The RcsCDB regulatory system plays a crucial role in the protection of *Salmonella enterica* serovar Typhimurium against oxidative stress

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Dps, the most abundant protein during the stationary growth phase, in *Salmonella enterica* is required for resistance to reactive oxygen species produced by the host during infection. It has been reported that in *Salmonella* *dps* expression is controlled by RpoS and Fur proteins. However, the regulation and function of Dps remain to be resolved. In the present work we demonstrate that activation of the complex RcsCDB regulatory system increases *dps* expression during exponential growth of *Salmonella*. In addition, we show that such *dps* upregulation produces high levels of H₂O₂ resistance. This phenotype allows the bacteria to avoid reactive oxygen species killing at early stages of growth, thus protecting its genetic material.

INTRODUCTION

*Salmonella enterica*, as an intracellular pathogen, is able to survive and recognize changes in the host tissues, promoting a coordinated regulation of virulence factors. It has been reported that when *Salmonella* is contained in the vacuole within the host, the expression of a large number of genes are induced to inactivate reactive oxygen species (ROS). This defence mechanism is controlled mainly by SoxRS, OxyR, σ₅, σ₅’, SlyA and RecA regulons (Farr & Kogoma, 1991; Lacour & Landini, 2004; Yoo et al., 2007). Furthermore, it has been reported that the *dps* gene product is also required for intracellular survival of the pathogen (Valdivia & Falkow, 1996). Dps was first described in *Escherichia coli* but is present in a wide range of bacteria, including eubacterial and archaeal species, as a 19 kDa non-specific DNA-binding protein. Dps protects the bacterial DNA against damage produced by the host ROS production during infection (Almiron et al., 1992; Burton et al., 2014; Chen & Helmann, 1995; Marjorette et al., 1995; Martinez & Kolter, 1997). Almiron et al. (1992) reported that expression of the *dps* gene is induced by the cessation of bacterial growth under control of RpoS, the σ₅ factor. However, subsequent studies in *E. coli* showed that this gene, as a member of the OxyR regulon, is also expressed in bacteria during exponential growth (Altuvia et al., 1994).

In this paper, we report that in *S. enterica* serovar Typhimurium (*S. Typhimurium*) the RcsCDB regulatory system is also involved in control of the *dps* gene expression mainly during the exponential growth phase. The RcsCDB phosphorelay belongs to the family of two-component regulatory systems, but unlike most members of this family, it is composed of three proteins: RcsC, RcsD and RcsB (Majdalani & Gottesman, 2005). In this system, RcsC acts as a sensor kinase protein attached to the inner membrane, which is able to recognize a specific stimulus. The second inner-membrane protein, RcsD, functions as an intermediary in the transfer of the phosphoryl group in an RcsC-dependent pathway or as a sensor under certain Rcs activation conditions in which RcsC is not required (Majdalani & Gottesman, 2005; Pescaretti et al., 2013; Takeda et al., 2001). The last component of this system is the response regulator RcsB, a cytoplasmic protein, which when phosphorylated is able to bind the DNA to modulate gene expression (Majdalani & Gottesman, 2005). In previous studies, we have shown that in *Salmonella* there are at least three pathways of RcsB phosphorylation, which depend on the environmental changes detected by RcsC, RcsD or RcsC–RcsD interactions (Pescaretti et al., 2013; Takeda et al., 2001). These findings explain why we failed to identify a specific signal leading to system activation. However, it has been reported that Rcs system activation occurs under different growth conditions or by specific gene mutations such as deletion of the tolB gene (Chen et al., 2001; Clarke et al., 1997; Dominguez-Bernal et al., 2004; Ebel et al., 1997; Mouslim & Groisman, 2003; Mouslim et al., 2003; Pescaretti et al., 2010). In this regard, we have demonstrated that the rcsC11 mutant produces constitutive activation of the Rcs system and dramatically attenuates *Salmonella*
virulence in an animal model. This mutation is produced by a point change in the ATPase domain of the rcsC gene, which prevents RcsB dephosphorylation (Costa & Anton, 2001; Mouslim et al., 2004). We established that the rcsC11-attenuated phenotype is characterized by: (i) partial repression of the flhDC operon, required for flagellum synthesis; (ii) repression of hilA, invF, sipC and invG genes required to establish infection in mice after being orally inoculated; (iii) induction of the cps operon required for colanic acid capsule production; and (iv) overexpression of wzc, which controls formation of the O-antigen long region, increasing resistance to the bactericidal effect of serum complement (Delgado et al., 2006; Farizano et al., 2012; Mouslim et al., 2004; Penheiter et al., 1997). However, we observed that only elimination of the rcsB gene from the rcsC11 mutant fully restored virulence levels, suggesting that other RcsB-dependent gene products are involved in such an attenuation phenotype. As is well known, in the presence of a pathogen the host induces a large number of defence mechanisms, and therefore here we look for other RcsB-dependent genes whose products allow the rcsC11 mutant to resist the host defence mechanisms and allow successful survival within the vacuole.

Using a list of RcsB-dependent genes obtained from a microarray assay, we demonstrate experimentally that the dps gene is a new member of the RcsB regulon. We show that under Rcs system activation, dps expression is induced during the exponential growth phase. Moreover, we demonstrate that this dps induction leads to an increase in the resistance to ROS.

**METHODS**

**Bacterial strains, molecular techniques and growth conditions.** Bacterial strains and plasmids used in this work are listed in Table 1. The mutations employed were introduced into different genetic backgrounds by Phage P22-mediated transductions following the protocol described by Davis et al. (1980). The chromosomal $\Delta dps::lacZY$ gene fusion strain was constructed as described by Ellermeier et al. (2002). Briefly, after removing the Cm$^\text{r}$ cassette from strain SL3474, the pCE36 plasmid harbouring the lacZY transcriptional fusion was integrated into the FLP recombination target sequence located downstream of the gene by FLP-mediated recombination (Ellermeier et al., 2002). The recombinant DNA techniques and bacterial growth were performed according to standard protocols (Sambrook et al., 1989). β-Galactosidase activity was measured as described by Miller (1972) from bacteria growing in Luria–Bertani (LB) medium at 37°C. Kanamycin, ampicillin and chloramphenicol were used at final concentrations of 50, 50 and 25 µg ml$^{-1}$, respectively.

**Construction of the P$_{dps}::$lacZYA fusion plasmid.** The plasmid used to determine the promoter activity contains the promoter region of dps driving transcription of the promoterless lacZYA operon as reporter. To construct this plasmid, we amplified by PCR a region of 356 bp located upstream of the dps start codon using the primers #8031 [EcoRI-(5′-CGGAATTCTGGTTCTACCTAAGCATTCG-3′)] and #8033 [5′-CCGGATCCCTCATATCCTCTTGATGTTTGTGT-CC-3′]-BamHI, and the S. Typhimurium 14028s chromosomal DNA as a template. The 356 bp PCR product was cloned between the corresponding restriction enzyme sites of the pRS415 vector (Simons et al., 1987), resulting in the pP$_{dps}::$lacZYA derivative plasmid.

**Electrophoretic mobility shift assays (EMSAs).** EMSAs were carried out according the protocol described by Lehti et al. (2012). To this end, the DNA sequences containing or lacking the putative RcsB-binding site were amplified by PCR using wild-type 14028s

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

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Description*</th>
<th>Reference or source</th>
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<tbody>
<tr>
<td><strong>S. Typhimurium</strong></td>
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<tr>
<td>14028s</td>
<td>Wild-type</td>
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<tr>
<td>EG12711</td>
<td>ΔrcsB::Cm</td>
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<td>rcsC11</td>
<td>Mouslim et al. (2004)</td>
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<tr>
<td>EG13354</td>
<td>tolB::Tn10d-Cm</td>
<td>Mouslim &amp; Groisman (2003)</td>
</tr>
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<td>This work</td>
</tr>
<tr>
<td>SL3474</td>
<td>Δdps::aph</td>
<td>Halsey et al. (2004)</td>
</tr>
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<td><strong>Plasmid</strong></td>
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<td>pRS415</td>
<td>Multicopy vector for promoter cloning, rep$_{pMB1}$ ori, lacZYA ramB. Ap</td>
<td>Simons et al. (1987)</td>
</tr>
<tr>
<td>pP$_{dps}::$lacZYA</td>
<td>pRS415 vector containing 356 bp of P$_{dps}$ region fused to promoterless lacZYA genes</td>
<td>This work</td>
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*Gene designations are summarized by Sanderson et al. (1995).*
chromosomal DNA as a template. PCR of the dps promoter product containing the predicted RcsB-binding site (356 bp) used primers #8031 and #8033. The control PCR product of 234 bp (truncated version), where the predicted RcsB-binding site was not included, was amplified using primers #8032 (5′-GCGTATTACTTCGTC-3′) and #8033. In this assay, 2 pmol of DNA was incubated at room temperature with 0, 5, 10, 40 or 80 nM of RcsB-H6 protein purified as previously described (Delgado et al., 2006). The samples were analysed on a 6 % non-denaturing Tris/borate-EDTA polyacrylamide gel and run at 8 mA at 4 °C. The polyacrylamide gels were then stained with ethidium bromide and the DNA fragments were visualized under UV irradiation.

**Oxidative stress resistance assays.** Bacterial resistance to hydrogen peroxide treatment was measured as described by Halsey et al., (2004). Briefly, the tested strains were grown for 5 h in LB medium, the cultures were diluted to 1:100 in PBS and incubated at 37 °C with 0.5 mM H₂O₂. After 2 h of treatment, aliquots of 20 μl of each culture were collected to determine the number of viable cells by serial dilution and plating onto LB agar. The qualitative determination of viability after the H₂O₂ treatment was evaluated by spotting 5 μl of the each dilution on LB agar plates. Controls for these assays were performed by incubation of the cells without H₂O₂.

**RESULTS**

**Identification of dps as a member of the RcsB regulon**

We have previously demonstrated that the rcsC11 mutant displays an attenuated virulence phenotype, resulting from downregulating expression of certain genes such as hilA, invF, sipC and invG required for invasion and replication of the pathogen during host infection (Mouslim et al., 2004). In addition, in the rcsC11 mutant other genes are upregulated to allow the bacteria to survive within the eukaryotic cell (Delgado et al., 2006; Farizano et al., 2012; Mouslim et al., 2004). However, we hypothesized that RcsB modulates expression of other genes whose products allow the pathogen survive within epithelial cells. Here, we looked for those RcsB-dependent genes that could be involved in the resistance to ROS produced by the host. This was based on the results of Burton et al. (2014), who reported that in the presence of the pathogen, the host induces the production of ROS to regulate the innate immune response mechanisms (Burton et al., 2014). To this end, we used a list of RcsB-modulated genes previously obtained from a microarray assay carried out with mRNA from wild-type, rcsC11 and rcsC11 rcsB strains (C. Mouslim et al., unpublished data). In this assay we observed that dps gene expression was 5.6-fold increased in rcsC11 compared with wild-type levels, while in the rcsC11 rcsB double mutant expression was strongly reduced, indicating that RcsB is required for dps induction (C. Mouslim et al., unpublished data). To verify these results we first carried out a bioinformatics analysis using the dps promoter region to identify a putative RcsB-binding site. As shown in Fig. 1(a, b), we found a sequence homologous to the RcsB-binding site present in other well-characterized RcsB-regulated genes, located 272 nt upstream from the previously described dps +1 transcription site (Mouslim & Groisman, 2003; Yoo et al., 2007). In addition, EMSA showed that migration of a 356 bp PCR product was retarded when RcsB concentration was increased (Fig. 1c, RcsB–DNA complex). Note that the PCR product used in this assay included both the −35 and −10 promoter boxes as well as the predicted RcsB-binding site. When the EMSA was carried out using a truncated PCR variant, lacking only the predicted RcsB-binding box, this regulator was unable to modify the electrophoretic mobility of the 234 bp PCR product, even at the highest RcsB concentrations used (Fig. 1d, right-hand panel, RcsB–DNA complex). In addition, we performed competition experiments incubating RcsB with both full-length (356 bp) and truncated (234 bp) PCR products. In this assay, RcsB was able to bind only the 356 bp product but not the 234 bp product lacking the predicted RcsB-binding site (Fig. 1d, left-hand panel). These data demonstrated the high specificity of RcsB for the predicted binding site on the dps promoter. Taken together, our results confirm that RcsB promotes dps expression by directly binding to the promoter region of dps.

**RcsB controls dps expression during exponential growth phase**

The position of the RcsB-binding site related to both −35 and −10 promoter boxes suggests that the regulator might exert a positive effect on dps gene expression. To determine whether RcsCDB system activation affects dps gene transcription, the levels of β-galactosidase activity were determined in wild-type S. Typhimurium (14028s), rcsC11 (representing activation conditions, MDs1608) and rcsB (negative control, MDs1569) strains harbouring chromosomal lacZY transcriptional fusions to the dps gene during growth on LB medium (Table 1). As shown in Fig. 2(a), the transcription levels of dps in the rcsC11 mutant were twofold higher than in the wild-type strain. Interestingly, this increase was observed after 5 h, which according to the growth curve occurs during the exponential phase (Fig. 2a, b). By contrast, dps transcription levels in the rcsB mutant were twofold lower than those observed in the wild-type strain after 5 h of growth (Fig. 2a, c). The differences in dps expression levels observed between these three genetic backgrounds were maintained during late stationary phase growth (Fig. 2a, b).

To confirm the effect of RcsB on dps expression, we determined the levels of β-galactosidase produced after 5 h of bacterial growth by the dps::lacZY fusion in a tolB mutant, as a different activation condition from the Rcs system. As shown in Fig. 2(c), in the tolB and rcsC11 mutants, dps expression was twofold induced relative to wild-type levels, while such induction was abolished in the tolB rcsB double mutant.

Together, these and the bioinformatic and EMSA results demonstrated that when the Rcs system is activated expression of the dps gene is induced during the exponential growth phase.
Induction of $dps$ in the exponential phase depends on $P_{rcsDB}$ activation

It has been reported that the Dps protein has the ability to affect the expression of about 36 genes, due to its non-specific DNA-binding activity (Almiron et al., 1992; Altuvia et al., 1994; Frenkiel-Krispin et al., 2001). According to these data and to avoid any growth defect of the $dps$ chromosomal gene elimination, we constructed the $P_{dps}::\text{lacZYA}$ plasmidic transcripcional fusion. To this end, a 356 bp sequence containing the $dps$ promoter ($P_{dps}$) as well as the predicted RcsB-binding site was cloned in the pRS415 vector in the correct ORF to the promoterless $\text{lacZYA}$ operon (Table 1). Levels of $\beta$-galactosidase activity produced by p$P_{dps}::\text{lacZYA}$ were then determined in the wild-type, rcsB and tolB strains. As shown in Fig. 3(a), $\beta$-galactosidase activity was twofold increased in the tolB mutant relative to the wild-type strain after 5 h of growth, as was observed using $dps::\text{lacZY}$ chromosomal transcripcional fusion (Fig. 2a). However, we observed the largest differences in $\beta$-galactosidase activity between these strains when the cultures reached the stationary growth phase, at 8 and 24 h (Fig. 3a). In these assays we also determined that when the rcsB gene was eliminated from the chromosome, $\beta$-galactosidase activity decreased about 4.8-fold compared with the wild-type background (Fig. 3a). These data confirmed that RcsB positively controls $dps$ expression, mainly playing the role of an inductor during the exponential phase when the bacteria sense new changes in their environment.

We have previously reported that there are at least three pathways by which the RcsB regulator could be phosphorylated (Pescaretti et al., 2013). In addition, the synthesis of this regulator also depends on the activity of two promoters, $P_{rcsDB}$ and $P_{rcsB}$. Activation of $P_{rcsDB}$ is
dps (MDs1569) and rcsC11 genetic backgrounds: wild-type S. Typhimurium (14028s), rcsB (EG12711) and tolB (EG13354) strains, during growth in LB medium. Asterisks indicate significant differences between the parental and tolB related to rcsB strains (P = 0.001). (b) Effect of different RcsB levels, expressed from its two promoters, on P\textsubscript{dps} activity: β-galactosidase activity produced by the P\textsubscript{dps}::lacZYA fusion was determined in tolB (EG13354), rcsB tolB (MDs1458), tolB P\textsubscript{rcsDB} (MDs1453) and tolB P\textsubscript{rcsB} (MDs1457) mutants. The measurements were performed over bacterial growth in LB medium. The data correspond to mean values and SD of three independent experiments, carried out in duplicate.

**Fig. 2.** The dps gene transcription is regulated by the RcsCDB system. (a) β-Galactosidase activity (Miller units) expressed by strains harbouring chromosomal dps::lacZY transcriptional fusions following growth in LB medium as described in Methods. Transcriptional activity was investigated in the following genetic backgrounds: wild-type S. Typhimurium (MDs1568), rcsB (MDs1569) and rcsC11 (MDs1608) strains. (b) Bacterial growth curve of dps (SL3474), dps rcsB (MDs1571) and dps rcsC11 (MDs1633) mutants, determined in LB medium. (c) Effect of different RcsCDB activation conditions on dps expression measured as β-galactosidase activity (Miller units) and determined in wild-type (MDs1568), rcsB (MDs1569), rcsC11 (MDs1608), tolB (MDs1572) and rcsB tolB (MDs1573) strains. β-Galactosidase activity was investigated after 5 h of bacterial growth. The data correspond to mean values and SD of three independent experiments, carried out in duplicate. Different letters represent statistically different β-galactosidase activity values (Tukey test, P = 0.05).

**Fig. 3.** Effect of the RcsB regulator on dps promoter activity. (a) β-Galactosidase activity of pP\textsubscript{dps}::lacZYA fusion was determined in the wild-type S. Typhimurium (14028s), rcsB (EG12711) and tolB (EG13354) strains, during growth in LB medium. Asterisks indicate significant differences between the parental and tolB related to rcsB strains (P = 0.001). (b) Effect of different RcsB levels, expressed from its two promoters, on P\textsubscript{dps} activity: β-galactosidase activity produced by the P\textsubscript{dps}::lacZYA fusion was determined in tolB (EG13354), rcsB tolB (MDs1458), tolB P\textsubscript{rcsDB} (MDs1453) and tolB P\textsubscript{rcsB} (MDs1457) mutants. The measurements were performed over bacterial growth in LB medium. The data correspond to mean values and SD of three independent experiments, carried out in duplicate.

observed at an early stage of the bacterial growth driving the production of a co-transcript rcsDB, while activation of P\textsubscript{rcsB} occurs during the stationary phase to control only the synthesis of rcsB transcript (Pescaretti et al., 2009, 2010). To determine which of the RcsB phosphorylation pathway is involved in the control of dps expression, levels of β-galactosidase produced from pP\textsubscript{dps}::lacZYA was determined in tolB rcsB, tolB P\textsubscript{rcsDB} and tolB P\textsubscript{rcsB} double mutants. In this assay, β-galactosidase activity decreased in the tolB P\textsubscript{rcsDB} and tolB P\textsubscript{rcsB} double mutants, reaching values six- and twofold lower than those observed in tolB, respectively (Fig. 3b). However, β-galactosidase activity in the tolB P\textsubscript{rcsB} mutant was higher than in the tolB rcsB mutant, while no significant differences were observed between the tolB P\textsubscript{rcsDB} and tolB rcsB mutants (Fig. 3b). These results indicated that the most important contribution of RcsB in dps induction is produced when the P\textsubscript{rcsDB} promoter is activated during the exponential phase.
Nevertheless, our data suggest that activation of the P_{rcsB} promoter controlling RcsB production also contributes to dps expression during a more prolonged stationary phase.

**The dps–RcsB-dependent induction is required for oxidative stress resistance**

Halsey et al. (2004) reported that the Dps protein is accumulated during the stationary phase and protects *Salmonella* from oxidative stress-dependent killing, allowing bacterial survival in murine macrophages (Halsey et al., 2004). To determine the physiological role of the RcsB-dependent dps induction we investigated oxidative stress resistance during the exponential growth phase (5 h) of wild-type, *rcsC*11, *rcsB* and *dps* strains. To this end, the strains were exposed to 0.5 mM H$_2$O$_2$ as described in Methods. As shown in Fig. 4(a), we found similar numbers of cell colonies in the control of all test strains (non-hydrogen H$_2$O$_2$ treatment), as confirmed by c.f.u. counts (data not shown). In accordance with Halsey et al. (2004), we observed that the c.f.u. number of the *dps* mutant was 1.5 orders of magnitude lower than the wild-type strain after H$_2$O$_2$ treatment (Fig. 4). By contrast, when the Rcs system was induced the resistance to ROS was increased, as the recount of c.f.u. of the *rcsC*11 mutant was similar in the control and treatment assays (Fig. 4b). Consequently, the c.f.u. recount of *rcsC*11 was higher than of the wild-type strain after treatment (Fig. 4). Moreover, the *rcsB* mutant was significantly more sensitive than the wild-type strain to H$_2$O$_2$ treatment (Fig. 4). These results suggest that the increased resistance of the *rcsC*11 mutant to H$_2$O$_2$ treatment is the result of *dps* gene induction in an RcsB-dependent manner. To validate this, we tested the resistance to oxidative stress at 5 h of the *rcsC*11 *dps* and *rcsB* *dps* double mutants, and of these strain complemented by pBAD::Dps plasmid harbouring the *dps* gene [provided by Dr S. J. Libby (Halsey et al., 2004)]. Fig. 4(a) shows that the higher resistance displayed by *rcsC*11 to oxidative stress was decreased 1.5-fold in the *rcsC*11 *dps* mutant, to levels even lower than the wild-type strain (Fig. 4b). In these assays, we found that *rcsB* *dps* was the most sensitive strain to such stress as we obtained a c.f.u. recount 2.5- and 1.5-fold lower than wild-type and *rcsB*, respectively (Fig. 4). This *rcsB* *dps* phenotype could be explained assuming that expression of other RcsB-dependent genes is required for H$_2$O$_2$ resistance. All complemented mutants recovered the resistance patterns of the wild-type strain (Fig. 4b). Moreover, we observed that survival to H$_2$O$_2$ treatment of the wild-type strain harbouring pBAD::Dps was higher than without plasmid (3.98 × 10$^7$ and 9.54 × 10$^6$ c.f.u. ml$^{-1}$, respectively). These H$_2$O$_2$ resistance levels were similar to those observed in the *rcsC*11 mutant (Fig. 4b).

![Fig. 4. Hydrogen peroxide sensitivity of *dps* mutant cells. The effect of H$_2$O$_2$ on the viability of wild-type *S. Typhimurium* (14028s), *rcsC*11 (EG14873), *rcsB* (EG12711), *dps* (SL3474), *rcsC*11 *dps* (MDs1633) and *rcsB* *dps* (MDs1571) strains, as well as of double mutants harbouring pBAD::Dps plasmid induced with 0.2% l-arabinose (complementation assay) was determined by spot (a) or viable-cell counting as log c.f.u. ml$^{-1}$ (b) of cells harvested during the exponential growth phase (5 h) in LB medium and then exposed for 2 h to 0.5 mM H$_2$O$_2$. All assays were conducted at least three times and carried out in duplicate. Different letters represent statistically different values (Tukey test, P=0.05).](http://mic.sgmjournals.org)
Cumulatively, these results demonstrated that activation of the Rcs system is at least in part responsible for *Salmonella* resistance to oxidative stress-dependent killing, mainly when the bacteria enter the exponential growth phase, by inducing earlier transcription of the *dps* gene.

**DISCUSSION**

The Dps protein, first identified as a low-molecular-mass protein that accumulated during stationary growth phase, is capable of binding to DNA (Almiron *et al.*, 1992). This protein is encoded by the *dps* gene and shows homology to ferritin protein, which is responsible for iron sequestering from the intracellular medium (Grant *et al.*, 1998). Based on this observation, it was suggested that Dps protects DNA against damage produced by ROS acting directly on DNA or sequestering iron and limiting Fenton-catalysed oxyradical formation (Chiancone & Ceci, 2010; Halsey *et al.*, 2004; Zhao *et al.*, 2002). In addition, resistance to ROS is essential for *S. Typhimurium* pathogenesis, which is enhanced through *dps* induction by those pathways controlled by SoxRS, OxyR, σ^54^, SlyA and RecA (Halsey *et al.*, 2004; Pacello *et al.*, 2008). By contrast, we previously established that constitutive activation of the RcsCDB system produced by the point mutation on the *rcsC* gene renders the *rcsC11* *Salmonella* strain avirulent in an animal model. This mutant is able to replicate within epithelial cells and at some time produces a kind of immunity in the host against virulent *Salmonella* strain infection (Mouslim *et al.*, 2004). Although we demonstrated that this phenotype results from strong repression of *hilA*, *invF*, *sipC* and *invG* RcsB-regulated genes, we consider that the attenuation requires expression of other genes involved in the bacterial resistance to the host immune system. The aim of this study was to determine whether resistance to ROS could be involved in the attenuation of virulence displayed by *rcsC11* or when the Rcs system is activated, by controlling the expression of genes such as *dps*, *sodAB* and *katG*.

Our microarray results demonstrated that among those genes required for ROS resistance, only expression of the *dps* gene was strongly induced when the Rcs regulatory system is activated, and abolished in the *rcsC11 rcsB* mutant (C. Mouslim *et al.*, unpublished data). These observations allowed us to postulate that *dps* would form part of the RcsB regulon. In addition, previous studies reported that when RcsB functions as an activator, in many cases it requires the RcsA co-activator to modulate gene expression (Gottesman, 1995; Gottesman & Stout, 1991). In this sense, our microarray results showed that RcsB induces transcription of *dps* in an RcsA-independent pathway, because gene expression was not affected in the *rcsC11 rcsA* mutant relative to those levels observed in *rcsC11* (C. Mouslim *et al.*, unpublished data).

RcsB functions as either a repressor or an activator of gene transcription dependent on the location of its binding site to the regulated promoter gene. Here we first verified the effect of RcsB on *dps* transcription by bioinformatics and *in vitro* EMSAs. We further identified the RcsB-regulatory binding box on the *dps* regulatory sequence, which was located far from the −55 and −10 box elements, indicating that RcsB exerts a positive modulation (Fig. 5a). So, this binding allows the formation of a stable DNA/RNA polymerase complex to favour induction of *dps* transcription. Furthermore, when *dps* expression levels were determined under Rcs system activation we observed a strong induction during the exponential growth phase (Fig. 5b). In addition, these high levels were maintained after 48 h of culture (Fig. 2). Although Yoo *et al.* (2007) reported that *dps* expression is induced during the stationary phase by the Fur regulator, our results demonstrated that this gene is also regulated by RcsB but during the exponential phase of growth. These data provide the first evidence of such *dps* regulation in *Salmonella*, which increase our knowledge of synthesis of this protein. In addition, Yoo *et al.* (2007) suggested that, as in *E. coli*, the *Salmonella dps* gene could also be controlled by OxyR during exponential growth. However, this assumption was made based only on the presence of a potential OxyR binding box on the *dps* regulatory region and not experimentally by *in vivo* assays. Nevertheless, our results suggest that RcsB exerts its activator effect independently of OxyR because the binding sites of both regulators are separated by 207 bp, which rules out the possibility that a steric impediment occurs (Fig. 5a). In addition, this RcsB-binding site is far from the nucleotide sequences required for Fur and RpoS binding (177 and 126 bp, and 259 bp, respectively, Fig. 5a) (Yoo *et al.*, 2007). The distances observed between these different regulatory regions allow us to suggest that these proteins bind to the promoter and all can work independently at the same time to modulate *dps* expression during stationary growth. By contrast, it has been shown that Dps affects expression of a large number of genes and consequently affects bacterial growth (Almiron *et al.*, 1992). Thus, we further investigated the effect of RcsB on *dps* transcription, when its promoter was fused to the lacZYA reporter operon into a plasmid vector. We observed the same expression pattern as those produced from chromosomal *dps::lacZY* fusion under Rcs system activation (Figs 2a and 3a, *rcsC11* and *tolB* mutant, respectively). Together these results suggest that the RcsB-dependent modulation follows a bimodal induction behaviour not affected by any other component.

As we demonstrated that the RcsCDB phosphorelay is a complex mechanism mainly due to the presence of two promoters that control *rcsB* expression, during different growth phases (Pescaretti *et al.*, 2009, 2013), we attribute this bimodal behaviour to the presence of different levels of the phosphorylated RcsB form. Thus, the first *dps* expression peak observed is due to the RcsB phosphorylation by the RcsC→RcsD→RcsB pathway during the exponential phase when *rcsD* and *rcsB* are co-transcribed from the *P_{rcsDB}* promoter as an operon. This hypothesis
We also observed that the decreased phosphorelay pathway and RcsDB abolished the elimination of such a promoter in the tolB mutant even after 24 h of culture. This suggests that the amount of RcsB produced by PrcsB was not enough to induce dps expression in stationary phase (Fig. 3b). However, in the tolB PrcsB mutant dps transcription was sevenfold higher than that observed in the tolB rcsB mutant after 24 h, confirming that the RcsC→RcsD→RcsB phosphorelay pathway and PrcsDB promoter activity are required for early modulation of dps. Nevertheless, our data suggest that RcsB also contributes to dps expression during a more prolonged stationary phase, when PrcsB starts to be active. In this last situation the RcsC→RcsB phosphorelay is the pathway that modulates dps transcription. Furthermore, the β-galactosidase activity observed during stationary phase suggests that in Salmonella RcsB, Fur and RpoS work cooperatively to increase dps transcription. Consequently, the high levels of Dps ensure the protection of DNA from damage caused mainly by ROS, which are increased in this bacterial growth period.

Additionally, we investigated the correlation between dps expression and its resistance to ROS damage, when the Rcs system is activated during the exponential growth phase. For the first time, to our knowledge, we demonstrate that in S. Typhimurium the strains growing exponentially become more resistant to H2O2 when the Rcs system is activated (Fig. 4b, rcsC11). Accordingly, we conclude that this phenotype is due to the dps RcsB-dependent induction, because under our conditions the rcsC11 dps double mutant displayed less resistance to H2O2 than the wild-type and rcsC11 strains (Fig. 4b). Moreover, when the rcsC11 dps double mutant was complemented with pBAD::Dps, resistance levels were restored to wild-type values (Fig. 4b). Note that the rcsC11 dps (pBAD::Dps) strain failed to achieve the rcsC11 resistance levels to H2O2, which could be explained assuming that the pBAD::Dps plasmid lacks the dps RcsB-regulatory region. These results confirm that early dps transcription induction and consequently resistance to ROS killing during the exponential phase are strongly dependent on RcsB control (Fig. 5b).

Taken together, our findings highlight the role of the RcsCDB regulatory system in the induction of dps expression and demonstrate for the first time that in Salmonella such modulation occurs during the exponential growth phase. Then, in the transition to stationary phase growth, other regulators, such as Fur and RpoS, contribute to increase dps transcription to up to 200,000 molecules per cell (Almiron et al., 1992; Yoo et al., 2007). We therefore suggest that successful survival of the rcsC11 mutant within epithelial cells requires, in part, dps upregulation increasing the resistance to host ROS damage; this is currently being studied further in our laboratory.

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