcsB* (EG12711), P_{rcsDB} (MDs1017), P_{rcsB} (MDs1018) strains
586 harboring the p*slyA* plasmid. Colonies from LB agar medium containing (+) or not (-) 0.5 mM
587 IPTG were picked using toothpick into motility medium and incubated at 37°C. The migration
588 diameter (mm) was measured after 4 h of incubation. The data correspond to the average values
589 of three independent experiments. The error bars correspond to standard deviation. The asterisk
590 represent statistically different values (Tukey test, p=0.05). (**B**) A picture of representative
591 swimming plates is shown. (**C**) The *dps* gene transcription is modulated by the SlyA regulator in
592 RcsB-dependent pathway. The ß-galactosidase activity (Miller units) of the *dps*::*lacZY*

chromosomal transcriptional fusion was investigated in the wild type (MDs1568) and *rcsB* (MDs1569) strains, harboring the p*slyA* plasmid and grown at 37 ºC in LB medium in absence (-) or presence (+) of 0.5 mM IPTG. Here the strains containing the pUHE2-21 vector were used as control. The data correspond to the average of three independent experiments, performed in duplicate. The error bars correspond to standard deviation.

Fig. 6. Model of the regulatory overlapping effect of RcsB and SlyA in the *Salmonella* virulence control. Different levels of RcsB act as a switch between the virulent or attenuated state of the bacteria: high concentrations of the regulator incline the balance towards the attenuated state (**A**), while in the absence or low concentrations do this towards the virulent state (**B**). SlyA represent one additional mechanism by which *Salmonella* controls this balance through the RcsCDB system, since SlyA represses the *rcsB* expression tilting the balance to the virulent state. ↑ induction; ↓ repression.

Figure 1

A)



B)

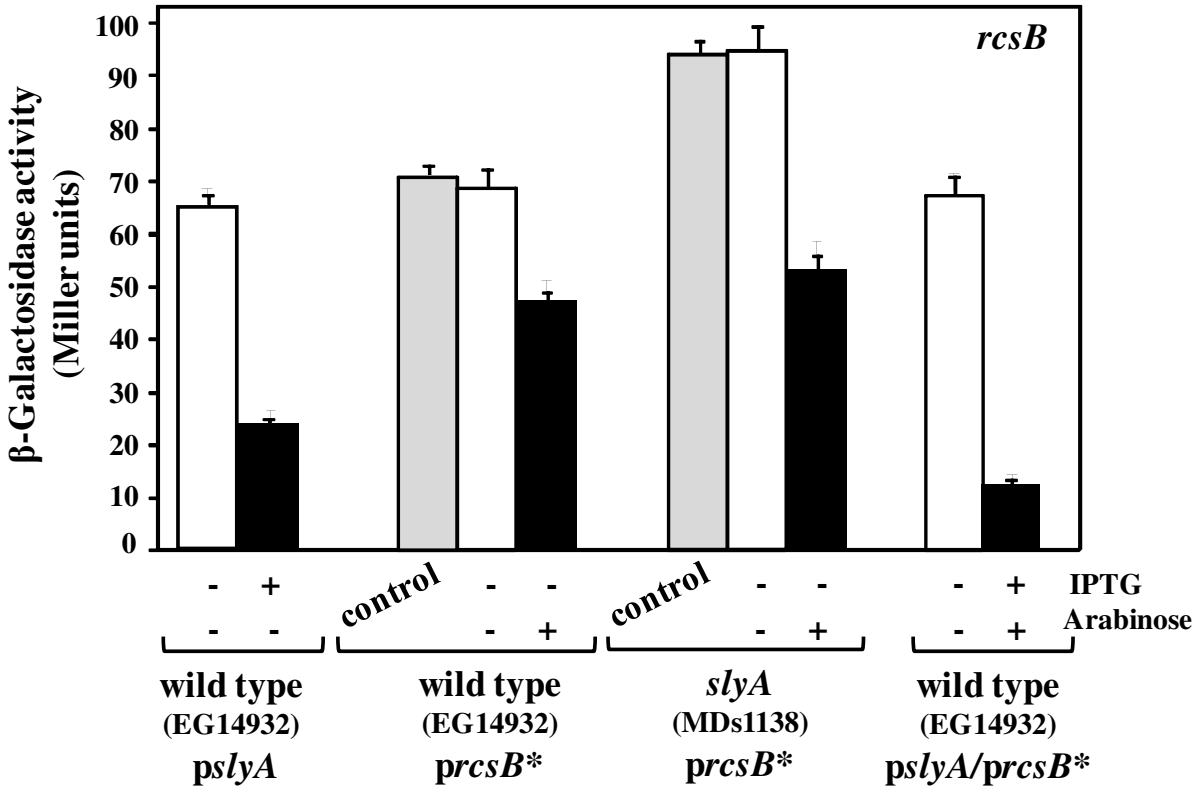
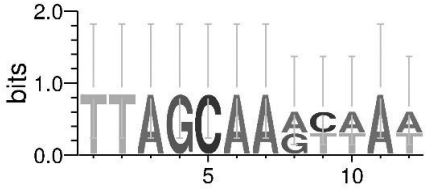


Figure 2

A)

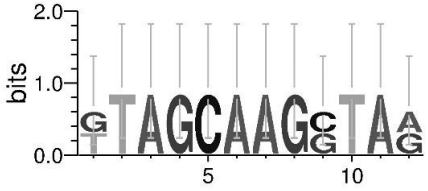
```
-248  GTTATTCACTACACTCCCCTGCTCGACCGTTCGTAAGAC
-209  ATTAGCAAATAATTTCTTGATATTTAGTGCTAAACATTT
-170  ATAAGTAGTCTTTATATTTGTACCGTTCTGCGAAGGTTG
-131  TACGCTTTTCCCGTCCCGCCGACGGAGCGCGTGTTAAGT
 -92  TGCCCGGCATGACGTAAGAGTCTGGAAATTCATTCATTA
                                -35
 -53  CCCTTTATACTGCCCTTCACCTTCAGCGTTGCTTTTACA
          -10
 -14  GGTCGTAAACATAAATGAGTC
```



SlyA consensus box

B)

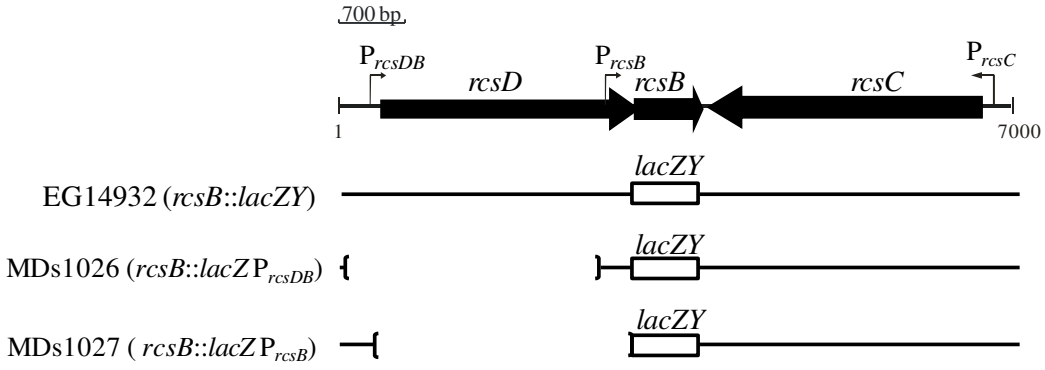
```
-163 CGCCTGAAAGGGGTGTTTGCCATGCTTAATCTGGTACCC
-124 GGCAAGCAGTTATGTGAAACGCTGGAACATCTGATTCGT
                                        -35
 -85 GAGAAAGATGCTCCAGGTATAGAAAAATATATCAGCGAC
                      -10
 -46 ATTGACGCCTACGTCAAAAGCTTGCTGTAGCAAGGTACC
  -7 CCAATACATGAACA
```



SlyA consensus box

Figure 3

A)



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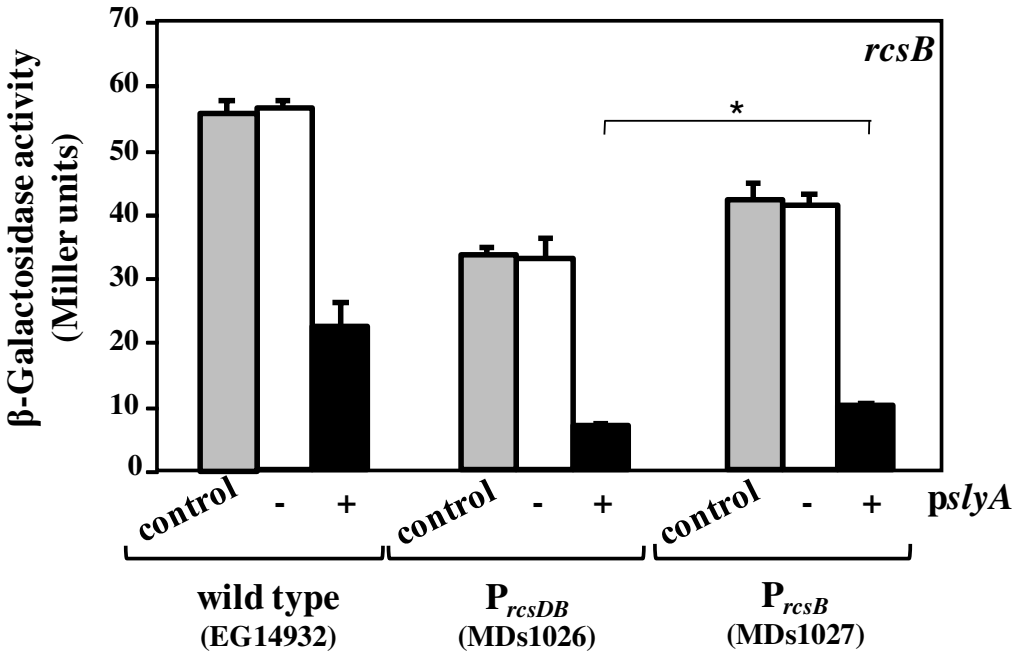

Figure 4

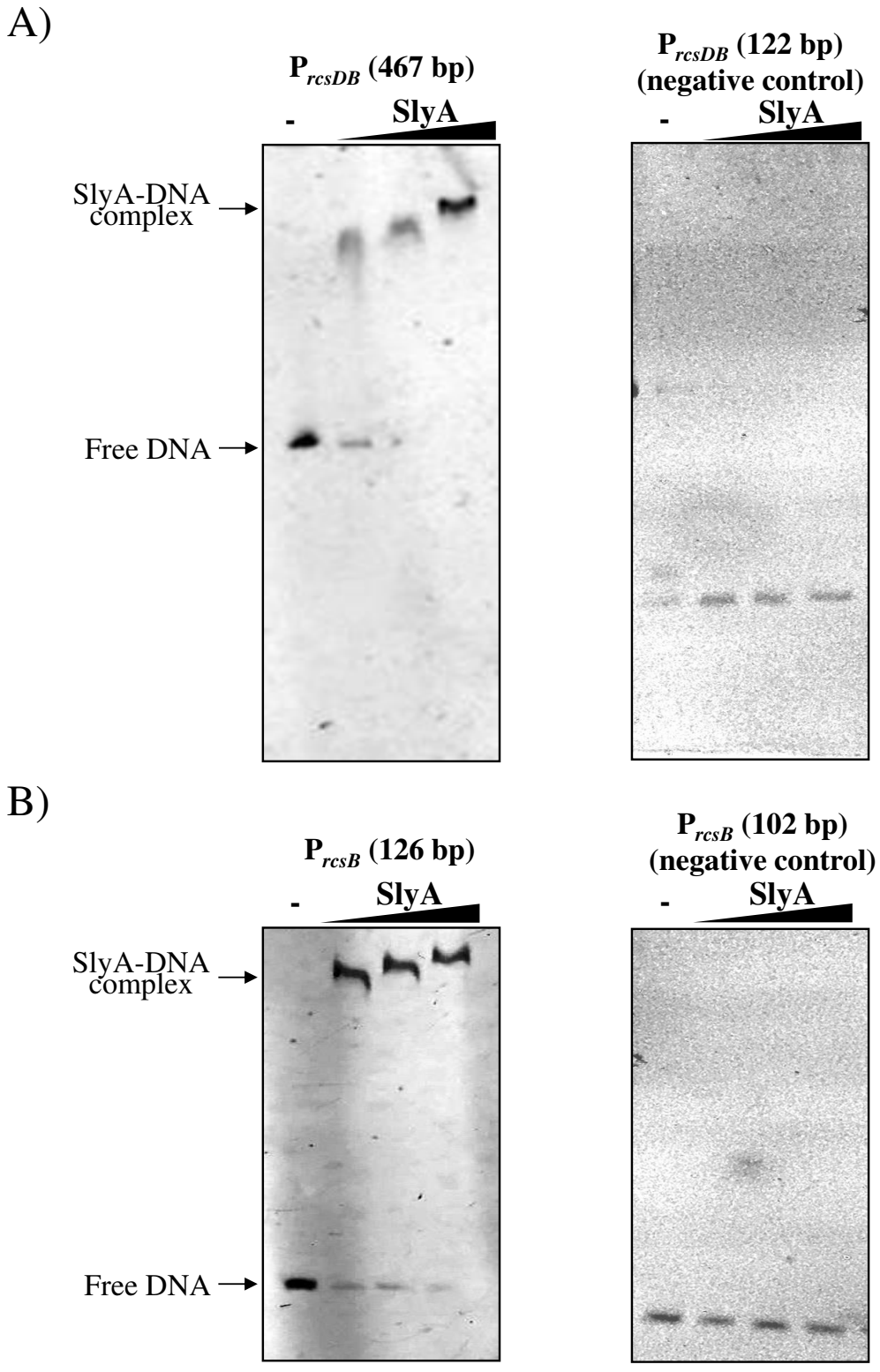

Figure 5

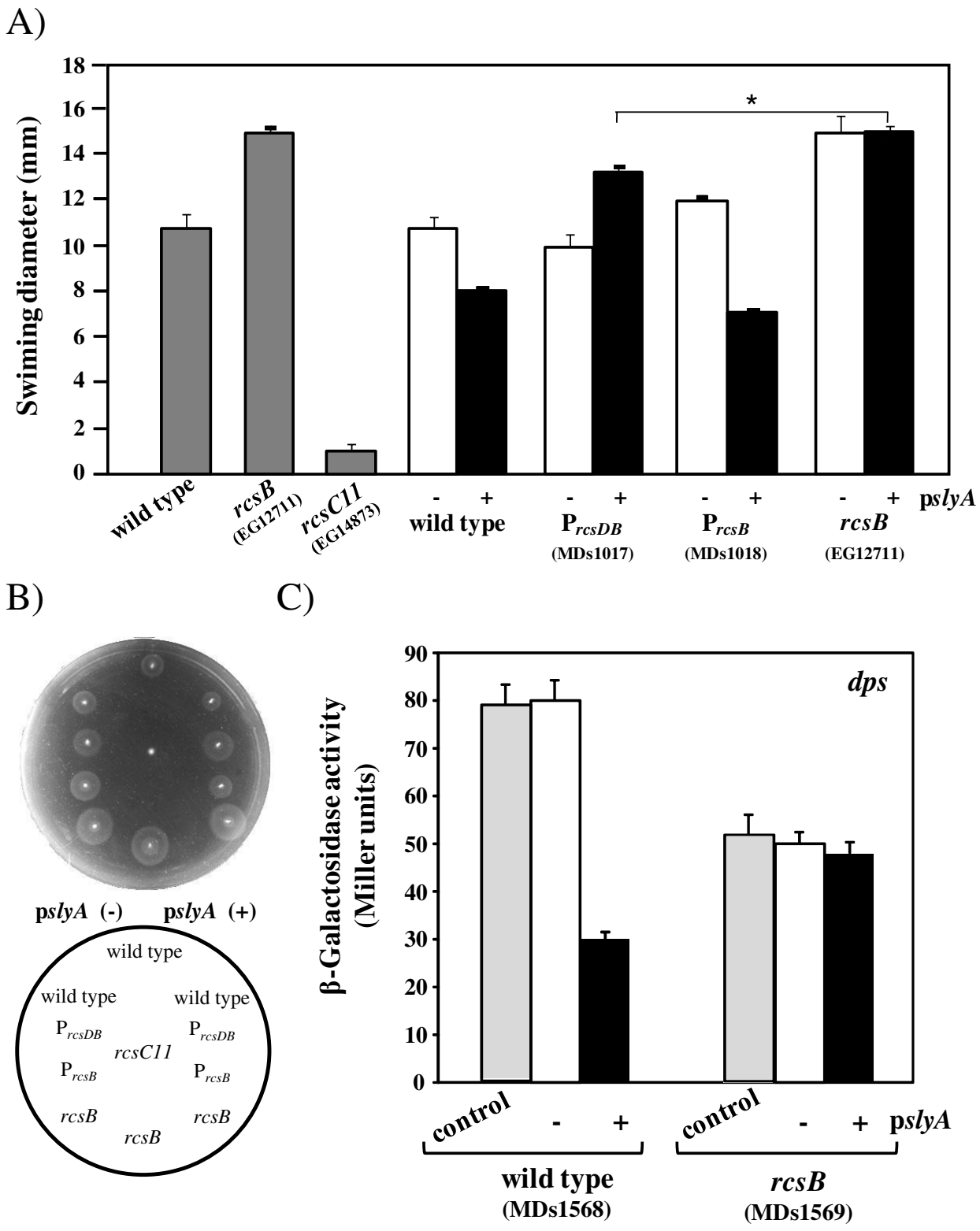

Figure 6