Dam Methylation Participates in the Regulation of PmrA/PmrB and RcsC/RcsD/RcsB Two Component Regulatory Systems in Salmonella enterica Serovar Enteritidis

https://doi.org/10.1371/journal.pone.0056474

Published in:
PLoS One

Document Version:
Publisher's PDF, also known as Version of record

Queen's University Belfast - Research Portal:
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Abstract

The absence of Dam in Salmonella enterica serovar Enteritidis causes a defect in lipopolysaccharide (LPS) pattern associated to a reduced expression of wzz gene. Wzz is the chain length regulator of the LPS O-antigen. Here we investigated whether Dam regulates wzz gene expression through its two known regulators, PmrA and RcsB. Thus, the expression of rcsB and pmrA was monitored by quantitative real-time RT-PCR and Western blotting using fusions with 3xFLAG tag in wild type (wt) and dam strains of S. Enteritidis. Dam regulated the expression of both rcsB and pmrA genes; nevertheless, the defect in LPS pattern was only related to a diminished expression of RcsB. Interestingly, regulation of wzz in serovar Enteritidis differed from that reported earlier for serovar Typhimurium; RcsB induces wzz expression in both serovars, whereas PmrA induces wzz in S. Typhimurium but represses it in serovar Enteritidis. Moreover, we found that in S. Enteritidis there is an interaction between both wzz regulators: RcsB stimulates the expression of pmrA and PmrA represses the expression of rcsB. Our results would be an example of differential regulation of orthologous genes expression, providing differences in phenotypic traits between closely related bacterial serovars.

Introduction

The lipopolysaccharide (LPS) of Salmonella enterica is the most abundant component of outer membrane of Gram negative bacteria which structure is divided in three regions: O-antigen polysaccharide, core oligosaccharide, and lipid A [1]. LPS synthesis is a complex process involving various steps. In particular, O-antigen production and assembly in Salmonella occurs by mechanisms that require Wzy (polymerase of the repeating subunits), Wzx (flippase that translocated subunit across the membrane) and Wzz (a chain length determinant) (previously Cld or Rol) [1,2,3,4,5,6,7,8]. Even though there is a significant amount of information on biochemistry and genetics of the LPS synthesis, the regulatory mechanisms that modulate its production are complex and poorly understood. However, it is known that LPS structure is dynamic, showing changes in response to local microenvironment signal. Many of these signals are detected as stimuli by signal transduction cascades. Usually, these systems are composed by a histidine kinase (HK) (sensor protein) that transmits the signal, through a phosphorylation cascade, to a second component, named response regulator [9,10,11,12,13,14,15]. Often, the response regulator is a transcription factor, thereby the result of its phosphorylation is the activation or repression of gene transcription which product is involved in the adaptation to that given microenvironment. The most important two-component regulatory systems involved in LPS modification are PhoP/PhoQ, PmrA/PmrB and RcsC/RcsD/RcsB. PmrA/PmrB and RcsC/RcsD/RcsB two-component regulatory systems in Salmonella enterica serovar Typhimurium (S. Typhimurium), each activated by different stimuli, independently promote transcription of the wzz gene [16]. The expression of wzz is also regulated by PhoP/PhoQ via PhoP-mediated upregulation of PmrD, which binds to the phosphorylated form of PmrA protecting it from dephosphorylation by PmrB [17,18]. In Salmonella, regulation of the long chain distribution of the O-antigen contributes not only to an effective barrier [19] but also affect serum resistance and entry into eukaryotic cells [20,21,22,23,24]. Furthermore, O-antigen length can also modulate acquired immunity. Indeed, Phalipon and coworkers demonstrated that in Shigella flexneri induction of an O-antigen-specific antibody response depends on the length of the polysaccharide chain [25]. Also, Helicobacter pylori alters its O-antigen structure expressing O-antigen of high molecular weight in...
response to acidic pH; an important adaptation that would facilitate colonization of the acidic gastric environment [26].

In gammaproteobacteria the DNA adenine methyltransferase (Dam) introduces a methyl group at the N6 position of the adenine of GATC sequence in the newly synthesized DNA strand after DNA replication, generating methylated DNA [27,28,29,30]. DNA methylation status can affect interactions between DNA and proteins such as RNA polymerase or transcription factors [30] that regulate (activate or repress) gene expression generating a plethora of effects. Thus, Dam mutants of S. enterica has shown to have many defects particularly in virulence and they have been proposed as candidate vaccines [31,32,33,34,35,36,37,38]. We have previously shown that a dam null mutant of S. Enteritidis presents a reduced expression of wzz gene and a defective O-antigen polysaccharide chain length distribution [39].

In this work we study the regulation of pmrA and rcsB expression by Dam methylation in S. enteritidis. In addition, we found that both wzz regulators have a regulatory influence on each other.

Materials and Methods

Bacterial strains, plasmids, strain construction, and growth conditions

Bacterial strains and plasmids used are listed in Table 1. S. Enteritidis #5694 was kindly given by Dr. Anne Morris Hooke, Miami University; originally from Dr. F. Collins’ collection, Trudeau Institute, Saranac Lake, New York. Strains #SS218, #SS219 and #SS220 are S. enteritidis isolates from poultry collected from Argentinean farms. Wild type strains were used to construct mutant strains listed in Table 1. Gene deletions were performed as described by Datsenko and Wanner [40]. Addition of a DNA fragment encoding 3xFLAG epitope tag at the 3′ end of protein-coding DNA sequences was carried out as previously described using plasmid pSUB11 as a template [41] and oligonucleotides pmrA-3×FLAG-5′ and pmrA-3×FLAG-3′ for PmrA, and rcsB-3×FLAG-5′ and rcsB-3×FLAG-3′ for RcsB. The mutagenic primers used are listed in Table 2. S. enteritidis was transformed by electroporation as previously described [42]. Gene deletion and the correct fusion of the ORF with 3xFLAG coding sequence were confirmed by sequencing (Macrogen Inc.), and analyzed with Sequencher (Gene Codes Corporation) and Vector NTI software. Bacteria were grown in Luria-Bertani (LB) broth [43] supplemented, as required, with antibiotics at the following final concentrations: ampicillin, 100 μg/ml; chloramphenicol, 30 μg/ml; kanamycin, 40 μg/ml; and tetracycline, 20 μg/ml. For PmrA and RcsB overproduction experiments bacteria were grown at 37°C in N-minimal medium [44], supplemented with 0.2% (w/v) glucose, 0.1 mg/ml casaminoacids, 2 μg/ml Vitamin B1 and 10 mM MgCl₂ (high Mg²⁺ concentration) or 10 μM MgCl₂ 100 μM FeSO₄ (low Mg²⁺ concentration plus Fe³⁺) [16]. Dam mutants were evaluated, phenotypically, determining the absence of methylated GATC sequences [39]. To confirm pmrA deletion and pmrA functionality, the resistance to the antimicrobial peptide Polymyxin B assay was carried out as previously described [45].

Molecular cloning of Salmonella pmrA and rcsB genes

DNA extracted from the parental strains of S. Enteritidis was used as template for PCR reaction to amplify pmrA and rcsB genes. PCR amplification was performed with either Pfx polymerase (Roche) (for amplification cloning fragments) or Taq polymerase (Qiagen). PCR fragments products were separated in agarose gels, purified using a Gel Extraction kit (Qiagen), and then digested using EcoRI restriction enzyme (Roche Diagnostics). Ligation with T4 DNA ligase (Rapid Ligation kit, Roche Diagnostics) into pUC18, also digested with EcoRI, and dephosphorylated with shrimp alkaline phosphatase (Roche Diagnostics) was performed. Competent E. coli DH5α cells were transformed with the ligation mixture by the calcium chloride protocol [46]. Colonies with a white color phenotype from plates with ampicillin and 0.2% (w/v) X-Gal were pooled and screened by PCR using the primers oligonucleotides pmrA-5′ and pmrA-3′ or rcsB-5′ and rcsB-3′ for pmrA or rcsB. Also, pooled colonies were screened by restriction digestion to preliminary identify the orientation of the inserts (with respect the plasmid promoter on sense or antisense). The integrity of the inserts were confirmed by DNA sequencing (Macrogen Inc.), using the sequencing primer M13 forward and M13 reverse, and the inserts were analyzed with Sequencher (Gene Codes Corporation) and Vector NTI software.

LPS analysis

LPS was extracted as described by Marolda et al [47]. Briefly, from overnight plate culture, samples were adjusted to OD₆₀₀ of 2.0 in a final volume of 100 μl. Then, samples were suspended in lysis buffer containing proteinase K as described by Hitchcock and Brown [48], followed by hot phenol extraction and a subsequent extraction of the aqueous phase with ether. LPS was resolved by electrophoresis in 14% polyacrylamide gels using a tricine-sodium dodecyl sulfate (SDS) system [49,50] and visualized by silver staining. Each well was loaded with the same LPS concentration determined by the keto-deoxoyctulosonic (KDO) assay [51]. A densitometry analysis was performed using ImageJ software. The ratio of the relative intensity of the lipid A-core band to the average intensity of the bands corresponding to total O-antigen and core was calculated by quantifying the pixels in a narrow window across the center of each lane. The densitometric analysis was calibrated by determining the ratio of the relative intensity of the lipid A-core region to the average intensity of the O-antigen bands.

Reverse transcription-PCR and quantitative real-time PCR

Bacteria were grown at 37°C with agitation to an OD₆₀₀ of 0.6. Cells were lysed, and total RNA was isolated using Trizol reagent (Invitrogen) according to the method described by the manufacturer. Contaminating DNA was digested with RNase-free DNase I (Epicentre Biotechnologies), and the purity of all RNA preparations was confirmed by subjecting them to PCR analysis using primers specific for the gene encoding the 16S rRNA (Table 2). After inactivation of DNase, RNA was used as a template for reverse transcription-PCR. Complementary cDNA was synthesized using random hexamer primers (Invitrogen), deoxyribonucleoside triphosphates, and Moloney murine leukemia virus M-MLV reverse transcriptase (Invitrogen). Relative quantitative real-time PCR was performed with an appropriate primer set, cDNAs, and Mezcla Real (Biodynamics) that contained nucleotides, polymerase, reaction buffer, and Green dye, using a Rotor-Gene 6000 real-time PCR machine (Corbett Research). The amplification program consisted of an initial incubation for 3 min at 95°C, followed by 40 cycles of 95°C for 20 s, 60°C for 30 s, and 72°C 20 s. The primers used are depicted in Table 2. A no-template control was included for each primer set. Melting curve analysis verified that each reaction contained a single PCR product. For the relative gene expression analysis, a comparative cycle threshold method (ΔΔCt) was used [52]. The number of copies of each sample transcript was determined with the aid of the software. Briefly, the amplification efficiencies of the genes of interest and the 16S rRNA gene used for normalization were tested. Each sample was first normalized for the amount of template added by
Cells were pelleted by centrifugation and resuspended with Protein extracts and Western blotting analysis

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

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<th>Strain</th>
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<th>Reference/Source</th>
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<td>Wild type</td>
<td>Dr. F. Collins collection</td>
</tr>
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<td>#5521</td>
<td>Wild type</td>
<td>Poultry isolate</td>
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<td>#5522</td>
<td>Wild type</td>
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<td>#5523</td>
<td>Wild type</td>
<td>Poultry isolate</td>
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<td>#5694 Δdam</td>
<td>[39]</td>
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**Escherichia coli** K-12

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doi:10.1371/journal.pone.0056474.t001

comparison to the 16S rRNA gene (endogenous control). The normalized values were further normalized using the wild-type sample (calibrator treatment). Hence, the results were expressed relative to the value for the calibrator sample, which was 1. Student’s t test was used to determine if the differences in retrotranscribed mRNA content observed in different backgrounds were statistically significant.

**Protein extracts and Western blotting analysis**

Total protein extracts were prepared from bacterial cultures grown at 37°C in LB medium and harvested at an OD<sub>600</sub> of 0.6. Cells were pelleted by centrifugation and resuspended with Laemmli buffer [53]. Three independent extractions for each sample were added together to minimize differences in protein recovery from sample to sample. For Western blot assays total proteins were boiled for 5–10 min in Laemmli sample buffer, and each lane was loaded with material from approximately 10<sup>6</sup> CFU before resolved by 12% SDS-polyacrylamide gel electrophoresis (PAGE) gel. Prestained SDS-PAGE standards (Bio-Rad) were used as molecular weight markers (not shown). The gels were blotted onto a Hybond-P membrane (GE Health-care, Madrid, Spain). Ponceau S red staining was used as loading control before blocking in 5% (w/v) dried skimmed milk in PBS. Finally, 3×FLAG fusion proteins were immunodetected using mouse-monoclonal anti-FLAG M2-peroxidase (HRP) antibodies (1:5,000, Sigma, St Louis,
The reacting bands were detected by enhanced chemiluminescence (ECL) (Luminol, Santa Cruz Biotechnology, Santa Cruz, CA) in an Image Quant 300 cabinet (GE Healthcare) following the manufacturer instructions. Blots were photographed, and the intensity of the signals expressed in arbitrary units was determined by densitometry analysis using the public domain NIH Image J software (http://rsb.info.nih.gov/nihimage/). We randomly selected three different bands from the Ponceau S stained membrane to normalize the intensity of the band of interest. Data were analyzed for statistical significance using a nonparametric Mann-Whitney test.

**Results**

Dam methylation participates in the regulation of pmrA and rcsB genes

PmrA and RcsB two-component regulatory system are the only two known wzz regulators described in S. Typhimurium. To determine whether the LPS phenotype of the dam mutant of S. Enteritidis (SEdam) is related to a diminished expression of these two regulators we analyzed the effect of overproduction of either RcsB or PmrA on the LPS pattern in the dam background. Recombinant plasmids containing the rcsB and pmrA genes cloned into pUC18 were transferred by electroporation in SEdam.

<table>
<thead>
<tr>
<th>Gene Targeted</th>
<th>Primer⁺</th>
<th>Sequenceᵇ (5’→3’)</th>
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<td><strong>Gene deletion</strong></td>
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<td><em>dam</em></td>
<td>*dam:*Cm (F)</td>
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Primer sequences were purchased from Invitrogen Inc. and were designed according to the DNA sequence information available for the S. Enteritidis strain (Salmonella spp. comparative sequencing blast server BLAST Server Database at www.sanger.ac.uk).

*F*, forward primer; *R*, reverse primer.

Underlined nucleotides indicate the sequence homologous to pKD3, pKD4 or pSUB11. Underlined and italicized nucleotidic regions indicate the restriction endonuclease enzyme cut sites (*EcoRI*) incorporated into the primer sequence.

Note: OE3266 (SEdam) was used to evaluate the contribution of Dam methylation to the regulation of pmrA and rcsB genes.

For the in vivo experiments, the control strain used was OE3266 (SEdam). This strain was selected based on its ability to support the growth of recombinant plasmids containing the rcsB or pmrA genes and their ability to express these genes in the absence of Dam methylation. In addition, OE3266 (SEdam) was selected as a control strain because it has been shown to be proficient in Dam methylation and is capable of supporting the growth of recombinant plasmids containing the rcsB or pmrA genes.

**Table 2.** Oligonucleotides primers used in this study.

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<td><em>rcsB</em></td>
<td><em>rcsB:</em>-3 × FLAG-5’</td>
<td>CTAATTCTTCTGCTACCCGACAGACAAAGCTACAAAAGACCATG</td>
</tr>
<tr>
<td></td>
<td><em>rcsB:</em>-3 × FLAG-3’</td>
<td>ATAAGGTTTraGCGGATGGAATAGTTAGT</td>
</tr>
</tbody>
</table>

MO). The reacting bands were detected by enhanced chemiluminescence (ECL) (Luminol, Santa Cruz Biotechnology, Santa Cruz, CA) in an Image Quant 300 cabinet (GE Healthcare) following the manufacturer instructions. Blots were photographed, and the intensity of the signals expressed in arbitrary units was determined by densitometry analysis using the public domain NIH Image J software (http://rsb.info.nih.gov/nihimage/). We randomly selected three different bands from the Ponceau S stained membrane to normalize the intensity of the band of interest. Data were analyzed for statistical significance using a nonparametric Mann-Whitney test.

**Results**

Dam methylation participates in the regulation of pmrA and rcsB genes

PmrA and RcsB two-component regulatory system are the only two known wzz regulators described in S. Typhimurium. To determine whether the LPS phenotype of the dam mutant of S. Enteritidis (SEdam) is related to a diminished expression of these two regulators we analyzed the effect of overproduction of either RcsB or PmrA on the LPS pattern in the dam background. Recombinant plasmids containing the rcsB or pmrA genes cloned into pUC18 were transferred by electroporation in SEdam and...
wild type strains. As we previously described, the LPS pattern of the dam mutant showed many more visible bands in the intermediate region of the gel (Fig. 1, lane 2) compared with the banding pattern of the wild-type LPS (Fig. 1, lane 1). The LPS O-antigen profiles of the transformed strains were analyzed in bacteria cultured in LB and under growing conditions known to activate the PmrA/PmrB two-component regulatory system. Results are depicted in Fig. 1. Regardless the culture media used, high Mg$^{2+}$ or low Mg$^{2+}$ + Fe$^{3+}$, we found that RcsB overexpression in SE$\Delta$dam mutant (Fig. 1A, lanes 4 and 7) generates an LPS banding pattern comparable to that of the wild type (Fig. 1A, lanes 1 and 5). Similar results were observed when bacteria were cultured in LB medium (not shown). These data would indicate that the changes in the O-antigen LPS pattern of any strain studied (data not shown). Transformation with plasmids bearing the genes rcsB or pmrA in antisense orientation to the P$\_\text{lac}$ promoter; (pIZ833, ppmrAas, pprcsB), or with empty plasmid vector (pUC18) produced no changes in the O-antigen LPS pattern of any strain studied (data not shown). These data would indicate that the dam mutant produces a reduced amount of RcsB protein, suggesting that rcsB gene expression is up-regulated by Dam.

Next, we analyzed LPS pattern in the absence of RcsB and PmrA. For this purpose we constructed rcsB and pmrA deletion mutants of S. Enteritidis (SE$\Delta$rcsB and SE$\Delta$pmrA strains, respectively) using the lambda Red recombination system. As shown in Fig. 2A, the LPS phenotype of SE$\Delta$rcsB is similar to that observed in SE$\Delta$dam mutant (lanes 2 and 3, respectively). Complementation with the plasmid bearing the rcsB gene restored LPS pattern to that found in the wild type strain of S. Enteritidis (Fig. 2A, lane 4). The lack of pmrA did not modify LPS pattern in S. Enteritidis. As shown in Fig. 2B, deletion mutant SE$\Delta$pmrA (lane 2) presents an LPS pattern similar to that of the wild type strain (lane 1). Collectively, these experiments indicate that the reduced wzz gene expression observed in SE$\Delta$dam mutant correlates with a diminished expression of rcsB rather than pmrA.

In silico analysis has shown the presence of GATC motifs in the coding sequence and/or surrounding nucleotides of pmrA and rcsB genes [39]. Then we investigated whether Dam methylation regulates the expression of pmrA, rcsB or both by analyzing the transcription of these genes in the dam mutant and the parental strain of S. Enteritidis grown to exponential phase in LB medium. By real-time quantitative PCR, the relative expression of both pmrA and rcsB genes in SE$\Delta$dam is reduced (56% and 59%, respectively) compared with the parental strain (Fig. 3). Complementation of dam mutation with plasmid pIZ833 restored the expression of pmrA, rcsB and wzz genes to wild type levels (Fig. 3). Thus, a functional Dam results in upregulation of the expression of pmrA and rcsB genes in S. Enteritidis. To analyze whether the reduction in the amount of pmrA and rcsB mRNA observed in the absence of Dam correlated with the amount of proteins, we quantified PmrA and RcsB in SE$\Delta$dam mutant. Because murine anti PmrA or anti RcsB antibodies are not commercially available, we constructed SE$\Delta$dam mutants harboring either pmrA::3×Flag or rcsB::3×Flag transcriptional fusions in the chromosome. Total

**Figure 1.** LPS analysis of S. Enteritidis strains overexpressing RcsB (A) or PmrA (B) protein. Equal amount of LPS was loaded in each lane and analyzed by Tricine/SDS-PAGE on a 14% (w/v) acrylamide gel followed by silver staining. The concentration of LPS was determined by measuring KDO using the purpald assay. A. Lanes 1–4: bacteria grown in N-minimal medium containing 10 mM MgCl$_2$; lanes 5–8: bacteria grown in N-minimal medium containing 10 μM MgCl$_2$ 100 μM FeSO$_4$. B. Lanes 1–4: bacteria grown in N-minimal medium containing 10 mM MgCl$_2$; lanes 5–7: bacteria grown in N-minimal medium containing 10 μM MgCl$_2$ 100 μM FeSO$_4$. Plasmids pIZ833, pprcsB and pppmrA bears the dam, rcsB and pmrA genes respectively.

doi:10.1371/journal.pone.0056474.g001
bacterial proteins were extracted and the relative amount of PmrA and RcsB was determined by Western blot developed with anti-FLAG antibodies (Fig. 4). Densitometry analysis showed that the amount of PmrA produced by the dam mutant (as well as the complemented strains) was similar to that produced by the wild type strain (Fig. 4A). On the other hand the relative amount of the RcsB produced by the dam mutant was significantly reduced to 63% compared with that of the parental strain (Fig. 4B).

RcsB induces the expression of wzz and pmrA, whereas PmrA represses the expression of wzz and rcsB.

Next we analyzed to what extent the expression of wzz was reduced in the absence of its two regulators in S. Enteritidis. To do that, real-time quantitative PCR was performed using mRNA obtained from knockout rcsB and pmrA mutants and from wild type strains grown in LB medium. As shown in Fig. 5A, the expression of wzz was reduced to 29% in SEΔrcsB mutant compared with the wild type (strain #5694). In contrast, we observed 50% increased expression of wzz in SEΔpmrA with respect to the wild type (strain #5694) (Fig. 5A). These features would not be exclusive to wild type strain #5694, since similar results were found using pmrA and rcsB mutants constructed from clinical isolates of S. Enteritidis (data not shown).

These findings prompted us to investigate whether an interaction exists between both wzz regulators. Therefore, we determined the expression of rcsB in the absence of pmrA (SEΔpmrA) and the
expression of pmrA in the absence of rcsB (SEΔrcsB). As shown in Fig. 5B, the expression of rcsB in the mutant lacking pmrA was increased by 24% with respect to the parental strain cultured in LB medium. In contrast, deficiency in rcsB diminished the expression of pmrA to 30% compared with the wild type strain grown in the same medium (Fig. 5C). The expression of rcsB and pmrA was restored in complemented strains (data not shown). To further investigate these interactions, we analyzed wzz expression in the wild type, rcsB and pmrA mutants grown in conditions that stimulate or repress pmrA. As shown in Fig. 5D, similar patterns in the expression of wzz were found between bacteria cultured under conditions known to activate (low Mg²⁺; low Mg²⁺+Fe³⁺) or repress (high Mg²⁺) pmrA. We found that regardless the culture media utilized, wzz expression was reduced in SEΔrcsB mutant.

**Figure 5.** Relative expression of *wzz*, *rcsB* and *pmrA* mRNA in *pmrA* and *rcsB* mutant by real-time quantitative PCR. Total mRNA was harvested from cultures of SEΔrcsB, SEΔpmrA and S. Enteritidis wild type #5694 (wild type) grown in LB medium (A,B,C) or grown in low Mg²⁺, low Mg²⁺+Fe³⁺ and high Mg²⁺ (D). The relative amount of *wzz* mRNA was determined by reverse transcription real-time quantitative PCR and related to mRNA levels in wild type strain #5694 (A,B,C) or in wild type strain #5694 grown in low Mg²⁺ (D), set as 1. Values are means ± SD of five independent mRNA extractions performed in triplicates. * significant difference p<0.01 with respect to wild type strain #5694 grown in the same media; $ significant difference p<0.01 with respect to the same strain grown in pmrA-inducing conditions (low Mg²⁺ and low Mg²⁺+Fe³⁺); ¥ significant difference p<0.05 with respect to the same strain grown in pmrA-inducing conditions (low Mg²⁺ and low Mg²⁺+Fe³⁺).

doi:10.1371/journal.pone.0056474.g005
and increased in SEΔpmrA mutant compared with the parental strain. Interestingly, when the wild type strain was cultured under conditions that repress pmrA (high Mg\(^{2+}\)), the expression of wzz was 3 or 2 fold higher compared with the wild type grown in low Mg\(^{2+}\) or low Mg\(^{2+}\)+Fe\(^{3+}\), respectively. This increase was even higher in the absence of the pmrA gene for any culture medium tested (Fig. 5D). Additional experiments revealed that concurrent with the augmented expression of wzz (Fig. 5D), the wild type strain increased the expression of pmrA and reduced the expression of ptcA in high Mg\(^{2+}\) compared with low Mg\(^{2+}\) (data not shown). These results confirm that wzz expression is induced by RcsB and repressed by PmrA. In all cases, the expression of wzz was restored in complemented strains (data not shown).

Is there a third regulator of wzz in S. Enteritidis?

Results presented in Fig. 5D also show that the expression of wzz is induced in the absence of pmrA by high Mg\(^{2+}\) (pmrA repressive condition). This finding is interesting since it suggests the existence of another wzz regulator; therefore, we decided to investigate the expression of wzz in a double mutant of S. Enteritidis lacking pmrA and rcsB genes (SEΔrcsBΔpmrA strain). As shown in Fig. 6A and B, this double mutant was able to express wzz mRNA. We found that regardless the culture condition used the expression of wzz was decreased significantly in the double mutant compared with the parental strain. Nevertheless, it is worth noting that for the double mutant the expression of wzz was 2.5 fold higher in high Mg\(^{2+}\) than in low Mg\(^{2+}\) (Fig. 6B). Moreover, LPS analysis showed that - the double mutant was able to express O-antigen (Fig. 6B, lane 2). Altogether, our results indicate that, in addition to PmrA and RcsB, another regulator(s) exists in S. Enteritidis.

Discussion

We have reported earlier that the absence of Dam in S. Enteritidis causes a defect in the O polysaccharide chain length distribution associated to reduced wzz gene expression. Here we investigated whether Dam regulates wzz gene expression through its two known regulators, PmrA and RcsB. We found that Dam regulates the expression of both rcsB and pmrA genes; nevertheless, the SEΔdam LPS phenotype of S. Enteritidis is only associated with RcsB. The fact that SEΔdam mutant exhibits reduced levels of rcsB mRNA and a diminished amount of RcsB indicates that the expression of rcsB gene is controlled (directly or indirectly) by Dam methylation. The lack (SEΔrcsBΔpmrA) or even a diminished amount (SEΔdam strain) of RcsB resulted in an increased amount of shorter polysaccharide chains similar to the dam LPS phenotype. Furthermore, we found that overproduction of RcsB in SEΔdam mutant restores the O-antigen LPS pattern back to that of S. Enteritidis wild type. The involvement of RcsB in the regulation of polymerization was reported earlier in S. Typhimurium [16]. It was shown that the lack of RcsB affects the mobility in those bands containing 6–10 and 16–22 O-antigen subunits. Unlike serovar Enteritidis, no increase in the amount of shorter polysaccharides was reported for the rcsB mutant of S. Typhimurium. These subtle differences in the regulation of the O-antigen chain length between two serovars of Salmonella enterica would allow them to colonize specific ecological and immunological niches [54].
In *S. Typhimurium*, PmrA not only stimulates *wzz* expression, regulating the O-antigen chain length, but also participates in core and lipid A modifications [16,55,56,57,58,59,60]. Therefore, it would be reasonable to expect a direct participation of PmrA in the O polysaccharide chain length phenotype of *S. Enteritidis*; we found, however, that the absence of PmrA does not cause alterations in the LPS pattern. This is in agreement with the fact that overproduction of PmrA in the *dam* mutant does not restore the defective LPS pattern. On the other hand, our data indicate that Dam methylation (directly or indirectly) does modulate *pmaC* expression. Indeed, *pmaC* mRNA was reduced in the *dam* mutant. This finding is in agreement with microarray analysis data reported by Balboutin *et al.* in *S. Typhimurium dam* mutants [31]. Interestingly, despite the diminished amount of *pmaC* mRNA found in the *dam* mutant, PmrA levels remained unchanged. Discrepancies between mRNA transcription and protein translation have been reported earlier [61,62,63]. In this regard, different mechanisms related to mRNA stability have been proposed to play a critical role in this phenomenon. Therefore, we conclude that, in *S. Enteritidis*, a functional Dam is required for adequate levels of *pmaC* and *rcsB* gene expression. Also, the diminished amount of RcsB in SE*Dam* strain could explain the reduced *wzz* gene expression found earlier in this mutant [39]. We also analyzed the individual participation of PmrA and RcsB in the expression of *wzz* gene in *S. Enteritidis*. As expected, we found that the relative amount of *wzz* is reduced in *rcsB* mutant, indicating that RcsB induces *wzz* gene expression. Surprisingly, in *pmaC* deletion mutant the amount of *wzz* mRNA was higher than in the wild type, indicating that, unlike RcsB, PmrA represses *wzz* gene expression. This finding could explain the normal LPS phenotype of SE*pmaC* (this mutant would not lack Wzz protein).

In order to investigate a putative regulatory effect between both *wzz* regulators, we determined the expression of *rcsB* in a *pmaC* mutant, and *pmaC* expression in an *rcsB* mutant. We found that both regulators affect each other expression. The relative expression of *pmaC* mRNA decreases in the absence of *rcsB*, whereas in the absence of *pmaC*, the relative amount of *rcsB* mRNA increases. These results would indicate that, under the growth conditions used, RcsB stimulates *pmaC* whereas PmrA represses *rcsB*. Also, these findings could explain the elevated expression of *wzz* found in the *pmaC* mutant; in the absence of PmrA, RcsB is derepressed and therefore *wzz* is induced. Regulatory interactions between two-component regulatory systems, coordinating responses to diverse stimuli, have been described. The mechanisms involved in these regulations include phosphatases interrupting phosphorlyl transfer in phosphorylases and transcriptional and post-transcriptional modifications [64,65,66,67,68]. Then, it is possible that an interaction between both PmrA/PmrB and RcsC/RcsD/RcsB two-component regulatory systems would exist in *S. Enteritidis*. In favor of a direct RcsB-mediated regulation of *pmaA*, alignment analysis revealed a potential RcsB protein binding site in *pmaA* gene of *S. Enteritidis* (see Fig. S1 for the bioinformatics analysis performed). Similar results were obtained when the alignment analysis was performed between the conserved regulatory sequences of PmrA binding sites and a putative PmrA binding motif found in *rcsB* gene (supplemental data). Altogether these results would indicate a direct regulation of PmrA protein on *rcsB* gene and RcsB protein on *pmaA* gene. The balance between the expression and repression of *pmaA* and *rcsB* in response to environmental signals suggests a fine tuning of selective genes required for the adaptation to a specific niche.

The experiments performed using double mutant *rcsB pmaC* of *S. Enteritidis* indicate that *wzz* gene is expressed even in the absence of both regulators. Early studies on serovar Typhimurium showed that in the absence of *rcsB* and *pmaA* genes (both *wzz* inducers), the activity of the *wzz* promoter is barely detected and consequently the O-antigen is not synthesized. In fact, the LPS phenotype of *rcsB pmaA* double mutant of *S. Typhimurium* closely resembles that of a *wzz* mutant [16]. On the contrary, our experiments demonstrate that the LPS pattern of *S. Enteritidis* lacking both *rcsB* and *pmaA* genes (*wzz* inducer and repressor, respectively) does conserve O-antigen. These results indicate that, in *S. Enteritidis*, full expression of *wzz* would not depend exclusively on PmrA and RcsB. Although the *wzz* mRNA amount found in *rcsB pmaA* double mutant could be related to a basal expression of *wzz* (but still enough to allow the synthesis of O-antigen), the induction of *wzz* by high Mg²⁺ observed in *rcsB* mutant as well as in *rcsB pmaA*

**Figure 7. Schematic diagram of the proposed regulatory network of *wzz* gene expression in *S. Enteritidis*.** The regulatory cascade for *wzz* gene expression involves Dam methylation, PmrB/PmrA and RcsC/RcsD/RcsB two-component regulatory system and a putative third regulator (X). Proteins are indicated by ovals, whereas genes are symbolized by block arrows. Black dots indicate methylation sites (5′-GATC-3′ sequences). Dashed lines indicate direct interactions demonstrated in *S. Typhimurium*. Positive regulation (induction) is labeled with ↑ and (+), whereas negative regulation (repression) is labeled with ↓ and (−). The question mark indicates a putative regulation. doi:10.1371/journal.pone.0056474.g007
double mutant strongly support the possibility of a third gene regulating \textit{wzz} expression in \textit{S. Enteritidis}. In summary, we showed that in \textit{S. Enteritidis} Dam methylation regulates \textit{wzz} expression through \textit{rcsB} and \textit{pmrA} genes; whereas \textit{RcsB} induces \textit{wzz} gene expression \textit{PmrA} represses it. We also present evidence that \textit{rcsB} and \textit{pmrA} genes regulate each other; \textit{RcsB} stimulates the expression of \textit{pmrA} and \textit{PmrA} represses \textit{rcsB} gene expression. Finally, our results support the existence of a third gene regulating \textit{wzz} expression in \textit{S. Enteritidis}, that can be induced when bacteria is grown in high Mg\textsuperscript{2+}. The regulatory network of \textit{wzz} gene expression proposed, including the involvement of the hypothetical third \textit{wzz} regulator, is shown in Fig. 7. Thereby, results presented here would be an example of differential regulation of orthologous genes expression providing differences in phenotypic traits between closely related bacterial serovars.

Supporting Information

Figure S1  Bioinformatics analysis. A. Conserved sequence of \textit{PmrA}-binding motif. The conserved nucleotides of the sequences corresponding to \textit{PmrA} binding motif are boxed. B. Molecular analysis of \textit{rcsB} gene region. Diagram of the DNA sequence corresponding to \textit{rcsB} region based on Refseq NC_011294 sequence of \textit{S. enterica} serovar Enteritidis. Alignment analysis performed between the conserved regulatory sequences of \textit{PmrA} motif and the potential \textit{PmrA} protein binding site sequences found in \textit{rcsB} gene region are depicted in the correspondent localization. The two know \textit{rcsB} promoters \textit{Pre1B} (located within \textit{rcsD} coding region) and \textit{Pre2B} (located at \textdegree{}32 pb upstream of the \textit{rcsD ORF}) are marked with arrows. C. Alignment analysis of one of the potential \textit{RcsB}-binding motifs found in \textit{pmrA} gene region with the reported \textit{RcsB}-dependent regulatory sequences of different enterobacteria. Homologous sequences of the potential \textit{RcsB}-binding site found in comparison with the reported \textit{RcsB} motif are in bold. D. Molecular analysis of \textit{pmrA} gene region. Diagram of the DNA sequence corresponding to \textit{pmrA} region based on Refseq NC_011294 sequence of \textit{S. enterica} serovar Enteritidis. Potential \textit{RcsB} protein binding site sequences found in \textit{pmrA} gene are depicted in the correspondent localization. Next to each potential sequence is indicated the orientation (direct, + or complementary, −), the position relative to the ATG sequence of the gene and the amount of mismatches found in the alignment (mm).

(TIF)

Acknowledgments

We are very grateful to Maria Isabel Bernal for excellent technical assistance.

Author Contributions

Conceived and designed the experiments: SHS MRAC MAV MCC. Performed the experiments: SHS MRAC. Analyzed the data: SHS MRAC MCC. Contributed reagents/materials/analysis tools: SHS MAV MCC. Wrote the paper: SHS MAV MCC.

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