Salmonella biofilm development depends on the phosphorylation status of RcsB

Cristina Latasa[1], Begoña García[1], Maite Echeverz[1], Alejandro Toledo-Arana[1], Jaione Valle[1], Susana Campoy[2], Francisco García-del Portillo[3], Cristina Solano[1] and Iñigo Lasa[1,*]

[1]Instituto de Agrobiotecnología, Idab, Universidad Pública de Navarra-CSIC-Gobierno de Navarra, Campus de Arrosadía, Pamplona-31006, Navarra, Spain; [2]Departament de Genètica i Microbiologia, Universitat Autònoma de Barcelona, 08193 Barcelona, Spain. [3]Departamento de Biotecnología Microbiana, Centro Nacional de Biotecnología-Consejo Superior de Investigaciones Científicas (CNB-CSIC), Darwin 3, 28049 Madrid, Spain.

[*] Corresponding author: Instituto de Agrobiotecnología. Universidad Pública de Navarra. Pamplona-31006, SPAIN

Tel: 34 948 168007

Fax: 34 948 232191

Email: ilasa@unavarra.es

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Abstract

Rcs phosphorelay is a complex signalling pathway involved in the regulation of many cell surface structures in enteric bacteria. In response to environmental stimuli, the sensor histidine kinase (RcsC) autophosphorylates and then transfers the phosphate through intermediary steps to the response regulator (RcsB), which once phosphorylated, regulates gene expression. Here, we show that *Salmonella* biofilm development depends on the phosphorylation status of RcsB. Thus, unphosphorylated RcsB, hitherto assumed as inactive, is essential to activate the expression of the biofilm matrix compounds. Preventing RcsB phosphorylation either by disrupting the phosphorelay at RcsC or RcsD level or by producing a non-phosphorylatable RcsB allele induces biofilm development. On the contrary, phosphorylation of RcsB by the constitutive activation of Rcs pathway inhibits biofilm development, an effect that can be counteracted by the introduction of a non-phosphorylatable RcsB allele. Inhibition of biofilm development by phosphorylated RcsB is due to the repression of CsgD expression, by a mechanism dependent on the accumulation of the small non-coding RNA RprA. Our results indicated that unphosphorylated RcsB plays an active role for integrating environmental signals, and more broadly that RcsB phosphorylation acts as a key switch between planktonic and sessile lifestyle in *S.* Typhimurium.

Introduction

Salmonella enterica spp *Enterica* ser. Typhimurium (*S.* Typhimurium) is a principal agent of gastroenteritis in humans. It is an ubiquitous bacteria with a cyclic lifestyle involving passage from the gastrointestinal track of animal hosts into the external environment and back into a new host (23, 57). It is now recognized that part of the ecological success of *S.* Typhimurium facing such highly variable environmental conditions, lies in its ability to grow as organized multicellular complexes embedded in a protective extracellular matrix, currently known as biofilms (38-42, 48, 59). The extracellular matrix that encloses *S.* Typhimurium biofilm is made of several components among which three, fimbriae, cellulose and the surface protein BapA, have been characterized (29, 40, 48, 59). Given that the synthesis of such a complex matrix is a highly energy-consuming process, an extremely tight regulation appears to be critical to efficiently integrate multiple environmental signals with an appropriate biofilm-related gene expression profile (7, 57).

The RcsCDB phosphorelay is a complex signalling pathway exclusively present among members of the *Enterobacteriaceae* family that coordinates the expression of a large number of genes important for maintenance of cell wall integrity, cell division, stationary phase sigma factor activity, biofilm development, motility and virulence (for a review, see (4, 30). The precise nature of the inducing signals are still unknown, but mounting data reinforce the idea that the Rcs phosphorelay-signalling system responds to changes in the integrity of the peptidoglycan layer by remodelling the bacterial surface (4, 5, 35). In contrast to the majority of TCS that consists of a direct phospho-transfer cascade between a membrane-associated sensor protein (HK) and a response regulator (RR), this pathway involves three members: a hybrid sensor kinase (RcsC), a phospho-transferase (RcsD), and a response regulator (RcsB). Signal transduction

begins with the autophosphorylation of RcsC at the conserved histidine residue H479. The phosphoryl group is then transferred to a conserved aspartate D875 residue of RcsC and subsequently to the histidine residue H841 of the intermediary protein RcsD. Finally, the phosphoryl group is transferred to the aspartate residue (D56) of the response regulator protein RcsB and this modification facilitates DNA-binding and changes in the expression of RcsB-regulated genes (30). In addition, an auxiliary protein, RcsA, cooperates with RcsB for the binding to some target promoters like those responsible for capsule or flagella synthesis (30).

In *Escherichia coli*, the activation of the Rcs phosphorelay results in an increase in the expression of the extracellular polysaccharide colanic acid (20) and the inhibition of the expression of genes encoding surface adhesins, such as antigen 43 and curli, and the *flhDC* operon, encoding the master regulators of flagella biosynthesis (Ferrieres & Clarke, 2003; Francez-Charlot et al., 2003; Vianney et al., 2005). As these genes are involved in attachment to surfaces (antigen 43, curli fimbriae and flagella) and biofilm matrix production (colanic acid), a simple model that the Rcs phosporelay gradually represses the production of proteinaceous appendages while increasing the production of EPS has been proposed. Thus, the inactivation of RcsC in *E. coli* results in a biofilm defective phenotype (12, 25). In the case of *S.* Typhimurium, production of colanic acid and repression of motility are also regulated by Rcs phosphorelay (1). However, *S.* Typhimurium biofilm matrix contains cellulose instead of colanic acid as a major exopolysaccharidic compound (48, 59). Remarkably, the contribution of the Rcs system to the regulation of not only cellulose production but also other components of the biofilm, namely fimbriae and BapA, has never been established and thus, the regulation of biofilm development in *Salmonella* has been considered to occur independently of the Rcs phosphorelay pathway.

The synthesis of the biofilm matrix formed by *S.* Typhimurium is synchronized by a complex network whose key point is represented by the regulator of the LuxR family, CsgD (40, 59). CsgD activates the transcription of (i) the *csgBA* operon, responsible for the biosynthesis of curli fimbriae (21, 42); (ii) *adrA*, a gene encoding a protein of the GGDEF family, involved in the synthesis of bis-(3'-5')-cyclic dimeric guanosine monophosphate (c-di-GMP), which is required for allosteric activation of the cellulose synthase (41, 46, 59) and (iii) *bapA*, a gene encoding for a large surface protein, whose deletion causes the loss of the capacity to form a biofilm in LB media (29). The activity of CsgD is in turn regulated at two different levels. On the one hand, the expression of CsgD is controlled at the transcriptional level by environmental conditions (18, 19) through the intervention of global regulatory proteins like RpoS, members of the two-component signalling system such as OmpR and CpxR and members of the GGDEF/EAL domain proteins involved in c-di-GMP signalling (28, 37, 40, 42, 46). On the other hand, CsgD activity appears to be regulated by phosphorylation. Thus, unphosphorylated CsgD efficiently activates fimbriae and cellulose production whilst phosphorylated CsgD is unable to activate *csgBA* and *adrA* transcription (58). The source of the phosphoryl group has not been specified.

In this study we investigated the role of Rcs phosphorelay in *S. enterica* biofilm development. We show that deletion of RcsB or constitutive activation of Rcs phosphorelay inhibits biofilm development. In contrast, prevention of RcsB phosphorylation either by impeding phosphorelay from RcsC or RcsD or by producing a non-phosphorylatable variant of RcsB enhances biofilm development. We also show that unphosphorylated RcsB is a positive regulator of *csgD,* while accumulation of phosphorylated RcsB represses *csgD* expression. In summary, our findings demonstrate

that phosphorylation status of RcsB mediates the switch between a planktonic and a sessile lifestyle in *S.* Typhimuirum.

Results

Deletion of rcsC and rcsB have opposite effects on Salmonella biofilm formation capacity

Previous studies have shown that the Rcs phosphorelay predominantly regulates genes involved in the production of cell surface-associated structures such as exopolysaccharides, fimbriae, lipopolysaccharide and flagella. Since some of these compounds are necessary to build the *Salmonella* biofilm matrix, we thus were interested in studying the contribution of the RcsCDB phosphorelay pathway to biofilm development. For that, we constructed deletion mutants of each of the genes encoding components of the Rcs phosphorelay: the sensor-histidine kinase, RcsC, the phospho-transfer, RcsD, the response regulator, RcsB, and the auxiliary protein, RcsA, in the strain *S.* Typhimurium 14028, generating strains 14028Δ*rcsC*, 14028Δ*rcsD*, 14028Δ*rcsB* and 14028Δ*rcsA*. We confirmed the capacity of our deletion mutants to interfere with an active RcsCDB phosphorelay system by introducing each mutation into a *S.* Typhimurium 14028 strain harbouring a punctual mutation in *igaA (igaA1)* that provokes a constitutive activation of the RcsCDB pathway (1). As expected, the resultant *igaA1*Δ*rcsC, igaA1*Δ*rcsB* and *igaA1*Δ*rcsA* strains lost the mucoid phenotype characteristic of an active Rcs phosphorelay pathway, indicating that RcsCDB phosphorelay was impeded in each individual mutant (data not shown).

We then tested several phenotypes associated with *Salmonella* multicellular behaviour such as the rdar (red, dry and rough) morphotype on Congo-Red agar plates, which reflects the coexpression of curli fimbriae and cellulose and pellicle development at the air-broth interface in LB medium, which requires the production of cellulose, curli fimbriae and the surface protein BapA.

Results revealed that *S*. Typhimurium 14028Δ*rcsC* and 14028Δ*rcsD* displayed stronger biofilm phenotypes compared to the wild type strain, characterized by a strong rdar morphotype on congo red agar plates and a thick pellicle in LB media (Fig. 1A). On the contrary, the *rcsB* defective strain showed a diminished capacity to bind the congo red dye and lost the capacity to develop the pellicle at the air-liquid interface in LB medium. The strain lacking *rcsA* displayed biofilm phenotypes indistinguishable to those of the wild type strain (Fig. 1A).

As the mutation of *rcsC* and *rcsB* produced opposite effects, we constructed a strain lacking both the histidine kinase and response regulator, referred to as 14028Δ*rcsBC*. The negative multicellular behaviour displayed by the double *rcsBC* mutant strain confirmed the dominance of the effect caused by the absence of *rcsB* (data not shown). Complementation of 14028Δ*rcsB* and 14028Δ*rcsBC* strains with a wild type RcsB gene restored normal biofilm patterns and discarded possible pleiotropic effects (Fig. 1B).

To confirm that phenotypes displayed by the mutants did not depend on the strain genetic background, deletions were P22-transduced into the clinical *S*. Enteritidis 3934 isolate generating *S*. Enteritidis 3934Δ*rcsC, S*. Enteritidis 3934Δ*rcsD, S*. Enteritidis 3934Δ*rcsB* and *S*. Enteritidis 3934Δ*rcsA* strains. All mutants behaved the same way than *S*. Typhimurium 14028 mutants supporting the requirement of RcsB in the *Salmonella* biofilm formation process (data not shown).

In summary, these results reveal that depending on the member of the Rcs system that is deleted, the consequences on the capacity of *Salmonella* to develop a biofilm are different. Thus, the absence of RcsB impairs biofilm formation phenotypes whereas absence of RcsC and RcsD causes the opposite effects.

Inactivation of Rcs phosphorelay induces biofilm development

To investigate the reasons why the deletion of RcsC enhanced biofilm formation capacity whereas the absence of RcsB impaired biofilm development, we first excluded the possibility that RcsB levels might be altered in the absence of RcsC. To test this hypothesis, the RcsB protein was tagged with the 3xFLAG epitope and its synthesis was monitored by Western blot. We found no significant differences in RcsB protein levels between strains *S.* Typhimurium 14028 and 14028Δ*rcsC* (Fig. 2A).

We next examined whether the transfer of the phosphoryl group from RcsC and RcsD to RcsB could play a role in the regulation of biofilm development. To experimentally determine the contribution of the phosphorelay, we generated two different strains harbouring chromosomal H479A (strain 14028RcsC_H479A) and D875A (strain 14028RcsC_D875A) substitutions in RcsC transmitter and receiver domains respectively (6, 50). The first aminoacidic change impairs autophosphorylation of the RcsC protein, while the substitution of the aspartic residue impairs the translocation of the phosphoryl group to RcsD. We also tested a third strain with a punctual substitution in H841R of RcsD that renders a protein unable to receive the phosphoryl group from RcsC and consequently to transfer it to RcsB (Fig. 2B). Analysis of the biofilm formation capacity of these strains revealed that 14028RcsC_H479A, 14028RcsC_D875A and 14028RcsD_H841R strains assembled strong biofilms, just as those formed by strains 14028Δ*rcsC* and 14028Δ*rcsD* (Fig. 2C). Together, these results indicate that the Rcs phosphorelay could control negatively *S.* Typhimurium multicellular behaviour.

Biofilm formation is positively regulated by the unphosphorylated RcsB system

The exacerbated multicellular behaviour showed by the strains defective in the phosphorelay, namely 14028Δ*rcsC*, 14028Δ*rcsD*, 14028RcsC_H479A, 14028RcsC_D875A and 14028RcsD_H841R, whose pool of RcsB is supposed to be

mostly unphosphorylated, was consistent with a positive effect of the unphosphorylated isoform of RcsB on biofilm development. To test this, a strain harbouring a mutation in *rcsB* that replaced the aspartic residue by a non-phosphorylatable glutamic residue was constructed. This strain was referred to as 14028RcsB_D56Q. In order to discard the possibility that RcsB_D56Q could be phosphorylated in any other residue and confirm its permanent unphosphorylated status, we tested the capacity of both RcsB and the RcsB_D56Q variant proteins to bind radioactive acetyl phosphate *in vitro*. To do so, purified RcsB and RcsB_D56Q, together with the positive control RR462 protein from *Thermotoga maritima* (2), were incubated in the presence of radioactive acetyl phosphate. As shown in Figure 3**,** the presence of a signal of a molecular weight coinciding with RcsB in the case of the wild type protein and its absence in the case of RcsBD56Q strongly suggested that the RcsB_D56Q allele cannot be phosphorylated.

Analysis of the biofilm phenotypes displayed by strain 14028RcsB_D56Q revealed that this RcsB isoform led to the formation of a thick pellicle in LB medium and rdar colonies on congo red plates (Fig. 2D). These phenotypes were comparable to those displayed by the strains defective in the phosphorelay pathway indicating that the accumulation of unphosphorylated RcsB allele in these mutants was responsible for their enhanced biofilm formation capacity.

Constitutive activation of the Rcs phosphorelay inhibits biofilm formation

Taking advantage of previous studies that identified single point mutations in RcsC that result in the constitutive activation of the Rcs phosphorelay pathway (16), we next investigated the role of phosphorylated RcsB on the regulation of biofilm matrix development. For that, we analyzed the multicellular behaviour of a strain that harbours a constitutively active RcsC allele due to a single aminoacidic change (T903A) in the

receiver domain that inhibits RcsC phosphatase activity (Figure 4A) (16). This strain will be referred to as 14028RcsC*. *S*. Typhimurium 14028RcsC* strain produced RcsB protein levels similar to that of wild type strain indicating that RcsB synthesis is not affected by the constitutive activation of RcsC (Fig. 4B). *S*. Typhimurium 14028RcsC* displayed a mucoidy phenotype on solid media like calcofluor and Congo-Red agar plates, consistent with a constitutive activation of the Rcs phosphorelay pathway and thus, colanic acid capsule overproduction. This constitutive activation of the system led to an impairment in building a biofilm at the air-liquid interface in LB (Fig. 4C).

Since large amounts of colanic acid could impair cell-to-cell interactions between adhesins (22) we aimed to discard the overproduction of capsule itself as the major cause that negatively affected matrix formation. To do so, the gene involved in colanic acid biosynthesis *wcaA* was deleted in the 14028RcsC* strain. The resulting 14028RcsC*Δ*wcaA* strain remained unable to develop a biofilm and displayed morphotypes similar to that of 14028RcsC* strain indicating that overproduction of colanic acid was not responsible for the biofilm deficiency caused by the constitutive activation of RcsC (Fig. 4C). Finally, we aimed to check if the introduction of a non-phosphorylatable isoform of RcsB could counteract the negative effects of the constitutive activation of RcsC. For that, we performed a chromosomal RcsB_D56Q substitution in 14028RcsC* strain. Confirming our supposition, the resulting strain 14028 RcsC*RcsB_D56Q regained the ability to synthesize the pellicle in LB, to produce rdar colonies on Congo-Red agar plates and to bind calcofluor in a way indistinguishable to that shown by the wild type strain (Fig. 4C). These results indicate that RcsB can regulate different processes depending on its phosphorylation status. Thus, phosphorylated RcsB allele induces the synthesis of colanic acid capsule whereas unphosphorylated RcsB is necessary for the synthesis of biofilm matrix compounds.

RcsBC regulate the expression of the CsgD master regulator

CsgD represents the checkpoint of biofilm formation under inducing conditions given that it governs the synthesis of curli, cellulose and BapA (29, 40, 59). We thus found conceivable to test whether RcsCDB phosphorelay could be somehow affecting the expression of *csgD*. Firstly, we verified that the thick matrix formed by the strain lacking RcsC was being synthesized via CsgD. To do that, we constructed a double Δ*rcsC*Δ*csgD* mutant strain. The resulting strain was unable to develop the distinctive pellicle on LB and lost the rdar phenotype both on Congo Red and calcofluor agar plates (Fig. 5A).

We then analyzed the transcription of *csgD* and its target gene *csgA* by real time PCR in the wild type and its corresponding 14028Δ*rcsB*, 14028Δ*rcsC* and 14028Δ*csgD* mutants. In comparison to the transcriptional levels shown by the wild type strain, the levels of *csgD* mRNA, and subsequently of *csgA*, were dramatically decreased in the RcsB defective strain compared to those of the wild type strain, while they were slightly, though not significantly increased in the absence of RcsC (Fig. 5B).

We then measured the transcription of *csgD* in strains harbouring single point mutations that resulted in changes in the phosphorylation status of different members of the Rcs pathway. As it is shown in figure 5B, the mRNA profiles of *csgD* were in accordance with the phenotypes previously observed. Thus, mRNA levels of *csgD* were significantly higher in the strain harbouring the unphosphorylatable RcsB isoform (14028RcsB_D56Q) compared to those shown by the wild type strain. Strains in which the Rcs phosphorelay had been disrupted (14028RcsC_H479A, 14028RcsC_D875A), showed a slight increase in *csgD* mRNA levels. It is important to notice that the effects on the amount of *csgD* mRNA were not as pronounced as in the case of the strain

expressing the unphosphorylatable RcsB, probably due to a residual degree of phosphorylation of RcsB proceeding from the acetyl phosphate cytoplasmic pool. On the other hand, *csgD* mRNA levels were significantly reduced in the strain where RcsC was constitutively active (strain 14028RcsC*), being these levels restored to those of wild type background in the strain harbouring unphosphorylatable RcsB (14028RcsC*RcsB_D56Q). To confirm that the changes on *csgD* mRNA levels correlated with CsgD protein levels, CsgD protein was tagged with the c-Myc epitope in the different Rcs mutant strains and its synthesis was monitored by Western blot. The results revealed a strong correspondence between *csgD* mRNA levels and the amount of CsgD protein (Fig. 5C). Taken together, these results indicate that non-phosphorylated RcsB is required for expression of CsgD and hence, CsgD is not expressed when RcsB is deleted or when RcsB is mainly present in its phosphorylated form.

RprA, a small RNA induced by the RcsCDB activation, negatively regulates CsgD.

The consensus RcsB box sequence has been identified and described as not very reliable to infer the genes that may be directly regulated by RcsB (55). Thus, even though we did not detect a RcsB binding site in the *csgD* intergenic region, where many other regulatory proteins actually bind (18, 37), and taking into account that unphosphorylated RcsB may recognize a different binding site, we aimed to determine whether unphosphorylated or phosphorylated RcsB could be regulating *csgD* transcription by means of direct binding to its promoter. To do so, we assayed binding of purified RcsB and RcsBD56Q proteins to a DNA fragment containing the *csgD* regulatory intergenic region using acetyl phosphate as the potential phosphodonor molecule. Gel retardation assays failed to detect any interaction between RcsB and

RcsBD56Q proteins with the *csgD* promoter region, independently of the presence of acetyl phosphate (data not shown).

In view of this result, we decided to focus on the premise that RcsB-mediated regulation of *csgD* transcription might be indirect. A very recent work has provided evidence that RprA binds to the 5´-UTR region of *csgD* and downregulates its translation by an antisense mechanism (27). A target prediction program suggested a region located 100 nucleotides upstream to the ATG translation initiation codon of CsgD that showed a high-scoring interaction value for the sequence-related RprA sRNA. Interestingly, even though alignment of *Salmonella* and *E. coli csgD* 5´-UTR regions showed significant differences between both species (Fig. 6D), the RprA binding site characterized in the *E. coli* orthologe gene by Jorgensen and colleagues (27) was highly conserved. Thus, we decided to explore the possibility that RcsB- mediated regulation could be brought about by silencing of CsgD by RprA. As a starting point we measured RprA levels by northern blot in our genetic backgrounds that lead to different phosphorylation levels of RcsB. As shown in Figure 6A, the levels of RprA present in the cell were strictly dependent on the phosphorylated status of RcsB. Thus, on one hand, RprA intensity decayed in the Δ*rcsC* and RcsB_D56Q strains, whose RcsB pool is mostly or totally unphosphorylated. On the other hand, the constitutive activation of RcsC provoked an impressive overexpression of RprA, similar to the one previously described in *E. coli* (31). To further characterize the role of RprA, we overexpressed this small RNA in a wild type genetic background in our aim to mimic the endogenous high RprA levels caused by Rcs activation. The resulting SL14028 p[RprA] strain formed colonies with less intense colour in Congo Red agar and was unable to synthesise the LB-associated pellicle (Fig. 6B).

This last result prompted us to consider whether RprA could be inhibiting *csgD* expression by an anti-sense mediated mechanism. To test this hypothesis, we first tagged the chromosomal csgD gene with a N-Terminal c-Myc epitope and the resulting strain was transformed with the plasmid that overexpressed RprA. As shown in figure 6C, high RprA levels caused a strong reduction in the levels of CsgD, an effect that was also observed when RcsC was constitutively active.

In addition, to explore the possibility that RprA could target *csgD* mRNA and impede its translation, we constructed a plasmid-borne *csgD*::*gfp* translational fusion between the 5' UTR region of *csgD* and GFP reporter gene (53). The detection of GFP levels by western-blot showed that overexpression of RprA either by the constitutive activation of RcsC or by producing RprA from a multicopy plasmid inhibits the expression of GFP. On the contrary, when the same construction was introduced in the strain harbouring the chromosomal non-phosphorylatable RcsB isoform, an intense band with a molecular weight that coincide with GFP could be detected (Fig. 6E).

In conclusion, these results evidence that the activation of the Rcs system leads to the overexpression of RprA that contributes to the inhibition of CsgD expression at posttranscriptional level by an antisense mediated mechanism exerted over the 5´-UTR region of *csgD* mRNA.

Discussion

The Rcs phosphorelay cascade, one of the most deeply studied signalling pathway in bacteria, is known to be involved in *E. coli* biofilm formation by mediating the remodelling of the bacterial surface during growth on a solid surface (12). In this study, we have shown that RcsB inversely regulates the expression of CsgD, the master regulator of *Salmonella* biofilm development, depending on its phosphorylation status.

Such conclusion was initially raised by the at first sight contradictory effects of *rcsC* and *rcsB* mutations on the multicellular behaviour of *Salmonella*. According to our results, the presence of RcsB was necessary for pellicle development whereas RcsC was dispensable. To reconcile both phenotypes, we hypothesized that unphosphorylated RcsB might be required to induce biofilm development. In support of this hypothesis, we found that interruption of the phosphorelay at RcsC or RcsD levels acquired through the mutations RcsC_H479A, RcsC_D875A and RcsD_H841R enhanced the capacity of the bacteria to form a biofilm. On the contrary, single point mutations that provoke a constitutive activation of the phosphorelay due to the loss of the RcsC phosphatase function turned the bacteria incapable to show an aggregative communal behaviour. From these results, we inferred that unphosphorylated RcsB was contributing to positively regulate biofilm development whereas phosphorylated RcsB was inhibiting the process. However, by using this approach we could not exclude that low levels of phosphorylated RcsB generated by phosphodonors such as acetyl phosphate might be sufficient to activate biofilm development in the absence of functional RcsC and RcsD proteins. To raise this point, we generated a strain producing a nonphosphorylatable RcsB (D56Q) allele. This strain was characterized by a very strong biofilm phenotype, undoubtedly demonstrating the capacity of unphosphorylated RcsB to activate biofilm formation. Furthermore, the insertion of the unphosphorylatable isoform of RcsB in the genome of the strain producing a constitutively active RcsC allele restored the biofilm formation capacity of this strain, indicating that phosphorylation of RcsB has a negative effect in biofilm development.

How does unphosphorylated RcsB regulate biofilm formation in *Salmonella*? CsgD is the master regulator of the biofilm matrix compounds of *Salmonella* (29, 42, 46, 48, 59), and it was, therefore, the first candidate through which RcsB might affect the

synthesis of biofilm matrix compounds. In agreement with the biofilm phenotypes, analysis of *csgD* mRNA and CsgD protein levels in the different mutant strains showed that CsgD expression decreases when Rcs phosphorelay is active or in the absence of *rcsB*. In contrast, CsgD was expressed at higher levels compared to the wild type strain when the phosphorelay was impeded or in the strain producing the non-phosphorylatable variant of RcsB. The simplest explanation for these results is that unphosphorylated RcsB is required to activate CsgD expression. Evidences supporting a positive regulatory role for unphosphorylated RcsB, which had previously assumed to be inactive, were first provided by Mariscotti and García del Portillo (33). These authors unveiled that unphosphorylated RcsB isoform caused a more-pronounced positive effect on *spvA* expression than the phosphorylated one, though both phosphorylated and unphosphorylated RcsB isoforms were required for attaining proper *spvA* expression. Yet, in the case of *csgD*, our study has shown that unphosphorylated RcsB itself is sufficient to induce an increase in *csgD* mRNA, and thus CsgD levels. Consequently, the strain exclusively harbouring a chromosomal RcsB_D56Q variant displayed positive biofilm phenotypes under all conditions tested. In addition, since CsgD promotes the synthesis of c-di-GMP, these data indicate the existence of a link between Rcs and c-di-GMP signalling network. Altogether, these results lead to a complex scenario where Rcs phosphorelay pathway plays a key role transmitting environmental signals to properly timed biofilm development in *Salmonella*.

It is worth mentioning that the introduction of a non-phosphorylatable isoform of RcsB, and in a lower extent the deletion of RcsC, led to the production of fluorescent colonies on calcofluor agar plates, even when a deletion of the *bcs* operon, responsible for the synthesis of cellulose, was undertaken (data not shown). Since our previous results reported evidence about the existence of a second polysaccharide that takes part of the

S. Enteritidis biofilm matrix (48), it seems plausible that unphosphorylated RcsB might be also contributing to the synthesis of this second exopolysaccharide.

It was previoulsy described that RprA synthesis is regulated by the RcsC/RcsB phosphorelay system in *E. coli* (Majdalani and Gottesman 2002). More recently, a studied aimed at the discovery of novel sRNAs that target *csgD* mRNA in *E. coli* has provided evidence that RprA, together with McaS and GcvB, repress csgD translation via an antisense-mediated mechanism (27). Given that prediction programs at the Freiburg RNA tools web page detected a potential binding of RprA to the 5'-untranslated region of *csgD* mRNA in *Salmonella* that partially matches with the RprA binding sequence described in *E. coli* and that RprA expression is induced when RcsC phosphorelay is active, we reasoned that biofilm inhibition in RcsC constitutive mutants of *Salmonella* could result from the repression of RprA over *csgD*. In support of this hypothesis, we found out that phenotypes resulting from increasing the amount of RprA in a wild type strain were indicative of a distorted multicellular behaviour. The demonstration of an antisense RNA-driven translational attenuation was achieved both indirectly, using a tagged chromosomal version of CsgD, and directly through a plasmid-beared *csgD-gfp* fusion. A curiousness of RprA-*csgD* regulation in *Salmonella* is the long distance between the target sequence and the RBS of *csgD*, which would make a steric interference with initiating ribosomes very unlikely (9).

The RcsB phosphorylation shift mediated by the dual kinase/phosphatase activity of RcsC has also been proposed as a key feature for the regulation of the biofilm formation in *E. coli* (13). However, the Rcs-dependent regulation of biofilm development in *E. coli* differs from that of *S.* Typhimurium as the mutation in *E. coli* of RcsB alone has no effect on biofilm formation, whilst null and point mutations that impede RcsC activity results in a negative phenotype. This situation seems to be exactly the opposite to that

found in *Salmonella*. One possible explanation is that inactivation of *rcsC* might result in the accumulation of phosphorylated RcsB in *E. coli* (13), whereas the same mutation might result in the accumulation of unphosphorylated RcsB in *S.* Typhimurium. We can envision at least two different mechanisms that could lead to this different outcome; first, the phosphatase activity of RcsC could prevail over the kinase one in *E. coli*, while the opposite balance would take place in *S.* Typhimurium. Alternatively, RcsB might be prone to accept phosphoryl groups from a higher number of phosphodonors in *E. coli* compared to *S.* Typhimurium. Further studies and quantification of the RcsB phosphorylation levels *in vivo* in wild type strains as well as in the absence of RcsC will be necessary to confirm this hypothesis. As additional data supporting that the accumulation of phosphorylated RcsB is also the reason for the biofilm deficiency in *E. coli*, we could confirm that the introduction of a non-phosphorylatable isoform of RcsB in *E. coli* 55989 Δ*rcsC* strain restored the capacity of the strain to ensemble a biofilm matrix (data not shown). In both bacteria, the displacement of RcsB ratio to the phosphorylated isoform would trigger an excess of RprA that silenced csgD translation. RprA of *E. coli* and *Salmonella* show a 100% of similarity, whereas *csgD* 5´ UTRs in both bacteria show significant differences. Besides, the overexpression of RprA in *E. coli* fails to reduce plasmid-borne GFP fusion that harbours the 5´UTR of *csgD* amplified from *Salmonella* and vice versa (data not shown) suggesting that additional species-specific sRNAs or regulators might bind to the upstream element of *csgD*. In view of the differences between Rcs-mediated regulation in *E. coli* and *S.* Typhimurium, it seems that slight divergences at Rcs phosphorelay level could represent key features for supporting their different lifestyles thanks to a specialization in the coupling of environmental signals with multicellular behaviour.

Unquestionably, protein phosphorylation is a fundamental strategy used for harmonizing a great diversity of stimuli and responses (56). Up to date, it has been generally assumed that response regulators have two states, the inactive unphosphorylated form, and the active phosphorylated status. In this way, the output response would depend on the activation of gene transcription by the phosphorylated response regulator or the relief from the inhibitory effect exerted by the response regulator upon the acceptance of the phosphoryl group. Nonetheless, Dyer and Dahlquist (11) provided structural evidence of a new intermediate conformation of the unphosphorylated regulator CheY, which was still able to bind to a peptide of its effector target protein FliM. Furthermore, other studies have also highlighted the importance of the unphosphorylated isoform of response regulators like DegU of *Bacillus subtilis*, which is required for competence development and binds to the promoter region of the master regulator encoding gene *comK* (45) or CpdR of *Caulobacter,* whose unphosphorylated form is responsible for the activation and localization at the cell pole of ClpXP and therefore drives cell-cycle progression of this bacterium (26). In *S.* Typhimurium, apart from the regulation exerted by unphosphorylated RcsB on the virulence plasmid encoded *spvA* gene (33), a recent study has proved that unphosphorylated CsgD regulates biofilm formation in *S.* Typhimurium, whereas the phosphorylation of CsgD negatively affects the function of this protein (58). Since CsgD is an orphan response regulator, we find conceivable the possibility that RcsC, apart from regulating CsgD expression, might also contribute to the phosphorylation of CsgD, fact that would turn this protein less stable.

Altogether, our results agree with a model in which RcsB would inversely regulate the production of important cell surface-associated structures in a phosphorylation-dependent manner (Fig. 7). In *Salmonella*, under environmental conditions where the

Rcs system is OFF, RcsB would be mainly unphosphorylated and it would induce CsgD expression. Then, CsgD will activate the synthesis of AdrA, curli fimbriae and the Bap surface protein. AdrA, one of the most active c-di-GMP cyclases of *S.* Typhimurium, will subsequently elevate c-di-GMP levels that will switch on cellulose synthesis through the binding to BcsA and will brake flagella motility upon binding to YcgR (36, 43). Under environmental conditions where Rcs is ON, accumulation of phosphorylated RcsB would lead to an increase of RprA, a decrease in CsgD expression, and the ensuing decrease in c-di-GMP levels would lead to the inhibition of cellulose, fimbriae and Bap production. At the same time, phosphorylated RcsB will induce genes required for colanic acid capsule synthesis and persistent infection and will repress the synthesis of flagella and genes involved in the first stages of infection.

In a more general sense, our results anticipate that response regulators with regulatory activities at both phosphorylated and unphosphorylated states will be more common than has hitherto been imagined, opening a novel perspective in the regulatory capacities of these proteins.

Experimental procedures

Bacterial strains, plasmids and culture conditions

The most relevant bacterial strains and plasmids used and constructed in this study are listed in Table1. *E. coli*, *S.* Typhimurium and *S.* Enteritidis cells were grown in Luria-Bertani (LB) broth, in trypticase soy broth (TSB) and on LB agar (Pronadisa) with appropriate antibiotics at the following concentrations: kanamycin (Km) 50 μg ml^{-1}, chloramphenicol (Cm) 25 μg ml^{-1} and tetracycline 20 (Tc) μg ml^{-1}. *E. coli* 55989 strain and derivatives were grown at 37ºC in M63B1 0.4% glucose-minimal medium (M63B1-glu).

Phenotypic assays for biofilm formation

All the *Salmonella* strains obtained throughout the study were tested for their ability to produce a biofilm in LB, visualized as a floating pellicle at the air-broth interface that totally blocked the surface of the culture and could not be dispersed by shaking. The analysis of the capacity for cellulose production was carried out by qualitatively assessing the level of calcofluor (Fluorescent Brightener 28; Sigma) binding of colonies grown on LB agar supplemented with calcofluor 200 μg ml^{-1}, at room temperature for 48h (48). LB agar without salt and complemented with Congo red (40 μg ml^{-1}) and Coomassie brilliant blue (20 μg ml^{-1}) was also used to determine the Congo red-binding properties of the strains included in the study. Static biofilm formation assays were performed in 96-well poly(vinyl chloride) microtiter plates (Falcon; Becton Dickinson Labware) for 24 h as described before (34)

DNA manipulations

Routine DNA manipulations were performed using standard procedures described elsewhere (29) unless otherwise indicated. Plasmid DNA from *E. coli* was purified with a Quantum Prep plasmid kit (Bio-Rad). Plasmids were transformed into *E. coli* and *S.* Enteritidis by either heat shock or electroporation. Transformants carrying the Red helper plasmid were made electrocompetent with the following protocol. Cells were grown overnight in LB broth Amp at 30°C and then used to inoculate 500 ml of LB broth Amp that was incubated with aeration at 30°C to an OD_{600} of 0.2. Then, L-arabinose (Sigma) was added to a final concentration of 0.08%, and incubation continued until the OD_{600} reached 0.7. The suspension was cooled down on ice for 15 min, and cells were made electrocompetent by washing twice with the same volume of water and then once with 40 ml of ice-cold 10% glycerol. Cells were finally resuspended in 1.5 ml of ice-cold 10% glycerol. Restriction enzymes were purchased from Boehringer Mannheim and used according to the manufacturer's instructions. Oligonucleotides were obtained from Thermo Scientific Biopolymers (Table 2). Phage P22 HT105/1 int-201 (44) was used to carry out transductions between strains according to recommended protocols (32) To obtain phage-free isolates, transductants were purified on EBU plates and phage sensitivity was tested with the clear-plaque mutant P22 (17)

One-step inactivation of chromosomal genes

For the insertion of kanamycin and tetracycline resistance cassettes in *rcsC* and *rcsB* genes of *S.* Typhimurium 14028 and *E. coli* 55989, PCR-generated linear DNA fragments in combination with a helper plasmid were used. The Red helper plasmid pKOBEGA is a low-copy-number plasmid that contains an ampicillin resistance gene, a temperature-sensitive origin of replication and the Red system, which includes three

genes expressing Exo, Bet and Gam functions of λ phage (3). Plasmid pKOBEGA was introduced into *S.* Enteritidis 3934 by electroporation, and transformants were selected on LB agar Amp after incubation for 24 h at 30°C. One transformant carrying the Red helper plasmid was made electrocompetent as described above. A selectable antibiotic resistance gene was generated by PCR from a freshly isolated colony of *E. coli* MC4100 *ybeW*::km and *S.* Typhimurium TT3699 *ara651*::tn10, using primer pairs of 80-nucleotide (nt)-long primers that included 60 nt homology extensions for the targeted locus and 20 nt priming sequences for the kanamycin and tetracycline resistance genes as template. The genes mutated using this method and the corresponding primers are described in Table 2. Electroporation (25 µF, 200 W, 2.5 kV) was carried out according to the manufacturer's instructions (Bio-Rad) using 50 µl of cells and 1-5 µg of purified and dialysed (0.025 mm nitrocellulose filters; Millipore) PCR product. Shocked cells were added to 1 ml of LB broth, incubated for 1 h at 30°C and then spread onto LB agar supplemented with the appropriate antibiotic to select Km^R or Tc^R transformants after incubation at 30°C for 24 h. Mutants were then grown on LB broth supplemented with Km or Tc at 43°C for 24 h and incubated overnight on LB agar Amp at 30°C to test for the loss of the helper plasmid.

In frame deletion of RcsD

The pKO3blue shuttle vector (47) was used to perform the markerless in-frame deletion of *rcsD* gene. For that construction, DNA fragments corresponding to the upstream (fragment AB) and downstream (fragment CD) regions of *rcsD* were amplified with oligonucleotide pairs specified in table 2 using chromosomal DNA from strain *S.* Typhimurium 14028 as a template. Both fragments were cloned in the pGEMT-easy vector (Promega), digested with *SphI* and *XhoI* enzymes in the case of AB fragment and

XhoI and *BamHI* in the case of CD fragment. The AD fragment was then subcloned in pKO3blue vector digested with *SphI* and *BamHI*, confirmed by sequencing and electroporated into strain *S*. Typhimurium 14028. As pKO3blue contains a temperature-sensitive origin of replication, the construction was integrated into the chromosome through homologous recombination at non-permissive temperature (44ºC). Five colonies grown at 44ºC were picked into 5 ml of LB and incubated for 24 hours at 30ºC. Ten fold serial dilutions of these cultures were plated on LB plates containing 5% of sucrose. After 24 hours at 30ºC, sucR colonies were replica plated on LB agar plates supplemented with chloramphenicol and LB agar plates supplemented with X-gal (150 $\mu g\ ml^{-1}$). White colonies that were able to grow in the presence of sucrose but resulted chloramphenicol sensitive due to the loss of pKO3blue plasmid were selected. The deletion was tested by PCR, using the external *rcsD*.ok oligo pair.

Construction of punctual aminoacidic changes

To perform the substitutions RcsB_D56Q, RcsC_H479A and RcsC_D875A in *S*. Typhimurium 14028 and RcsB_D56Q in *E. coli* 55989, two separate PCR products with overlapping sequences including the targeted sequence were combined. The reverse oligonucleotide of the PCR generating AB fragment and the forward oligonucleotide generating CD fragment were complementary to allow the PCR products AB and CD to anneal.

One µl of each purified PCR product were mixed and a second PCR using external primers was performed to obtain a single fragment. The fusion product was purified and cloned in the pGEMT-easy vector (Promega). Once the construction was confirmed by sequencing, the fragment was cloned into the *Sph*I and *BamH*I sites of the pKO3blue plasmid, except for the fragments corresponding to changes RcsB_D56Q, RcsC_H463A

and RcsC_D859A that were cloned into the *Bgl*II and *Not*I sites of pKO3Blue. The following steps consisting of pKO3blue::AD integration and excision were performed in the same way to that described for *rcsD* deletion. Aminoacidic replacements were tested by PCR, using an external forward oligo (ok.Fw) and a reverse oligonucleotide whose 3´extreme matches with the changed codon (ok.Rv).

Protein labelling

RcsB labelling was carried out applying the epitope tagging method described by Uzzau *et al.* (54). Using the plasmid pSUB11 as template, a DNA module containing the 3xFLAG epitope and the kanamycin resistance marker was amplified by PCR with primers *rcsB*.Flag.Fw and *rcsB*.Flag.Rv (Table 2). Transformation of the wild type and *rcsC* mutant strains expressing lambda red functions yielded recombinants carrying the *rcsB* gene fused to the 3xFLAG sequence. The resulting carboxy-terminal-tagged RcsB protein was detected by Western blot analysis.

The pKO3blue plasmid (47) was also used for tagging CsgD protein with the c-Myc epitope. For that construction, DNA fragments corresponding to the upstream (fragment AB) and downstream (fragment CD) regions of the c-Myc insertion site were amplified with oligonucleotide pairs specified in table 2 (primer AB.Rv contains the c-Myc sequence), using chromosomal DNA from strain *S*. Typhimurium 14028 as a template. Both fragments were cloned in the pGEMT-easy vector (Promega), digested with *SphI* and *XhoI* in the case of AB fragment and *Xho*I and *BamH*I in the case of CD fragment. The AD fragment was then sub-cloned in pKO3blue vector digested with *Sph*I and *BamH*I and confirmed by sequencing. The following steps consisting of pKO3blue::AD integration and excision were performed in the same way to that described for *rcsD* deletion. The insertion of the c-Myc sequence was tested by PCR, using an external

forward oligo (ok.Fw) and a reverse oligonucleotide containing the c-Myc sequence (ok.Rv).

Construction of CsgD::GFP translational fusion

To construct p*csgD::gfp*, a fragment of the *csgD* gene from -143 up to +33 positions with respect to the ATG start codon was amplified by PCR using the oigos *csgD*.UTR.Fw and *csgD*.UTR.Rv. Chromosomal DNA from strain *S*. Typhimurium 14028 served as a template. This DNA segment was subcloned in pGEMTeasy and digested with *Nsi*I and *Nhe*I for its insertion into pXG-10 as described previously (53).

Western Blot analysis

Samples for western analysis were prepared as follows. For obtaining whole bacterial lysates, cells were grown in LB under static conditions for 72 hours at room temperature and after centrifugation of 1 ml of culture, cells were harvested, washed, and finally resuspended in 50 µl of PBS. An equal volume of Laemmli sample buffer was added to each sample and they were boiled at 100ºC for 5 min. Proteins were separated on SDS-Polyacrylamide gels (10%-4.5%) and stained with Coomassie brilliant blue R250 (0.25%; Sigma). For Western blotting, proteins were transferred to Hybond-ECL Nitrocellulose membranes (Amersham Biosciences) by electroblotting. Probing was carried out with anti-FLAG or anti-c-Myc phospatase alkaline-labelled antibodies (Sigma) diluted 1:500 for 90 min at room temperature. Bound ligands were detected using the ECL^{TM} Western Blotting Analysis system (Amersham Biosciences).

Radioactive phosphorylation assays

In vitro phosphorylation assays were performed as described before (58) with some modifications. Briefly, [^{32}P] acetyl phosphate (acP) was synthesized with *E.coli* acetate kinase enzyme (Sigma Aldrich) and ^{32}PγATP (γ-[^{32}P] ATP, 6000Ci mmol^{-1}, Perkin Elmer) in a reaction mixture that contained 10μl of ^{32}PγATP, 5 μl of 10x triethaolamine buffer pH 7,6, 9 units of Acetate kinase and deionised water up to 50 μl. Upon incubation at 25ºC for 2 hours, the radioactive acP was separated from the enzyme using a Microcon-10 microconcentrator (Millipore). Then, 10 μl of this elute were mixed with 2 μg of RcsB, RcsB_D56Q and RR462 proteins respectively and incubated for 30 minutes at 30ºC. The reaction was stopped by addition of 2XSDS loading buffer and samples were charged and electrophoresed in a criterion XT Bris-Tris 10% gel (BioRad). Radioactivity was detected by direct exposition using a high performance autoradiography film (GE Healthcare). In parallel, 2 μg of non-radioactive RcsB, RcsB_D56Q and RR462 were also subjected to electrophoresis and protein lanes were further visualized with Coomassie stain for ensuring both size and equal quantities.

Production and purification of RcsB and RcsB_D56Q

The *rcsB* and *rcsB_D56Q* CDS DNA fragment were amplified from DNA extracted from *S.* Typhimurium 14028 and *S.* Typhimurium 14028RcsB_D56Q, respectively with high-fidelity thermophilic DNA polymerase (Dynazyme Ext, Finnzymes) using the primers *rcsB*.pet.Fw and *rcsB*.pet.Rv (Table 2). The resultant 650bp fragment was first cloned in pGEMT easy Vector (Promega), digested with *Eco*RI and *Hind*III, and subcloned in pET-28b(+) (Novagen). The resulting construction was verified by sequencing and introduced through electroporation into the *E.coli* BL21(DE3) strain. 250 ml of LB supplemented with Km were inoculated with 2 ml of an overnight LB cultures of BL21-pET28b::*rcsB* and BL21-pET28b::*rcsB*_D56Q. The cells were grown

at 37ºC until a DO_{600} value of 0,5. At this moment the inductor molecule IPTG was added at a concentration of 1mM and the cell growth continued for 5 hours under the same conditions. After a centrifugation at 5000g (30 min) the pellet obtained was resuspended in 10 ml of Bug Buster HT (Novagen) and incubated 20 min at room temperature. A new centrifugation step (16000g, 20 min) was carried out in order to separate the soluble fraction (supernatant) and inclusion bodies (pellet). The soluble fraction was incubated on ice during 30 min in the presence of DNAse and RNAse (100 μg/ml; Gibco-BRL). The lysate was then filtered (Filtropur S 0.45, Sarstedt) and the peptide was purified with the Protino Ni-TED packed columns (Macherey-Nagel) following the manufacturer's instructions.

Real time PCR

Total RNA from bacterial cells grown under biofilm forming conditions was obtained by a Trizol reagent method previously described (51). Two micrograms of each RNA sample were subjected in duplicate to DNAse I (Invitrogen) treatment for 30 min at 37ºC. The enzyme was inactivated at 65ºC in the presence of EDTA 0.25mM during 10 minutes. The RNA samples were reverse transcribed in the presence or absence of the enzyme SuperScript Reverse Transcriptase (Invitrogen) respectively in order to verify the absence of contaminating genomic DNA. All preparations were purified using CentriSep spin columns (Princeton separations). One twentieth of each reaction was used for real-time semiquantitative PCR using the SYBR Green PCR Master Mix (Applied Biosystems) in the ABI Prism 7900 HT (Applied Biosystems). The transcripts were amplified using the primers described in table 2, and the *gyr*B gene was selected as the endogenous control of the experiment as it is constitutively expressed. The primer concentration was previously optimized and standard curves were obtained for every

gene in order to verify that the amplification efficiency was similar so the formula $2^{-\Delta\Delta ct}$ could be applied. To monitor the specificity, final PCR products were analysed by melting curves. Only samples with no *gyr*B amplification of the minus reverse transcriptase aliquot were considered in the study. The amount of RNA was expressed as the n-fold difference relative to the control gene ($2^{-\delta Ct}$, where ΔCt represents the difference in threshold cycle between the target and control genes).

Northern Blot

The detection of the small RNA RprA by Northern blot was performed as described elsewhere (51) with some modifications. RNA samples (5-10 μg) were loaded in duplicate in a 5% pre-cast urea-acrylamide gel (Bio Rad) and electrophoresed at 100V for 60 min in 1xTBE buffer. After electrophoresis, the gel was electroblotted overnight onto a Nytran membrane (0.2 μm pore size; Sigma) at 4°C and 250 mA. The RNA was UV cross-linked to the membrane using the UV Stratalinker 1800 (Stratagene) according to the manufacturer's specifications. Prehybridization was performed in ULTRAhyb solution (Ambion). RprA and 5S small RNAs were developed using [^{32}P]-5´-end-labelled oligonucleotides *rprA*NB, and 5SNB (Table 2), respectively, that were labelled with the use of T4 polynucleotide kinase (New England Biolabs). The hybridization step was carried out at 39ºC during 16 hours. Following two washes with 2× SSC containing 0.1% SDS for 10 min at 39°C, blots were washed twice with 2× SSC for 5 min at room temperature. Finally, blots were exposed for autoradiography with Hyperfilm MP (GE Healthcare).

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Figure legends

Figure 1. Biofilm phenotypes of *S.* Typhimurium 14028 and *rcs* mutants.

Pellicle formation capacity in LB at room temperature and morphotype on Congo-Red agar and fluorescence on calcofluor agar plates displayed by strains *S.* Typhimurium 14028 wt and its derivatives *rcsC*, *rcsD*, *rcsA* and *rcsB* mutants (A). The same phenotypes displayed by the *rcsB* mutant strain complemented with an *rcsB* wild type allele expressed *in trans* are also shown (B).

Figure 2: Change of phosphorylatable residues in the Rcs pathway

A- Western blot analysis of RcsB expression in *S.* Typhimurium 14028 wild type and 14028Δr*csC* strains. Samples for protein analysis were withdrawn after 72 hours of incubation under LB biofilm forming conditions.

B- Schematic diagram showing the Rcs transduction signal. The residues involved in phosphate transfer from the RcsC kinase via RcsD to the response regulator RcsB and the changes undertaken are indicated. The possibility of RcsB phosphorylation via acetyl phosphate is also sown.

C- Biofilm phenotypes: Pellicle formation capacity in LB at room temperature and colony morphology on Congo-Red agar plates of *S.* Typhimurium 14028 wild type, 14028Δ*rcsC*, 14028RcsC_H479A, 14028 RcsC_D875A 14028 RcsD_H841R strains.

D- Pellicle formation capacity in LB at room temperature and colony morphology on Congo-Red agar plates of *S.* Typhimurium 14028RcB_D56Q strain.

Figure 3: Phosphorylation of RcsB and RcsB_D56Q *in vitro*

An autoradiogram of RcsB and RcsB_D56Q electrophoresed upon incubation with radioactive acetyl phosphate (see experimental procedures) is show. The exposition was

prolonged up to 24 hours. As a technical control of *in vitro* phosphorylation, an autoradiogram of RR468 phosphorylated under the same conditions is included (A). Coomassie staining following a parallel non-radioactive electrophoresis for ensuring equal protein amounts and appropriate molecular weight is also exposed (B).

Figure 4: Effects of constitutive activation of the Rcs pathway

A- Schematic diagram representing the constitutive activation of the Rcs pathway. The residues involved in the transfer of phosphoryl groups from the RcsC kinase via RcsD to the response regulator RcsB and the T903A mutation in the receiver domain of RcsC that renders a constitutive activation state are indicated.

B- Western blot analysis of RcsB expression. Samples for protein analysis were withdrawn after 72 hours of growth under LB biofilm forming conditions.

C- Biofilm phenotypes: Pellicle formation capacity in LB at room temperature and colony morphology on Congo-Red agar plates of *S.* Typhimurium 14028RcsC* strain, 14028RcsC*Δ*wcaA* and 14028RcsC*RcsB_D56Q strains.

Figure 5: Effects of Rcs phosphorelay on *csgD* expression

A- Colony morphology on Congo-Red agar plates of *S.* Typhimurium 14028 wild type, 14028Δ*rcsC*, 4028Δ*csgD* and 14028Δ*rcsC* Δ*csgD*.

B- Real time quantification of *csgD* mRNA in the *S.* Typhimurium 14028 wild type strain and its derivative *rcsC* and/or *rcsB* mutants as a result of complete deletion or aminoacidic changes. Asterisk in *rcsC* denotes the T903A mutation, which raises a constitutive activation of the phosphorelay. Real time quantification of the CsgD-regulated gene *csgA* in strains *S.* Typhimurium 14028 wild type, 14028Δ*rcsB*,

14028Δ*rcsC* and 14028Δ*csgD* is also shown. Asterisks over the graphic bars denote an associate p value <0.05 in Mann-Whitney tests.

C- Western blot analysis of CsgD in *S.* Typhimurium 14028 wild type, 14028Δ*rcsB*, 14028Δ*rcsC,* 14028RcsB_D56Q, 14028RcsC* and 14028RcsC*RcsB_D56Q strains. The band corresponding to low weight unspecific signal is shown as a loading control.

Figure 6. Negative regulation of *csgD* by *RprA*

A- Northern blot analysis of RprA and 5S rRNA levels.

B- Biofilm phenotypes: Pellicle formation capacity in LB at room temperature and colony morphology on Congo-Red agar plates of *S.* Typhimurium 14028 wild type and 14028 p[RprA] strains.

C- Alignment of 5´-UTR mRNA sequences of *E. coli* and *Salmonella csgD* gene. RprA binding-sites described in *E. coli* (27) and those predicted for *Salmonella* are highlighted in yellow. SD denotes Shine Dalgarno and ATG codon is underlined.

D- Western blot analysis of CsgD in *S.* Typhimurium 14028 wild type, 14028p[RprA] and 14028RcsC* strains.

E- Western blot analysis of GFP in *S.* Typhimurium 14028 wild type, 14028 p*csgD:gpf* 14028 p*csgD:gpf* p[RprA] , 14028RcsC* p*csgD:gpf* and 14028RcsBD56Q p*csgD:gpf* strains.

Figure 7. Model for the regulation of *Salmonella* biofilm formation by the Rcs phosphorelay

In the model, when the Rcs system is OFF, the unphosphorylated form of RcsB induces the expression of CsgD. CsgD ensuing induces the expression of genes encoding curli fimbriae and the diguanylate cyclase AdrA. Accumulation of AdrA enhances the levels

of the secondary messenger c-di-GMP, which activates the synthesis of cellulose and biases flagellar rotation towards CCW direction through the binding to YcgR (36). When Rcs system is ON, the levels of phosphorylated RcsB increases, leading to the repression of CsgD in a mechanism partially dependent on the small non coding RNA RprA. Besides, the flagellar master genes *flhDC* and some genes required for intestinal phase of infection are repressed whereas genes involved in synthesis of the colanic-acid capsule and factors required for persistent infection are upregulated.

Table 1. Strains and plasmids used in this study.

Strains	Characteristics	Source/Reference
S. Typhimurium		
14028	Wild type clinical isolate	(14)
14028Δ*rcsB*	Δ*rcsB*::Km^R	This study
14028Δ*rcsC*	Δ*rcsC*::Tc^R	This study
14028Δ*rcsD*	In frame markerless deletion	This study
14028Δ*rcsBC*	Δ*rcsCB*::Tc^R	This study
14028Δ*rcsA*	Δ*rcsA*::*mudQ* Clo^R	Gift from F. García-del Portillo
14028Δ*rcsB*	Δ*rcsB*::*mudQ* Clo^R	Gift from F. García-del Portillo
14028Δ*rcsC*	Δ*rcsC*::mudQ Clo^R	Gift from F. García-del Portillo
14028Δ*rcsB*[pIZ1589]	Δ*rcsB*::*mudQ* complemented with pBAD::*rcsB* wt, Clo^R Amp^R	This study
14028*igaA**	*igaA* _R188H	(10)
14028*igaA**Δ*rcsA*	*igaA* _R188H Δ*rcsA*::*mudQ* Clo^R	This study
14028*igaA**Δ*rcB*	*igaA* _R188H Δ*rcsB*::*mudQ* Clo^R	This study
14028*igaA**Δ*rcsC*	*igaA* _R188H Δ*rcsC*::mudQ Clo^R	This study
14028*rcsC**	*rcsC*T903A (allele RcsC55)	(16)
14028*rcsB*::3xFLAG	*rcsB*::3xFLAG Km^R	This study
14028**Δ***rcsC rcsB*::3xFLAG	Δ*rcsC*::*mudQ rcsB*::3xFLAG Clo^R Km^R	This study
14028*rcsC***rcsB*::3xFLAG	*rcsC*_T903A *rcsB*::3xFLAG Km^R	This study
14028*rcsC*_H479A		This study
14028*rcsC*_D875A		This study
14028*rcsD*_H841R		Gift from J. Casadesús
14028*rcsB*D56Q		This study
14028*rcsC** *rcsB*D56Q		This study
14028*rcsC** Δ*wcaI*	*rcsC*_T903A Δ*wcaI*::Km^R	This study
14028Δ*csgD*	Δ*csgD*::Km^R	(15)
14028Δ*rcsC* Δ*csgD*	Δ*csgD*::Km^R Δ*rcsC*::*mudQ* Clo^R	This study
14028*csgD::cMyc*	Insertion of cMyc epitope at position 53 of CsgD by homologous recombination	This study
14028*csgD::cMyc* Δ*rcsC*	Insertion of cMyc epitope at position 53 of CsgD by homologous recombination; Δ*rcsC*::mudQ Clo^R	This study
14028*csgD::cMyc* Δ*rcsB*	Insertion of cMyc epitope at position 53 of CsgD by homologous recombination; Δ*rcsB*::mudQ Clo^R	This study
14028*csgD::cMyc rcsB*_D56Q	Insertion of cMyc epitope at position 53 of CsgD by homologous recombination; *rcsB*D56Q	This study
14028*csgD::cMyc rcsC**	Insertion of cMyc epitope at position 53 of CsgD by homologous recombination; *rcsC*T903A	This study
14028*csgD::cMyc rcsC*rcsB_D56Q*	Insertion of cMyc epitope at position 53 of CsgD by homologous recombination; *rcsC*T903A*; rcsB*D56Q	This study
TT3699*ara651*::tn10	Used as template for Tetracycline cassette resistance amplification	Gift from J. Casadesús
14028 p[RprA]	Overexpression of the sRNA RprA; Amp^R	This study
14028*csgD::cMyc* p[RprA]	Insertion of cMyc epitope at position 53 of CsgD by homologous recombination; complemented with p[RprA]; Amp^R	This study

14028 pXG-10::*csgD* SL	GFP translational fusion with the 5`UTR mRNA region of *csgD* Clo^R	This study
14028 pXG-10::*csgD* p[RprA]	GFP translational fusion with the 5`UTR mRNA region of *csgD* ; RprA expressed in a multicopy plasmid Amp^R Clo^R	This study
14028 RcsC* pXG-10::*csgD* SL	GFP translational fusion with the 5`UTR mRNA region of *csgD; rcsC*T903A Clo^R	This study
14028 RcsBD56Q pXG-10::*csgD* SL	GFP translational fusion with the 5`UTR mRNA region of *csgD; rcsB*D56Q Clo^R	This study
14028 pCsgD::GFP	GFP translational fusion with the 5`UTR mRNA region of *csgD* amplified from *E. coli* Clo^R	This study
S. Enteritidis 3934	Wild type clinical isolate	(49)
3934Δ*rcsA*	Δ*rcsA*::*mudQ* Clo^R	This study
3934Δ*rcsB*	Δ*rcsB*::*mudQ* Clo^R	This study
3934Δ*rcsC*	Δ*rcsC*::*mudQ* Clo^R	This study
E.coli		
MC4100 *ybe*W:Km	Used as template for Kanamicin cassette resistance amplification	Gift from J.M. Ghigo
XL1 Blue	*rec*A1 *end*A1 *gyr*A96 *thi*-1 *hsd*R17 *sup*E44 *rel*A1 *lac* [F´*pro*AB *lacI*^{q}ZΔ*M15* Tn10(Tet^r)]	Stratagene
BL21(DE3)	B F– dcm ompT hsdS(r – m –) gal λ (DE3)	Novagen
55989	*E. coli* enteroaggregative pathogenic stain	(8)
55989Δ*rcsC*	Δ*rcsC*::km^R	This study
55989Δ*rcsB*	Δ*rcsB*::Tc^R	This study
55989*RcsBD56Q*		This study
55989Δ*rcsCRcsBD56Q*	Δ*rcsC*::km^R	This study
Plasmids		
pIZ1589	pBAD containing rcsB from SL14028; Amp^R	(33)
pET28-b(+)	T7 expression vector; Km^R	Novagen
pET28-b(+)::*rcsB*	pET28-b (+) containing *rcsB* from SL14028; Km^R	This study
pET28-b(+)::*rcsB*D56Q	pET28-b (+) containing *rcsB* from SL14028*rcsB*D56Q; Km^R	This study
pKOBEGA	Vector for recombination experiments; Amp^R	Gift from J.M. Ghigo
pXG-10	Cloning vector for translational GFP fusions; Cm^R	(52)
pCsgD::GFP	pXG-10 derivative containing 5´-UTR *csgD* mRNA region from *E .coli;* Cm^R	(24)
pXG-10::*csgD* SL	pXG-10 derivative containing 5´-UTR *csgD* mRNA region from SL14028; Cm^R	This study
p[RprA]	pGEMT easy sRNA containing RprA from SL14028; Amp^R	This study
pKO3blue	Shuttle vector for homologous recombination; Clo^R	(47)

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Table 2. Oligonucleotides used in this study

Gene/Region	Primer	SEQUENCE
RcsB Salmonella	*rcsB*.km.Fw	ATGAACAATATGAACGTAATTATTGCCGATGAC CACCCGATTGTACTGTTCGGTATTCGCAAAGCC ACGTTGTGTCTCAA
	rcsB.km.Rv	ACGATCCCTTCAATATCAAGATCCAACACGGCG CTCAGGATCGCCGGGTTGTTGTTCATGGCGCTG AGGTCTGCCTCGTG
	*rcsB*D56QAB.Fw	GGATCCAGATGAAAATGCCGAGCT
	*rcsB*D56Q.AB.Rv	CATGGACAGCTGAGTGATCAA
	*rcsB*D56Q.CD.Fw	TTGATCACTCAGCTGTCCATG
	*rcsB*D56Q.CD.Rv	GCATGCAAAGATGAGTCGACTGGTA
	*rcsB*D56Q.ok.Fw	CTTATCGAAGAGCAGCTGG
	*rcsB*D56Q.ok.Rv	TCTCCCGGCATGGAGACTG
	rcsB.Flag.Fw	AATGATATCGCGCTGCTCAACTATCTCTCTTCTG TCACCCTGAGTCCGACAGACAAAGAAGACTACA AAGACCATGACGG
	rcsB.Flag.Rv	TGAGTCGACTGGTAGGCCTGATAAGCGTAGCGC CATCAGGCTGGGTAACATAAAAGCGATCATATG AATATCCTCCTTAG
	*rcsB*okFlag.Fw	CGCTGCTCAACTATCTCT
	*rcsB*okFlag.Rv	TCAGGCTGGGTAACATAAA
	rcsB.pet.Fw	GAATTCGATGAACAATATGAACGTAATT
	rcsB.pet.Rv	AAGCTTTTCTTTGTCTGTCGGACTCAG
RcsB E. coli	*rcsBcoli*.Tc.Fw	ATGAACAATATGAACGTAATTATTGCCGATGAC CATCCGATAGTCTTGTTCGGTATTCGCCGCTGTT AATCACTTTACTT
	rcsBcoli.T*c*.Rv	GGCGATATCGTTCTCGACACCCAGCTTCATCATC GCAGATTTCTTCTGGCTACTGATGGTGGTTATCA AGAGGGTCATTA
	rcsBcoli.Fw	TAATTGAAGTGCAACTGGCGC
	rcsBcoli.Rv	TTAGTCTTTATCTGCCGGACT
rcsC Salmonella	*rcsC*.Tc.Fw	CTTAATCGCCTTTGTTTCGGTGTTTTACATCGTC AATGCCCTGCACCAGCGGGAGTCTGACTGTTAA TCACTTTACTT

Gene/Region	Primer	SEQUENCE
	rcsC.Tc.Rv	ACAGGCTTCGACAAACAGCTGTCCATACCGGAC TCCAGGCAACGTTGTTTCTCTTCCGCCGGTTATC AAGAGGGTCATTA
	rcsC.Fw	CTTATTCAGAGCGTTAGCGT
	rcsC.Rv	ATACACCGCCAGCGTCTGTT
	*rcsC*H479AAB.Fw	GGATCCGATGGTGTCAATATTCTGAGT
	*rcsC*H479AAB.Rv	GTACAGCGGTGTGCGCAATTCCGCGCTGACCGT CGCAAGGAACAT
	*rcsC*H479CD.Fw	ATGTTCCTTGCGACGGTCAGCGCGGAATTGCGC ACACCGCTGTAC
	*rcsC*H479CD.Rv	GCATGCCTCCGAATCGACGGAAATAT
	*rcsC*H479ok.Fw	AGCATGAACAGTTCAACCGTA
	*rcsC*H479ok.Rv	GTACAGCGGTGTGCGCAATTCCGC
	*rcsC*D875AAB.Fw	GGATCCATATTTCCGTCGATTCGGAG
	*rcsC*D875AAB.Rv	ATACCCCAGCGATCCCAATTGCGCGGCCAGCAA GCGGCGATTGAT
	*rcsC*D875ACD.Fw	ATCAATCGCCGCTTGCTGGCCGCGCAATTGGGA TCGCTGGGGTAT
	*rcsC*D875ACD.Rv	GCATGCTTATGCCCGCGTTTTACGTA
	*rcsC*D875Aok.Fw	GATTATCTCAGCATTCGCGT
	*rcsC*D875Aok.Rv	ATACCCCAGCGATCCCAATTGCGC
rcsC E. coli	*rcsCcoli*.Km.Fw	TCGTACAACCCTGAAAGCCTCGCGCTACATGTT CAGAGCATTGGCGTTAGTGCTCTGGCT AAAGCCACGTTGTGTCTCAA
	rcsCcoli.Km.Rv	CGGCATATAACGTCAGCGTCTGTTTTATCACATC CAGCGTTACCGGCTTCGACAGGCAGC GCGCTGAGGTCTGCCTCGTG
	rcsCcoli.Fw	AGTCGATGTAGAGATCATAG
	rcsCcoli.Rv	TTATCTGGCATTTGCACCGAT
rcsCBC	*rcsCDM*.Tc.Fw	GTTAGCGTTACTCATTTGGCTCTTAATCGCCTTT GTTTCGGTGTTTTACATCGTCAATGCCGCTGTTA ATCACTTTACTT
	rcsBDM.Tc.Rv	CTGTCTATTATCGTTCTGACCATGAACAACAACC CGGCGATCCTGAGCGCCGTGTTGGATGGTTATC AAGAGGGTCATTA

Gene/Region	Primer	SEQUENCE
	rcsCDM.Fw	TTGAAATACCTTGCTTCCTTT
	rcsBDM.Rv	ATGAACAATATGAACGTAATT
rcsD	*rcsD*.ok.Fw	AACAGAATCTTCATTCGCAAC
	rcsD.AB.Fw	GCATGCCATCATTAACTTTATTTATTA
	rcsD.AB.Rv	CTCGAGCACAATGATCAGCAATAAGAA
	rcsD.CD.Fw	CTCGAGTATGCGCTATTTGTAGACACA
	rcsD.CD.Rv	GGATCCATGGAGAGGTCAGTGATCAAC
	rcsD.ok.Rv	ATAGACAGGCTCGGAAAATGA
csgD	*csgD*.c-Myc.AB.Fw	GGATCCAGCGAAATGTACAACTTTACT
	csgD.c-Myc.AB.RV	CTCGAGCAGATCTTCTTCAGAAATAAGTTTTTGT TCCGAGATATCTTCCAGAGAACG
	csgD.c-Myc.CD.Fw	CTCGAGTGCATTGTTTTAATGGATATG
	csgD.c-Myc.CD.RV	GGATCCAACTTCATTGGCATGCAGGTT
	csgD.c-Myc.ok.Fw	AAGACGTGACACACTTCGTTT
	csgD.c-Myc.ok.Fw	CAGATCTTCTTCAGAAATAAGTTTTTGTTC
	csgD.rt.Fw	GCAGGATAATTTAAGCCGCA
	csgD.rt.Rv	TAATCCGCTGACCACGTGTTC
	csgD.UTR.Fw	AGTTAAAAGTATTTTCGTAAATA
	csgD.UTR.Rv	CCGGCTAGCGTGACCATGAATACTATGGACTT
csgA	*csgA.rt.Fw*	CAAACGATGCCCGTAAATC
	csgA.rt.Rv	TTTAGCGTTCCACTGGTCGA
gyrB	*gyrB*.rt.Fw	CGGTAGTCAACGCTCTGTC
	gyrB.rt.Rv	GGCCAGAAACGTACCATCGT
rprA	*rprA.Fw*	CATCTCATTTCTGTCGCAAAT
	rprA.Rv	GACTTGAACAGAATCACACT
	*rprA*NB	CACACAGCAATTCGTTGTTTCACTCAGGG
5S RNA	*5S*NB	CTACGGCGTTTCACTTCTGAGTTC

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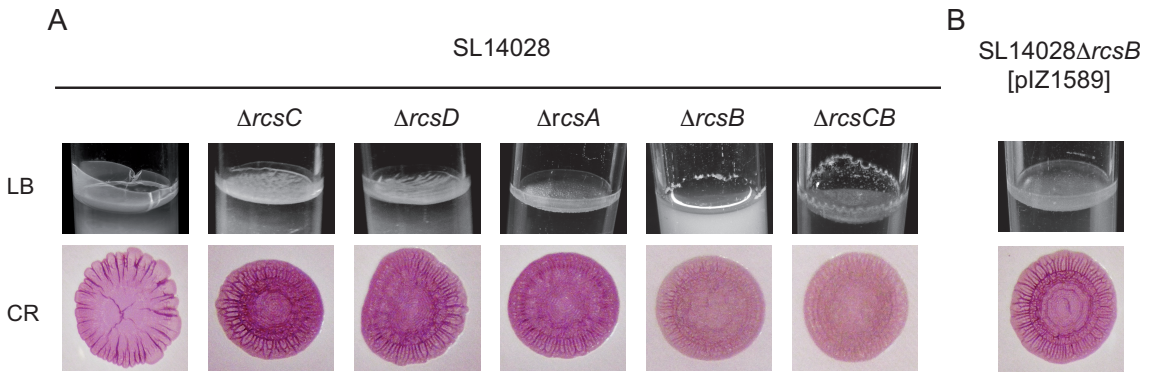
Figure 1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SL14028
B
SL14028Δ*rcsB*
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Δ*rcsB*
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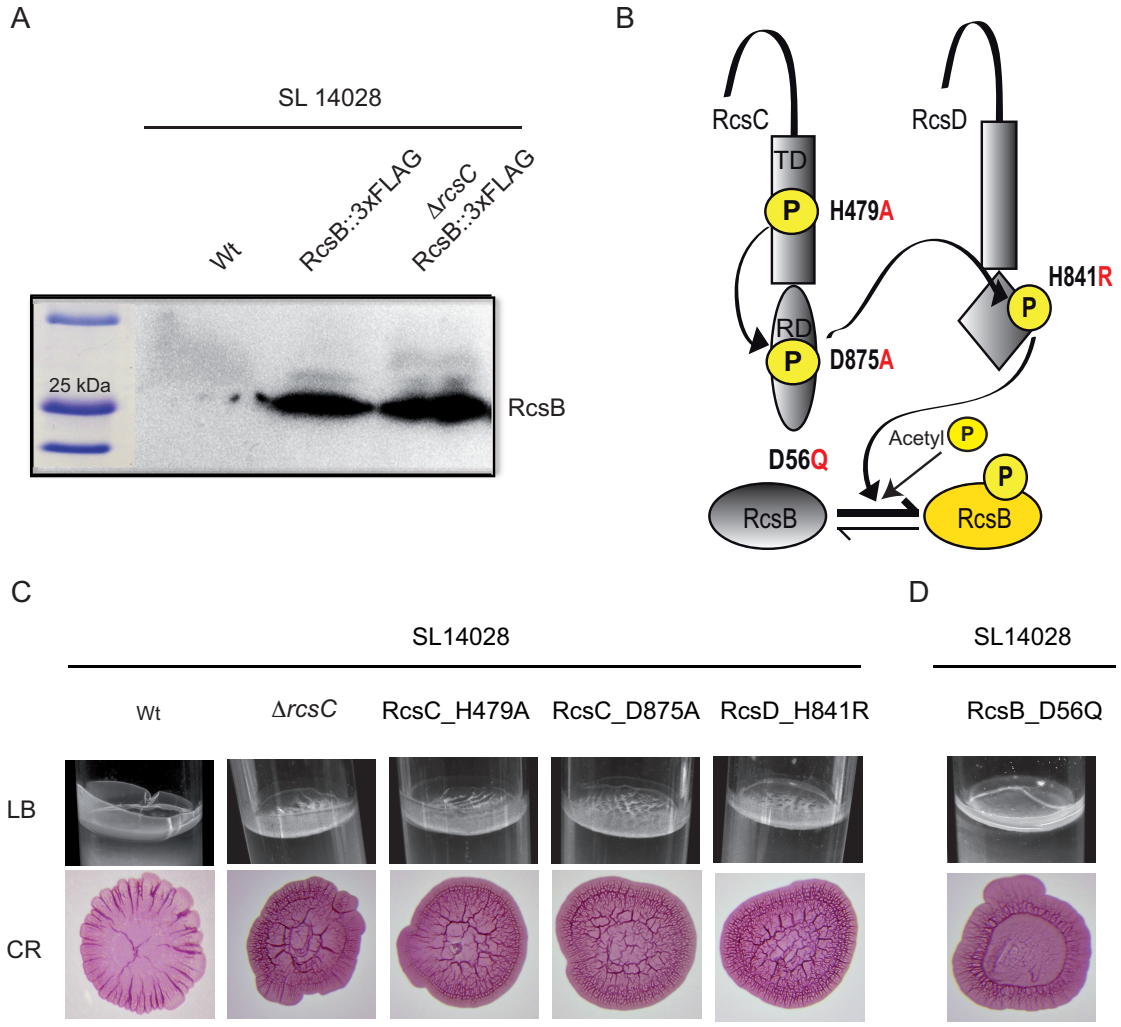
LB
CR

Figure 2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ΔrcsC RcsB::3xFLAG
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B
RcsC
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H479A
RD
P
D875A
H841R
P
Acetyl
P
D56Q
RcsB
P
RcsB
C
SL14028
Wt
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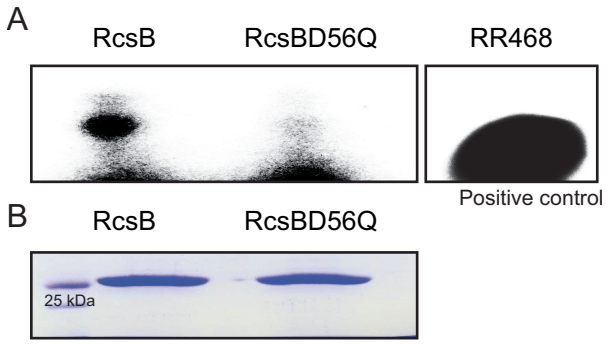
SL14028
RcsB_D56Q

Figure 3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Positive control
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RcsB
RcsBD56Q
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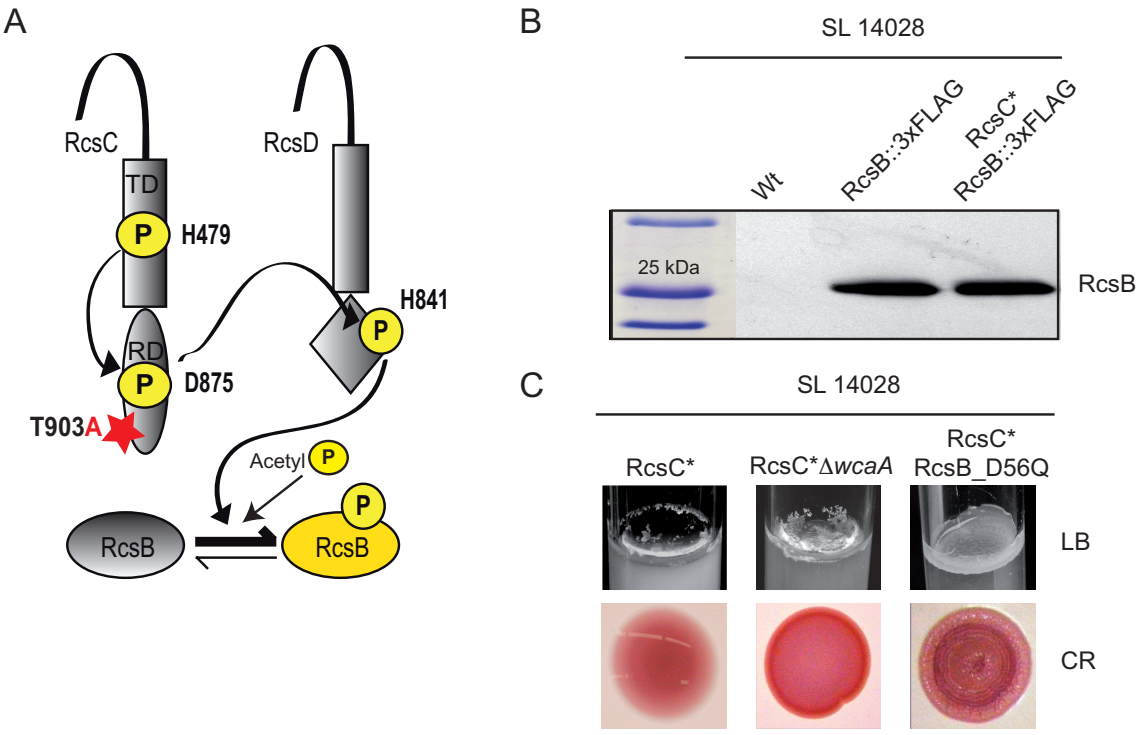
Figure 4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Acetyl
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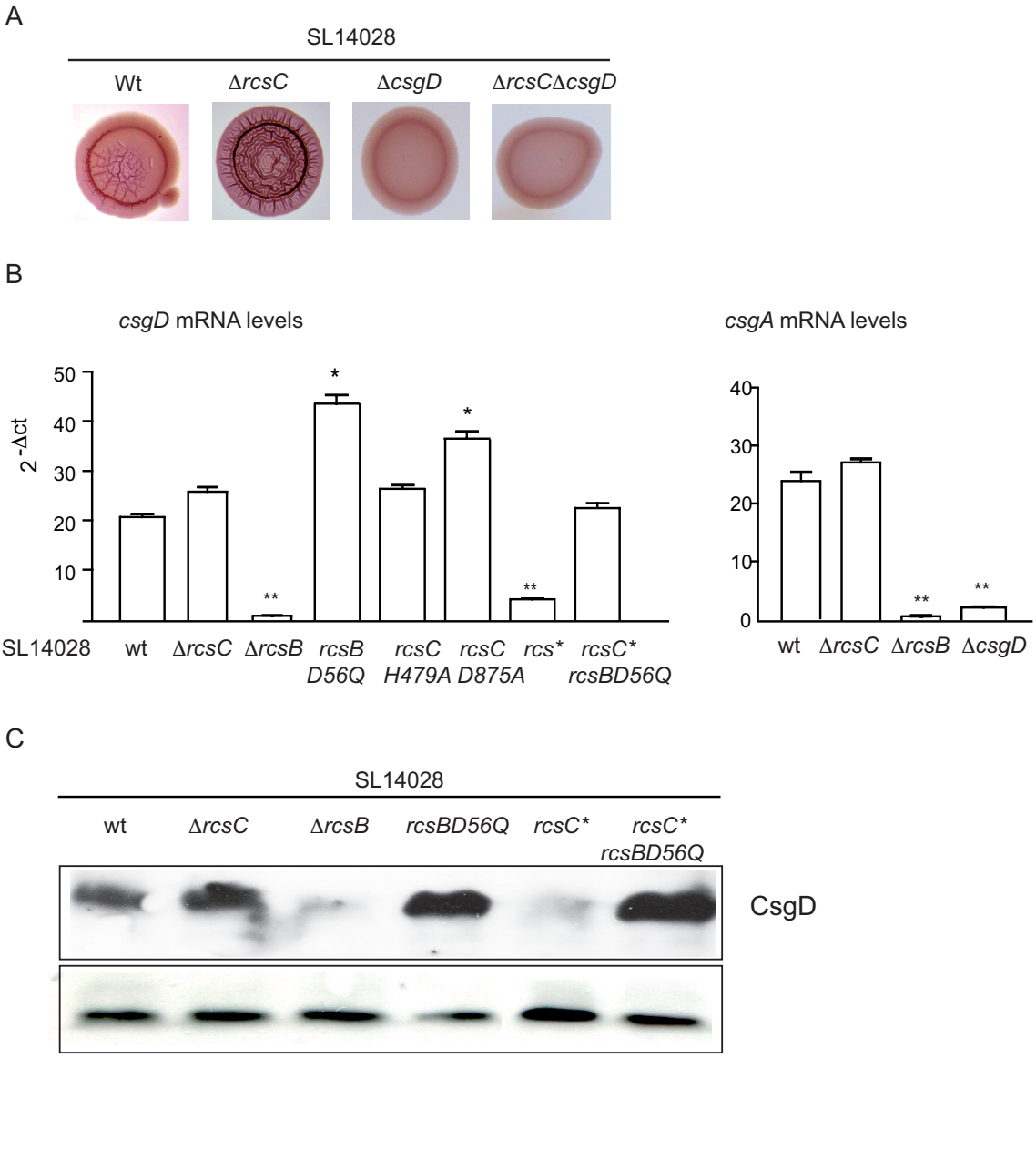
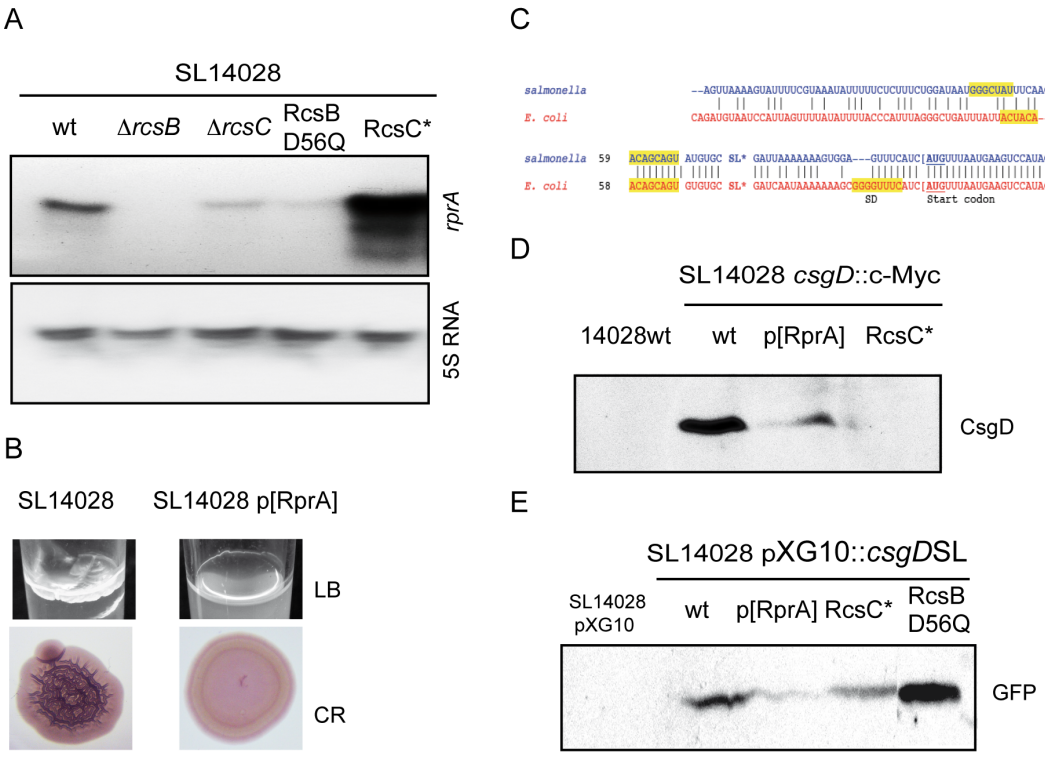
RcsB
B
SL 14028
Wt
RcsB::3xFLAG
RcsC*
RcsB::3xFLAG
25 kDa
RcsB
C
SL 14028
RcsC*
RcsC*ΔwcaA
RcsC*
RcsB_D56Q
LB
CR

Figure 5


A
SL14028
Wt
ΔrcsC
ΔcsgD
ΔrcsCΔcsgD
B
csgD mRNA levels
2-Δct
50
40
30
20
10
SL14028
wt
ΔrcsC
ΔrcsB
rcsB D56Q
rcsC H479A
rcsC D875A
rcs*
rcsC* rcsBD56Q
csgA mRNA levels
40
30
20
10
0
wt
ΔrcsC
ΔrcsB
ΔcsgD
C
SL14028
wt
ΔrcsC
ΔrcsB
rcsBD56Q
rcsC*
rcsC* rcsBD56Q
CsgD

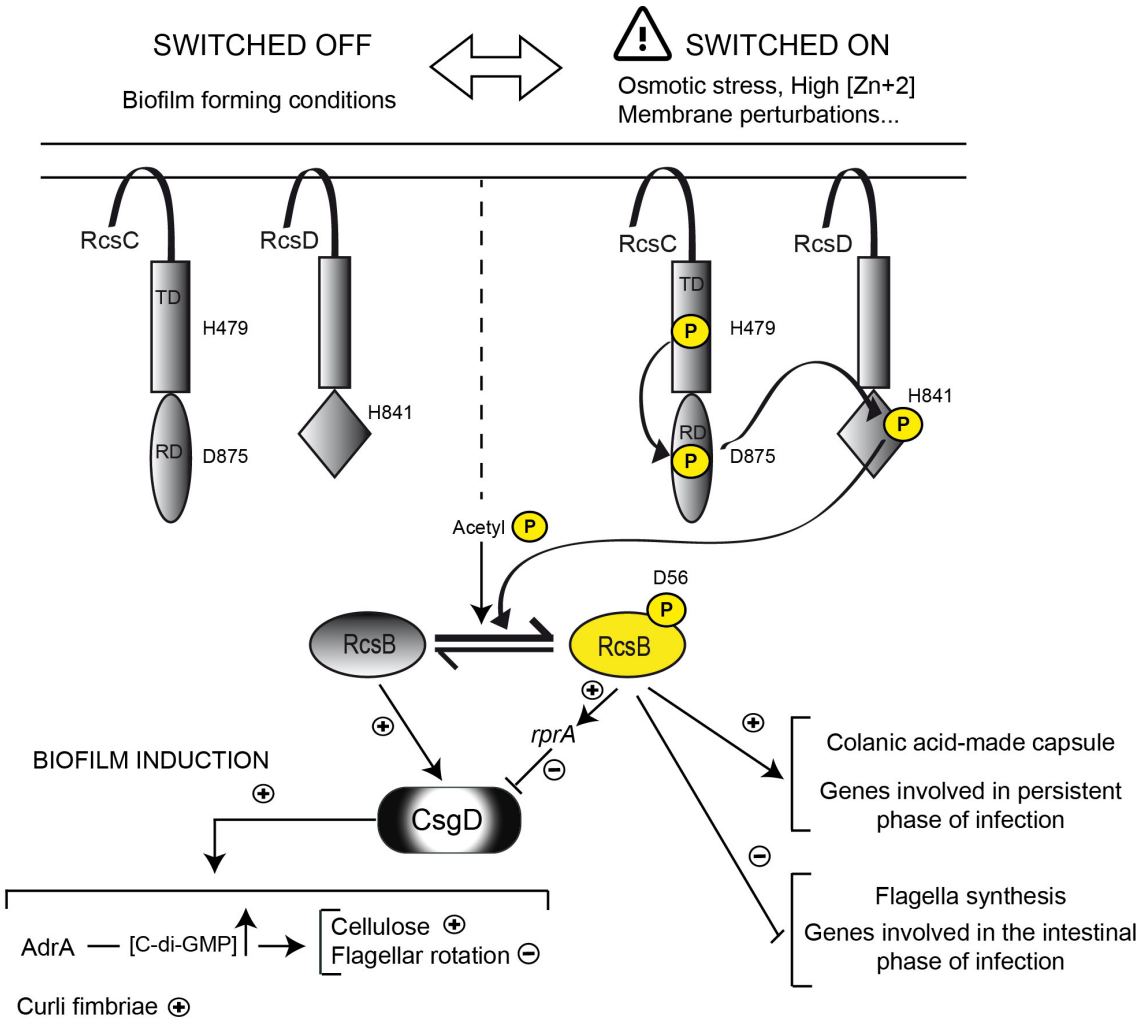
1 **Figure 6**



2 E

3

Figure 7


SWITCHED OFF
Biofilm forming conditions
SWITCHED ON
Osmotic stress, High [Zn+2]
Membrane perturbations...
RcsC
RcsD
TD
H479
RD
D875
H841
P
Acetyl
D56
RcsB
rprA
BIOFILM INDUCTION
CsgD
AdrA
[C-di-GMP]
Cellulose
Flagellar rotation
Curli fimbriae
Colanic acid-made capsule
Genes involved in persistent phase of infection
Flagella synthesis
Genes involved in the intestinal phase of infection