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The PmrAB System-inducing Conditions Control Both Lipid A Remodeling and O-antigen Length Distribution, Influencing the *Salmonella* Typhimurium-Host Interactions*

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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, which influences host interaction.

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 the host immunity barrier. The aim of this study was to define the role and interaction of PmrAB-dependent gene products required for the lipopolysaccharide component synthesis or modification mainly during the *Salmonella* infection. The PmrAB two-component system activation promotes a remodeling of lipid A and the 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 is induced, and their products are required to determine the O-antigen chain length. Here we 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.

Like other Gram-negative bacteria, the lipopolysaccharide (LPS) in *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium)[4] is the major surface constituent located in the outer leaflet of the 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, whereas the O-antigen is a polymer of three to six sugar repeat units extended out from the cell surface (3, 4).

To survive microenvironmental changes, pathogenic bacteria are able to remodel their outer membrane, mainly at the level of lipid A and O-antigen. The remodeling 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 such as 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, which is responsible for addition of

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[4] The abbreviations used are: *S.* Typhimurium, *Salmonella enterica* serovar Typhimurium; L-Ara4N, 4-aminoarabinose; pEtN, phosphoethanolamine; S, short; L, long; VL, very long; Bis-Tris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol; Cm, chloramphenicol; IPTG, isopropyl 1-thio-β-D-galactopyranoside; BACTH, bacterial adenylate cyclase two-hybrid.

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 the PmrAB system 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 16–35 subunits (L-type) (4, 29), and the Wzz_{fepE} protein, which is responsible for the very long O-antigen containing >100 subunits (VL-type) (30).

We reported previously 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. 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 low molecular weight region (1–15 subunits) and that the interaction of both gene products is necessary to control its formation. The latter allows us to propose that in *S.* Typhimurium the O-antigen subunits are distributed in the following regions: (i) low molecular weight 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}*. Here we conclude that the PmrAB system is the master regulator of the LPS remodeling 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 described previously (31). Bacteria were grown at 37 °C in Luria-Bertani (LB) (32) or in N-minimal medium (0.5 M Trizma (Tris 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, 50 μg/ml; ampicillin, 50 μg/ml; and polymyxins B and E, 10 μg/ml.

Introduction of Gene Mutations in the Salmonella Chromosome and Plasmid Construction—The one-step gene inactivation method (34) was used to generate deletion of the *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 XbaI restriction enzyme, resulting in derivative plasmids listed in Table 1. The correct orientation of the insert 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 an A_{600} of 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 cells/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 were 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).

TABLE 1
Bacterial strains and plasmids used in this study

	Description[a]	Ref. or source
Strains		
S. enterica serovar Typhimurium		
14028s	Wild type	60
EG13307	Δ*pmrA*::Cm	53
EG14929	Δwzz_{st}::Cm	24
EG13633	Δ*pmrC*::Cm	8
EG13678	Δ*pagP*::Cm	17
MDs1443	Δwzz_{fepE}::Cm	25
MDs1015	Δwzz_{st} Δ*pmrC*::Cm	This work
MDs1016	Δwzz_{st} Δ*pagP*::Cm	This work
MDs1102	Δ$pbgE_2$::Cm	This work
MDs1103	Δ$pbgE_3$::Cm	This work
E. coli		
DHM1	*F*−, *cya-854*, *recA1*, *endA1*, *gyrA96* (Nal^r), *thi1*, *hsdR17*, *spoT1*, *rfbD1*, *glnV44* (AS)	37
Plasmids		
pUHE2–2*lacI*^{q}	rep$_{pMB1}$ Ap^r *lacI*^{q}	61
p$pbgE_2$	pUHE2–2*lacI*^{q} containing $pbgE_2$	This work
p$pbgE_3$	pUHE2–2*lacI*^{q} containing $pbgE_3$	This work
pwzz_{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	37
pKT25	Km^r, p15A *ori*, vector for fusion to C terminus of Cya-T25	37
pUT18C-Zip	pUT18 with the leucine zipper domain of the yeast GCN4 activator	37
pKT25-Zip	pKT25 containing leucine zipper domain of the yeast GCN4 activator	37
pKD3	*bla* FRT *cat* FRT PS1 PS2 oriR6K	34
pKD46	*bla* P$_{BAD}$ *gam bet exo* pSC101 oriTS	34
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.* (59). Nal^r, nalidixic acid resistance; Ap^r, ampicillin resistance; Km^r, kanamycin resistance.

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 an A_{600} of 0.3–0.4. 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, cfu were 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 KpnI/XbaI restriction enzymes, whereas the wzz_{st} PCR product was cloned using KpnI/BamHI. The plasmid derivatives were controlled by DNA sequencing and co-transformed in all compatible combinations into *Escherichia coli* DHM1 strain. To determine the potential protein interactions, β-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 zipper GCN4 domain, whereas the strain harboring empty vectors served as the negative control. β-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 sample 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 the 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 the wild-type 14028s strain and its 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),

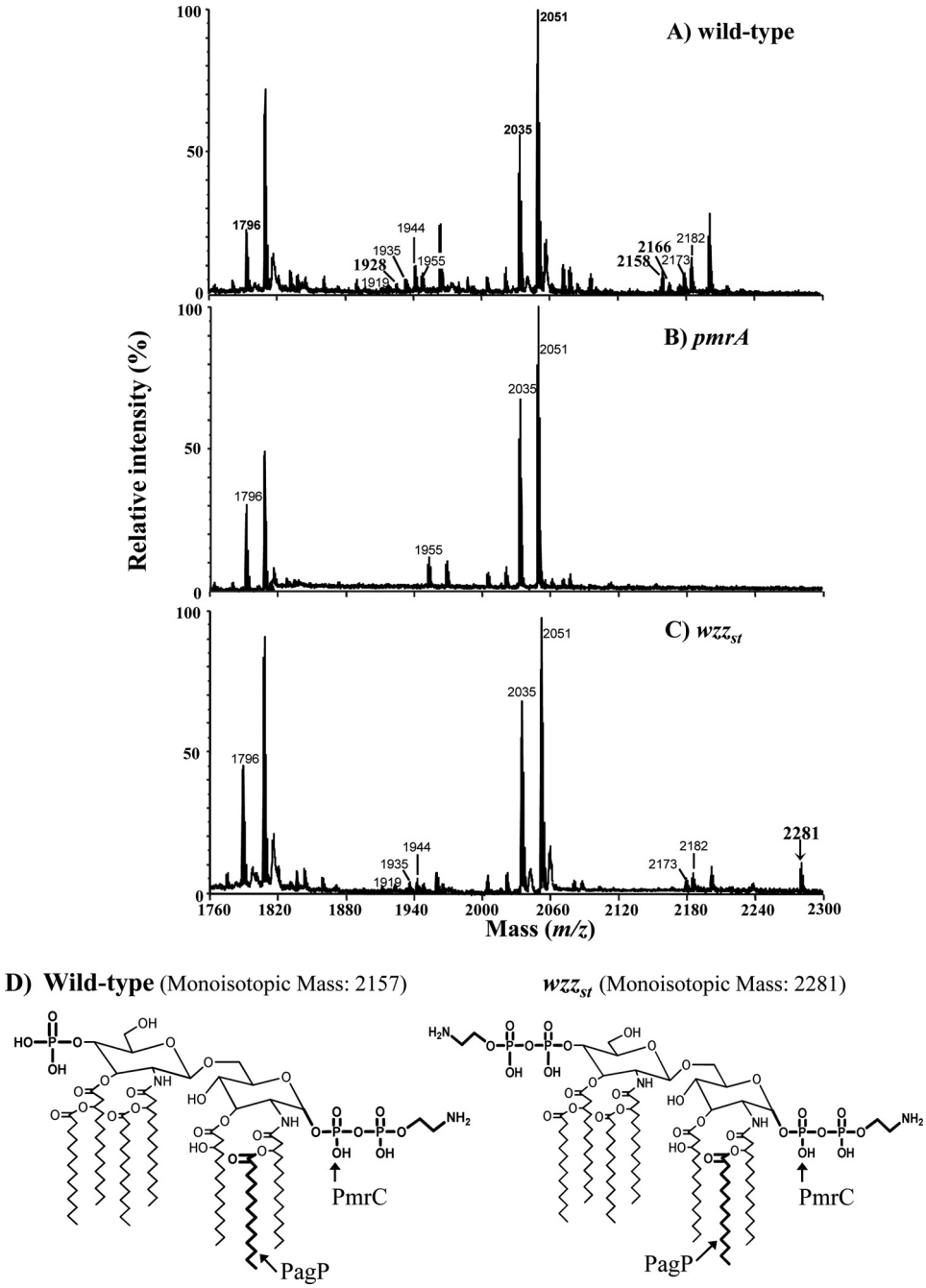

FIGURE 1. **Analysis of the lipid A profiles from wzz_{st} mutant.** The lipid A obtained from wild-type (14028s) (*A*), *pmrA* (EG13307) (*B*), and wzz_{st} (EG14929) (*C*) strains grown to logarithmic phase in N-minimal medium with low Mg^{2+} were analyzed by negative ion mode MALDI-TOF mass spectrometry. 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*, *pmrC*, and wzz_{st} gene regulation, respectively (6, 8–10, 58). The PagP protein catalyzes the palmitic acid transfer to lipid A (9, 10), whereas PmrC is required for modification of lipid A with pEtN, resulting in a lipid A species of *m/z* 2158 (*left*). In the absence of wzz_{st}, a new pEtN group (≅123 average mass units) could be added at the 1- or 4′-position of the diphosphorylated *m/z* 2158 lipid A, leading to the *m/z* 2281 species formation (*right*).

under this assay condition, the wild-type strain (Fig. 1*A*) 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. 1*B*) where the prototype lipid A (*m/z* 1796) was modified only by palmitic acid addition (*m/z* 1955, 2035, and 2051) because 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. 1*C*) 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, whereas the species at m/z 2158 was absent (Fig. 1*C*). 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 species 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. 1*D*) (8, 9, 40). As the chemical structures for most of the lipid A species in *S. enterica* have been determined previously (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 Fig. 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. 2, *A* 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 species 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, as in wzz_{st}, species harboring L-Ara4N (m/z 1928 and 2166) were also absent (Fig. 2, *B* and *D*). Taken together, our results suggest that the 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—Because the above results demonstrated that wzz_{st} is involved in lipid A modification and we previously observed that an O-antigen of low molecular weight 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. 3*A*) (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 low molecular weight as compared with that from wild-type, wzz_{st}, or wzz_{fepE} strains (Fig. 3*B*). 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. 3*B*). 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 Hölzer *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 low molecular weight region named the S region (1–15 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_4SPK X_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 of both proteins, was found to be essential for their function (46, 47). It was of interest to investigate whether this domain is also present in $PbgE_2$ and $PbgE_3$. The bioinformatics analysis of $PbgE_2$ and $PbgE_3$ sequences, carried out using the Transmembrane Prediction Server (Stockholm University, Sweden), suggested that both are inner membrane proteins bearing four transmembrane domains 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 transmembrane domains comprising residues 49–92 from $PbgE_2$ and residues 70–107 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. 3*C*, *filled line box*), whereas the second portion, the "GGMXGAG" motif, was conserved in $PbgE_3$ (Fig. 3*C*, *dotted line box*). Because 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, 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 XbaI and KpnI restriction sites (Table 2). To investigate interactions with $PbgE_2$ and $PbgE_3$, we also cloned the wzz_{st} gene with primers containing BamHI and KpnI 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. The *E. coli* DHM1 strain co-transformed with empty vectors, which was used as a negative control, showed basal levels of β-galactosidase activity (~100 Miller units). A positive control generated by using pKT25-zip and pUT18C-zip plasmids harboring the leucine zipper GCN4 domain displayed high levels of β-galactosidase activity (≅6000 Miller units). We observed an increase in β-galactosidase activity compared with the negative control when the $pbgE_2$ and

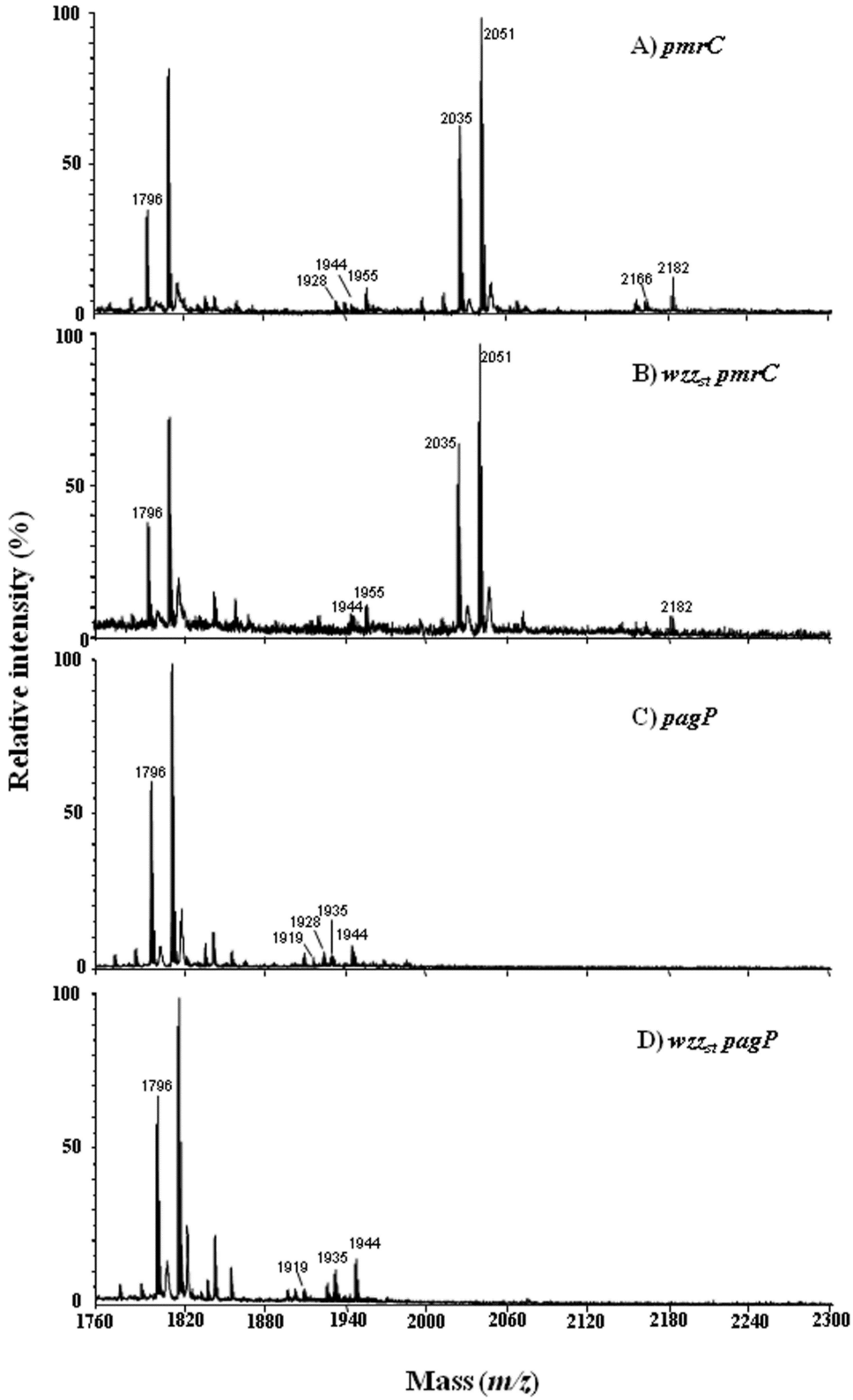

FIGURE 2. **The formation of *m/z* 2281 lipid A species requires *pmrC* and *pagP*.** Shown is negative ion mode MALDI-TOF mass spectrometry analysis of lipid A obtained from *pmrC* (EG13633) (*A*), wzz_{st} *pmrC* (MDs1015) (*B*), *pagP* (EG13678) (*C*), and wzz_{st} *pagP* (MDs1016) (*D*) mutants grown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

$pbgE_3$ 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 $PbgE_2$ and $PbgE_3$ are not self-interacting proteins because in the *E. coli* DHM1 strain

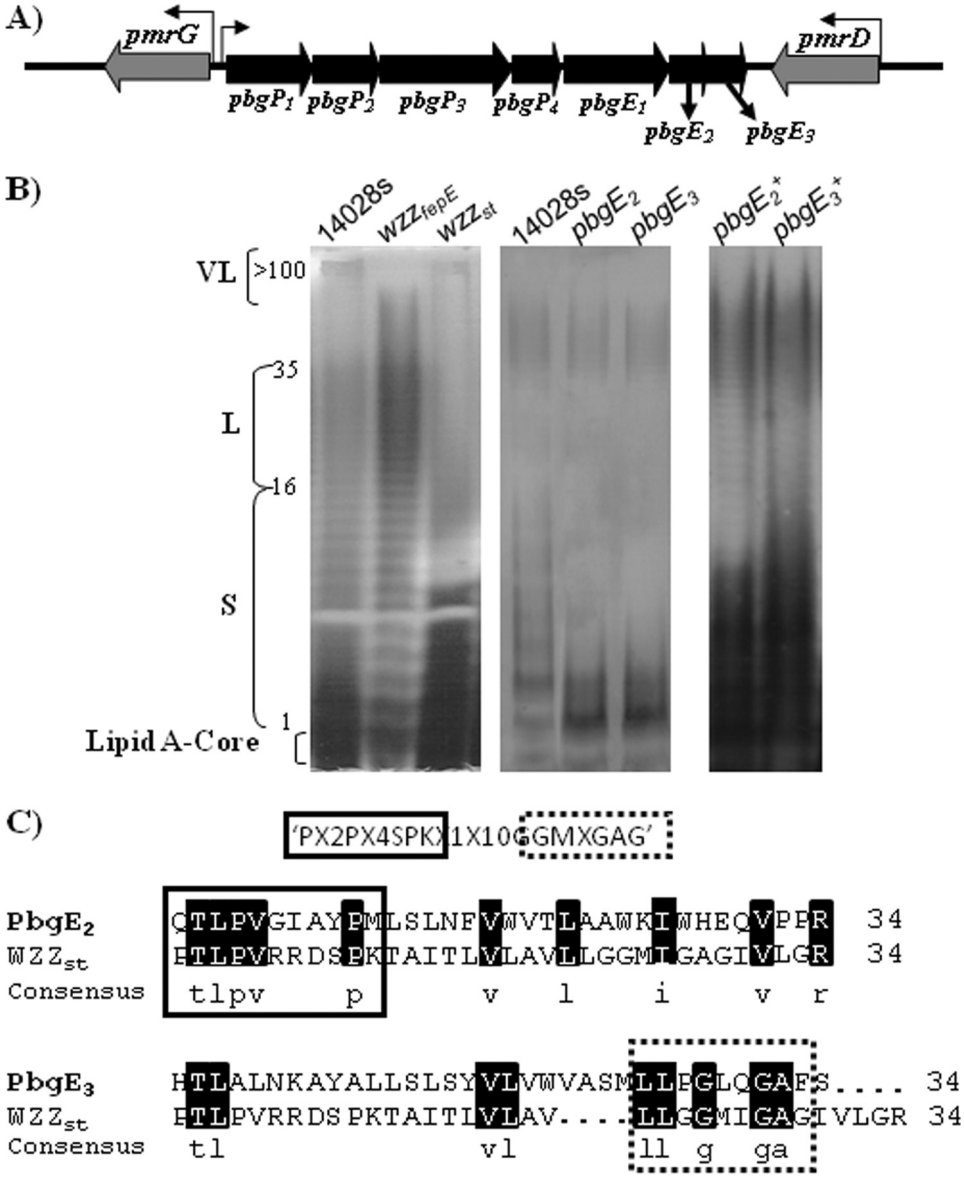

FIGURE 3. **Role of $pbgE_2$ and $pbgE_3$ genes in the O-antigen length distribution.** *A*, graphic representation of the *pbgPE* operon harboring seven genes required for L-Ara4N incorporation into lipid A. *B*, production of VL-, L-, and S-type O-antigen in response to PmrAB system-inducing conditions. The SDS-PAGE analysis of LPS was performed using samples isolated from $\approx 1 \times 10^8$ 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* and *middle panels*) 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 panel*). 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.

harboring the pT25-pbgE_2/pT18C-pbgE_2 or pT25-pbgE_3/pT18C-pbgE_3 combinations the β-galactosidase activity levels were hardly increased as compared with the 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 $PbgE_3$ protein. 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 the 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 the 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 for 1 h. As shown in Fig. 5*A*, 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% 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. 5*A*, *gray bars*). We observed that the resistance of the complemented mutants was higher than in the wild-type strain probably due 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 $pbgE_3$

TABLE 2
Primers used in this work

No.	Name[a]	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

[a] Fwd, forward; Rev, reverse.

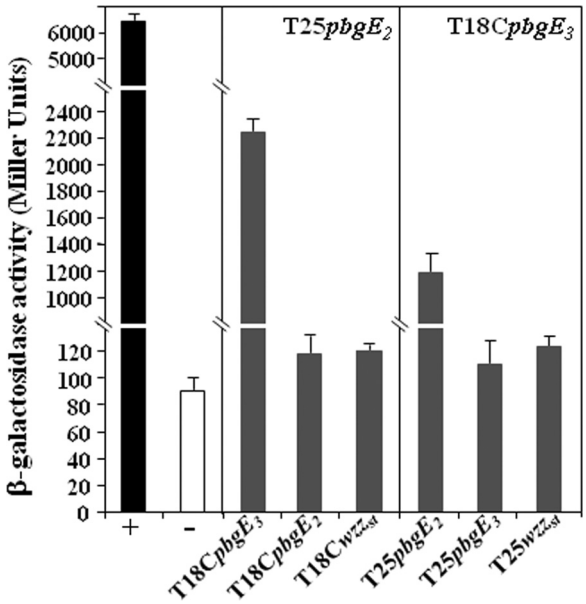

FIGURE 4. **Analysis of *S.* Typhimurium $PbgE_2$ and $PbgE_3$ protein-protein interaction.** The BACTH system was used to determine interaction among $PbgE_2$, $PbgE_3$, and Wzz_{st} proteins. The β-galactosidase activity expressed by *E. coli* DHM1 strain co-transformed with plasmids harboring the $pbgE_2$, $pbgE_3$, 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 zipper GCN4 domain was used as a positive control (+), whereas the strain harboring empty vectors serves as a negative control (−). Data correspond to mean values of three independent experiments performed in duplicate. *Error bars* correspond to the S.D.

gene products are involved in the formation of the S region, which is required for serum resistance. We reported previously that deletion of *pmrA* did not abolish resistance to serum complement and that the wzz_{st} and wzz_{fepE} mutants are more sensitive than the wild-type strain but not as sensitive as the *pmrA* mutant (24, 25). Here we noticed that the $pbgE_2$ and $pbgE_3$ mutants displayed the same sensitivity patterns as 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 the wild-type strain when the PmrAB system is activated.

As the PmrA regulator is also required during bacterial replication within macrophages (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. 5*B*, 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. 5*B*, 6 and 18 h). It must be noted that the replication ability of the *pmrA* mutant decreased 18 h postinfection (Fig. 5*B*). 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 pwzz_{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.

Because $pbgE_2$ 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 polymyxins B and E. The bacteria were grown in low Mg^{2+} conditions and then treated with the antibiotics as described under "Experimental Procedures." Strains lacking $pbgE_2$ or $pbgE_3$ exhibited less resistance than the wild-type strain to both cationic peptides (Fig. 5*C*). However, they were more resistant than *pmrA* (Fig. 5*C*). These results demonstrated that the $pbgE_2$ and $pbgE_3$ mutant 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, the distal region of the Gram-negative bacteria LPS, protects against the bactericidal action of serum complement and cationic peptides (26–28). Early work

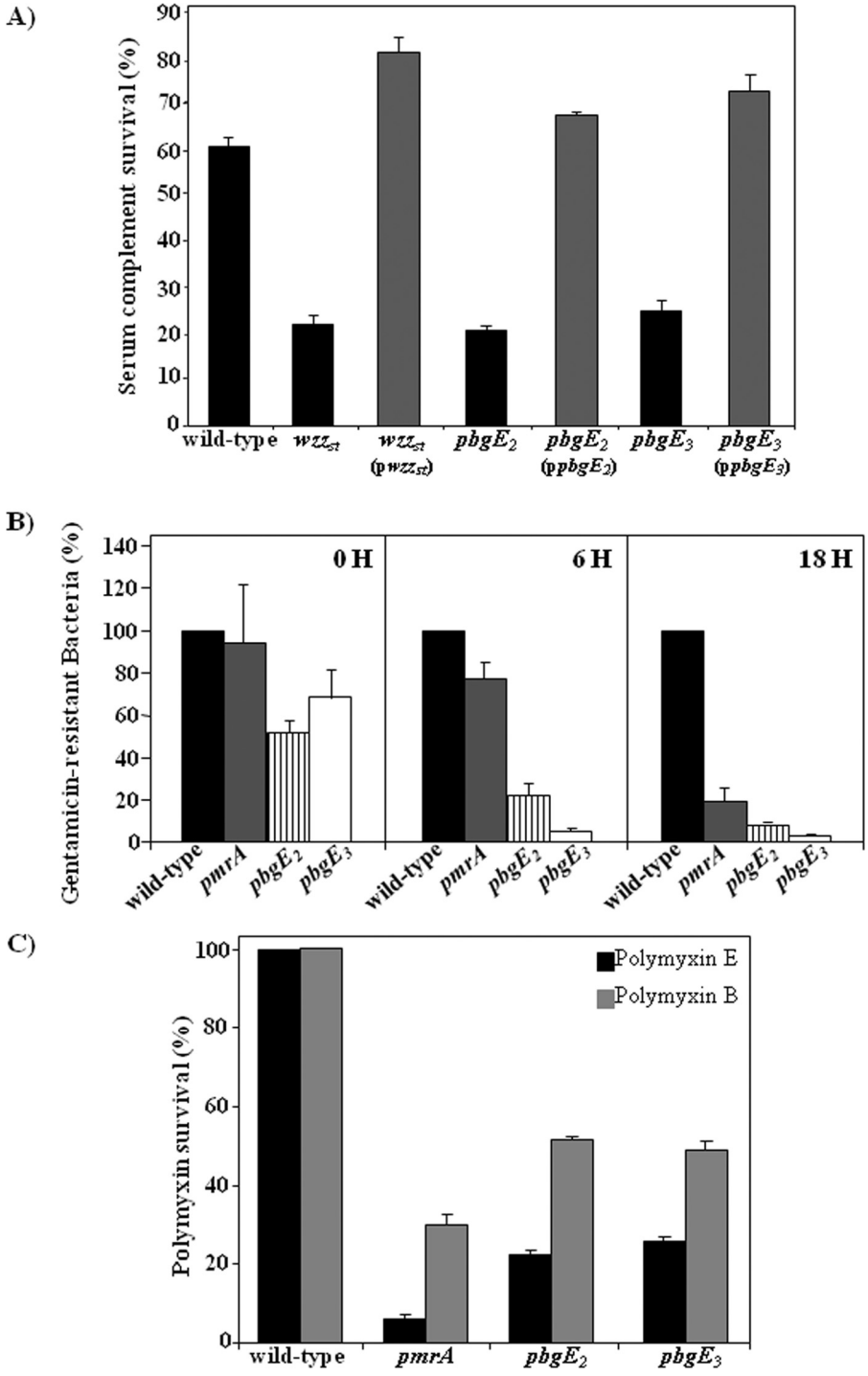

FIGURE 5. **Functional role of $pbgE_2$ and $pbgE_3$ genes.** *A*, resistance to complement-mediated killing. The wild-type (14028s), wzz_{st} (EG14929), $pbgE_2$ (MDs1102), and $pbgE_3$ (MDs1103) strains grown in low Mg^{2+} were exposed to 20% human serum for 1 h, and counts of viable bacteria (*black bars*) were determined as described under "Experimental Procedures." The *gray bars* represent the levels of serum complement resistance of the wzz_{st}, $pbgE_2$, and $pbgE_3$ mutants when they were complemented by pwzz_{st}, p$pbgE_2$, or p$pbgE_3$ plasmids and 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) (*gray bars*), $pbgE_2$ (MDs1102) (*striped bars*), and $pbgE_3$ (MDs1103) (*white bars*) mutants were determined at 0 or 6 and 18 h postinfection, 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 (*gray bars*). The number of surviving bacteria was represented as described under "Experimental Procedures." Data correspond to mean values of three independent experiments performed in duplicate. *Error bars* correspond to the S.D.

directed to the study of synthesis, composition, and distribution of this LPS portion demonstrated 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 has been 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 the 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 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 the 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 the 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 with 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 is the regulation of wzz_{st} gene required for this new function because no changes were observed in the lipid A profile of the *rcsB* mutant as compared with 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 to form 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 low molecular weight was maintained in these mutants (24, 25). This observation is in accordance with the reported by Hölzer *et al.* (45), who demonstrated that the *S.* Typhimurium O-antigen displays 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 to insect larvae.

We demonstrated that there are regions in $PbgE_2$ and $PbgE_3$ that 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, whereas the second part is 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 demonstrated by the fact that they confer resistance to complement-mediated killing. Here we 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. 5*A*). 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. 5*B*). Interestingly, this reduction was even more marked than in the *pmrA* mutant (Fig. 5*B*). In addition, the $pbgE_2$ and $pbgE_3$ mutants were less resistant to polymyxins B and E than the 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 the $pbgE_3$ 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 the different experimental conditions (mainly in the culture media) used. 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 remodeling through L-Ara4N and pEtN incorporation. Remarkably, the *pbgPE* operon products are also involved in this process. Furthermore, here we 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 conditions 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 contribute 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 allows bacteria to survive adverse conditions.

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REFERENCES

1. Brade, H., Opal, S. M., Vogel, S. N., and Morrison, D. C. (1999) *Endotoxin in Health and Disease*, pp. 31–38, Marcel Dekker, Inc., New York
2. Raetz, C. R. (1993) Bacterial endotoxins: extraordinary lipids that activate eucaryotic signal transduction. *J. Bacteriol.* **175,** 5745–5753
3. Nikaido, H. (2003) Molecular basis of bacterial outer membrane permeability revisited. *Microbiol. Mol. Biol. Rev.* **67,** 593–656

4. Raetz, C. R., and Whitfield, C. (2002) Lipopolysaccharide endotoxins. *Annu. Rev. Biochem.* **71,** 635–700
5. Groisman, E. A., Kayser, J., and Soncini, F. C. (1997) Regulation of polymyxin resistance and adaptation to low-Mg^{2+} environments. *J. Bacteriol.* **179,** 7040–7045
6. Gunn, J. S., Lim, K. B., Krueger, J., Kim, K., Guo, L., Hackett, M., and Miller, S. I. (1998) PmrA-PmrB-regulated genes necessary for 4-aminoarabinose lipid A modification and polymyxin resistance. *Mol. Microbiol.* **27,** 1171–1182
7. Helander, I. M., Kilpeläinen, I., and Vaara, M. (1994) Increased substitution of phosphate groups in lipopolysaccharides and lipid A of the polymyxin-resistant *pmrA* mutants of *Salmonella typhimurium*: a 31P-NMR study. *Mol. Microbiol.* **11,** 481–487
8. Lee, H., Hsu, F. F., Turk, J., and Groisman, E. A. (2004) The PmrA-regulated *pmrC* gene mediates phosphoethanolamine modification of lipid A and polymyxin resistance in *Salmonella enterica*. *J. Bacteriol.* **186,** 4124–4133
9. Bishop, R. E., Lo, E. I., Khan, M. A., El Zoeiby, A., and Jia, W. (2004) Enzymology of lipid A palmitoylation in bacterial outer membranes. *J. Endotoxin Res.* **10,** 107–112
10. Guo, L., Lim, K. B., Poduje, C. M., Daniel, M., Gunn, J. S., Hackett, M., and Miller, S. I. (1998) Lipid A acylation and bacterial resistance against vertebrate antimicrobial peptides. *Cell* **95,** 189–198
11. Kawasaki, K., Ernst, R. K., and Miller, S. I. (2004) Deacylation and palmitoylation of lipid A by Salmonellae outer membrane enzymes modulate host signaling through Toll-like receptor 4. *J. Endotoxin Res.* **10,** 439–444
12. Kawasaki, K., Ernst, R. K., and Miller, S. I. (2005) Purification and characterization of deacylated and/or palmitoylated lipid A species unique to *Salmonella enterica* serovar Typhimurium. *J. Endotoxin Res.* **11,** 57–61
13. Chamnongpol, S., and Groisman, E. A. (2000) Acetyl phosphate-dependent activation of a mutant PhoP response regulator that functions independently of its cognate sensor kinase. *J. Mol. Biol.* **300,** 291–305
14. Gunn, J. S., Ryan, S. S., Van Velkinburgh, J. C., Ernst, R. K., and Miller, S. I. (2000) Genetic and functional analysis of a PmrA-PmrB-regulated locus necessary for lipopolysaccharide modification, antimicrobial peptide resistance, and oral virulence of *Salmonella enterica* serovar Typhimurium. *Infect. Immun.* **68,** 6139–6146
15. Roland, K. L., Martin, L. E., Esther, C. R., and Spitznagel, J. K. (1993) Spontaneous *pmrA* mutants of *Salmonella typhimurium* LT2 define a new two-component regulatory system with a possible role in virulence. *J. Bacteriol.* **175,** 4154–4164
16. Wösten, M. M., Kox, L. F., Chamnongpol, S., Soncini, F. C., and Groisman, E. A. (2000) A signal transduction system that responds to extracellular iron. *Cell* **103,** 113–125
17. Shi, Y., Cromie, M. J., Hsu, F. F., Turk, J., and Groisman, E. A. (2004) PhoP-regulated *Salmonella* resistance to the antimicrobial peptides magainin 2 and polymyxin B. *Mol. Microbiol.* **53,** 229–241
18. Kato, A., and Groisman, E. A. (2004) Connecting two-component regulatory systems by a protein that protects a response regulator from dephosphorylation by its cognate sensor. *Genes Dev.* **18,** 2302–2313
19. Kox, L. F., Wösten, M. M., and Groisman, E. A. (2000) A small protein that mediates the activation of a two-component system by another two-component system. *EMBO J.* **19,** 1861–1872
20. Breazeale, S. D., Ribeiro, A. A., and Raetz, C. R. (2003) Origin of lipid A species modified with 4-amino-4-deoxy-L-arabinose in polymyxin-resistant mutants of *Escherichia coli.* An aminotransferase (ArnB) that generates UDP-4-deoxyl-L-arabinose. *J. Biol. Chem.* **278,** 24731–24739
21. Trent, M. S., Ribeiro, A. A., Lin, S., Cotter, R. J., and Raetz, C. R. (2001) An inner membrane enzyme in *Salmonella* and *Escherichia coli* that transfers 4-amino-4-deoxy-L-arabinose to lipid A: induction on polymyxin-resistant mutants and role of a novel lipid-linked donor. *J. Biol. Chem.* **276,** 43122–43131
22. Nishino, K., Hsu, F. F., Turk, J., Cromie, M. J., Wösten, M. M., and Groisman, E. A. (2006) Identification of the lipopolysaccharide modifications controlled by the *Salmonella* PmrA/PmrB system mediating resistance to Fe(III) and Al(III). *Mol Microbiol* **61,** 645–654
23. Tamayo, R., Choudhury, B., Septer, A., Merighi, M., Carlson, R., and Gunn, J. S. (2005) Identification of *cptA*, a PmrA-regulated locus required for phosphoethanolamine modification of the *Salmonella enterica* serovar Typhimurium lipopolysaccharide core. *J. Bacteriol.* **187,** 3391–3399
24. Delgado, M. A., Mouslim, C., and Groisman, E. A. (2006) The PmrA/PmrB and RcsC/YojN/RcsB systems control expression of the *Salmonella* O-antigen chain length determinant. *Mol. Microbiol.* **60,** 39–50
25. Pescaretti, M. M., Lopez, F. E., Morero, R. D., and Delgado, M. A. (2011) The PmrA/PmrB two component system controls the expression of wzz_{fepE} gene from *Salmonella typhimurium*. *Microbiology* **157,** 2515–2521
26. Burns, S. M., and Hull, S. I. (1998) Comparison of loss of serum resistance by defined lipopolysaccharide mutants and an acapsular mutant of uropathogenic *Escherichia coli* O75:K5. *Infect. Immun.* **66,** 4244–4253
27. Joiner, K. A. (1985) Studies on the mechanism of bacterial resistance to complement-mediated killing and on the mechanism of action of bactericidal antibody. *Curr. Top. Microbiol. Immunol.* **121,** 99–133
28. Skurnik, M., and Bengoechea, J. A. (2003) The biosynthesis and biological role of lipopolysaccharide O-antigens of pathogenic Yersiniae. *Carbohydr. Res.* **338,** 2521–2529
29. Morona, R., van den Bosch, L., and Manning, P. A. (1995) Molecular, genetic, and topological characterization of O-antigen chain length regulation in *Shigella flexneri*. *J. Bacteriol.* **177,** 1059–1068
30. Murray, G. L., Attridge, S. R., and Morona, R. (2003) Regulation of *Salmonella typhimurium* lipopolysaccharide O antigen chain length is required for virulence; identification of FepE as a second Wzz. *Mol. Microbiol.* **47,** 1395–1406
31. Davis, R. W., Bolstein, D., and Roth, J. R. (1980) *Advanced Bacterial Genetics*, Cold Spring Harbor Laboratory. Cold Spring Harbor, NY
32. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual.*, 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
33. Snavely, M. D., Gravina, S. A., Cheung, T. T., Miller, C. G., and Maguire, M. E. (1991) Magnesium transport in *Salmonella typhimurium.* Regulation of *mgtA* and *mgtB* expression. *J. Biol. Chem.* **266,** 824–829
34. Datsenko, K. A., and Wanner, B. L. (2000) One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. *Proc. Natl. Acad. Sci. U.S.A.* **97,** 6640–6645
35. Marolda, C. L., Welsh, J., Dafoe, L., and Valvano, M. A. (1990) Genetic analysis of the O7-polysaccharide biosynthesis region from the *Escherichia coli* O7:K1 strain VW187. *J. Bacteriol.* **172,** 3590–3599
36. Mouslim, C., Delgado, M., and Groisman, E. A. (2004) Activation of the RcsC/YojN/RcsB phosphorelay system attenuates *Salmonella* virulence. *Mol. Microbiol.* **54,** 386–395
37. Karimova, G., Pidoux, J., Ullmann, A., and Ladant, D. (1998) A bacterial two-hybrid system based on a reconstituted signal transduction pathway. *Proc. Natl. Acad. Sci. U.S.A.* **95,** 5752–5756
38. Miller, J. H. (1972) *Experiments in Molecular Genetics.*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
39. Soncini, F. C., and Groisman, E. A. (1996) Two-component regulatory systems can interact to process multiple environmental signals. *J. Bacteriol.* **178,** 6796–6801
40. Steinberg, D. A., Hurst, M. A., Fujii, C. A., Kung, A. H., Ho, J. F., Cheng, F. C., Loury, D. J., and Fiddes, J. C. (1997) Protegrin-1: a broad-spectrum, rapidly microbicidal peptide with *in vivo* activity. *Antimicrob. Agents Chemother.* **41,** 1738–1742
41. Guo, L., Lim, K. B., Gunn, J. S., Bainbridge, B., Darveau, R. P., Hackett, M., and Miller, S. I. (1997) Regulation of lipid A modifications by *Salmonella typhimurium* virulence genes *phoP-phoQ*. *Science* **276,** 250–253
42. Zhou, Z., Lin, S., Cotter, R. J., and Raetz, C. R. (1999) Lipid A modifications characteristic of *Salmonella typhimurium* are induced by NH_4VO_3 in *Escherichia coli* K12. Detection of 4-amino-4-deoxy-L-arabinose, phosphoethanolamine and palmitate. *J. Biol. Chem.* **274,** 18503–18514
43. Zhou, Z., Ribeiro, A. A., and Raetz, C. R. (2000) High-resolution NMR spectroscopy of lipid A molecules containing 4-amino-4-deoxy-L-arabinose and phosphoethanolamine substituents. Different attachment sites on lipid A molecules from NH_4VO_3-treated *Escherichia coli versus* kdsA mutants of *Salmonella typhimurium*. *J. Biol. Chem.* **275,** 13542–13551
44. Bennett, H. P., and Clarke, D. J. (2005) The *pbgPE* operon in *Photorhabdus luminescens* is required for pathogenicity and symbiosis. *J. Bacteriol.* **187,**

77–84
45. Hölzer, S. U., Schlumberger, M. C., Jäckel, D., and Hensel, M. (2009) Effect of the O-antigen length of lipopolysaccharide on the functions of Type III secretion systems in *Salmonella enterica*. *Infect. Immun.* **77,** 5458–5470
46. Daniels, C., and Morona, R. (1999) Analysis of *Shigella flexneri wzz* (Rol) function by mutagenesis and cross-linking: *wzz* is able to oligomerize. *Mol. Microbiol.* **34,** 181–194
47. Franco, A. V., Liu, D., and Reeves, P. R. (1998) The *wzz* (*cld*) protein in *Escherichia coli*: amino acid sequence variation determines O-antigen chain length specificity. *J. Bacteriol.* **180,** 2670–2675
48. Dasgupta, T., de Kievit, T. R., Masoud, H., Altman, E., Richards, J. C., Sadovskaya, I., Speert, D. P., and Lam, J. S. (1994) Characterization of lipopolysaccharide-deficient mutants of *Pseudomonas aeruginosa* derived from serotypes O3, O5, and O6. *Infect. Immun.* **62,** 809–817
49. Joiner, K. A., Hammer, C. H., Brown, E. J., Cole, R. J., and Frank, M. M. (1982) Studies on the mechanism of bacterial resistance to complement-mediated killing. I. Terminal complement components are deposited and released from *Salmonella minnesota* S218 without causing bacterial death. *J. Exp. Med.* **155,** 797–808
50. Murray, G. L., Attridge, S. R., and Morona, R. (2005) Inducible serum resistance in *Salmonella typhimurium* is dependent on *wzz*(*fepE*)-regulated very long O antigen chains. *Microbes Infect.* **7,** 1296–1304
51. Whitfield, C., Amor, P. A., and Köplin, R. (1997) Modulation of the surface architecture of gram-negative bacteria by the action of surface polymer: lipid A-core ligase and by determinants of polymer chain length. *Mol. Microbiol.* **23,** 629–638
52. Gottesman, S., and Stout, V. (1991) Regulation of capsular polysaccharide synthesis in *Escherichia coli* K12. *Mol. Microbiol.* **5,** 1599–1606
53. Mouslim, C., and Groisman, E. A. (2003) Control of the *Salmonella ugd* gene by three two-component regulatory systems. *Mol. Microbiol.* **47,** 335–344
54. Mouslim, C., Latifi, T., and Groisman, E. A. (2003) Signal-dependent requirement for the co-activator protein RcsA in transcription of the RcsB-regulated *ugd* gene. *J. Biol. Chem.* **278,** 50588–50595
55. Francez-Charlot, A., Laugel, B., Van Gemert, A., Dubarry, N., Wiorowski, F., Castanié-Cornet, M. P., Gutierrez, C., and Cam, K. (2003) RcsCDB His-Asp phosphorelay system negatively regulates the *flhDC* operon in *Escherichia coli*. *Mol. Microbiol.* **49,** 823–832
56. Harshey, R. M., and Matsuyama, T. (1994) Dimorphic transition in *Escherichia coli* and *Salmonella typhimurium*: surface-induced differentiation into hyperflagellate swarmer cells. *Proc. Natl. Acad. Sci. U.S.A.* **91,** 8631–8635
57. Zhou, Z., Ribeiro, A. A., Lin, S., Cotter, R. J., Miller, S. I., and Raetz, C. R. (2001) Lipid A modifications in polymyxin-resistant *Salmonella typhimurium*: PmrA-dependent 4-amino-4-deoxy-L-arabinose, and phosphoethanolamine incorporation. *J. Biol. Chem.* **276,** 43111–43121
58. Wösten, M. M., and Groisman, E. A. (1999) Molecular characterization of the PmrA regulon. *J. Biol. Chem.* **274,** 27185–27190
59. Sanderson, K. E., Hessel, A., and Rudd, K. E. (1995) Genetic map of *Salmonella typhimurium*, edition VIII. *Microbiol. Rev.* **59,** 241–303
60. Fields, P. I., Swanson, R. V., Haidaris, C. G., and Heffron, F. (1986) Mutants of *Salmonella typhimurium* that cannot survive within the macrophage are avirulent. *Proc. Natl. Acad. Sci. U.S.A.* **83**, 5189–5193
61. Soncini, F. C., Vescovi, E. G., and Groisman, E. A. (1995) Transcriptional autoregulation of the *Salmonella typhimurium phoPQ* operon. *J. Bacteriol.* **177,** 4364–4371