Introduction

Species of the genus Shigella are the cause of shigellosis, a diarrheal disease that is endemic in developing countries [1]. The major site of Shigella pathology is the colon, where bacteria invade the intestinal mucosa, spread to the adjacent epithelial cells and cause much tissue damage, fluid secretion and inflammation, producing the clinical manifestations of shigellosic diarrhea with blood and mucus [2]. Before colonization and invasion of the colonic mucosa, Shigella must overcome gastric acidity. In contrast to other bacterial enteric pathogens, such as Vibrio cholerae or Salmonella Typhi, species of Shigella are able to initiate infection of humans following ingestion of 10 to 100 bacteria, without neutralization of gastric acid [3–5]. For this reason, acid survival is considered an important pathogenic characteristic of these species [4].

Shigella has evolved sophisticated mechanisms for acid resistance that are induced under conditions of moderate acidity [pH 5.5] and are essential for its subsequent resistance to extreme acidity [pH 2.5]. Two major acid-resistance pathways have been described in Shigella. The acid-resistance pathway 1, or oxidative system, is similar to that of Escherichia coli and requires complex media, oxidative growth and acid induction. This system is regulated by the RpoS sigma factor and the cyclic AMP receptor protein. The acid-resistance pathway 2, in turn, is a stationary-phase, glutamate-dependent pathway induced by growth in mildly acidic condition in the absence of oxygen [6–8]. A third system, which is induced in minimal media under fermentative conditions, has been described in S. flexneri 2457T [8].

Global gene expression profiling of the pH response in S. flexneri 2a [9], E. coli K-12 [10] and Campylobacter jejuni [11] has revealed a large number of genes that are differentially regulated by medium pH. The most pH-dependent genes were those involved in energy metabolism, as well as heat shock and oxidative stress genes. Also, pH differentially regulated a large number of periplasmic proteins and envelope components. This was not unexpected, since surface structures are exposed to the external pH and, thus, most probably play a role in the protective response of the cell to pH variations. Among S. flexneri pH-regulated genes, porin ompF and other outer membrane proteins genes were identified [9]. In E. coli, expression of transport proteins, redox modulators, chaperones and other outer membrane and periplasmic proteins was found to be pH-dependent [10], whereas in C. jejuni, capsular, flagellar and chemotaxis proteins expression was influenced by acid shock [11].

In Helicobacter pylori, acidity down-regulated 12 putative outer membrane proteins [12] and, in Vibrio cholerae, the OmpT porin was repressed by acid while the OmpU porin was required for acid resistance [13]. In addition, proteins involved in production of long fatty acids have been shown to be acid-induced in Streptococcus mutans [14,15] and Lactobacillus lactis [16]. In the latter, proteomic...
analysis revealed that several proteins involved in the biosynthesis of the cell wall and of capsular polysaccharides were regulated by the pH of the medium [16].

The lipopolysaccharide (LPS) is the major component of the outer membrane that mediates bacterial interaction with the environment; however, not much is known about the role that this complex glycolipid plays in resistance to extreme acidity. LPS is composed of three covalently-linked domains: lipid A, which is embedded in the outer membrane, the central oligosaccharide core and the O polysaccharide or O antigen (OAg), which is exposed to the bacterial surface. In S. flexneri, the OAg has two preferred chain lengths, a short OAg (S-OAg) of 11 to 17 repeat units and a very long OAg (VL-OAg) of about 90 repeat units. These chain lengths, or modal distributions, are controlled by the WzzB and WzzAR1 regulators, respectively [17,18]. Also, the addition of glucose residues to the OAg changes its conformation making it more compact and short [19].

It has been described that, under moderate acid conditions, the lipid A domain of LPS is modified by the addition of polar groups that vary between species. For instance, in E. coli and Salmonella Typhimurium, mildly acidic pH (6.0) activates the PmrAB two-component system. The PmrA transcription factor activates the expression of the eptA and araT genes, which code for enzymes that attach phosphoethanolamine (PEtN) and L-amino arabinose (L-Ara4N), respectively, to the lipid A [20–25]. L-Ara4N is added to the 4’ phosphate of lipid A and sometimes to the 1’ phosphate, while PEtN can be attached to the 1 position. In Salmonella, but not in E. coli, PmrAB can be indirectly activated by low pH through the PhoPQ two-component system, which responds to low Mg²⁺ and Ca²⁺ and to low pH growth conditions [26,27]. PhoPQ activation results in addition of palmitate to form hepta-acylated lipid A, incorporation of 2-OH myristate at position 3 and removal of 3-OH myristate from position 3 of lipid A [25,28]. In vivo, these changes in LPS structure are believed to contribute to bacterial resistance to antimicrobial peptides and to the moderate acidic environment found within macrophages [21,25,28,29].

With regard to the OAg, a few studies have suggested that the structure of this domain of LPS is modified in response to the pH of the medium. Delgado et al [30] reported that PmrAB activation in S. Typhimurium results in upregulation of the cld (wzzST) gene, increasing the L-OAg chain length distribution [30], whereas in H. pylori, exposure to low pH induced the expression of one unidentified gene related to OAg biosynthesis. A mutant lacking this gene was significantly more sensitive to acid stress (pH 3.5) than the wild-type strain. In addition, changes in the LPS electrophoretic profiles were observed [31]. The authors suggested that these modifications could contribute to the adaptation to resist extreme acidity, but this was not verified.

Together, these studies demonstrate that moderate low pH induce modifications of LPS in other enteropathogens, increasing their resistance to this environmental condition. However, they have not addressed whether these and/or other alterations of LPS structure play a role in resistance to extreme acid conditions. In this paper, we establish that both the OAg and the lipid A domains of S. flexneri LPS are modified by exposure to moderate acid conditions, we determine the nature of these modifications and show their importance in the acid-resistance phenotype of this pathogen.

**Results**

The O antigen contributes to acid resistance of S. flexneri

**Figure 1. Contribution of the O antigen to S. flexneri 2a acid resistance.** LPS profiles (A) and acid resistance (B) of S. flexneri 2457T (wt), MSF1210 (∆waaL) and MSF1210/pMM112 (∆waaL/eptA®) LPS samples from equal numbers of bacterial cells (1×10⁷ CFU) were loaded in each lane and were analyzed by Tricine-SDS-polyacrylamide gel electrophoresis on a 14% (w/v) acrylamide gel followed by silver staining. Brackets indicate the VL-OAg, the S-OAg and the lipid A-core region. For acid resistance assays, cells were grown overnight in citrate-buffered LB (pH 5.5) and diluted 1:1000 into the acid-challenge media. Survival is stated as a percentage of the inoculum. Averages ± standard errors (error bars) are shown. Statistical significance was determined by a Student’s t test. (*, P < 0.05).

doi:10.1371/journal.pone.0025557.g001
OAg bands was observed (Figure 1A, lane 3). This profile was similar to that of the wild type (Figure 1A, lane 1). We compared the ability of the ΔωzzD mutant and the wild-type 2457T to survive an exposure to pH 2.5 for 30 min, after overnight adaptation to pH 5.5, as described in Materials and Methods. As shown in Figure 1B, the mutant lacking OAg was significantly more sensitive to extreme acidity than the wild-type strain. The mutant harboring pMM112 recovered the ability to survive under this condition. A similar result was obtained for ΔωzzD and ΔωzzD/pMM112 derivatives of S. flexneri serotype 5a strain M90T (data not shown). Thus, the presence of polymerized OAg molecules increases resistance to extreme acidity of S. flexneri previously adapted to moderate acid pH.

The S-OAg is required for acid resistance

Because the results described above indicated that the OAg contributes to acid resistance, we investigated whether a particular length of the OAg molecules was required for this property. For this purpose, we constructed defined deletion mutants in genes ωzzB (strain MSF102) and ωzzD/pMM107, which encode proteins that regulate the S-OAg and VL-OAg chain length distributions, respectively. We also generated a double mutant lacking both regulators (strain MSF209). LPS was obtained and analyzed by SDS-PAGE and silver staining. As shown in Figure 2A, the ΔωzzB mutant was devoid of S-OAg but it produced VL-OAg (lane 2), whereas the ΔωzzD/pMM107 mutant exhibited the opposite phenotype (lane 3). The double mutant showed a random distribution of OAg molecules (lane 4), in contrast to the wild type that produced both S-OAg and VL-OAg (lane 1). Transformation of the double mutant with each intact gene cloned in a multicopy plasmid restored the expression of the corresponding chain length (lanes 5 and 6). Acid resistance of each of these strains was assayed for 30 min at pH 2.5 after overnight adaptation to pH 5.5. As shown in Figure 2B, ΔωzzB and the double mutant (strains MSF102 and MSF209, respectively) were significantly more sensitive to extreme acid conditions, with survival levels less than 20% of the wild type. In contrast, the ΔωzzD/pMM107 mutant (strain MSF107) showed acid resistance levels similar to the wild type. In accordance with these results, the double mutant transformed with a multicity plasmid including the intact ωzzB gene, but not the intact ωzzD/pMM107 gene, recovered the wild-type levels of resistance to extreme acidity.

In order to ascertain whether the S-OAg chain length that is characteristic of S. flexneri 2a is required for acid resistance, strain MSF209 was transformed with multicopy plasmids expressing each OAg chain length regulator of Salmonella Typhimurium. Similar to S. flexneri, this bacterium shows a bi-modal distribution of the OAg expressing a long (L-)OAg of 16 to 35 repeat units regulated by WzzT and a very long (VL-)OAg of more than 100 repeat units regulated by WzzEPK [32–36]. The S. flexneri double mutant expressing the heterologous chain regulators (strains MSF209/pMM110 and MSF209/pMM110 and MSF209/pMM110 and MSF209/pMM110 and MSF209/pMM110) showed acid resistance levels similar to those of the wild-type 2457T and of the mutant expressing S. flexneri WzzB (Figure 2B). Together, these results indicate that S-OAg distinctive of S. flexneri 2a is essential to confer acid resistance.

Glucosylation of the O antigen also contributes to acid resistance

The OAg of S. flexneri 2a is glucosylated in the last rhamnose residue [37]. It has been described that this modification affects the conformation of the polymer in such a way that LPS molecules containing glucosylated OAg are more compact and shorter than molecules of non-glucosylated OAg [38]. Because we found that the length of the OAg was critical to acid resistance (S-OAg), we...
decided to investigate whether the degree of glucosylation could also influence this property. To this end, we constructed a deletion mutant in the gtrABII operon encoding proteins responsible for glucosylation of the OAg. The electrophoretic profile of this mutant (strain MSF2743) showed OAg bands migrating slightly faster than those of the wild-type 24577T. This observation was more evident when the electrophoresis was performed on a longer polyacrylamide gel (Figure 3A, lanes 2 and 1, respectively). Acid resistance experiments showed that MSF2743 was about 50% more sensitive to pH 2.5 than the wild type. When this mutant was transformed with the intact gtrABII operon cloned in a multicopy plasmid, the acid resistant phenotype was restored to wild-type levels (Figure 3B).

A mild acidic condition modifies the LPS structure of S. flexneri

The mechanisms for resistance to extreme acidity are induced by previous adaptation to a moderate acidic pH [6–9]. In other enterobacteria, it has been demonstrated that this latter condition causes changes in the LPS structure [31]. We decided to investigate whether adaptation to pH 5.5 altered the LPS of S. flexneri 2a, and, if that was the case, whether these changes contributed to resistance to extreme acidity. We first compared the LPS profiles of bacteria grown at pH 7.0 and pH 5.5. As shown in Figure 4, the LPS of bacteria grown at pH 5.5 had a slightly lower mobility of the bands corresponding to OAg than bacteria grown at pH 7.0 (lanes 2 and 1, respectively). It was observed that at pH 5.5, this region contained lower amounts of OAg bands ranging from 11–15 units, but higher amounts of OAg bands of 16–18 units (see densitogram in Figure 4, upper right). In order to ascertain whether this modification was relevant to acid resistance, we performed acid-resistance assays using the ΔwzzB mutant (strain MSF102) transformed with plasmids containing site-directed mutations in the gene encoding the WzzB regulator (Table 1). When strain MSF102/pRMCD108 (mutation WzzB<sub>K267N</sub>) was grown at pH 7.0, produced an LPS profile similar to that observed in the wild type grown at pH 5.5 (Figure 5A, compare lanes 2 and 3). In contrast, when strain MSF102/pRMCD127 (mutation WzzB<sub>ΔN6</sub>) was grown at pH 5.5, it produced an LPS profile similar to the wild type grown at pH 7.0 (Figure 5A, compare lanes 1 and 4). Thus it was expected that, if the shift of the S-OAg that occurs at pH 5.5 was relevant to acid resistance, strain MSF102/pRMCD108 would be resistant to extreme acid pH without previous adaptation to pH 5.5. On the other hand, strain MSF102/pRMCD127 would be sensitive to acid even after adaptation.

The results showed that MSF102/pRMCD108 was as sensitive as the wild-type strain grown at pH 7.0 (non-adapted), whereas MSF102/pRMCD127 showed resistance levels similar to the wild-type grown at pH 5.5 (adapted) (Figure 5B). These results indicated that the change in the electrophoretic mobility of the S-OAg induced by moderate acidic conditions is not relevant to resistance to extremely acidic pH.

We also noticed that the OAg of bacteria grown at acid pH migrated as double bands (Figure 4, see densitogram lower right). The double bands were not attributable to glucosylated OAg molecules because they were also present in the ΔgtrABII mutant grown at pH 5.5 (data not shown). The double bands were more evident in the OAg molecules containing one, two, three and four repeat units and in the lipid A-core region. The double band in this region was also evident in LPS obtained from bacteria grown at pH 7.0, although it was much fainter than at pH 5.5 (compare lanes 1 and 2 in Figure 4, and lanes 1 and 3 in Figure 5). With these observations, we hypothesized that this modification actually occurred in the lipid A-core region and, as a consequence, the OAg profile appeared altered. In order to test this notion, we analyzed the LPS profiles of defined mutants in the polymerization or ligation of the OAg, and with different degrees of truncation of the outer core [ΔwaaD, ΔwaaJ and ΔwaaL]. The LPS patterns of the mutants and the structure of the LPS outer core [39,40] are shown in Figures 6A and B, respectively. The ΔwaaJ mutant exhibited the lipid A-core region substituted by one OAg unit (lanes 1 and 2), whereas the ΔwaaL mutant synthesized only lipid A-core (lanes 3 and 4). The ΔwaaD mutant showed a core region that migrated slightly faster than that of ΔwaaL (lanes 5 and 6) consistent with the lack of the GlcNac residue in the outer core (Figure 6B). Deletion of waaJ and waaL genes resulted in additional truncations of the core region (lanes 7 and 8; 9 and 10, respectively). The waaJ gene product adds Glc II while the waaL product adds the Gal residue to the outer core (Figure 6B). All strains exhibited the double band when grown at pH 5.5.

These results indicated that the changes in the LPS structure by exposure to moderate acidic condition affected the lipid A and/or the inner core regions of LPS.

In S. Typhimurium, moderate acidic pH activates the PhoPQ two-component system. Phosphorylated PhoP activates expression of the pagP gene, encoding a protein responsible for the addition of palmitate to lipid A [25,41,42]. To define whether a similar regulation occurs in S. flexneri, we constructed a phoP deletion mutant and analyzed the LPS profiles of bacteria grown at pH 7.0 and 5.5. The results showed that double bands were still present in the ΔphoP mutant (data not shown). Thus, it was unlikely that the double bands were due to the addition of palmitate to lipid A.
The 32P-labelled lipid A was isolated and subjected to samples from equal numbers of bacterial cells (1 \times 10^7 CFU) were loaded in each lane and were analyzed by Tricine-SDS-polyacrylamide gel electrophoresis on a 14% (w/v) acrylamide gel followed by silver staining. Bracket shows the S-OAg region. Arrow heads point to the double bands observed in LPS from bacteria grown at pH 5.5. The right panels show the densitograms of the bands in the gel. Upper graph shows the bands corresponding to the S-OAg (11 to 18 units) and lower panels show the densitograms of the bands in the gel. Upper graph shows the bands corresponding to the Lipid A-core substituted with a second phosphate group (1-PP) and L-Ara4N were not detected (Figure 7, lane 3). This last spot was missing in the chromatogram of lipid A from strain MSF1650 (Figure 7, lane 4). Interestingly, in this mutant genetic background, PEtN was detected in lipid A from bacteria grown at pH 7.0. Finally, we analyzed lipid A obtained from a \( \Delta \text{petA} \) mutant (strain MSF1666). As expected, this mutant lacked L-Ara4N; however, it was able to add PEtN to lipid A at pH 7.0 (Figure 7, lane 5).

A less negative bacterial surface charge is produced by lipid A modification under mild acidic condition

To evaluate whether the modifications of lipid A by the addition of polar groups alter the cell surface charge, we measured the cytochrome C binding affinity of the wild type cells grown at pH 7.0 and 5.5. The results (Table 1) showed that binding was lower when bacteria were grown at pH 5.5. That binding was lower when bacteria were grown at pH 5.5, suggesting that addition of PEtN to lipid A at moderate acidic pH confers a less negative charge to the bacterial surface. To confirm this notion, cytochrome C binding affinity of the wild type cells overexpressing the \( \text{petA} \) gene, grown at pH 7.0, was measured. As shown in Table 1, these cells bound about one half the amount of cytochrome C than the wild type grown under this condition.

| Strain (growth pH) | % Cytochrome C bound |
|-------------------|----------------------|
| 2457T (7.0)       | 27.1 ± 2.5           |
| 2457T (5.5)       | 8.8 ± 2.4            |
| 2457T/pMM113 (7.0)| 15.0 ± 3.8           |

Values represent percent binding of cytochrome C after incubation with bacteria for 10 min at room temperature. Data are the means and standard deviations from two independent experiments run in duplicate.

doi:10.1371/journal.pone.0025557.t001

To elucidate the chemical nature of the modifications of lipid A, \( S. \text{flexneri} \) was grown in LB broth in the presence of \( ^{32}\text{P} \), at pH 7.0 and pH 5.5. The \( ^{32}\text{P} \)-labelled lipid A was isolated and subjected to TLC as described in Materials and Methods. The results showed that \( S. \text{flexneri} \) grown at pH 7.0 produces lipid A molecules partially substituted with a second phosphate group (1-PP) and L-Ara4N (Figure 7, lane 1). In contrast, when bacteria were grown at pH 5.5 the lipid A was modified by the addition of PEtN, while 1-PP and L-Ara4N were not detected (Figure 7, lane 2). The chemical nature of these species was confirmed by analyzing the lipid A profiles of the \( \Delta \text{arnT} \) mutant (strain MSF1650) and of the wild-type strain transformed with the \( \text{petA} \) gene cloned in a multicopy plasmid (strain 2457T/pMM113) grown at pH 7.0. This strategy was used because we were unable to obtain an \( \Delta \text{petA} \) mutant either by the Red swap methodology or by using the suicide vector pGP704 containing an internal region of the gene.

In strain 2457T/pMM113, spots corresponding to lipid A substituted with one and two molecules of PEtN (double) were observed. As in the wild-type grown at pH 7.0, 1-PP and L-Ara4N were also detected (Figure 7, lane 3). This last spot was missing in the chromatogram of lipid A from strain MSF1650 (Figure 7, lane 4). Interestingly, in this mutant genetic background, PEtN was detected in lipid A from bacteria grown at pH 7.0. Finally, we analyzed lipid A obtained from a \( \Delta \text{pmrA} \Delta \text{wzzB} \) mutant (strain MSF1666). As expected, this mutant lacked L-Ara4N; however, it was able to add PEtN to lipid A at pH 7.0 (Figure 7, lane 5).

Lipid A modifications induced under mild acidic conditions confer resistance to extreme acidity

To investigate whether the modification of the lipid A region induced by growth at pH 5.5 is involved in resistance to extreme acid conditions, we tested acid resistance of the wild-type strain transformed with pMM113. As shown in Figure 8, overexpression of the \( \text{petA} \) gene significantly increased acid resistance after 30 and 60 min of exposure to pH 2.5, compared to the parental strain. This result indicates that the addition of PEtN to the lipid A region of LPS, induced by a moderate pH, contributes to resistance to extreme acid. In contrast, deletion of the \( \text{arnT} \) gene did not alter this property. As shown in Figure 8, the \( \Delta \text{arnT} \) mutant (strain MSF1650) showed levels of acid resistance identical to the wild-type strain.

Finally, we transformed the \( \Delta \text{waaL} \Delta \text{wzzB} \) and \( \Delta \text{petA}\text{II} \) mutants with plasmids pMM112 or pMM113 and assayed the acid resistance of the resulting strains. The results showed that the transformed strains were as sensitive to extreme acidity as the original mutants (data not shown), indicating that, even though lipid A modification contributes to acid resistance, the presence of glucosylated OAg is fundamental to this property.

Discussion

Remodelling of the bacterial envelope is an important strategy used by bacteria to adapt to stressful conditions. Global expression studies carried out in other enteropathogens have revealed that a great number of genes involved in the biosynthesis of surface structures are regulated by the pH of the environment [9–13]. In the present study, we investigated whether the lipopolysaccharide of \( S. \text{flexneri} \) 2a is modified by exposure to a moderate acidic pH, and if these modifications increase bacterial resistance to extreme acidity.

Our results clearly show that the presence of OAg is required for acid resistance, since a mutant that does not bind the polymer
to the lipid A-core was highly sensitive to pH 2.5, even after being adapted to pH 5.5. These results are consistent with the work of other authors, who demonstrated that the lack of OAg or diminished amount of this polysaccharide impaired the ability of *Rhizobium leguminosarum* biovar trifolii [43], *H. pylori* [31] and *Salmonella enterica* serovar Dublin [44] to grow or persist under acidic conditions. In an *E. coli* O157:H7, a strain closely related to *Shigella*, it has also been demonstrated the involvement of LPS in the resistance to organic acid [45]. Although the acid stress used in that study was different from our conditions (pH 4.5 versus pH 2.5), their results support our conclusion that the OAg is essential to acid resistance. More interestingly, we found that a particular length of the OAg, the S-OAg modal distribution, is required for resistance to extreme acidity. In contrast, neither the VL-OAg modal length nor OAg lengths characteristic of *S. Typhimurium* contributed significantly to this property.

McGowan et al [31] proposed that the OAg could act as a physical barrier to prevent entry of protons to the bacterial cell. Our results do not agree with this notion, because the mutant lacking VL-OAg (Δ*wzzB*) was as resistant to extreme acid as the wild-type strain, meaning that LPS molecules of high molecular weight do not confer higher levels of resistance, as would be expected if the OAg polymer acted as a physical barrier for protons.

Another explanation for the role of the OAg in the acid stress response could be that the polysaccharide may be positively charged repelling protons from the surrounding medium. However, at the pH values used in this study, the −OH groups of the rhamnose residues in the *S. flexneri* OAg are protonated and neutral [46] and, hence, they do not confer a positive charge to the OAg molecules. On the other hand, the amide group in the N-acetylglucosamine residue is deprotonated and neutral at the pH values used here. In consequence, the OAg would not be charged.
under the conditions of this study and, thus, it would not act as an
electrostatic barrier for the influx of protons.

Because it is known that moderate acidic conditions produce
changes in the expression of outer membrane proteins [47,48] and
that the absence of some porins affects resistance to acidic pH
[49,50], we propose that the presence of OAg, in particular the S-
OAg modal length, may be necessary for assembly or function of a
protein in the bacterial envelope required for acid resistance. In
support of this idea, it has been suggested that a preferential
association between certain outer membrane proteins and LPS
molecules of a particular length may occur [35]. For example, the
IcsA protein, involved in actin polymerization that allows
S. flexneri spreading between epithelial cells, requires the presence of S-OAg
to be assembled at one pole of the bacterial surface [35, 51 52]. Alternatively, or in addition, synthesis of S-OAg may slow down
polymerization of very long OAg molecules (VL-OAg), which
could mask a membrane protein needed for acid resistance. In this
context, our previous work [33] demonstrated that the two Shigella
chain-length regulators compete to control the degree of O
antigen polymerization; hence, increase in one modal length
results in a parallel decrease in the other.

Our results also show that glucosylation of the OAg contribute
to acid resistance. Our data indicates that this modification is also
present in bacteria grown under standard conditions (LB, pH 7.0),
in agreement with a previous study conducted by West et al [19].
Interestingly, these authors demonstrated that glucosylation of the
OAg is required for the proper function of the Type Three
Secretion System (T3SS) of S. flexneri, because glucosylated OAg is
more compact and short than non-glucosylated LPS, allowing a
proper exposure of the needle complex [38]. Additionally, a more
compact and dense OAg favours the interaction between OAg
molecules, contributing to stabilize the outer membrane [54] and
protecting it from the damage caused by acidic pH and entry of
protons. Given all these observations, we speculate that glucosy-
lated OAg containing mainly S-OAg molecules is required for
optimal functioning of one or more proteins that contribute to the
acid stress response.

The current work also demonstrates that moderate acidic pH
induces modifications in the lipid A region of S. flexneri. Previous
work by Gibbons et al [29] showed that lipid A of S. Typhimurium
is covalently modified by the addition of acyl and/or polar
substituents in response to low Mg\(^{2+}\) concentrations and mild
acidic pH. Under these conditions, lipid A was derivatized with
phosphoethanoamine (PEtN), aminoarabinose (L-Ara4N), 2-hy-
droxytryristate and/or palmitate moieties. It is believed that these
modifications confer resistance to cationic antimicrobial peptides
by masking negative phosphate groups with positively charged
moieties [55–58]. In E. coli K12 W3110, lipid A is modified in
response to growth under acidic but no low Mg\(^{2+}\) conditions. The
main change is the addition of PEtN to the phosphate molecule at
position 1 of N-acetyl glucosamine. The addition of two PEtN to
each phosphate molecule at positions 1 and 4 was also detected in
a small fraction of lipid A molecules. In contrast to S. Typhimurium, no L-Ara4N was detected. At pH 7.5, lipid A

**Figure 7.** Effect of pH on lipid A modifications in S. flexneri. \(^{32}\)P-
labelled lipid A was isolated from bacteria grown in N-minimal medium
at pH 7.0 (lanes 1, 3, 4 and 5) or 5.5 (lane 2). Lipid A species were
resolved by TLC with the solvent system chloroform/pyridine/88%
formic acid/water (50:50:16:5, v/v). The strains are 2457T (wt), 2457T/
pMM113 (wt/eptA\(^+\)), MSF1650 (ΔarnT) and MSF1666 (ΔpmrA).
doi:10.1371/journal.pone.0025557.g007

**Figure 8.** Effect of lipid A modifications on acid resistance of S.
flexneri. The strain are 2457T (wt), 2457T/pMM113 (wt/eptA\(^+\)) and
MSF1650 (ΔarnT). Cells were grown overnight in citrate-buffered LB
(pH 5.5) and diluted 1:1000 into the acid-challenge media. Survival is
stated as a percentage of counts at time zero. Averages ± standard
errors (error bars) are shown. Statistical significance was determined by
the two-way ANOVA and Bonferroni post test (*, \(P < 0.05\), ***, \(P
< 0.001\)).
doi:10.1371/journal.pone.0025557.g008
was mainly unmodified or, a low fraction of molecules, carried a 1-PP group [29,59].

Our results demonstrate that similar modifications occur in S. flexneri. As in E. coli, the main change induced at pH 5.5 was the addition of PEtN to lipid A, whereas L-Ara4N was not detected. In contrast, at pH 7.0 the lipid A molecules were derivatized with 1-PP and L-Ara4N. The regulatory pathways involved in these modifications also appear to be different to E. coli and S. Typhimurium, because a ΔpmrA mutant lacked L-Ara4N but it was able to add PEtN to lipid A, showing a chromatographic profile similar to ΔwzaT. This result suggests that modification of lipid A with PEtN in response to acid pH is not mediated by the PmrAB system. Future work will be needed to uncover the regulatory system involved in this modification.

The presence of L-Ara4N does not seem to add to extreme acid resistance. In contrast, addition of PEtN to lipid A protects S. flexneri from extreme acid pH. It has been reported that PEtN lipid A modification reduces the net charge of the phosphate group from $-1.5$ to $-1$ at neutral pH [54]. Here we demonstrate that addition of PEtN confers a less negative charge to bacterial surface. We speculate that at pH 2.5 the presence of positively charged amine groups increases the net charge to reach positive values. Thus, we hypothesize that lipid A modification with polar substituents generate an electrostatic barrier that acts repelling protons and diminishing their influx to the bacterial cytoplasm.

To our knowledge, this is the first report that addresses the role of LPS in resistance of S. flexneri to extreme acid conditions. Our data demonstrate that both modification of the lipid A region and the presence of glucosylated OAg molecules containing the S-OAg modal length are required to confer S. flexneri its high level of acid resistance. Our results strengthen the hypothesis proposed by others [35,38] that, during evolution of S. flexneri as a successful pathogen, selection of preferred OAg chain lengths and glucosylation of the polysaccharide was optimized. LPS modifications that occur in response to environmental conditions contribute to adaptation of bacteria to the different stages of infection.

### Materials and Methods

#### Bacterial strains, plasmids, media and growth conditions

Table 2 summarizes the properties of the bacterial strains and plasmids used in this study. Bacteria were grown at pH 7.0 in Luria-Bertani medium (LB, 10 g/l Bacto tryptone, 5 g/l Bacto yeast extract, 5 g/l NaCl) or in citrate-buffered LB medium.

| Table 2. Strains and plasmids used in this study. |
|-----------------------------------------------|
| **Strain or plasmid** | **Relevant properties** | **Source or reference** |
| **S. flexneri** | | |
| 2457T | wild-type strain | ISP<sup>a</sup> |
| MSF107 | Δwzz<sub>gtrABII</sub>Δzph<sub>gtrABII</sub>aph<sub>R</sub>, Km<sup>R</sup> | [50] |
| MSF102 | 2457T Δwzz<sub>B</sub> | [50] |
| MSF209 | 2457T Δwzz<sub>8</sub> Δwzz<sub>gtrABII</sub>Δzph<sub>gtrABII</sub>aph<sub>R</sub>, Km<sup>R</sup> | [50] |
| MSF1749 | 2457T Δwzy | This study |
| MSF1210 | 2457T Δwzd<sub>L</sub> | This study |
| MSF1144 | 2457T Δwaa<sub>D</sub> | This study |
| MSF1009 | 2457T Δwaa<sub>L</sub> | This study |
| MSF1015 | 2457T Δwaa<sub>I</sub> | This study |
| MSF2743 | 2457T ΔgtrAB<sub>II</sub> | This study |
| MSF1650 | 2457T Δarn<sub>T</sub> | This study |
| MSF1666 | 2457T Δpmr<sub>A</sub> | This study |
| **Plasmids** | | |
| pKD46 | bla PBAD gam bet exo pSC101 ori<sub>T</sub>S | [57] |
| pKD4 | bla FRT ahp FRT P51 P52 oriR6K | [57] |
| pCP20 | bla cat cI857 ΔPr<sub>o</sub> flp pSC101 ori<sub>T</sub>S | [58] |
| pGEM-T-Easy | TA cloning vector, Ap<sup>R</sup> | Promega |
| pJC139 | S. flexneri 2457T wzz<sub>B</sub> cloned into pGEM-T-Easy, Ap<sup>R</sup> | [63] |
| pJC142 | S. Typhimurium LT2 wzz<sub>四世</sub> cloned into pGEM-T-Easy, Ap<sup>R</sup> | [67] |
| pMM110 | S. Typhimurium LT2wzz<sub>四世</sub> cloned into pGEM-T-Easy, Ap<sup>R</sup> | This study |
| pJC144 | S. flexneri 2457T wzz<sub>四世</sub> cloned into pGEM-T-Easy, Ap<sup>R</sup> | [66] |
| pMM112 | S. flexneri 2457T waa<sub>L</sub> cloned into pGEM-T-Easy, Ap<sup>R</sup> | This study |
| pMM113 | S. flexneri 2457T eptA cloned into pGEM-T-Easy, Ap<sup>R</sup> | This study |
| pCC1 | Single copy cloning vector, oril, ori<sub>R</sub>, CM<sup>R</sup> | EPICENTRE |
| pMM111 | S. flexneri 2457T gtrAB<sub>II</sub> cloned into pCC1, CM<sup>R</sup> | This study |
| pRMCD108 | Wzz<sub>B</sub>(K267N) | [68] |
| pRMCD127 | Wzz<sub>B</sub>(M327T) | [68] |

<sup>a</sup>Km<sup>R</sup>, kanamycin resistant; Ap<sup>R</sup> ampicillin resistance; Cm<sup>R</sup>, chloramphenicol resistance.

<sup>b</sup>ISP, Institute of Public Health, Santiago, Chile.

doi:10.1371/journal.pone.0025557.t002
Mutagenesis of waaD, waaJ, waaL, gtrABII, arnT and pmrA genes

Mutagenesis was performed by the Red/Swap method to create chromosomal mutations by homologous recombination using PCR products [60]. Shigella flexneri 2a strain 2457T containing pKD46, which expresses the λ Red recombinase system, was transformed with PCR products that were generated using as template plasmid pKD4, which contains the FRT-flanked kanamycin-resistance gene (aph). Primers were designed according to the DNA sequence available for the S. flexneri 2457T strain. Each primer pair also carried 30 bases that were homologous to the edge of the gene targeted for disruption. The sequences of the oligonucleotide primers are shown in Table 3. The kanamycin-resistant transformants were replica plated in the absence of antibiotic selection at 37°C and finally assayed for ampicillin sensitivity to confirm loss of pKD46. To obtain non-polar deletion mutants, the antibiotic resistance gene was removed by transforming the gene replacement mutants with pCP20, which encodes the FLP recombinase [61]. Correct insertional gene replacements and the deletion of the antibiotic gene cassettes were confirmed by PCR.

Construction of expression plasmids

DNA fragments containing the S. flexneri 2457T waaL, arnT, eptA, gtrABII genes and S. Typhimurium LT2 wzzST strain were used to clone into the one copy vector pCC1 (Table 1).

LPS analysis

Culture samples were adjusted to an optical density at 600 nm of 2.0 in a final volume of 100 µl. Then, proteinase K-digested whole-cell lysates were prepared as described previously [62], and LPS was separated on 12% w/v acrylamide gels using a Tricine-sodium dodecyl sulfate (SDS) buffer system [63]. Gel loadings were normalized so that each sample represented the same number of cells. Gels were silver stained by a modification of the procedure of Tsai & Frasch [64]. Densitometry analysis was performed using the UN-SCANT-IT gel software (Silk Scientific).

Acid resistance assay

For acid-resistance assays bacteria were aerobically grown overnight in LB (pH 7.0) or citrate-buffered LB (pH 5.5) media. Cultures grown to stationary phase were diluted 1:1000 into 5 ml of E medium [64]. Cultures grown to stationary phase were diluted 1:1000 into 5 ml of E medium 0.2 g/l MgSO4·7H2O, 2 g/l citric acid monohydrate, 10 g/l K2HPO4·3 H2O, 3.5 g/l NaH2PO4·4H2O, acidified with HCl to pH 2.5. This medium was supplemented with 0.2% glucose as carbon source, 20 µg/ml asparagine acid and 20 µg/ml nicotinic acid. The pH 2.5 cultures were incubated at 37°C with shaking; acid challenge was stopped at 0 or 30 min by dilution in LB and cell viability was immediately determined by plating appropriate dilutions in LB plates in triplicate. At least

Table 3. Primers used in this study.

| primers | Sequence (5’-3’) | purpose |
|-----------------|-----------------|----------|
| W_sf_waa1       | accctcaacttattttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttt...
three repetitions were performed for each experiment. Acid resistance was calculated as percent survival, taking the bacterial counts obtained at time 0 as 100%.

Analysis of $^{32}$Pi labelled lipid A
An overnight culture grown at $37^\circ$C on LB was diluted 1000-fold into 5 ml of fresh LB medium at pH 7.0 or 5.3. Labelling was started by addition of 5 $\mu$Ci/ml of $^{32}$HPO$_4$ (Chilean Commission of Nuclear Energy), and allowed to continue growing overnight at $37^\circ$C with shaking. The $^{32}$P-labeled bacteria were collected by centrifugation and washed twice with 5 ml of phosphate-buffered saline (PBS) to pH 7.4. The bacterial pellets were resuspended in 0.8 ml of PBS and the lipid A fractions were extracted as described by Zhou et al [65]. The lipid A samples were dissolved in chloroform/methanol (4:1, v/v), and several microliters ($5,000$ cpm per lane) were applied to the origin of a Silica Gel TLC plate. The $^{32}$P-labeled lipid A species were resolved with the solvent system chloroform/pyridine/88% formic acid/water (50:50:16:5, v/v). The plate was developed by autoradiography.

Cytochrome C binding assay
An overnight culture grown at $37^\circ$C on LB was diluted 1000-fold into 5 ml of fresh LB medium at pH 7.0 or 5.5. Bacteria were collected by centrifugation and washed twice with 10 mM phosphate buffer (PB), pH 6.8. Bacterial pellets were resuspended in 1 ml of PB and the OD$_{600}$ nm was measured. Then, samples were diluted to 5 OD units and 30 $\mu$l of cytochrome C (10 mg/ml in PB) were added to 1.2 ml of bacterial suspensions. Samples were incubated for 10 min at room temperature and then centrifuged for 2 min at 13,000 rpm. The supernatants (1 ml) were recovered and the OD$_{530}$ nm was measured.

Acknowledgments
We thank Renato Morona and Luisa Van Den Bosch for the gift of plasmids pRMCD127 and pRMCD108. We thank Carla Delporte for her help with the TLC experiments and Carlos Santiviago for helpful discussions.

Author Contributions
Conceived and designed the experiments: MM SAA IG. Performed the experiments: MM AH SAA. Analyzed the data: MM AH SAA IG. Wrote the paper: MM SAA IG.

References
1. Kotloff KL, Winickoff JP, Ivanoff B, Clemens JD, Swerdlow D, et al. (1999) Global burden of shigellosis infections: implications for vaccine development and implementation of control strategies. Bull W H O 77: 651–666.
2. Schroeder GN, Hilbi H (2008) Molecular pathogenesis of Shigella spp.: controlling host cell signaling, invasion and death by type III secretion. Clin Microbiol Rev 21: 134–156.
3. Blaser MJ, Newman LS (1982) A review of human salmonellosis: I. Infective dose. Rev Infect Dis 4: 1096–1106.
4. Gorden J, Small PLC (1993) Acid resistance in enteric bacteria. Infect Immun 61: 364–367.
5. Audia JP, Webb CG, Foster JW (2001) Breaking through the acid barrier: an orchestrated response to proton stress by enteric bacteria. Int J Med Microbiol 29: 97–106.
6. Liu J, Lee IS, Frey J, Sloczewska JL, Foster JW (1995) Comparative analysis of extreme acid survival in Salmonella typhimurium, Shigella flexneri, and Escherichia coli. J Bacteriol 177: 4097–4104.
7. Foster JW (2004) Escherichia coli acid resistance: tales of an amusel acidophile. Nat Rev Microbiol 2: 898–907.
8. Jensenison AV, Verma NR (2007) The acid-resistance pathways of Shigella flexneri 2457T. Microbiology 153: 2593–2602.
9. Cheng F, Wang J, Peng J, Yang J, Fu H, et al. (2007) Gene expression profiling of the pH response in Shigella flexneri 2a. FEMS Microbiol Lett 270: 12–20.
10. Maurer LM, Yohana E, Boudarian SS, Radmacher M, Sloczewska JL (2005) pH regulates genes for flagellar motility, catalysis, and oxidative stress in Escherichia coli K-12. J Bacteriol 187: 304–319.
11. Reid AN, Pandey R, Palyada K, Whithour I, Doukhanean E, et al. (2008) Identification of Campylobacter jejuni genes contributing to acid adaptation by transcriptional profiling and genome-wide mutagenesis. Appl Environ Microbiol 74: 1590–1612.
12. Merrell DS, Goodrich ML, Otto G, Tompkins LS, Falkow S (2003) pH-regulated gene expression of the gastric pathogen Helicobacter pylori. Infect Immun 71: 3529–3539.
13. Merrell DS, Bailey G, Kaper JB, Camilli A (2001) The ToxR-mediated organic acid tolerance response of Vibrio cholerae requires OmpU. J Bacteriol 183: 2746–2754.
14. Quivey RG Jr., Faustofreri R, Monahan K, Marquis R (2000) Shifts in membrane fatty acid profiles associated with acid adaptation of Streptococcus mutans. FEMS Microbiol Lett 189: 89–92.
15. Fosso EM, Quivey RG Jr. (2004) Shifts in the membrane fatty acid profile of Streptococcus mutans enhance survival in acidic environments. Appl Environ Microbiol. 70: 929–936.
16. Budin-Verneuil A, Pichereau V, Affray Y, Ehrlich D, Maguin E (2007) Proteome phenotyping of acid-stress-resistant mutants of Lactobacillus delbrueckii MG1363. Proteomics 7: 2038–2046.
17. Morona R, Van den Bosch L, Manning PA (1995) Molecular, genetic, and topological characterization of O-antigen chain length regulation in Shigella flexneri. J Bacteriol 177: 1059–1068.
18. Stevenson G, Kessler A, Reeves PR (1995) A plasmid-borne O-antigen chain length determinant and its relationship to other chain length determinants. FEMS Microbiol Lett 125: 25–30.
19. West NP, Sansonetti P, Mounier J, Exley RM, Parsot C, et al. (2005) Optimization of virulence functions through glucosylation of Shigella LPS. Science 307: 1313–1317.
20. Helander IM, Kilpelainen I, 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: 481–488.
21. Nummila K, Kilpelainen I, Zahringer U, Vaara M, Helander IM (1995) Lipopolysaccharides of polymyxin B-resistant mutants of Escherichia coli are extensively substituted by 2-aminoethyl pyrophosphate and contain aminoarabinose in lipid A. Mol Microbiol 16: 271–278.
22. Gunj JS, Ryan SS, Van Velkinburgh JC, Ernst RK, Miller SI (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.
23. Zhou Z, Ribeiro AA, Lin S, Cotter RJ, Miller SI, et al. (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.
24. Lee H, Hsu FF, Turk J, Grosman EA (2004) The PmrA-regulated pmrC gene mediates phosphoethanolamine modification of lipid A and polymyxin resistance in Salmonella enterica. J Bacteriol 186: 4124–4133.
25. Gunj JS (2008) The Salmonella PmrA/B regulon: lipopolysaccharide modifications, antimicrobial peptide resistance and more. Trends Microbiol 16: 284–290.
26. Garcia Vescovi E, Soncini FG, Grosman EA (1996) Mg$^{++}$ as an extracellular signal: environmental regulation of Salmonella virulence. Cell 84: 163–174.
27. Beaaron BL, Wilson L, Foster JW (1998) A low pH-inducible, PhoPQ-dependent acid tolerance response protects Salmonella typhimurium against inorganic acid stress. J Bacteriol 180: 2409–2417.
28. Ernst RK, Guina T, Miller SI (2001) Salmonella typhimurium outer membrane remodeling: role in resistance to host innate immunity. Microbes Infect 3: 1327–1334.
29. Gibbons HS, Kalb SR, Cotter RJ, Raetz CR (2005) Role of Mg$^{++}$ and pH in the modification of Salmonella lipid A after endocytosis by macrophage tumour cells. Mol Microbiol 55: 425–440.
30. Delgado MA, Mouslim C, Grosman EA (2006) The PmrA/PmrB and RcsC/RcsB systems control expression of the Salmonella O-antigen chain length determinant. Mol Microbiol 60: 39–50.
31. McGowan CC, Necheva A, Thompson SA, Cover TL, Blaser MJ (1998) Acid-induced expression of an LPS-associated gene in Helicobacter pylori. Mol Microbiol 30: 19–31.
32. Barin DA, Stevenson G, Brown PK, Haase A, Reeves PR (1995) Repeat unit polysaccharides of bacteria: a model for polymerization resembling that of ribosomes and fatty acid synthetase, with a novel mechanism for determining chain length. Mol Microbiol 20: 685–694.
33. Batchelor RA, Alifano P, Biffali E, Hall SI, Hall RA (1992) Nucleotide sequences of the genes regulating O-polysaccharide antigen chain length (olv) from Escherichia coli and Salmonella typhimurium: protein homology and functional complementation. J Bacteriol 174: 5226–5236.
34. McClelland M, Sanderson KE, Speir J, Clifton SW, Latreille P, et al. (2001) Complete genome sequence of Salmonella enterica serovar Typhimurium LT2. Nature 413: 852-856.

35. Morona R, Daniell C, Van Den Bosch L (2003) Genetic modulation of Shigella flexneri 2a lipopolysaccharide O antigen modal chain length reveals that it has been optimized for virulence. Microbiology 149: 925-939.

36. Murray GL, Attridge SR, Morona R (2003) Regulation of Salmonella typhimurium lipopolysaccharide O antigen chain length is required for virulence; identification of RfaH as Wzz. Infect Immun 71: 3529-3539.

37. Allison GE, Verma NK (2000) Serotype-converting bacteriophages and O-antigen modification in Shigella flexneri. Trends Microbiol 8: 17-23.

38. West NP, Sansonetti P, Mounier J, Edey RM, Parnet C, et al. (2005) Optimization of virulence functions through glycosylation of Shigella LPS. Science 307: 1315-1317.

39. Jin Q, Yuan Z, Xu J, Wang Y, Shen Y et al (2002) Genome sequence of Shigella flexneri 2a insights into pathogenicity through comparison with genomes of Escherichia coli K12 and O157. Nucleic Acid Res 30: 4432-4441.

40. Kamin N, Vinogradov E, Li J, Monteiro MA, Whitleaf C (2004) Chromosomal and plasmid-encoded enzymes are required for assembly of the R3-type core oligosaccharide in the lipopolysaccharide of Escherichia coli O157:H7. J Biol Chem 279: 31237-31250.

41. Bishop RE, Gibbons HS, Guina T, Trent MS, Miller SI, et al. (2000) Transfer of plasmid from the cell wall to the outer membrane of Salmonella enterica serovar Typhimurium LT2. Mol Microbiol 39: 439-447.

42. Thomsen LE, Chadfield MS, Bispham J, Wallis TS, Olsen JE (2003) Reduced acid tolerance response and protein amounts of LPS affect both stress tolerance and virulence of Salmonella enterica serovar Typhimurium. Environ Microbiol 5: 1216-1227.

43. Chen H, Gartner E, Rolfe BG (1993) Involvement of genes on a megaplasmid in the organic acid resistance of Shiga toxin-producing Escherichia coli O157:H7. Mol Microbiol 7: 979-988.

44. Jiang T, Tran AX, Hankins JV, Mengin-Lecreulx D, Trent MS (2008) Periplasmic phosphorylation of lipid A is linked to the synthesis of undecaprenyl phosphate. Mol Microbiol 67: 264-277.

45. Zhou Z, Lin S, Cotter RJ, Raetz CR (1999) Lipid A modifications characteristic of Salmonella enterica serovar Typhimurium. J Biol Chem 274: 18503-18514.

46. Roberts KA, Wanner BL (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.

47. Thompson LE, Chadfield MS, Bispham J, Wallis TS, Olsen JE (2003) Reduced amounts of LPS affect both stress tolerance and virulence of Salmonella enterica serovar Dublin. FEMS Microbiol Lett 228: 225-231.

48. Raetz CR, Reynolds CM, Trent MS, Bishop RE (2007) Lipid A modification systems in gram-negative bacteria. Annu Rev Biochem 76: 295-329.

49. Chang H, Gartner E, Rolfe BG (1995) Involvement of Genes on a Megaplasmid in the Organic Acid Resistance of Shiga toxin-producing Escherichia coli O157:H7. J Biol Chem 270: 31791-31799.

50. Morona R, Daniels C, Van Den Bosch L (2005) 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 73: 6139-6146.

51. Montoya R, Choudhury B, Septer A, Merighi M, Carlsson R, et al. (2005) Identification of qepA, a PmrA-regulated locus required for phosphoethanolamine modification of the Salmonella enterica serovar typhimurium lipopolysaccharide core. J Bacteriol 187: 3391-3399.

52. Perez JC, Grossman KA (2007) Acid pH activation of the PmrA/PmrB two-component regulatory system of Salmonella enterica. Mol Microbiol 63: 283-293.

53. Herrera CM, Hankins JV, Trent MS (2010) Activation of PmrA inhibits LpxT-dependent phosphorylation of lipid A promoting resistance to antimicrobial peptides. Mol Microbiol 76: 1444-1460.

54. Zhou Z, Lin S, Cotter RJ, Raetz CR (1999) Lipid A modifications characteristic of Salmonella enterica serovar Typhimurium. J Biol Chem 274: 18503-18514.

55. Chen H, Gartner E, Rolfe BG (1993) Involvement of genes on a megaplasmid in the organic acid resistance of Shiga toxin-producing Escherichia coli O157:H7. Mol Microbiol 7: 979-988.

56. Tamayo R, Choudhury B, Septer A, Merighi M, Carlsson R, et al. (2005) Identification of qepA, a PmrA-regulated locus required for phosphoethanolamine modification of the Salmonella enterica serovar typhimurium lipopolysaccharide core. J Bacteriol 187: 3391-3399.

57. Perez JC, Grossman KA (2007) Acid pH activation of the PmrA/PmrB two-component regulatory system of Salmonella enterica. Mol Microbiol 63: 283-293.

58. Herrera CM, Hankins JV, Trent MS (2010) Activation of PmrA inhibits LpxT-dependent phosphorylation of lipid A promoting resistance to antimicrobial peptides. Mol Microbiol 76: 1444-1460.

59. Touzé T, Tran AX, Hankins JV, Mengin-Lecreulx D, Trent MS (2008) Periplasmic phosphorylation of lipid A is linked to the synthesis of undecaprenyl phosphate. Mol Microbiol 67: 264-277.

60. Datsenko KA, Wanner BL (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.

61. Nesterenko PP, Wackernagel W (1995) Gene disruption in Escherichia coli: Tn5 and Km8 cassettes with the option of Fap-mediated excision of the antibiotic resistance determinant. Gene 158: 9-14.

62. Hitchcock PJ, Brown TM (1983) Morphological heterogeneity among Salmonella lipopolysaccharide chemotypes in silver-stained polyacrylamide gels. J Bacteriol 154: 269-277.

63. Lesse AF, Campagnari AA, Bittner WE, Apicella MA (1990) Increased resolution of lipopolysaccharides and lipopoligosaccharides utilizing tricine-sodium dodeyl sulfate-polyacrylamide gel electrophoresis. J Immunol Methods 126: 109-117.

64. Zhou Z, Lin S, Cotter RJ, Raetz CR (1999) Lipid A modifications characteristic of Salmonella enterica serovar Typhimurium. J Biol Chem 274: 18503-18514.

65. Carter JA, Blonder CJ, Zaltívar M, Alvarez SA, Marolda CL, et al. (2007) O-antigen modal chain length in Shigella flexneri 2a is growth-regulated through RfaH-mediated transcriptional control of the wzz gene. Microbiology 153: 3499-3507.

66. Bravo D, Silva C, Carter JA, Hoare A, Alvarez SA, et al. (2008) Growth-phase regulation of lipopolysaccharide O-antigen chain length influences serum resistance in serovars of Salmonella. J Med Microbiol 57: 938-946.

67. Daniell C, 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.