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

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

Sebastián Hernán Sarnacki¹, María del Rosario Aya Castañeda¹, María Angeles Noto Llana¹, Mónica Nancy Giacomodonato¹, Miguel Ángel Valvano²*, María Cristina Cerquetti¹*

¹Instituto de Investigaciones en Microbiología y Parasitología Médica, Universidad de Buenos Aires-Consejo Nacional de Investigaciones Científicas y Técnicas (IMPaM, UBA-CONICET), Buenos Aires, Argentina. ²Department of Microbiology and Immunology, Centre for Human Immunology, University of Western Ontario, London, Ontario, Canada

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 3×FLAG tag in wild type (wt) and dam strains of *S. enterica*. 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. enterica* 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.


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* E-mail: sebasnarnacki@yahoo.com.ar (SHS); ccerquetti@fmed.uba.ar (MCC)

Current address: Centre for Infection and Immunity, Queen's University of Belfast, Belfast, United Kingdom.

Introduction

The lipopolysaccharide (LPS) 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 divided in three regions: O-antigen polysaccharide, core oligosaccharide, and lipid A [25]. Also, LPS modification are PhoP/PhoQ, PmrA/PmrB and RcsC/RcsD/RcsB two-component regulatory systems of *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 affects 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 gamma proteobacteria 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 [27,29,30]. 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 have 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 argentine 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 3×FLAG 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-F×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 3×FLAG 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 casamino acids, 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 Pwo 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 downlac218 combined with rcsB-F and rcsB-R for rcsB, and pmrA-F or pmrA-R for pmrA. 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 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-deoxyoctulosonic (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+LPS 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 (Epitrend 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 for 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. Then 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. Student’s relative to the value for the calibrator sample, which was 1. Hence, the results were expressed as normalized values were further normalized using the wild-type comparison to the 16S rRNA gene (endogenous control). The grounds were statistically significant.

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

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant characteristic(s)</th>
<th>Reference/Source</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>S. enterica</em> serovar Enteritidis</td>
<td>#5694 Wild type</td>
<td>Dr. F. Collins collection</td>
</tr>
<tr>
<td>#55218</td>
<td>Wild type</td>
<td>Poultry isolate</td>
</tr>
<tr>
<td>#55219</td>
<td>Wild type</td>
<td>Poultry isolate</td>
</tr>
<tr>
<td>#55220</td>
<td>Wild type</td>
<td>Poultry isolate</td>
</tr>
<tr>
<td>SEΔdam</td>
<td>#5694 Δdam</td>
<td>[39]</td>
</tr>
<tr>
<td>SEΔrcsB</td>
<td>#5694 ΔrcsB</td>
<td>This work</td>
</tr>
<tr>
<td>SEΔpmrA</td>
<td>#5694 ΔpmrA</td>
<td>This work</td>
</tr>
<tr>
<td>SEΔrcsBΔpmrA</td>
<td>#5694 ΔrcsB ΔpmrA</td>
<td>This work</td>
</tr>
<tr>
<td>SEΔwzz</td>
<td>#5694 Δwzz</td>
<td>[39]</td>
</tr>
<tr>
<td>SEpmrA3×FLAG</td>
<td>#5694 pmrA-3×FLAG, Km&lt;sup&gt;+&lt;/sup&gt;</td>
<td>This work</td>
</tr>
<tr>
<td>SEpmrA3×FLAG Δdam</td>
<td>#5694 pmrA-3×FLAG, Δdam, Km&lt;sup&gt;+&lt;/sup&gt;</td>
<td>This work</td>
</tr>
<tr>
<td>SErcsB3×FLAG</td>
<td>#5694 rcsB-3×FLAG, Km&lt;sup&gt;+&lt;/sup&gt;</td>
<td>This work</td>
</tr>
<tr>
<td>SE218ΔrcsB</td>
<td>#55218 ΔrcsB</td>
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<tr>
<td>SE218ΔpmrA</td>
<td>#55218 ΔpmrA</td>
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</tr>
<tr>
<td>SE219ΔrcsB</td>
<td>#55219 ΔrcsB</td>
<td>This work</td>
</tr>
<tr>
<td>SE219ΔpmrA</td>
<td>#55219 ΔpmrA</td>
<td>This work</td>
</tr>
<tr>
<td>SE220ΔrcsB</td>
<td>#55220 ΔrcsB</td>
<td>This work</td>
</tr>
<tr>
<td>SE220ΔpmrA</td>
<td>#55220 ΔpmrA</td>
<td>This work</td>
</tr>
<tr>
<td>SErcsB3×FLAG Δdam</td>
<td>#5694 rcsB-3×FLAG, Δdam, Km&lt;sup&gt;+&lt;/sup&gt;</td>
<td>This work</td>
</tr>
</tbody>
</table>

**Escherichia coli** K-12

| DH5α          | F- · 80lacZ1M15 endA recA hsdR17(rK<sup>−</sup> m<sub>K</sub> <sup>−</sup> S) supE thi gyrA relA (ΔlacZYA-argF) U169 | Laboratory stock |

**Plasmids**

| pCP20         | FLP<sup>+</sup>, λcI857<sup>+</sup>, λPr<sup>+</sup>, Rep<sup>+</sup>, Amp<sup>+</sup>, Cm<sup>−</sup> | [40]            |
| pKZ333        | E. coli dam gene, Amp<sup>+</sup> | [69]            |
| pKD3          | Template plasmid for mutagenesis, Amp<sup>+</sup>, Cm<sup>−</sup> | [40]            |
| pKD4          | Template plasmid for mutagenesis, Amp<sup>+</sup>, Km<sup>+</sup> | [40]            |
| pKD46         | γ-δ and exo from λ phage, araC-P<sub>ara</sub>, Amp<sup>+</sup> | [40]            |
| pU8B11        | 3′×FLAG FRT apr FRT bla R6KoriV | [41]            |
| pUC18         | High copy number cloning vector, Amp<sup>+</sup> | GenBank/EMBL sequence accession number L09136 |
| prcsB         | rcsB; Amp<sup>+</sup> (pUC18 backbone) | This work |
| prcsBAs       | rcsB cloned in antisense orientation to the P<sub>-inc</sub> promoter; Amp<sup>+</sup> (pUC18 backbone) | This work |
| ppmrA         | pmrA; Amp<sup>+</sup> (pUC18 backbone) | This work |
| ppmrAs        | pmrA cloned in antisense orientation to the P<sub>inc</sub> promoter; Amp<sup>+</sup> (pUC18 backbone) | This work |

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 back-grounds 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 (SE dam)* 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.

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

<table>
<thead>
<tr>
<th>Gene Targeted</th>
<th>Primer&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Sequence&lt;sup&gt;b&lt;/sup&gt; (5′→3′)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Gene deletion</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>dam</em></td>
<td><em>dam</em>:Cm (F)</td>
<td>TTTTGCACAGCCGGAAGGTTGAATTAGTTAGTCACGATGTCAGCTGAGCTGAGCTCTCC</td>
</tr>
<tr>
<td><em>pmrA</em></td>
<td><em>pmrA</em>:Cm (R)</td>
<td>GGCAATCAAATTCGTGTTCTCAAGGACTAGAAATAGATATATCTTCCCTTAG</td>
</tr>
<tr>
<td><em>rcsB</em></td>
<td><em>rcsB</em>::Km (F)</td>
<td>CTTACGTCAAAAGCTTGCTGTAGCAAGGTAGCCCAATACCATAGATATATCTTCCCTTAG</td>
</tr>
<tr>
<td><strong>Gene epitope tagging</strong></td>
<td></td>
<td></td>
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<tr>
<td><em>pmrA</em></td>
<td><em>pmrA</em>-3 × FLAG-5′</td>
<td>TCGCGGTTTGGCTACATGCTGTTGCAACAGGAAAGGAGAAACGACTAAAGGACCATGACGGT</td>
</tr>
<tr>
<td><em>rcsB</em></td>
<td><em>rcsB</em>-3 × FLAG-5′</td>
<td>CTTACGTCAAAAGCTTGCTGTAGCAAGGTAGCCCAATACCATAGATATATCTTCCCTTAG</td>
</tr>
<tr>
<td><strong>Gene cloning</strong></td>
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<td><em>pmrA</em></td>
<td><em>pmrA</em>-F</td>
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</tr>
<tr>
<td><em>rcsB</em></td>
<td><em>rcsB</em>-F</td>
<td>GATCGAATTCATAGAAAATACTGATTGTGGAGATGACGGCA</td>
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<tr>
<td><strong>Verification of predicted construction</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>dam</em></td>
<td>Rpe</td>
<td>TACGCGAATTCATAGAAAATACTGATTGTGGAGATGACGGCA</td>
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<tr>
<td><em>pmrA</em></td>
<td><em>pmrA</em>-F</td>
<td>GATCGAATTCATAGAAAATACTGATTGTGGAGATGACGGCA</td>
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<tr>
<td><em>pmrA</em></td>
<td><em>pmrA</em>-R</td>
<td>GATCGAATTCATAGAAAATACTGATTGTGGAGATGACGGCA</td>
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<tr>
<td><em>rcsB</em></td>
<td><em>rcsB</em>-F</td>
<td>GATCGAATTCATAGAAAATACTGATTGTGGAGATGACGGCA</td>
</tr>
<tr>
<td><em>rcsB</em></td>
<td><em>rcsB</em>-R</td>
<td>GATCGAATTCATAGAAAATACTGATTGTGGAGATGACGGCA</td>
</tr>
<tr>
<td><strong>Real-time PCR</strong></td>
<td></td>
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<tr>
<td>16S rRNA gen</td>
<td>q-16s-F</td>
<td>GCCGCAAGGTATTAAACCTCAA</td>
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<tr>
<td><em>rcsB</em></td>
<td>q-rcsB-F</td>
<td>ACCGCAGCTAATTGGATT</td>
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<tr>
<td><em>pmrA</em></td>
<td>q-pmrA-F</td>
<td>AACCAGCATGTAGCCAAACC</td>
</tr>
<tr>
<td><em>wzz</em></td>
<td>q-wzz-F</td>
<td>CGTCGGTTGCTTGCTGTGAG</td>
</tr>
<tr>
<td><strong>Primers 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 <a href="http://www.sanger.ac.uk">www.sanger.ac.uk</a>).</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;sup&gt;a&lt;/sup&gt;F, forward primer; R, reverse primer.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;sup&gt;b&lt;/sup&gt;Underlined nucleotides indicate the sequence homologous to pKD3, pKD4 or pSUB11. Underlined and italicized nucleotidic regions indicate the restriction endonuclease enzyme cut sites (EroII) incorporated into the primer sequence.</td>
<td></td>
<td></td>
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<tr>
<td>doi:10.1371/journal.pone.0056474.t002</td>
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</table>
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\(_{\text{dam}}\) 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). It seems that the presence of high amounts of RcsB in a dam background reduces the intermediate region bands observed for SE\(_{\text{dam}}\) mutant (Fig. 1A, lanes 2 and 6). On the contrary, no changes were evident in the LPS pattern of SE\(_{\text{dam}}\) mutant overexpressing PmrA regardless the growth environmental condition, high Mg\(^{2+}\) or low Mg\(^{2+}\)+Fe\(^{3+}\) (Fig. 1B, lanes 4 and 7). ppmrA plasmid functionality was confirmed by Polymyxin B resistance assay as described in materials and methods (data not shown). Again, similar results for LPS pattern were obtained when bacteria were cultured in LB medium (data not shown). Transformation with plasmids bearing the genes cloned in antisense orientation to the P\(_{\text{lac}}\) promoter; (p\(\text{pmrA-lac}\), p\(\text{rcsB-lac}\), 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\(_{\text{ΔrcsB}}\) and SE\(_{\text{ΔpmrA}}\) strains, respectively) using the lambda Red recombination system. As shown in Fig. 2A, the LPS phenotype of SE\(_{\text{ΔrcsB}}\) is similar to that observed in SE\(_{\text{dam}}\) 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\(_{\text{ΔpmrA}}\) (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\(_{\text{dam}}\) 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\(_{\text{dam}}\) is reduced (56% and 59%, respectively) compared with the parental strain (Fig. 3). Complementation of dam mutation with plasmid pIZ333 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\(_{\text{dam}}\) mutant. Because murine anti PmrA or anti RcsB antibodies are not commercially available, we constructed SE\(_{\text{dam}}\) 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 pIZ333, prcsB and ppmrA bears the dam, rcsB and pmrA genes respectively.

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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 \textit{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 \textit{dam} mutant was significantly reduced to 63% compared with that of the parental strain (Fig. 4 B).

RcsB induces the expression of \textit{wzz} and \textit{pmrA}, whereas PmrA represses the expression of \textit{wzz} and \textit{rcsB}

Next we analyzed to what extent the expression of \textit{wzz} was reduced in the absence of its two regulators in \textit{S. Enteritidis}. To do that, real-time quantitative PCR was performed using mRNA obtained from knockout \textit{rcsB} and \textit{pmrA} mutants and from wild type strains grown in LB medium. As shown in Fig. 5A, the expression of \textit{wzz} was reduced to 29% in \textit{SE\textit{dam}}\textit{rcsB} mutant compared with the wild type (strain \#5694). In contrast, we observed 50% increased expression of \textit{wzz} in \textit{SE\textit{dam}}\textit{pmrA} 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 \textit{pmrA} and \textit{rcsB} mutants constructed from clinical isolates of \textit{S. Enteritidis} (data not shown).

These findings prompted us to investigate whether an interaction exists between both \textit{wzz} regulators. Therefore, we determined the expression of \textit{rcsB} in the absence of \textit{pmrA} (\textit{SE\textit{dam}}\textit{pmrA}) 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 Mg\(^2\)+, low Mg\(^2\)++Fe\(^{3+}\)) or repress (high Mg\(^2\)+) 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 Mg\(^2\)+, low Mg\(^2\)++Fe\(^{3+}\) and high Mg\(^2\)+ (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 Mg\(^2\)+ (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 Mg\(^2\)+ and low Mg\(^2\)++Fe\(^{3+}\)); ¥ significant difference p<0.05 with respect to the same strain grown in pmrA-inducing conditions (low Mg\(^2\)+ and low Mg\(^2\)++Fe\(^{3+}\)). 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 \textit{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 \textit{pmrA} gene for any culture medium tested (Fig. 5D). Additional experiments revealed that concurring with the augmented expression of \textit{wzz} (Fig. 5D), the wild type strain increased the expression of \textit{rcsB} and reduced the expression of \textit{pmrA} in high Mg\(^{2+}\) compared with low Mg\(^{2+}\) (data not shown). These results confirm that \textit{wzz} expression is induced by RcsB and repressed by PmrA. In all cases, the expression of \textit{wzz} was restored in complemented strains (data not shown).

Is there a third regulator of \textit{wzz} in \textit{S. Enteritidis}?

Results presented in Fig. 5D also show that the expression of \textit{wzz} is induced in the absence of \textit{rcsB} by high Mg\(^{2+}\) (\textit{pmrA} repressive condition). This finding is interesting since it suggests the existence of another \textit{wzz} regulator; therefore, we decided to investigate the expression of \textit{wzz} in a double mutant of \textit{S. Enteritidis} lacking \textit{pmrA} and \textit{rcsB} genes (SEΔrcsBΔpmrA strain). As shown in Fig. 6A and B, this double mutant was able to express \textit{wzz} mRNA. We found that regardless the culture condition used the expression of \textit{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 \textit{wzz} was 2.5 fold higher in high Mg\(^{2+}\) than in low Mg\(^{2+}\) (Fig. 6B). Moreover, LPS analysis showed that - concomitantly with the expression of \textit{wzz} - the double mutant was capable to synthesize O-antigen (Fig. 6C, lane 3). Note that in the absence of Wzz, \textit{S. Enteritidis} (SE\textit{wzz} mutant) is unable to generate O-antigen (Fig. 6B, lane 2). Altogether, our results indicate that, in addition to PmrA and RcsB, another regulator(s) exists in \textit{S. Enteritidis}.

Discussion

We have reported earlier that the absence of Dam in \textit{S. Enteritidis} causes a defect in the O polysaccharide chain length distribution associated to reduced \textit{wzz} gene expression. Here we investigated whether Dam regulates \textit{wzz} gene expression through its two known regulators, PmrA and RcsB. We found that Dam regulates the expression of both \textit{rcsB} and \textit{pmrA} genes; nevertheless, the \textit{dam} LPS phenotype of \textit{S. Enteritidis} is only associated with RcsB. The fact that SE\textit{dam} mutant exhibits reduced levels of \textit{rcsB} mRNA and a diminished amount of RcsB indicates that the expression of \textit{rcsB} gene is controlled (directly or indirectly) by Dam methylation. The lack (SEΔ\textit{rcsB} mutant) or even a diminished amount (SE\textit{dam} strain) of RcsB resulted in an increased amount of shorter polysaccharide chains similar to the \textit{dam} LPS phenotype. Furthermore, we found that overproduction of RcsB in SE\textit{dam} mutant restores the O-antigen LPS pattern back to that of \textit{S. Enteritidis} wild type. The involvement of RcsB in the regulation of polymerization was reported earlier in \textit{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 \textit{rcsB} mutant of \textit{S. Typhimurium}. These subtle differences in the regulation of the O-antigen chain length between two serovars of \textit{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 \cite{16,55,36,57,58,59,60}. 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 \( pmrA \) expression. Indeed, \( pmrA \) mRNA was reduced in the \( dam \) mutant. This finding is in agreement with microarray analysis data reported by Balbontin et al. in S. Typhimurium \( dam \) mutants \cite{31}. Interestingly, despite the diminished amount of \( pmrA \) mRNA found in the \( dam \) mutant, PmrA levels remained unchanged. Discrepancies between mRNA transcription and protein translation have been reported earlier \cite{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 \( pmrA \) and \( rcsB \) gene expression. Also, the diminished amount of RcsB in \( SEA\text{adam} \) strain could explain the reduced \( wzz \) gene expression found earlier in this mutant \cite{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 \( rcB \) mutant, indicating that RcsB induces \( wzz \) gene expression. Surprisingly, in \( pmrA \) 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 \( SEA\text{pmrA} \) (this mutant would not lack Wzz protein).

To investigate a putative regulatory effect between both \( wzz \) regulators, we determined the expression of \( rcsB \) in a \( pmrA \) mutant, and \( pmrA \) expression in an \( rcsB \) mutant. We found that both regulators affect each other expression. The relative expression of \( pmrA \) mRNA decreases in the absence of \( rcsB \), whereas in the absence of \( pmrA \), the relative amount of \( rcsB \) mRNA increases. These results would indicate that, under the growth conditions used, RcsB stimulates \( pmrA \) whereas PmrA represses \( rcsB \). Also, these findings could explain the elevated expression of \( wzz \) found in the \( pmrA \) 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 phosphoryl transfer in phosphorelays and transcriptional and post-transcriptional modifications \cite{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 \( pmrA \), alignment analysis revealed a potential RcsB protein binding site in \( pmrA \) 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 \( pmrA \) gene. The balance between the expression and repression of \( pmrA \) 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 \( rcsB \text{pmrA} \) 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 \( pmrA \) 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 \( rcsB \text{pmrA} \) double mutant of S. Typhimurium closely resembles that of a \( wzz \) mutant \cite{16}. On the contrary, our experiments demonstrate that the LPS pattern of S. Enteritidis lacking both \( rcsB \) and \( pmrA \) 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 \( rcsB \text{pmrA} \) 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 Mg\(^{2+}\) observed in \( rcsB \) mutant as well as in \( rcsB \text{pmrA} \)

![Figure 7. Schematic diagram of the proposed regulatory network of wzz gene expression in S. Enteritidis.](https://doi.org/10.1371/journal.pone.0056474.g007)
Serovars.

In summary, we showed that in S. Enteritidis Dam methylation regulates \( \omega_{zz} \) expression through \( \text{rcsB} \) and \( \text{pmrA} \) genes; whereas RcsB induces \( \omega_{zz} \) gene expression PmrA represses it. We also present evidence that \( \text{rcsB} \) and \( \text{pmrA} \) genes regulate each other; RcsB stimulates the expression of \( \text{pmrA} \) and PmrA represses \( \text{rcsB} \) gene expression. Finally, our results support the existence of a third gene regulating \( \omega_{zz} \) expression in S. Enteritidis, that can be induced when bacteria is grown in high Mg\(^{2+}\). The regulatory network of \( \omega_{zz} \) gene expression proposed, including the involvement of the hypothetical third \( \omega_{zz} \) 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 PmrA-binding motif. The conserved nucleotides of the sequences corresponding to PmrA binding motif are boxed. B. Molecular analysis of rcsB gene region. Diagram of the DNA sequence corresponding to \( \text{rcsB} \) region based on Refseq NC\_011294 sequence of \( \text{S. enterica} \) serovar Enteritidis. Alignment analysis performed between the conserved regulatory sequences of PmrA motif and the potential PmrA protein binding site sequences found in \( \text{rcsB} \) gene region are depicted in the correspondent localization. The two known \( \text{rcsB} \) promoters \( \text{Pre}_1 \) and \( \text{Pre}_2 \) are marked with arrows. C. Alignment analysis of one of the potential RcsB-binding motifs found in \( \text{pmrA} \) gene region with the reported RcsB-dependent regulatory sequences of different enterobacteria. Homologous sequences of the potential RcsB-binding site found in comparison with the reported RcsB motif are in bold. D. Molecular analysis of \( \text{pmrA} \) gene region. Diagram of the DNA sequence corresponding to \( \text{pmrA} \) region based on Refseq NC\_011294 sequence of \( \text{S. enterica} \) serovar Enteritidis. Potential RcsB protein binding site sequences found in \( \text{pmrA} \) 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 (\( \text{mm} \)).

(TIF)

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Author Contributions

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

References


