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The PmrAB-inducing conditions control both lipid A remodelling and O-antigen length distribution, influencing the *Salmonella* Typhimurium-host interactions.

Juan V. Farizano[1], María de las Mercedes Pescaretti[1], Fabián E. López[1], Fong-Fu Hsu[2], and Mónica A. Delgado[1]*

[1]Instituto Superior de Investigaciones Biológicas (Consejo Nacional de Investigaciones Científicas y Técnicas-Universidad Nacional de Tucumán) and Instituto de Química Biológica "Dr. Bernabe Bloj", San Miguel de Tucumán, Tucumán, Argentina.

[2]Mass Spectrometry Resource, Division of Endocrinology, Diabetes, and Metabolism, Department of Internal Medicine, Washington University School of Medicine, St. Louis, Missouri, USA.

Running title: wzz_{st}, $pbgE_2$ and $pbgE_3$, role in LPS modifications

To whom correspondence should be addressed: Mónica A. Delgado, Instituto Superior de Investigaciones Biológicas (Consejo Nacional de Investigaciones Científicas y Técnicas-Universidad Nacional de Tucumán) and Instituto de Química Biológica "Dr. Bernabe Bloj", Chacabuco 461, 4000 San Miguel de Tucumán, Tucumán, Argentina. Tel/Fax: 54-381-4248921; E-mail: monicad@fbqf.unt.edu.ar

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Background: *Salmonella* Typhimurium LPS structure is regulated by the PmrAB, PhoPQ and RcsCDB systems.
Results: Wzz_{st} is required for lipid A modifications. $PbgE_2$ and $PbgE_3$ control formation of short O-antigen region.
Conclusion: PmrAB system is the master regulator of LPS remodeling, modulating genes that modify lipid A, core and O-antigen.
Significance: *Salmonella* exhibits complex mechanisms to modulate its LPS that influence host interaction.

SUMMARY

The *Salmonella enterica* serovar Typhimurium lipopolysaccharide consisting of covalently linked lipid A, non-repeating core oligosaccharide and the O-antigen polysaccharide, is the most exposed component of the cell envelope. Previous studies demonstrated that all of these regions act against host immunity barrier. The aim of this study was to define the role and interaction of PmrAB-dependent gene products required for the lipopolysaccharide components synthesis or modification, mainly during the *Salmonella* infection. The PmrAB two-component system activation promotes a remodelling of lipid A and core region by addition of 4-aminoarabinose and/or phosphoethanolamine. These PmrA-dependent activities are produced by activation of *ugd*, *pbgPE*, *pmrC*, *cpta* and *pmrG* transcription. In addition, under PmrA regulator activation the expression of wzz_{fepE} and wzz_{st} genes are induced and their products are required to determine the O-antigen chain length. We here report for the first time that Wzz_{st} protein is necessary to maintain the balance of 4-aminoarabinose and phosphoethanolamine-lipid A modifications. Moreover, we demonstrate that the interaction of the PmrA-dependent $pbgE_2$ and $pbgE_3$ gene products is important for the formation of the short O-antigen region. Our results establish that PmrAB is the global regulatory system that controls lipopolysaccharide modification, leading to a coordinate regulation of 4-aminoarabinose incorporation and O-antigen chain length to respond against the host defense mechanisms.

INTRODUCTION

Like other Gram-negative bacteria, the lipopolysaccharide (LPS) in *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium) is the major surface constituent located in the outer

leaflet of outer membrane (1,2). This LPS is composed of: i) the hydrophobic lipid A, which anchors LPS to the outer membrane (3); ii) an oligosaccharide core; and iii) an O-antigen polysaccharide. In *S.* Typhimurium the lipid A is composed of sugars and fatty acids, while the O-antigen is a polymer of three to six sugar repeat units extended out from the cell surface (3,4).

In order to survive microenvironmental changes, pathogenic bacteria are able to remodel their outer membrane, mainly at the level of lipid A and O-antigen. The remodelling of lipid A is produced by palmitoylation and/or deacylation in a PhoPQ-dependent manner; and by addition of 4-aminoarabinose (L-Ara4N) and phosphoethanolamine (pEtN) depending on PmrAB system activation (5-12). These modifications allow bacteria to resist the host immunity barriers, like iron and cationic peptides (13-17). The PmrAB two-component system consists of the PmrA response regulator and the PmrB sensor which is able to sense Fe^{3+}, activating the system (16). This regulatory system can also be activated by low Mg^{2+} in a PhoPQ-dependent pathway, in which the PhoP-activated PmrD protein is required (18,19). These two systems control the above lipid A modification through the regulation of *pagP*, *ugd*, *pbgPE* and *pmrC* genes (5,6,8,11,12). It has been demonstrated that expression of the *pbgPE* operon (also called *arn* or *pmrHFIJKLM* operon) and *ugd* gene, involved in synthesis and incorporation of L-Ara4N into lipid A, are induced by PmrAB-activation (6,20,21). In addition, this system controls the expression of *pmrC* gene, responsible for addition of pEtN to lipid A (5,6,8), and *pmrG* and *cptA* genes, which modify the core-LPS region (22,23). These results suggest that activation of PmrAB systems is strongly necessary to respond against the host immunity barriers.

On the other hand, the normal assembly of *S.* Typhimurium O-antigen heteropolymer requires functional Wzz$_{fepE}$, Wzx, Wzy and Wzz$_{st}$ proteins (3,4). This LPS component plays a direct role in the resistance to phagocytosis, antimicrobial peptides, and serum complement, which are dependent on O-antigen chain length (5,6,8,11,12,24-28). It has been demonstrated that the O-antigen follows a bimodal distribution of its subunits attached to the lipid A-core (4,29,30). This distribution is controlled by the Wzz$_{st}$ protein (also known as Cld, WzzB or Rol), which mediates the production of long O-antigen containing 16 to 35 subunits (L-type) (4,29); and the Wzz$_{fepE}$ protein, which is responsible for the very long O-antigen (VL-type) containing >100 subunits (30).

We previously reported that the *wzz$_{st}$* gene is under control of the PmrA and RcsB regulators (24,25). This result raises the possibility that the Wzz$_{st}$ protein is required in more than one bacterial membrane modification process. In this connection, we demonstrated that when RcsB is activated the Wzz$_{st}$ protein is involved in the negative control of flagella in hyperflagellated bacteria (24). However, no new function was determined for this protein when it is expressed under PmrA activation. On the other hand, in previous studies we observed that *S.* Typhimurium displays a third O-antigen region of low molecular weight (LMW). This observation, and the fact that PmrA also regulates the expression of *wzz$_{fepE}$* and *wzz$_{st}$*, prompted us to investigate the possibility that other PmrA-dependent genes might control the formation of this third O-antigen region.

Taken together, the above observations suggest that there is a very specialized PmrA-dependent regulation mechanism acting on LPS components. This hypothesis led us to study the role of the PmrAB-dependent Wzz$_{st}$ protein in lipid A modification, the participation of other PmrAB-dependent gene products in the synthesis of the third O-antigen region, and their relevance in the *Salmonella* infection. Our results demonstrate for the first time that Wzz$_{st}$ protein is required to maintain the balance of L-Ara4N and pEtN modifications at the level of lipid A. We also found that the absence of the last two genes of *pbgPE* operon, *pbgE$_2$* and *pbgE$_3$*, results in an O-antigen without the LMW region (1 to 15 subunits), and that the interaction of both gene products is necessary to control its formation. The latter allow us to propose that in *S.* Typhimurium the O-antigen subunits are distributed in the following regions: i) LMW or Short (S), controlled by *pbgE$_2$* and *pbgE$_3$* genes; ii) Long (L), controlled by *wzz$_{st}$*; and iii) Very Long (VL), controlled by *wzz$_{fepE}$*. We here conclude that the PmrAB system is the master regulator of the LPS remodelling; and that both O-antigen formation and lipid A modifications occur simultaneously during *Salmonella* infection.

EXPERIMENTAL PROCEDURES

Bacterial strains, molecular techniques and growth conditions-Bacterial strains used in this study are listed in Table 1. Phage P22-mediated

transduction was used to introduce the gene mutations into different strains, following the protocol previously described (31). Bacteria were grown at 37 °C in Luria–Bertani (LB) (32) or in N-minimal medium (0.5 M Trizma base, 0.5 M Bis-Tris, 50 mM KCl, 75 mM $(NH_4)_2SO_4$, 5 mM K_2SO_4, and 10 mM KH_2PO_4, pH 7.4) (33), supplemented with 0.1% casamino acids, 38 mM glycerol and 10 mM $MgCl_2$ (high Mg^{2+}) or 10 µM $MgCl_2$ (low Mg^{2+}), as indicated. Antibiotics were used at the following final concentrations: chloramphenicol (Cm), 25 µg/ml; kanamycin (Km), 50 µg/ml; ampicillin (Ap), 50 µg/ml; and polymyxin B and E, 10 µg/ml.

Introduction of gene mutations in the Salmonella chromosome and plasmids construction-The one-step gene inactivation method (34) was used to generate deletion of *pbgE2* or *pbgE3* complete coding sequence. The Cm cassette was amplified by using pKD3 plasmid as template and primers #7008, #7009, #7010 and #7011 (Table 2). The correct insertion of the cassette in the mutant was confirmed by direct nucleotide sequencing.

The cloning of the *pbgE2* and *pbgE3* genes was carried out by PCR amplification using genomic DNA extracted from wild-type *S.* Typhimurium 14028s strain and primers #1001, #1002, #1003 and #1004 (Table 2). The PCR products were cloned into pUHE2-2*lacI*^{q} vector using *Xba*I restriction enzyme, resulting in derivative plasmids listed in Table 1. The insert correct orientation was confirmed by both PCR and direct nucleotide sequencing.

LPS analysis-The LPS was purified from wild-type *S.* Typhimurium 14028s, wzz_{st}, wzz_{fepE}, $pbgE_2$, $pbgE_3$, $pbgE_2$ (p$pbgE_2$) and $pbgE_3$ (p$pbgE_3$) strains grown to stationary phase in N-minimal medium with low Mg^{2+}, following the protocol described by Marolda *et al.* (35). Cultures were normalized to OD_{600}=1.0 in a final volume of 100 µl. Ten microliters of each LPS normalized sample were analyzed using Tris/glycine/SDS 12% acrylamide gels, which were silver stained as described (24).

Serum complement sensitivity assay-The analysis of serum sensitivity of the strains grown in N-minimal medium with low Mg^{2+} was carried out as described (24). Briefly, 10^4 cell/ml were incubated for 1 h at 37 °C with PBS buffer as control or with PBS containing 20% human serum (Sigma). The colony forming units (cfu) produced by each treatment was determined by serial dilutions plated on LB agar medium and incubated at 37 °C. The results were expressed as a percentage of the control (strains incubated in PBS buffer), as described previously (24).

Bacterial infection of eukaryotic cells-The strains grown overnight in N-minimal medium with low Mg^{2+} were used to infect the Raw 264.7 mouse macrophages as described (36). To test the macrophage phagocytic ability, the cells were lysed after 30 min of infection using 1% Triton X-100 and the number of viable bacteria that survived to the gentamicin treatment was determined by subsequent plating onto LB agar medium. The same procedure was used to evaluate replication ability, but the number of viable bacteria was determined after 6 and 18 h of infection. Results were expressed as a percentage of survival to gentamicin calculated as 100× (cfu ml^{-1} mutant bacteria)/(cfu ml^{-1} wild-type bacteria), at each time point.

Peptide killing assays-The polymyxin survival assay was carried out following the protocol described by Lee *et al.* (8). Overnight cultures of bacteria grown in N-minimal medium with high Mg^{2+} were diluted 1:100 in N-minimal medium with low Mg^{2+} and grown at 37 °C to reach OD_{600}=0.3 to 0.4. Then, 50 µl of a 1:100 dilution of these cultures were mixed with 50 µl of polymyxin B or E dissolved in PBS at 20 µg/ml (final concentration, 10 µg/ml), in a 96 well plate. After 1 h of incubation at 37 °C the cfu was determined by serial dilution on LB agar medium. The number of viable bacteria was represented as 100× (cfu ml^{-1} mutant bacteria polymyxin-treated)/(cfu ml^{-1} wild-type bacteria polymyxin-treated).

Protein-Protein interaction assay-This assay was performed as described by Karimova *et al.* (37). The $pbgE_2$, $pbgE_3$ and wzz_{st} genes were cloned into the pUT18, pUT18C, pKT25 and pKTN25 plasmids (Table 1). To this end, the gene sequences were amplified by PCR from wild-type *S.* Typhimurium 14028s genomic DNA using the primers listed in Table 2. The PCR products of $pbgE_2$ and $pbgE_3$ were digested and cloned with *Kpn*I/*Xba*I restriction enzymes, while the wzz_{st} PCR product was cloned using *Kpn*I/*Bam*HI. The plasmid derivatives were controlled by DNA sequencing, and co-transformed in all compatible combinations into *E. coli* DHM1 strain. To determine the potential protein interactions, ß-galactosidase activity from the co-transformant strains grown to stationary phase in LB medium

containing 0.5 mM IPTG was measured as described (38). The positive control was generated by transformation of *E. coli* DHM1 with pKT25-zip and pUT18C-zip plasmids containing the leucine zippers GCN4 domain, while the strain harboring empty vectors served as negative control. ß-galactosidase activities were expressed as the mean values of three independent experiments, done in duplicate.

Mass spectrometry analysis of lipid A-The lipid A samples were purified from the indicated bacterial strains grown in N-minimal medium with low Mg^{2+}, as described previously (17). The MALDI-TOF mass spectrometry assay, performed in the negative ion mode on a Voyager DE STR mass spectrometer (PerSeptive Biosystems) equipped with a 337 nm nitrogen laser and delayed extraction, was used for Lipid A samples analysis as described previously (17).

RESULTS

The wzz_{st} gene product is involved in lipid A modification in a PmrAB-dependent manner-In a previous work, we reported that expression of *wzz_{st}* is controlled by two regulatory systems, PmrAB and RcsCDB. We also observed that, following RcsCDB activation, the Wzz_{st} protein participates in the formation of L O-antigen region and in the bacterial swarming behavior (24). The working hypothesis of this section was that, under PmrAB system activation, the Wzz_{st} protein could be required in other physiological processes. As it is well known that the PmrAB system is required for lipid A modification and that the *wzz_{st}* gene is located close to *ugd*, which takes part in the synthesis and attachment of L-Ara4N to lipid A (6,14,21), we investigated whether the lipid A is modified by Wzz_{st}. To this end, we used negative-ion-mode MALDI-TOF mass spectrometry to analyze lipid A species as the $[M - H]^-$ ions from wild-type 14028s strain and their isogenic *pmrA* (EG13307) and *wzz_{st}* (EG14929) mutants (Table 1). These strains were grown in N-minimal medium at low Mg^{2+} conditions to promote the transcription of PmrA-activated genes in a PhoPQ-dependent pathway (18,39). Consistent with previous reports (8,17), under this assay condition the wild-type strain (Fig. 1A) and *rcsB* mutant (data not shown) displayed the same lipid A pattern. The profile of both strains showed the prototype lipid A at *m/z* 1796 and 2035, along with the ions at *m/z* 1928, 1944, 2166 and 2182 modified with L-Ara4N and those at *m/z* 1919, 1935, 2158 and 2173 modified with pEtN. It should be noted that the ions at *m/z* 2035, 2158, 2166, 2173 and 2182 are also modified with a palmitic acid. The *pmrA* mutant showed the expected lipid A profile (Fig. 1B), where the prototype lipid A (*m/z* 1796) was modified only by palmitic acid addition (*m/z* 1955, 2035 and 2051), since neither L-Ara4N nor pEtN addition can be carried out by this mutant. Interestingly, the lipid A profile observed in the *wzz_{st}* mutant (Fig. 1C) lacks the peaks at *m/z* 1928, 2158 and 2166 corresponding to prototype lipid A modified with either L-Ara4N or pEtN (8). Moreover, we observed that the lipid A diphosphorylated species (*m/z* 1928 and 2158) were affected mainly in the *wzz_{st}* mutant, suggesting that these species could be used as substrate for the Wzz_{st}-dependent modifications. These results indicate that expression of *wzz_{st}* leads to lipid A modifications in a PmrA-dependent manner.

Interestingly, a new lipid A peak at *m/z* 2281 appeared in the *wzz_{st}* mutant, while the species at *m/z* 2158 was absent (Fig. 1C). Based on previous reports, we suggest that the ion at *m/z* 2281 could be the result of an extra pEtN residue (≅123 average mass units) added to the 1- or 4′-phosphate of lipid A that peaks at *m/z* 2158. It is important to note that the *m/z* 2158 specie arises from the *m/z* 1796 ion modified by one pEtN and one palmitic acid group, when the *pmrC* and *pagP* genes are expressed under PmrAB and PhoPQ activation, respectively (Fig. 1D) (8,9,40). As the chemical structures for most of the lipid A species in *S. enterica* has previously been determined (41-43), we confirmed the extra pEtN residue hypothesis by MALDI-TOF analysis of the lipid A purified from *pmrC* (EG13633), *pagP* (EG13678), *wzz_{st} pmrC* (MDs1015) and *wzz_{st} pagP* (MDs1016) mutants, growing in low Mg^{2+}. As shown in Figure 2, the *m/z* 2281 peak is absent in the spectra arising from *pmrC* and *pagP* mutants and from *pmrC wzz_{st}* and *pagP wzz_{st}* double mutants. As expected, the peaks containing L-Ara4N and/or palmitic acid (*m/z* 1928, 1944, 1955, 2035, 2051, 2166 and 2182) or modified by L-Ara4N or pEtN (*m/z* 1928, 1944, 1919 and 1935) were maintained in the *pmrC* or in the *pagP* mutants, respectively (Fig. 2A and C). These results confirm that the *m/z* 2281 ion is formed by addition of a second pEtN residue to the *m/z* 2158 specie in the absence of Wzz_{st}, when the *pmrC* and *pagP* genes are expressed. Moreover, we observed that in the *wzz_{st} pmrC* and *wzz_{st} pagP* double mutants, like in *wzz_{st}*, species

harboring L-Ara4N (*m/z* 1928 and 2166) were also absent (Fig. 2B and D).

Taken together, our results suggest that *wzz_{st}* gene product is required to maintain the balance between modification in lipid A by L-Ara4N and pEtN, resembling the effect that Wzz_{st} exerts on the Wzx and Wzy balance required for O-antigen long chain determination.

The $pbgE_2$ and $pbgE_3$ gene products are necessary for O-antigen short chain length determination- Since the above results demonstrated that *wzz_{st}* is involved in lipid A modification and we previously observed that an O-antigen of LMW was retained in the *wzz_{st}* and *wzz_{fepE}* mutants (24,25), we studied the role of other PmrA-controlled genes in the formation of this O-antigen region. To this end, we first investigated the participation of the not well-characterized $PbgE_2$ and $PbgE_3$ proteins, encoded by the last two genes of the *pbgPE* operon (Fig. 3A) (14,22,44). To test whether *$pbgE_2$* or *$pbgE_3$* deletion affects the O-antigen chain length, we analyzed the distribution of LPS in the strains harboring *$pbgE_2$* or *$pbgE_3$* nonpolar gene deletions (Table 1), when the PmrAB system was activated. We noticed that the LPS obtained from *$pbgE_2$* and *$pbgE_3$* mutants lacked a silver staining material of LMW as compared with that from wild-type, *wzz_{st}* or *wzz_{fepE}* strains (Fig. 3B). Furthermore, the wild-type O-antigen chain length distribution was restored when *$pbgE_2$* and *$pbgE_3$* mutants were complemented by p*$pbgE_2$* and p*$pbgE_3$* plasmids, respectively (Fig. 3B). These results showed that deletion of *$pbgE_2$* exhibited no polarity effect on *$pbgE_3$* expression. Furthermore, our findings demonstrated that, in addition to Wzz_{st} and Wzz_{fepE}, the *$pbgE_2$* and *$pbgE_3$* gene products are involved in the control of O-antigen chain length distribution. In accordance with a previous report of Holzer *et al*. (45), our data allow us to establish that the O-antigen of *S.* Typhimurium follows a trimodal length distribution, and that formation of the poorly studied LMW region named Short or S region (1 to 15 sugar subunits attached to lipid A-core) is controlled by the $PbgE_2$ and $PbgE_3$ proteins.

Murray *et al*. (30) found that the domain '$PX_2PX_4SPKX_1X_{10}GGMXGAG$' is strongly conserved in both Wzz_{fepE} and Wzz_{st} proteins. This sequence, located in the C-terminal region and overlapping the second transmembrane domain (TMD) of both proteins, was found to be essential for their function (46,47). It was interesting to investigate whether this domain is also present in $PbgE_2$ and $PbgE_3$. The bioinformatic analysis of $PbgE_2$ and $PbgE_3$ sequences, carried out using Transmembrane Prediction Server (Stockholm University, Sweden, www.sbc.su.se/~miklos/DAS), suggested that both are inner membrane proteins bearing four TMD, comprising the amino acid residues 6-13, 38-61, 74-77 and 97-106 in $PbgE_2$ and 10-12, 51-57, 81-91 and 107-119 in $PbgE_3$. In addition, the multiple-alignment of these sequences with the '$PX_2PX_4SPKX_1X_{10}GGMXGAG$' domain from Wzz_{fepE} and Wzz_{st} showed a similarity of 48 and 43%, respectively. In both cases, this domain overlapped the third and fourth TMD, comprising residues 49 to 92 from $PbgE_2$ and 70 to 107 residues from $PbgE_3$. Interestingly, when the alignment was performed only with the Wzz_{st} protein domain, we noticed that $PbgE_2$ conserved the first part of the above domain, the 'PX_2PX_4SPK' motif (Fig. 3C, filled lines box); whereas the second portion, 'GGMXGAG' motif, was conserved in $PbgE_3$ (Fig. 3C, dotted lines box). Since, the $PbgE_2$ and $PbgE_3$ proteins are smaller than Wzz_{st}, we suggest that interaction of both proteins is necessary to control the S O-antigen modal distribution.

The $PbgE_2$ and $PbgE_3$ proteins are able to interact-To test the above notion, we investigated whether $PbgE_2$ and $PbgE_3$ act together or individually in the control of the S O-antigen region. We carried out an *in vivo* protein-protein interaction assay, following the Bacterial Adenylate Cyclase Two-Hybrid (BACTH) protocol (37). We constructed the pUT18 and pUT18C, pKT25 and pKTN25 derivative vectors, harboring the sequences of the T18 or T25 fragments *in frame* with the coding sequences of *$pbgE_2$* and *$pbgE_3$*. To this end these genes were amplified using wild-type *S*. Typhimurium 14028s DNA and primers containing *Xba*I and *Kpn*I restriction sites (Table 2). In order to investigate interactions with $PbgE_2$ and $PbgE_3$, we also cloned the *wzz_{st}* gene with primers containing *Bam*HI and *Kpn*I restriction sites to ensure the correct cloning orientation (Table 2). The resulting plasmids were introduced into *E. coli* DHM1, in all possible combinations. Functional interaction was determined by measuring the β-galactosidase activity produced by the transformed bacteria. *E. coli* DHM1 strain co-transformed with empty vectors, which was used as negative control, showed basal levels of β-galactosidase activity

(approximately 100 Miller units). A positive control, generated by using pKT25-zip and pUT18C-zip plasmids harboring the leucine zippers GCN4 domain, displayed high levels of β-galactosidase activity (≅6000 Miller units). We observed an increase in β-galactosidase activity, compared to negative control, when the *$pbgE_2$* and *$pbgE_3$* genes were fused to the C-terminal domain of either T18 or T25 fragments (Fig. 4, 2252 and 1190 Miller units). Similar results were obtained when both genes were cloned in the N-terminal region of these fragments (data not shown). These increased levels demonstrated that a strong protein-protein interaction occurs when *$pbgE_2$* and *$pbgE_3$* genes are expressed together. In these assays we observed that $PbgE_2$ or $PbgE_3$ are not self-interacting proteins, because in the *E. coli* DHM1 strain harboring the pT25-p*bgE_2*/pT18C-p*bgE_2* or pT25-p*bgE_3*/pT18C-p*bgE_3* combinations the β-galactosidase activity levels were hardly increased as compared to negative control (Fig. 4).

On the other hand, our results indicated that Wzz_{st} is unable to interact with itself or with the $PbgE_2$ or $PbgE_3$ proteins. Here, we observed only small differences in β-galactosidase activity levels when the corresponding fusion combinations were used (Fig. 4).

Physiological role of the $pbgE_2$ and $pbgE_3$ gene products-Previously, it has been reported that bacterial resistance to complement-mediated killing is directly related to O-antigen length, which acts against formation of a membrane attack complex (2,26-28). According to this, the resistance to serum complement of *$pbgE_2$* and *$pbgE_3$* mutants was used to examine the physiological importance of S O-antigen region in the *Salmonella* LPS. The susceptibility of isogenic strains, growing under PmrA-inducing conditions, to serum complement was determined by exposing them to 20% human serum during 1 h. As shown in Figure 5A, the *$pbgE_2$* and *$pbgE_3$* mutants, as well as *wzz_{st}*, were more sensitive to complement-mediated killing than the wild-type strain (20% of survival). In addition, when *$pbgE_2$*, *$pbgE_3$* and *wzz_{st}* mutants were complemented with plasmids harboring the corresponding genes, the resistance phenotype was restored (Fig. 5A, grey bars). We observed that the resistance of the complemented mutants was higher than in the wild-type strain, due probably to an increase in the expression of the genes when they are controlled by the IPTG-inducible promoter of the vector. These results confirmed that the *$pbgE_2$* and *$pbgE_3$* gene products are involved in the formation of the S region, which is required for serum resistance. We previously reported that deletion of *pmrA* did not abolish resistant to serum complement, and that the *wzz_{st}* and *wzz_{fepE}* mutants are more sensitive than wild-type strain but not as sensitive as the *pmrA* mutant (24,25). We here noticed that the *$pbgE_2$* and *$pbgE_3$* mutants displayed the same sensitivity patterns than *wzz_{st}* and *wzz_{fepE}*, thus we concluded that the *wzz_{fepE}*, *wzz_{st}*, *$pbgE_2$* and *$pbgE_3$* gene products have to act in concert to reach full serum resistance of wild-type strain, when the PmrAB system is activated.

As the PmrA regulator is also required during bacterial replication within macrophage (14,15), where both O-antigen and lipid A are involved, we investigated the importance of *$pbgE_2$* and *$pbgE_3$* gene products for *Salmonella* virulence. To this end, we studied the ability of the wild-type strain (14028s) and *pmrA* (EG13307), *$pbgE_2$* (MDs1102) and *$pbgE_3$* (MDs1103) mutants growing in low Mg^{2+} to infect and replicate within Raw 264.7 mouse macrophages. We observed that *$pbgE_2$* and *$pbgE_3$* mutants were less phagocytized, 45 and 35% respectively, by macrophages than wild-type and *pmrA* strains (Fig. 5B, 0 h). Moreover, the *$pbgE_2$* and *$pbgE_3$* mutants showed a decreased replication ability, 80 and 90%, with respect to the wild-type strain (Fig. 5B, 6 and 18 h). It must be noted that the replication ability of the *pmrA* mutant decreased after 18 h post-infection (Fig. 5B). To validate these results, we carried out complementation assays of *$pbgE_2$*, *$pbgE_3$* and *wzz_{st}* mutants with p*$pbgE_2$*, p*$pbgE_3$* and p*wzz_{st}* plasmids, respectively. We observed that the wild-type phagocytic and replicative capacities were restored in the three mutants (data not shown). These results suggest that *$pbgE_2$ and $pbgE_3$* gene products play an important role not only in bacterial intracellular replication, as happened with Wzz_{st} and Wzz_{fepE}, but also in the ability to enter eukaryotic cells (25). These functions could be attributed to their participation in the O-antigen trimodal distribution and in the lipid A modifications.

Since *$pbgE_2$ and $pbgE_3$* mutants were unable to replicate within macrophages, we investigated whether this phenotype results from defects in the resistance to cationic peptides. To test this possibility, we determined the ability of wild-type strain and *pmrA*, *$pbgE_2$* and *$pbgE_3$* mutants to survive the antimicrobial effects of polymyxin B

and E. The bacteria were grown in low Mg^{2+} conditions and then treated with the antibiotics as described in Experimental Procedures. Strains lacking *$pbgE_2$* or *$pbgE_3$* exhibited less resistance than the wild-type strain to both cationic peptides (Fig. 5C). However, they were more resistant than *pmrA* (Fig. 5C). These results demonstrated that the *$pbgE_2$* and *$pbgE_3$* mutants replication defects are in part due to their partial susceptibility to cationic peptides. In addition, our findings indicate that these gene products contribute to the wild-type polymyxin resistance under PmrA activation.

DISCUSSION

The O-antigen is the distal region of the Gram-negative bacteria LPS, which protects against bactericidal action of serum complement and cationic peptides (26-28). Early work was directed to the study of synthesis, composition and distribution of this LPS portion, demonstrating that the O-antigen length is important for the above mentioned protective effect and that the *S.* Typhimurium O-antigen follows a bimodal distribution pattern (4,29,48-51). In the past few years, our interest was focused on the regulatory mechanisms that control the expression of genes involved in O-antigen length (24,25). We previously established that *wzz_{fepE}* (VL O-antigen determinant) is positively regulated by the PmrAB system; whereas expression of *wzz_{st}* (L O-antigen determinant) is controlled by PmrAB and RcsCDB systems (24,25). Our findings led us to postulate that the Wzz_{st} protein may play some additional role, as occurs with the *ugd* gene product, which under RcsB activation participates in the colanic acid synthesis; but when induced by PmrA it is involved in the incorporation of L-Ara4N into lipid A (6,52-54). Previously, we investigated the above assumption for Wzz_{st} protein when only the RcsCDB system was activated, and demonstrated that the *wzz_{st}* mutation restored the precocious cell swarming behavior of an *rcsB* mutant (24,55,56). One of the aims of the present study was to examine the functions directed by Wzz_{st} when its gene is controlled by PmrAB system. We found that upon PmrA activation the L-Ara4N incorporation into specific Lipid A species is affected by the absence of *wzz_{st}*, resulting in the increase of pEtN-Lipid A species. We also found that the *wzz_{st}* mutant displays decreased levels of polymyxin E resistance as compared to the wild-type strain (data not shown). This phenotype may result from loss of the lipid A species modified by L-Ara4N, more than from the appearance of a new lipid A containing an extra pEtN group (peak at m/z 2281) (6,23). These observations were consistent with a previous report of Zhou *et al.* (57). It is important to highlight that only when PmrA is activated the regulation of *wzz_{st}* gene is required to this new function, since no changes were observed in the lipid A profile of the *rcsB* mutant as compared to that of the wild-type strain (data not shown). Our results confirm that the Wzz_{st} protein is involved in more than one function. We propose that the *wzz_{st}* gene product is required to maintain the balance between L-Ara4N and pEtN incorporation into lipid A. This probably implies an interaction with one or more proteins, forming a complex responsible for L-Ara4N and pEtN synthesis and(or) incorporation into lipid A. This would be similar to what is observed with Wzz_{st}, Wzx and Wzy in the control of L O-antigen region (4,51). Further studies to clarify this issue are currently in progress in our laboratory.

Although a bimodal distribution of *S.* Typhimurium O-antigen, controlled by Wzz_{st} and Wzz_{fepE} proteins has been proposed, we observed that in the absence of *wzz_{st}* and *wzz_{fepE}* genes an O-antigen portion of LMW was maintained in these mutants (24,25). This observation is in accordance with the reported by Holzer *et al.* (45), who demonstrated that the *S.* Typhimurium O-antigen display a trimodal distribution in the outer membrane. Taken together, our results not only confirm this O-antigen distribution but also clearly establish that the S region is under control of the *$pbgE_2$* and *$pbgE_3$* gene products. In agreement with our findings, Bennett and Clarke (44) reported that *$pbgE_2$* and *$pbgE_3$* participate in O-antigen synthesis in *Photorhabdus luminescens*, a Gram-negative bacterium pathogenic for insect larvae.

We demonstrated that there are regions in $PbgE_2$ and $PbgE_3$ which display similarity to the '$PX_2PX_4SPKX_1X_{10}GGMXGAG$' domain present in Wzz_{st} and Wzz_{fepE} proteins, required for functional control of VL and L O-antigen (30). We observed that in $PbgE_2$ the first part of this domain is highly conserved, while the second part is the one conserved in the $PbgE_3$ protein. These results, and the observation that both proteins are shorter than Wzz_{st} and Wzz_{fepE}, suggest that the complete functional '$PX_2PX_4SPKX_1X_{10}GGMXGAG$' domain might be formed only by interaction of $PbgE_2$ and $PbgE_3$ to control the S O-antigen formation. This hypothesis was confirmed by the *in vivo* protein-protein interaction assay (Fig. 4).

On the other hand, the physiological importance of the *$pbgE_2$* and *$pbgE_3$* genes in O-antigen length distribution was analyzed by the fact that they confer resistance to complement-mediated killing. We here established that deletion of *$pbgE_2$* and *$pbgE_3$* genes decreased the serum-complement resistance levels, as occurs with the *wzz_{st}* mutant, suggesting that the L and S O-antigen regions act more effectively than the VL region (Fig. 5A). Furthermore, we demonstrated that in *$pbgE_2$* and *$pbgE_3$* mutants both the susceptibility to phagocytosis and the ability to replicate within the host were reduced by several orders of magnitude relative to the wild-type strain (Fig. 5B). Interestingly, this reduction was even more marked than in the *pmrA* mutant (Fig. 5B). In addition, the *$pbgE_2$* and *$pbgE_3$* mutants were less resistant to polymyxin B and E than wild-type strain, but more resistant than the *pmrA* mutant. However, these results contradict the previous findings by Gunn *et al.* (14), who observed that *$pbgE_3$* strain was as sensitive to polymyxin B as the *pmrA* mutant. Also, these authors found that loss of *$pbgE_2$* left unchanged the wild-type resistance to this antibiotic. These discrepancies may be due to different experimental conditions used, mainly in the culture media. Collectively, our results suggest that the *$pbgE_2$* and *$pbgE_3$* mutations could lead to an attenuated virulence of *Salmonella*. Similar observations have been made by Bennett and Clarke (44) in *P. luminescens*.

In summary, in this work we have identified a novel function for Wzz_{st} in the lipid A remodelling through L-Ara4N and pEtN incorporation. Remarkably, the *pbgPE* operon products are also involved in this process. Furthemore, we here established that the *$pbgE_2$* and *$pbgE_3$* gene products, as well as Wzz_{st}, participate in the synthesis of the O-antigen, when they are under condition in which PmrA is activated. These findings support the working hypothesis that guided this study and underscore the importance of the PmrAB system in the LPS modifications that contributes to bacterial adaptation within the host. A challenge for future investigations will be the elucidation of the molecular mechanism involved in the balanced incorporation of the L-Ara4N and pEtN into lipid A, which allow bacteria to survive adverse conditions.

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FIGURE LEGENDS

FIGURE 1. Analysis of the lipid A profiles from *wzz_{st}* mutant. The lipid A obtained from wild-type (14028s) **(A)**, *pmrA* (EG13307) **(B)** and *wzz_{st}* (EG14929) **(C)** strains, grown to logarithmic phase in N-minimal medium with low Mg^{2+}, were analyzed by Negative-ion-mode MALDI-TOF mass spectrometry. These profiles show that the *wzz_{st}* mutant lacks diphosphorylated lipid A bearing L-Ara4N (*m/z* 1928); L-Ara4N and palmitic acid (*m/z* 2166), and pEtN and palmitic acid (*m/z* 2158) species. However, this mutant harbors a new lipid A species of *m/z* 2281. **(D)** *S.* Typhimurium lipid A modifications associated with PhoPQ and PmrAB activation, through *pagP*, and *pmrC* and *wzz_{st}* genes regulation, respectively (6,8-10,58). The PagP protein catalyzes the palmitic acid transfer to lipid A (9,10), while PmrC is required for modification of lipid A with pEtN, resulting in a lipid A species of *m/z* 2158 (left graph). In the absence of *wzz_{st}*, a new pEtN group (≅123 average mass units) could be added at 1 or 4' positions of the diphosphorylated *m/z* 2158-lipid A, leading to the *m/z* 2281 species formation (right graph, boxed group).

FIGURE 2. The formation of *m/z* 2281 lipid A species requires *pmrC* and *pagP*. Negative-ion-mode MALDI-TOF mass spectrometry analysis of lipid A obtained from *pmrC* (EG13633) **(A)**, *wzz_{st} pmrC* (MDs1015) **(B)**, *pagP* (EG13678) **(C)** and *wzz_{st} pagP* (MDs1016) **(D)** mutants, grown in low Mg^{2+} conditions to induce PmrAB system in a PhoPQ-dependent manner.

FIGURE 3. Role of *$pbgE_2$* and *$pbgE_3$* genes in the O-antigen length distribution. (A) Graphic representation of *pbgPE* operon, harboring seven genes required for L-Ara4N incorporation into lipid A. **(B)** Production of VL-, L- and S-type O-antigen in response to PmrAB system inducing conditions: the SDS-PAGE analysis of LPS was performed using samples isolated from ≈1 x 10^8 cells of wild-type (14028s), *wzz_{fepE}* (MDs1443), *wzz_{st}* (EG14929), *$pbgE_2$* (MDs1102) and *$pbgE_3$* (MDs1103) strains, grown in low Mg^{2+} (left picture), or from *$pbgE_2$* and *$pbgE_3$* mutants carrying p*$pbgE_2$* (*$pbgE_2{}^+$*) and p*$pbgE_3$* (*$pbgE_3{}^+$*) plasmids, respectively, grown in low Mg^{2+} and 0.5 mM IPTG (right picture). The VL-, L- and S-type O-antigen, and the number of O-subunits attached to the lipid A-core are indicated on the left. **(C)** Alignment of the '$PX_2PX_4SPKX_1X_{10}GGMXGAG$' domain conserved in $PbgE_2$ (filled line box) and $PbgE_3$ (dotted line box) proteins with the reported Wzz_{st} functional sequence domain of *S.* Typhimurium.

FIGURE 4. Analysis of *S.* Typhimurium $PbgE_2$ and $PbgE_3$ protein-protein interaction. The BACTH system was used to determine interaction between $PbgE_2$, $PbgE_3$ and Wzz_{st} proteins. The ß-galactosidase activity expressed by *E. coli* DHM1 strain co-transformed with plasmids harboring the *$pbgE_2$*, *$pbgE_3$* or *wzz_{st}* genes fused to complementary T18 or T25 fragments was measured in bacteria grown to stationary phase in LB medium containing 0.5 mM IPTG. The *E. coli* DHM1 strain transformed with pKT25-zip and pUT18C-zip plasmids containing the leucine zippers GCN4 domain was used as positive control (+), while the strain harboring empty vectors serves as negative control (-). Data correspond to mean values of three independent experiments performed in duplicate. Error bars correspond to the standard deviation.

FIGURE 5. Functional role of *$pbgE_2$* and *$pbgE_3$* genes. (A) Resistance to complement-mediated killing: the wild-type (14028s), *wzz_{st}* (EG14929), *$pbgE_2$* (MDs1102) and *$pbgE_3$* (MDs1103) strains grown in low Mg^{2+} were exposed to 20% human serum for 1 h and viable counts (black bars) were determined as described in Experimental Procedures. The grey bars represent the levels of serum complement resistance

of the *wzz_{st}*, *$pbgE_2$* and *$pbgE_3$* mutants when they were complemented by p*wzz_{st}*, p*$pbgE_2$* or p*$pbgE_3$* plasmids and its expression was induced by addition of 0.5 mM IPTG to low Mg^{2+}medium. **(B)** Effect of *$pbgE_2$*, *$pbgE_3$* and *wzz_{st}* deletion on the infection ability of *S.* Typhimurium: susceptibility to phagocytosis and replication ability within Raw 264.7 mouse macrophages of the parental (14028s) strain (black bars) and *pmrA* (EG13307) (grey bars), *$pbgE_2$* (MDs1102) (striped bars) and *$pbgE_3$* (MDs1103) (white bars) mutants were determined at 0 or 6 and 18 h post-infection, respectively. **(C)** Susceptibility of the O-antigen mutants to cationic peptides: antimicrobial peptide susceptibility was assayed by 1 h exposition of parental (14028s), *pmrA* (EG13307), *$pbgE_2$* (MDs1102) and *$pbgE_3$* (MDs1103) strains to 10 µg of polymyxin E (black bars) or polymyxin B peptides (grey bars). The number of surviving bacteria was represented as described in Experimental Procedures. Data correspond to mean values of three independent experiments performed in duplicate. Error bars correspond to the standard deviation.

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

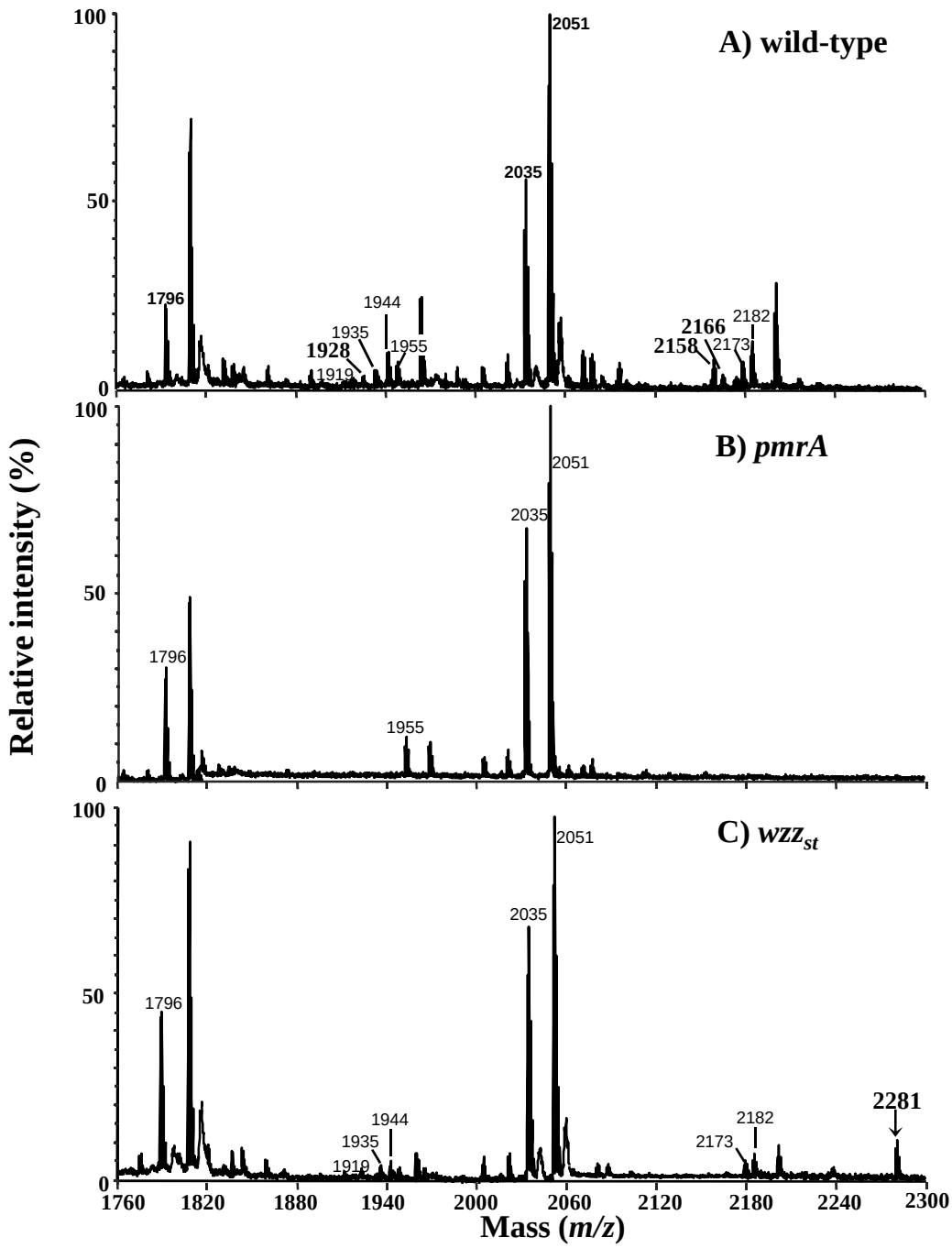
Strain	Description[a]	Reference or source
S. enterica serovar Typhimurium		
14028s	wild-type	Fields *et al.* (1986)
EG13307	Δ*pmrA*::Cm	Mouslim and Groisman (2003)
EG14929	Δ*wzz$_{st}$*::Cm	Delgado *et al.* (2006)
EG13633	Δ*pmrC*::Cm	Lee *et al.* (2004)
EG13678	Δ*pagP*::Cm	Shi *et al.* (2004)
MDs1443	Δ*wzz$_{fepE}$*::Cm	Pescaretti, *et al.* (2011)
MDs1015	Δ*wzz$_{st}$* Δ*pmrC*::Cm	This work
MDs1016	Δ*wzz$_{st}$* Δ*pagP*::Cm	This work
MDs1102	Δ*pbgE$_2$*::Cm	This work
MDs1103	Δ*pbgE$_3$*::Cm	This work
Escherichia coli		
DHMI	*F-, cya-854, recA1, endA1, gyrA96* (Nal^r), *thi1, hsdR17, spoT1, rfbD1, glnV44* (AS)	Karimova *et al.* (1998)
Plasmids		
pUHE2-2*lacI^q*	rep$_{pMB1}$ Ap^r *lacI^q*	Soncini *et al.* (1995)
p*pbgE$_2$*	pUHE2-2*lacI^q* containing *pbgE$_2$*	This work
p*pbgE$_3$*	pUHE2-2*lacI^q* containing *pbgE$_3$*	This work
p*wzz$_{st}$*	pUHE2-2*lacI^q* containing *wzz$_{st}$*	This work
pUT18C	Ap^r, Col E1 *ori*, vector for fusion to C-terminus of Cya-T18	Karimova *et al.* (1998)
pKT25	Km^r, p15A *ori*, vector for fusion to C-terminus of Cya-T25	Karimova *et al.* (1998)
pUT18C-Zip	pUT18 with the leucine zipper domain of the yeast GCN4 activator	Karimova *et al.* (1998)
pKT25-Zip	pKT25 containing leucine zipper domain of the yeast GCN4 activator	Karimova *et al.* (1998)
pKD3	*bla* FRT *cat* FRT PS1 PS2 oriR6K	Datsenko and Wanner (2000)
pKD46	*bla* P$_{BAD}$ *gam bet exo* pSC101 oriTS	Datsenko and Wanner (2000)
pT25-*pbgE$_2$*	pKT25 containing *pbgE$_2$*	This work
pT25-*pbgE$_3$*	pKT25 containing *pbgE$_3$*	This work
pT25-*wzz$_{st}$*	pKT25 containing *wzz$_{st}$*	This work
pT18C-*pbgE$_2$*	pUT18C containing *pbgE$_2$*	This work
pT18C-*pbgE$_3$*	pUT18C containing *pbgE$_3$*	This work
pT18C-*wzz$_{st}$*	pUT18C containing *wzz$_{st}$*	This work

a. Gene designations are summarized by Sanderson *et al.* (1995) (59). Nal^r, nalidixic acid resistance; Ap^r, ampicillin resistance; Km^r, kanamycin resistance.

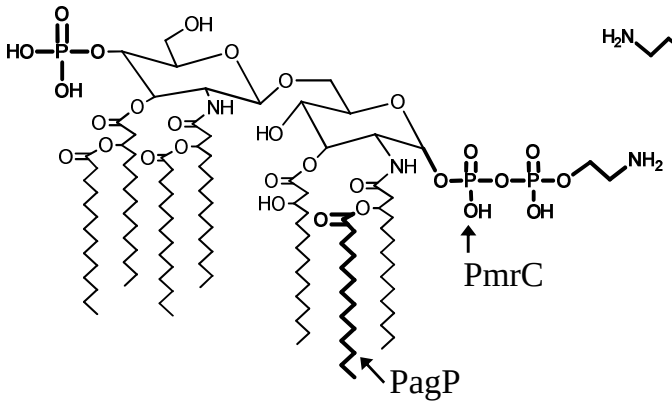
Table 2: Primers used in this work.

(#)	Name	5` to 3` sequence
1004	Fwd *$pbgE_2$*	TCT AGA ATG ATC GGC GTC GTT CTG
1001	Rev *$pbgE_3$*	TCT AGA TCA TGA TCT GGC GGG CAG
1002	Fwd *$pbgE_3$*	TCT AGA ATG GGC GTA ATG TGG GGA
1003	Rev *$pbgE_2$*	TCT AGA TTA TGC CGC ACT CCC CAG A
7008	Fwd Δ*$pbgE_2$*Cm	TGT AGG CTG GAG CTG CTT CGA TTA TCA GGG GCG TCT GGT GTT AAT TCA GTA TCG GCC TAA
7009	Rev Δ*$pbgE_2$* Cm	CAT ATG AAT ATC CTC CTT AGG AGG CAA TCG CAA CGC TTA TCA GTC CCC ACA TTA CGC CCA
7010	Fwd Δ*$pbgE_3$* Cm	TGT AGG CTG GAG CTG CTT CGT GAT TAT CAG CGG CAT TAT CAT TCT GGG GAG TGC GGC ATA
7011	Rev Δ*$pbgE_3$* Cm	CAT ATG AAT ATC CTC CTT AGT ATT CCT GCG ACG AAT GGC AGC GGA CGC GCA AGC CA
8040	BACTH-Fwd *$pbgE_2$*	CTA GTC TAG ACA TGA TCG GCG TCG TTC TGG TGC TTG CC
8041	BACTH-Fwd *$pbgE_3$*	CTA GTC TAG ACA TGG GCG TAA TGT GGG GAC TGA TAA GCG
8042	BACTH-Rev *$pbgE_3$*	CGG GGT ACC GGT GAT CTG GCG GGC AGA AAA ATC AGC
8043	BACTH-Rev *$pbgE_2$*	CGG GGT ACC GGT GCC GCA CTC CCC AGA ATG ATA ATG CCG C
8044	BACTH-Fwd *wzz_{st}*	CGC GGA TCC CAT GAC AGT GGA TAG TAA TAC GTC TTC CGG GCG TGG G
8045	BACTH-Rev *wzz_{st}*	CGG GGT ACC GGC AAG GCT TTT GGC TTA TAG CTA CGT AGC GCA TTG CGT CCC

Figure 1



D) Wild-type (Monoisotopic Mass: 2157)



***wzz**$_{st}$* (Monoisotopic Mass: 2281)

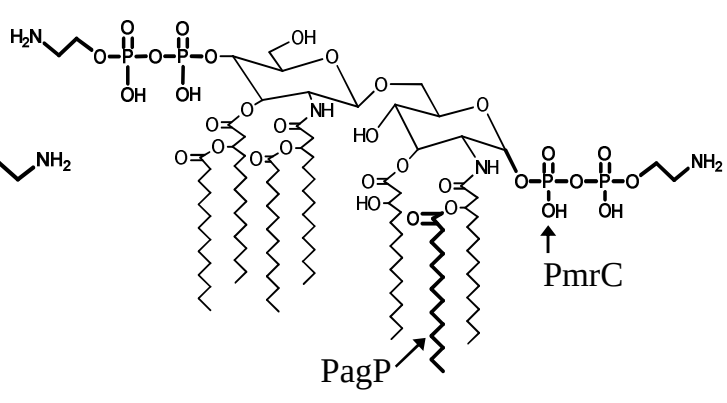


Figure 2

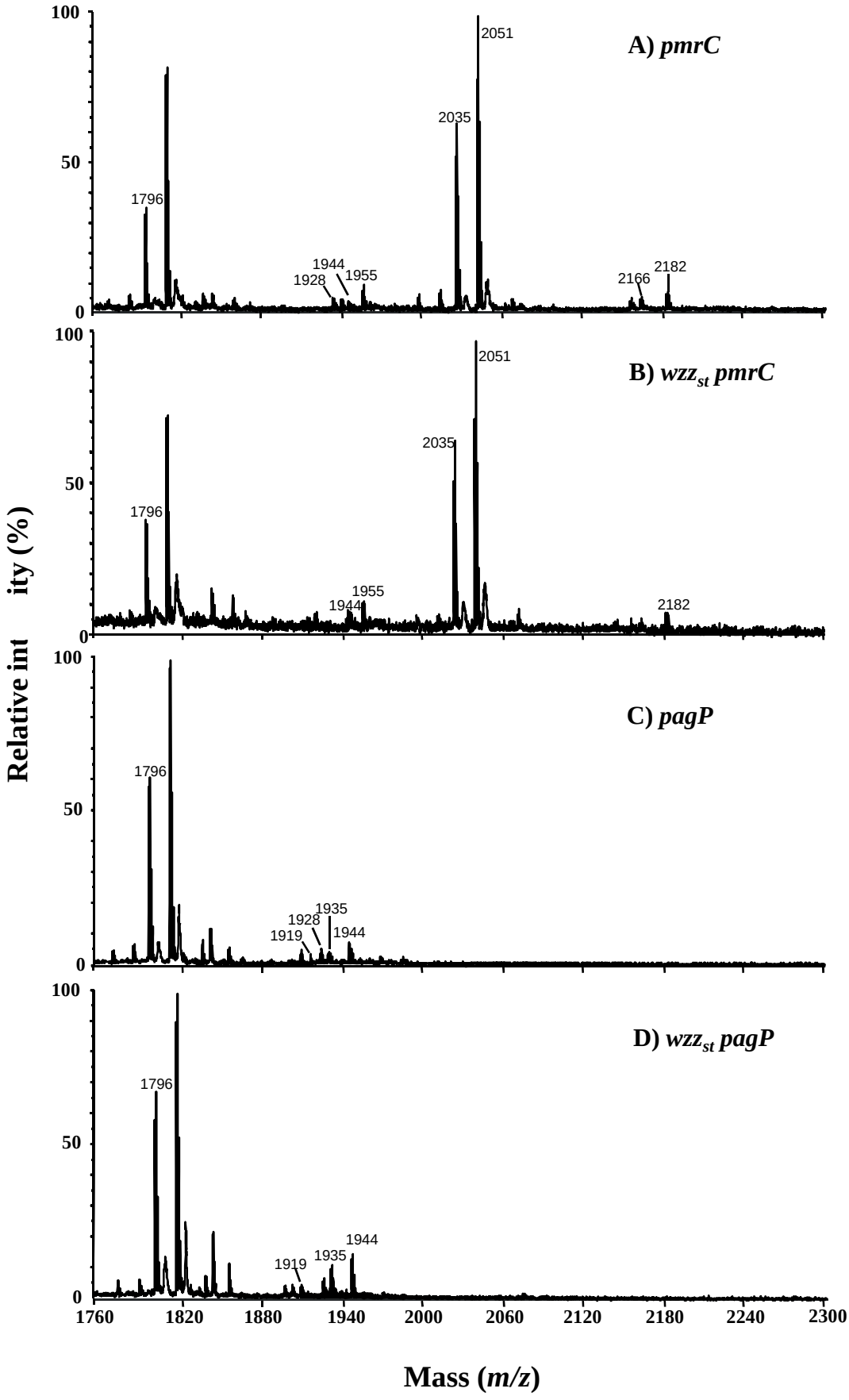
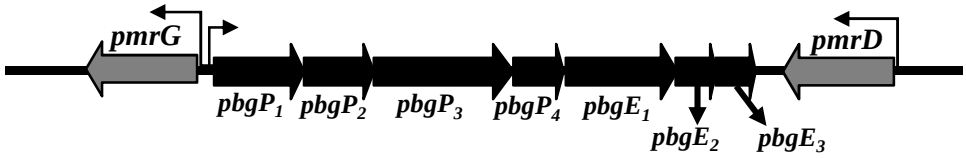
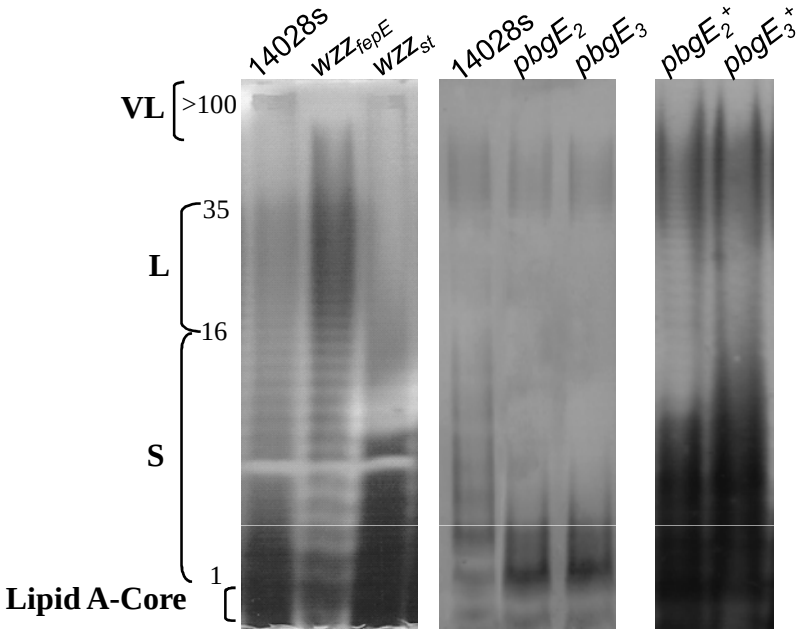


Figure 3

A)



B)



C)

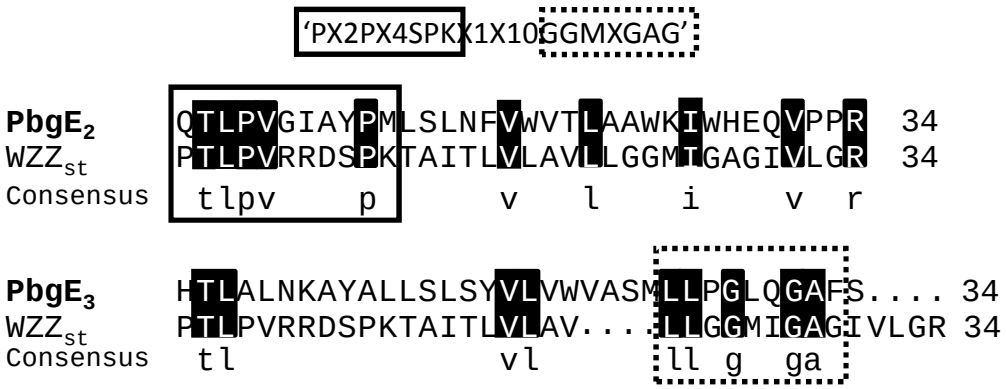


Figure 4

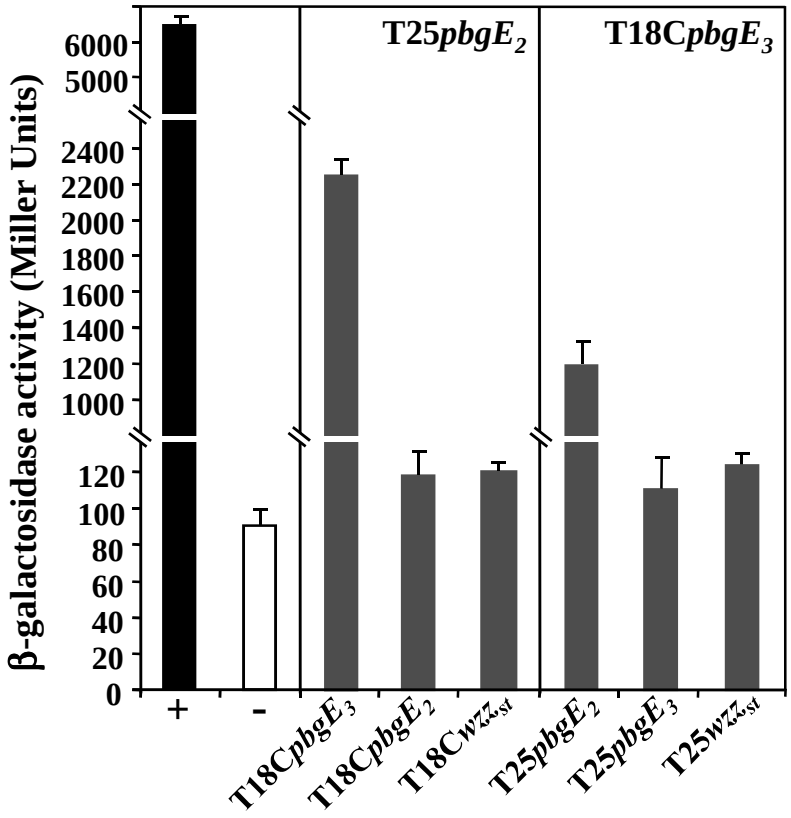
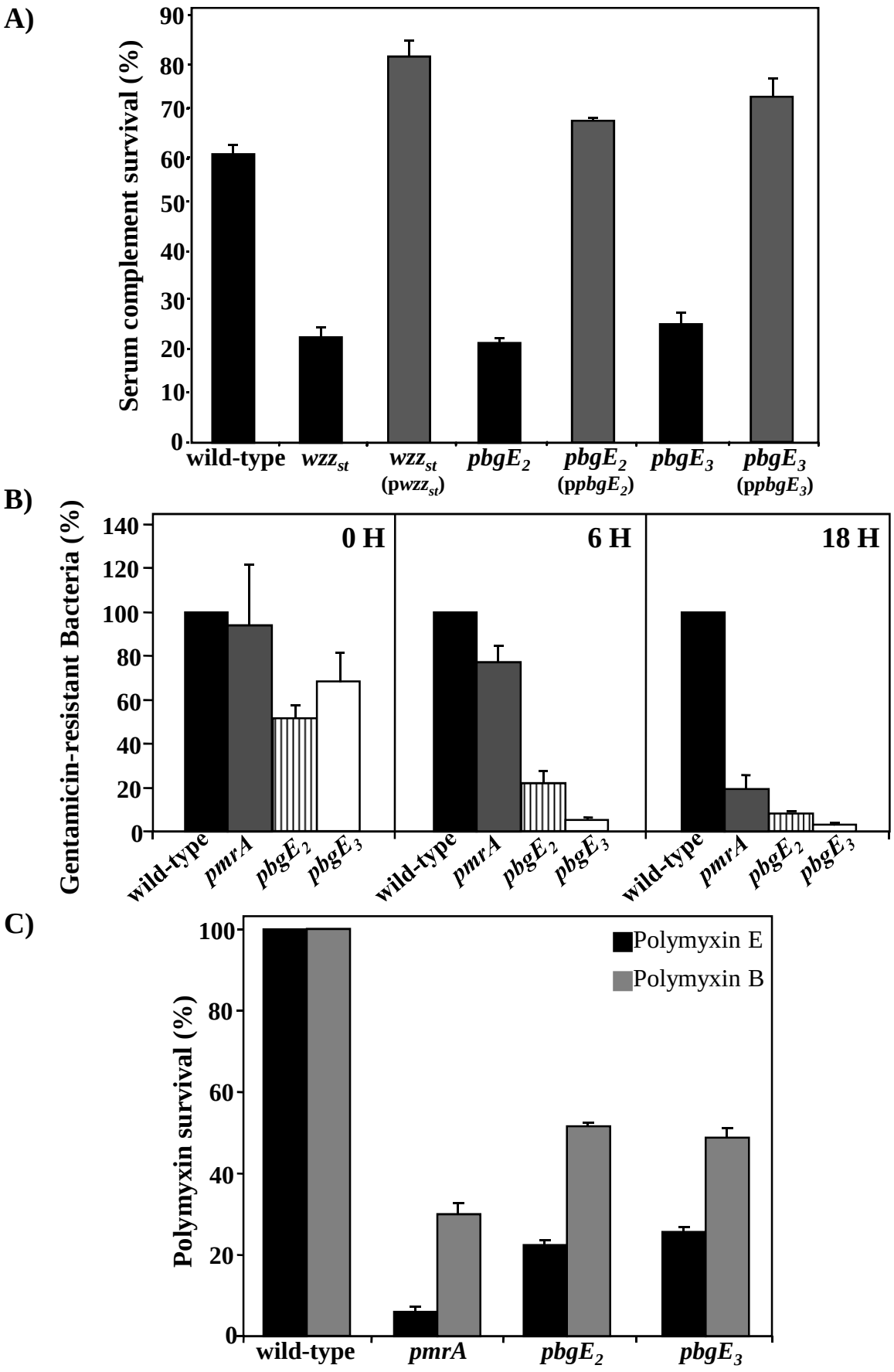


Figure 5



The PmrAB-inducing conditions control both lipid A remodelling and O-antigen length distribution, influencing the *Salmonella* Typhimurium-host interactions

Juan V. Farizano, Maria de las Mercedes Pescaretti, Fabian E. Lopez, Fong-Fu Hsu and Monica A. Delgado

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