The PmrA/PmrB regulatory system controls the expression of the \textit{wzz}^{\text{fepE}} gene involved in the O-antigen synthesis of \textit{Salmonella enterica} serovar Typhimurium

María de las Mercedes Pescaretti, Fabián E. López, Roberto D. Morero and Mónica A. Delgado

INTRODUCTION

The lipopolysaccharide (LPS) present in the outer membrane of Gram-negative bacteria is essential for the physical integrity and stability of the membrane (Raetz & Whitfield, 2002). Moreover, LPS plays an important role in the interaction of bacteria with their environment (Moran, 1994). The LPS is composed of three principal domains: a hydrophobic lipid A, a short non-repeating core oligosaccharide and a distal polysaccharide termed O-antigen (O-Ag). In \textit{Salmonella enterica} serovar Typhimurium (\textit{S. Typhimurium}) the O-Ag consists of a repeating polysaccharide chain of galactose, rhamnose and mannose (Raetz, 1996). The modal distribution of O-Ag chain lengths is controlled by the products of two genes, \textit{wzz}^{a} (also known as \textit{cld} or \textit{rol}) and \textit{wzz}^{fep} which confer to the O-Ag chain the feature to be long (L) or very long (VL), respectively (Morona \textit{et al.}, 1995; Murray \textit{et al.}, 2003; Raetz & Whitfield, 2002). The O-Ag chain length was previously correlated with bacterial virulence in mice as well as resistance to phagocytosis, cationic peptides and iron (Burns & Hull, 1998; Joiner, 1985; Skurnik & Bengoechea, 2003). Furthermore, several studies have determined that in \textit{Salmonella} the O-Ag is involved in resistance to the bactericidal activity of serum complement (Grossman \textit{et al.}, 1987; Joiner, 1985; Tomás \textit{et al.}, 1988). Murray \textit{et al.} (2005) reported enhanced survival of bacteria in serum when the VL O-Ag was increased by previous exposure to inactivated pig serum or iron limitation; however, this effect could not be correlated with change in the expression of the \textit{wzz}^{fepE} gene. More recently, it was confirmed that the VL O-Ag region is involved in the resistance of \textit{S. Typhimurium} to serum complement and to polymyxin B (Bravo \textit{et al.}, 2008; Hölzer \textit{et al.}, 2009). However, the regulation and the mechanism involved in the changes of the VL O-Ag structure by \textit{wzz}^{fepE} gene expression have not been determined.

The PmrA/PmrB system of \textit{S. Typhimurium} is activated directly by the presence of iron in the medium and indirectly by low magnesium concentration through the PhoP/PhoQ regulatory system (Kato & Groisman, 2004; Kox \textit{et al.}, 2000; Wösten \textit{et al.}, 2000). This system is involved in the modifications of LPS necessary for resistance to polymyxin B and iron (Gunn \textit{et al.}, 1998; Gunn \textit{et al.}, 2000; Nishino \textit{et al.}, 2006; Roland \textit{et al.}, 1993; Wösten \textit{et al.}, 2000). We previously established that activation of the PmrA/PmrB and the RcsCDB systems induces the transcription of \textit{wzz}^{a}, increasing the amount of L O-Ag and the resistance to serum complement (Delgado \textit{et al.}, 2006). Moreover, Sarnacki \textit{et al.} (2009) demonstrated that in \textit{Salmonella} Enteritidis, \textit{wzz}^{a} gene expression is activated by Dam methylation (Sarnacki \textit{et al.}, 2009).

Here we investigated the role of the PmrA/PmrB and RcsCDB systems in the control of \textit{wzz}^{fepE} gene expression...
correlated with the O-Ag modal lengths and its physiological role in Salmonella. Our results indicate that only the PmrA regulator binds to the wzzfepE promoter, inducing gene expression, which increases the amount of VL O-Ag. This effect results in an increase in the resistance to serum complement and polymyxin B, and in bacterial replication within macrophages.

**METHODS**

**Bacterial strains and growth conditions.** S. Typhimurium 14028s (parental strain) and its derivatives are detailed in Table 1. The ΔpmrAΔwzzfepE::Cm double mutant was obtained by phage P22-mediated transductions as described by Davis et al. (1980). The bacteria were grown at 37 °C in N-minimal medium containing either 10 mM MgCl₂ (H medium), 10 μM MgCl₂ (L medium) or 10 μM MgCl₂ and 100 μM FeSO₄ (LFe medium) to provide no, weak or strong PmrA-activation conditions, respectively. Chloramphenicol was used at a final concentration of 25 μg ml⁻¹.

**Mutation in the chromosomal wzzfepE locus.** Deletion of wzzfepE was achieved by the one-step gene-inactivation method (Datsenko & Wanner, 2000). The Cm cassette was amplified using plasmid pKD3 as template and primers ΔwzzfepE Fw (CTTTGATGAAAACATTGTTCCAGGTGACCTAGCTGCTCTC) and ΔwzzfepE Rv (GCCGGGATACGCTATCCGGCTTTTCGGGTAAACATATGAACTATCCTCCT) and ΔwzzfepE Rv (GCCGGGATACGCTATCCGGCTTTTCGGGTAAACATATGAACTATCCTCCT), the PCR product obtained was integrated into the chromosome by the Red system from plasmid pKD46 (Datsenko & Wanner, 2000). The correct insertion of the Cm cassette in the constructed mutant was confirmed by direct nucleotide sequencing.

**LPS analysis.** The LPS was purified from strains grown to stationary phase in L and LFe media following the procedure of Marolda pmrA (1990). The profile of LPS isolated from parental, 2516

**S1 mapping of the wzzfepE promoter.** The S1 mapping assay was performed following the protocol described by Garcia-Vescovi et al. (1996). The mRNA used in this assay was purified from parental strain 14028s grown to stationary phase in H, L or LFe medium; and from pmrA and wzzfepE mutants grown to stationary phase in LFe medium. The same amount of mRNA was used in each S1 reaction. The labelled DNA fragment used for S1 mapping was generated by PCR amplification of parental S. Typhimurium 14028s chromosomal DNA with primers [γ-3²P]ATP-wzzfepE S1-Fw (CGGGCGCCATGATCAGGCAA) and wzzfepE S1-Rv (CGGGCGCCATGATCAGGCAA). For coding strand protection the wzzfepE Foot-Fw primer was labelled with T4 polynucleotide kinase and [γ-3²P]ATP, while the wzzfepE Foot-Rv primer was labelled to determine the region protected for DNase I degradation on the non-coding strand. The PmrA-His₉ protein was purified as described by Delgado et al. (2006).

**DNAse I footprinting assay.** The footprinting assay was carried out according to Delgado et al. (2006). The DNA fragment used for DNase I protection assay was obtained by PCR amplification of parental 14028s chromosomal DNA with primers wzzfepE Foot-Fw (CGGTATACGGTCCGGAAG) and wzzfepE Foot-Rv (CGGGAA-CACATCTTTTACGCGG). For coding strand protection the wzzfepE Foot-Fw primer was labelled with T4 polynucleotide kinase and [γ-3²P]ATP, while the wzzfepE Foot-Rv primer was labelled to determine the region protected for DNase I degradation on the non-coding strand. The PmrA-His₉ protein was purified as described by Delgado et al. (2006).

**Serum complement sensitivity assay.** Strains were grown to the exponential phase in L or LFe medium, and then 10⁶ cells ml⁻¹ were incubated at 37 °C for 1 h with PBS or PBS containing 20 % activated human serum (Sigma). A higher concentration of activated human serum cannot be used because the bacterial viability is drastically diminished (Bravo et al., 2008; Murray et al., 2005; Delgado et al., 2006). Survival was determined by c.f.u. counts, expressed as a percentage of the control (strains incubated in PBS buffer) as described by Delgado et al. (2006).

**Bacterial infection of eukaryotic cells.** The bacterial infection of J774-A.1 mouse macrophages (American Type Culture Collection) was performed following the protocol described by Mouslim et al. (2004). Briefly, the macrophages were seeded in 24-well plates, and grown at 37 °C with 5 % CO₂. The bacteria were grown in LFe medium and added to the wells at a macrophage:bacteria ratio of 1:10. After 20 min, to eliminate bacteria that had not invaded macrophages, the cells were washed and incubated in fresh medium containing gentamicin (100 μg ml⁻¹). After 2 h of infection, the gentamicin concentration was lowered to 12 μg ml⁻¹ for the rest of the experiment, 24 h. The infectivity of each tested strain was determined by c.f.u. counts of macrophage lysates after 20 min of infection; the replication ability was determined in the same manner but after 2 and 24 h of infection.

**RESULTS**

**PmrA activation increases the O-Ag VL modal region**

As we previously demonstrated that activation of the PmrA/PmrB and RcsCDB systems induces production of the O-Ag L region, here we studied the effect of these regulators on O-Ag VL modal levels. We analysed the LPS profile of S. Typhimurium strain 14028s and its isogenic pmrA, rcsB and wzzfepE mutants, grown in L or LFe medium as activation condition. As indicated in Fig. 1(a), the parental strain grown in LFe medium showed a twofold increase in the relative intensity of the O-Ag VL region in comparison with bacteria grown in same medium without

---

**Table 1. Strains of S. Typhimurium used in this study**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Description</th>
<th>Reference or source</th>
</tr>
</thead>
<tbody>
<tr>
<td>14028s</td>
<td>Parental</td>
<td>Fields et al. (1986)</td>
</tr>
<tr>
<td>EG14929</td>
<td>ΔwzzfepE</td>
<td>Delgado et al. (2006)</td>
</tr>
<tr>
<td>EG13307</td>
<td>ΔpmrA</td>
<td>Mouslim &amp; Groisman (2003)</td>
</tr>
<tr>
<td>EG14931</td>
<td>ΔrcsB</td>
<td>Pescaretti et al. (2009)</td>
</tr>
<tr>
<td>MDx1443</td>
<td>ΔwzzfepE::Cm</td>
<td>This work</td>
</tr>
<tr>
<td>MDx1444</td>
<td>ΔpmrA ΔwzzfepE::Cm</td>
<td>This work</td>
</tr>
</tbody>
</table>
iron (L medium). The effect of iron was suppressed by deletion of the wzzfepE or pmrA gene but not of rcsB, suggesting that only the PmrA regulator was involved in the transcription of the wzzfepE gene (Fig. 1a). This hypothesis was tested by a qRT-PCR assay. The results of this assay indicated that the level of wzzfepE mRNA in the parental strain increased 1.5- and 4.5-fold when the bacteria were grown in L or LFe medium respectively, compared to those obtained in H medium as the no PmrA-activation condition (Fig. 1b). The pmrA mutant did not show differences in wzzfepE mRNA levels under activation conditions, indicating that PmrA is required to reach the levels observed in the parental strain under induction (Fig. 1b).

On the other hand, we observed an approximately 10-fold increase in the relative intensity of the O-Ag region in the wzzfepE mutant compared with the parental strain when both were grown in L medium (Fig. 1a). This is an unexpected result and the mechanism involved is currently being investigated.

**Molecular analysis of the wzzfepE promoter under PmrA regulation**

To study the regulation of the wzzfepE promoter at the molecular level in more depth, we performed an S1 mapping analysis. In this assay, a small amount of the S1 product was obtained when the mRNA was purified from the parental strain growing in H medium, whereas a marked increase of S1 product was detected when the same amount of mRNA from bacteria growing in L medium was used (Fig. 2a). We assume that the differences observed are due to changes in wzzfepE expression, because the experiment was repeated three times with identical results. In agreement with the results of Fig. 1(b), the increase was greater when bacteria were grown in LFe medium, producing full activation of PmrA (Fig. 2a). As expected, this S1 product was absent when the mRNA was obtained from pmrA or wzzfepE mutants growing in LFe medium (Fig. 2a). These data indicate that the presence of iron and a low concentration of magnesium in the growth medium activates the PmrA regulator, which induces wzzfepE transcription.

The S1 mapping results allowed us to identify the +1 transcription start site, which was located at 246 bp relative to the ATG codon (Fig. 2a, b). Based on the +1 site localization a putative PmrA-binding box that could serve as a positive regulation site for the wzzfepE gene expression was sought through bioinformatics analysis. This analysis revealed the presence of a sequence showing homology with the consensus PmrA-binding box (Marchal et al., 2004), located at 2145 bp relative to the +1 site (Fig. 2b, c). To confirm that the predicted box could serve as a PmrA-binding site, we performed a DNase I footprinting assay. This assay revealed that the PmrA protein was able to protect the region from positions 2107 to 2182 on the coding strand and from 298 to 2175 on the non-coding strand, relative to the +1 site (Fig. 2d). As shown in Fig. 2(b, d), the predicted PmrA-binding box was included in the protected sequences of PmrA, strongly suggesting that the positive regulation of wzzfepE transcription is produced by direct binding of the regulator to the gene promoter sequence.

**Physiological role of PmrA-dependent wzzfepE gene expression**

As mentioned above, the O-Ag is required to resist the bactericidal effect of human serum complement. We
investigated whether increased expression of the PmrA-dependent \textit{wzzfepE} gene is involved in this resistance. As shown in Fig. 3(a), the parental strain and \textit{wzzfepE} and \textit{pmrA} mutants exposed to human serum showed a 50\% higher survival value when growing in LFe medium than in L medium. Furthermore, in the presence of iron the parental strain showed 30–40\% higher survival than the \textit{wzzfepE} and \textit{pmrA} mutants (Fig. 3a). The observed survival levels of the \textit{wzzfepE} mutant and the parental strain clearly show that the iron induction effect on \textit{wzzfepE} expression is required for serum complement resistance. However it should be noted that other PmrA-dependent genes, such as
The wzzst gene could also participate in this resistance. An expected result was that the survival rate of the pmrA mutant did not reach values near zero (Fig. 3a). These data were in accordance with previously described experiments showing that the wzzst gene is also positively controlled by the Rcs system, in a pmrA mutant growing in the presence of iron, and that the product of this gene is also required for serum resistance (Delgado et al., 2006).

In addition, we studied the role of the wzzfepE gene product in the ability of the bacteria to replicate within host cells, a process for which the PmrA regulator is also required (Gunn et al., 2000; Roland et al., 1993). To achieve this objective we examined the ability of the parental strain and wzzfepE and wzzst mutants, previously grown in LFe medium, to replicate within J774-A.1 mouse macrophages. The capacity of infection of these strains was not determined here since macrophages are not appropriate for this study. However, we observed a marked decrease, 40%, in the replication ability of the wzzfepE mutant relative to the parental strain at both 2 and 24 h post-infection (Fig. 3b). The wzzst mutant replicated less effectively than the parental strain at 2 h of infection, but no difference was observed after 24 h (Fig. 3b). These results suggest that both gene products, WzzfepE and Wzzst, are required for bacterial replication within macrophages, the first of them being more important.

As the PmrA regulator controls the genes required for resistance to cationic peptides, such as polymyxin E and B, we tested the polymyxin B sensitivity of S. Typhimurium strains growing under PmrA induction. As shown in Table 2, PmrA activation (LFe medium) led to a twofold increase in the resistance of the parental strain to polymyxin B compared with the no-induction condition (H medium). When growing in LFe medium, the pmrA mutant displayed tenfold lower resistance levels than the parental strain, while the wzzfepE mutant was twofold less resistant (Table 2). As expected, the double mutant pmrA wzzfepE was the most polymyxin B-sensitive strain. These results clearly indicate that the wzzfepE gene product, as well as previously reported PmrA-dependent genes, also participates in polymyxin B resistance (Gunn et al., 1998).

Table 2. MICs of polymyxin B for S. Typhimurium 14028s and its mutants

<table>
<thead>
<tr>
<th>Strain</th>
<th>MIC (µg ml⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>H medium</td>
</tr>
<tr>
<td>pmrA</td>
<td>7.81</td>
</tr>
<tr>
<td>ΔpmrA</td>
<td>7.81</td>
</tr>
<tr>
<td>ΔwzzfepE</td>
<td>7.81</td>
</tr>
<tr>
<td>ΔpmrA ΔwzzfepE</td>
<td>7.81</td>
</tr>
</tbody>
</table>
**DISCUSSION**

The O-Ag plays an important role in *S. Typhimurium* infections, allowing the bacteria to resist the effects of serum complement and cationic peptides. Although many studies have been carried out to determine both the composition and distribution of O-Ag, little is known about its regulation mechanism. We previously demonstrated that *wzz* is under control of the PmrA/PmrB and Rcs systems; in the present work we have investigated the effect of both regulators on *wzz* gene transcription. It has been reported that the O-Ag is positively regulated by RpoN and RpoS in an RfaH-dependent pathway during the stationary growth phase (Bittner *et al.*, 2004). It is important to note that while the regulator RfaH study was focused on the control of the O-Ag assembly, our study and that of Sarmacki *et al.* (2009) were directed to the control of the O-Ag length. We here clearly demonstrate for the first time that *wzz* gene expression is induced by activation of the PmrA/PmrB system by direct binding of the PmrA regulator to the promoter region. In contrast to the study by Murray *et al.* (2005), we demonstrated that the presence of iron in the growth medium induces the transcription of the *wzz* gene in a PmrA-dependent manner. However, our results are not strictly comparable with those of Murray *et al.* (2005), due to the difference in the culture medium, the concentration of iron and the analyses used. The difference could be also attributable to the genetic background of the strains used in the studies, as was reported by Boyd *et al.* (2003) and Vernikos *et al.* (2007).

Here we have demonstrated that *wzz* as well as *wzz* is directly regulated by the PmrA/PmrB system. Both genes are responsible for the resistance of bacteria to serum complement. However, unlike *wzz*, *wzz* is not regulated by RcsB and therefore is not involved in the bacterial swarming behaviour (result not shown), but it is involved in survival within macrophages and also in resistance to polymyxin B. The regulation of *wzz* by the PmrA/PmrB system is relevant because it determines the environmental mechanism involved in the VL modal distribution of the O-Ag and suggests a role of *wzz* in the strategies of the bacteria against host defences.

In this work we have studied for the first time the role of the PmrA/PmrB system in the control of *wzz* gene expression and the importance of this control in terms of O-Ag serum complement resistance. The activation of the PmrA/PmrB system by iron and low magnesium increases the production of VL O-Ag with the concomitant enhancement of the serum and polymyxin B resistance. Furthermore, this increased resistance is enhanced by the presence of iron. Activation of the PmrA/PmrB system by high iron concentration is important for the survival of *Salmonella in vitro* and also in soil, in which iron is one of the most abundant metals (Chammongpol *et al.*, 2002). This high concentration is unlikely to occur in the human host, but could be important in the capabilities of this bacterium upon host entry.

In conclusion, our findings highlight the function of the PmrA/PmrB system in the regulation of the O-Ag VL region by the direct control of the *wzz* gene. We postulate that when the bacteria sense the environmental conditions within macrophages, such as low magnesium, the PmrA regulator is activated. This activation results in the induction of the *wzz* gene, which could allow bacteria to overcome the host defence mechanisms and to establish the infection. The modification of the O-Ag could also participate in other forms of resistance to host defences; such possibilities are currently being studied in our laboratory.

**ACKNOWLEDGEMENTS**

We thank E. A. Groisman for providing strains, C. L. Marolda for protocols of the purification and staining of the LPS and the PEB Latin American Fellow Program for the equipment donated. This work was supported by FONCYT grant 32124 and CONICET grant PIP 2518. M. M. P. and F. E. L. are Fellows of CONICET. R. D. M. and M. A. D. are Investigators of CONICET.

**REFERENCES**


Edited by: P. H. Everest

http://mic.sgmjournals.org