MICROBIOLOGY

Gene expression kinetics governs stimulus-specific decoration of the *Salmonella* outer membrane

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Lipid A is the innermost component of the lipopolysaccharide (LPS) molecules that occupy the outer leaflet of the outer membrane in Gram-negative bacteria. Lipid A is recognized by the host immune system and targeted by cationic antimicrobial compounds. In *Salmonella enterica* serovar Typhimurium, the phosphates of lipid A are chemically modified by enzymes encoded by targets of the transcriptional regulator PmrA. These modifications increase resistance to the cationic peptide antibiotic polymyxin B by reducing the negative charge of the LPS. We report the mechanism by which *Salmonella* produces different lipid A profiles when PmrA is activated by low Mg^{2+} versus a mildly acidic pH. Low Mg^{2+} favored modification of the lipid A phosphates with 4-amino-4-deoxy-L-aminoarabinose (L-Ara4N) by activating the regulatory protein PhoP, which initially increased the LPS negative charge by promoting transcription of *lpxT*, encoding an enzyme that adds an additional phosphate group to lipid A. Later, PhoP activated PmrA posttranslationally, resulting in expression of PmrA-activated genes, including those encoding the LpxT inhibitor PmrR and enzymes responsible for the incorporation of L-Ara4N. By contrast, a mildly acidic pH favored modification of the lipid A phosphates with a mixture of L-Ara4N and phosphoethanolamine (pEtN) by simultaneously inducing the PhoP-activated *lpxT* and PmrA-activated *pmrR* genes. Although L-Ara4N reduces the LPS negative charge more than does pEtN, modification of lipid A phosphates solely with L-Ara4N required a prior transient increase in lipid A negative charge. Our findings demonstrate how bacteria tailor their cell surface to different stresses, such as those faced inside phagocytes.

INTRODUCTION

Gram-negative bacteria are surrounded by an outer membrane that confers protection from various toxic agents (*1*). The outer membrane is composed of phospholipids in the inner leaflet and lipopolysaccharide (LPS) in the outer leaflet. The innermost portion of the LPS is lipid A, followed by a central core region and the outermost O-antigen (*2*, *3*). Phosphate residues in the lipid A and inner core are responsible for the negative charge of the LPS at neutral pH. This negative charge is normally neutralized by divalent cations, primarily Mg^{2+}. However, certain bacteria chemically modify their LPS in response to specific environmental signals (*2*, *4*), thereby altering permeability (*5*, *6*), resistance to antibacterial compounds (*7*–*10*), and activation of the immune system of host animals (*2*, *11*).

The Gram-negative bacterium *Salmonella enterica* serovar Typhimurium chemically modifies its lipid A phosphates with 4-amino-4-deoxy-L-aminoarabinose (L-Ara4N) and phosphoethanolamine (pEtN) (Fig. 1A) when experiencing conditions that activate PmrA, a transcriptional activator of genes encoding lipid A–modifying enzymes and an inhibitor of one such enzyme (*4*, *12*). PmrA decreases the negative charge of lipid A in several ways. First, it promotes transcription of the *pbgP* operon and the *ugd* gene, which are responsible for the modification of the lipid A phosphates with L-Ara4N, and of the *pmrC* gene, which mediates modification of the lipid A phosphates with pEtN (*9*, *13*) (*pbgP* is also referred to as *arnT* and *pmrK*; *pmrC* is also referred to as *eptA*). Second, PmrA directly activates expression of the *pmrR* gene, which encodes an inhibitor of LpxT (*12*), an enzyme responsible for the incorporation of an additional phosphate group at position 1 of lipid A (Fig. 1A) (*14*). Modification of the lipid A phosphates with L-Ara4N reduces the LPS negative charge more than does modification of the lipid A phosphates with pEtN. The lipid A phosphates are modified with both L-Ara4N and pEtN in *Salmonella* harvested from macrophages (*15*).

PmrA is a response regulator that functions with the sensor PmrB as a two-component system. PmrA is activated (phosphorylated) when its cognate sensor PmrB detects Fe^{3+} (*16*) or a mildly acidic pH (*17*) in the periplasm (Fig. 1B) or when the noncognate sensor PhoQ is activated by its specific signals such as low periplasmic Mg^{2+} (*18*, *19*). The latter activation entails PhoQ-mediated phosphorylation of its cognate response regulator PhoP (*20*), transcription of the PhoP-activated gene *pmrD* (*18*), and protection of phosphorylated PmrA by the PmrD protein (Fig. 1B) (*21*). Therefore, transcription of PmrA-activated genes is faster when the inducing signal is Fe^{3+} or a mildly acidic pH than when the inducing signal is low Mg^{2+} (Fig. 1, B and C) (*22*).

Modification of the lipid A phosphates with L-Ara4N and pEtN confers resistance to the cationic peptide antibiotic polymyxin B by reducing the negative charge of the lipid A (*7*, *13*). A *pmrA* null mutant is 10,000 times more sensitive to polymyxin B than is wild-type *Salmonella* (*16*). Polymyxins constitute a "last-resort" antibiotic against multidrug-resistant isolates of *Pseudomonas aeruginosa*, *Acinetobacter baumanii*, and *Klebsiella pneumoniae* (*23*) but are not used to treat *Salmonella* infections. However, the better understanding of the PmrA/PmrB and PhoP/PhoQ two-component systems uncovered in *Salmonella* and *Escherichia coli* (*4*, *24*) has provided a framework to understand the molecular basis for resistance to polymyxins in clinical isolates of other species where these systems have not been as thoroughly investigated (*25*).

Here, we provide a molecular explanation for how wild-type *Salmonella* decorates its lipid A phosphates with different chemicals depending on the signal that activates the PhoP and PmrA proteins (*16*–*18*). We establish that the different lipid A profiles reflect the

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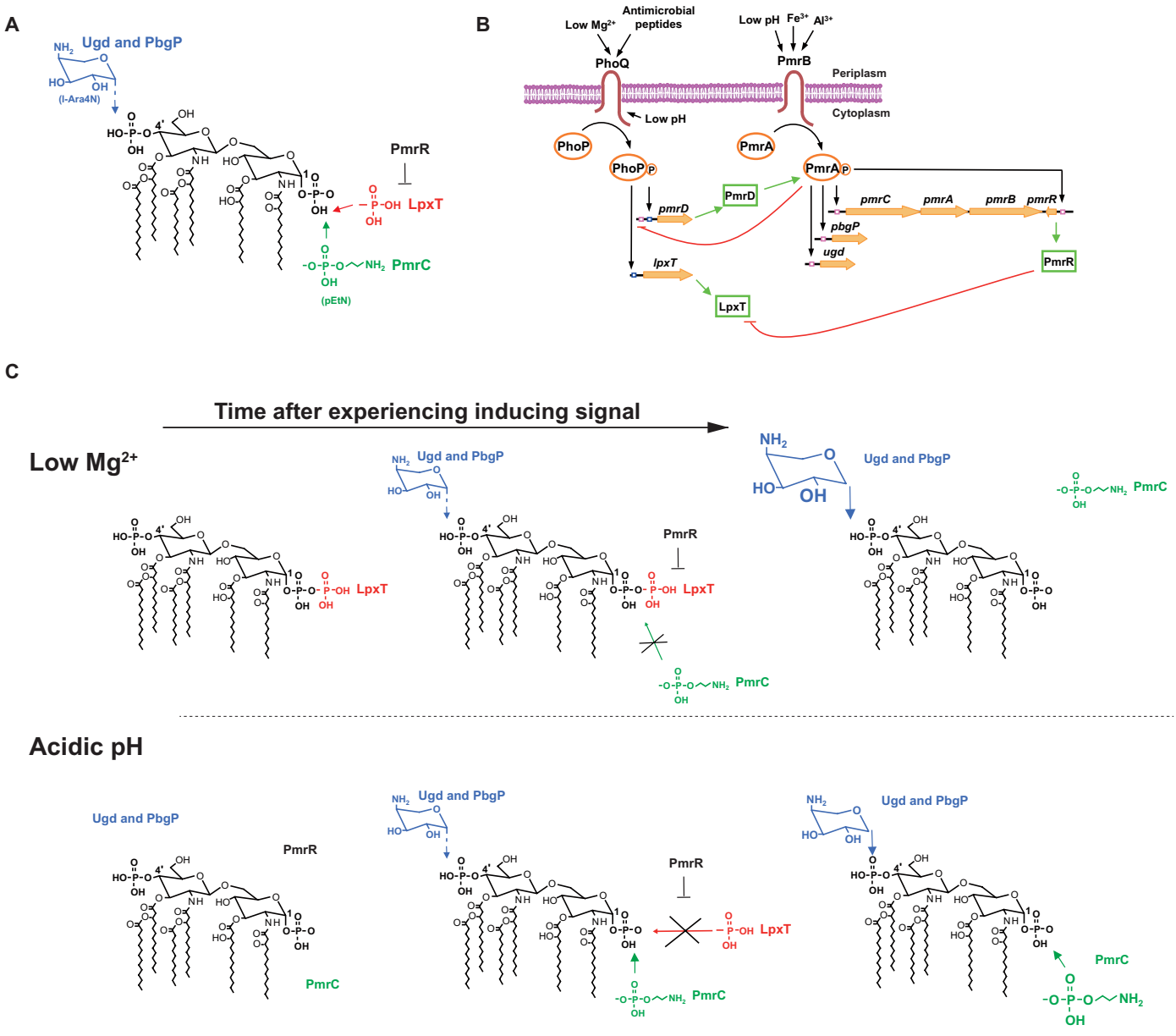

Fig. 1. Signal-dependent control of lipid A modifications. (**A**) Schematic representation of the lipid A moiety of the LPS and the lipid A modifications that alter its negative charge. In *Salmonella*, the predominant lipid A species is hexa-acylated and phosphorylated at the 1 and 4′ positions. LpxT adds a second phosphate group to the 1 position, resulting in a 1-diphosphorylated (1-PP) species. Alternatively, the lipid A 1-phosphate can be modified with pEtN by PmrC. The 4′-phosphate can be modified with L-Ara4N by Ugd and PbgP. Alternatively, but to a lesser extent, the 4′-phosphate may be modified by the addition of pEtN and the 1-phosphate modified by the addition of L-Ara4N. PmrR binds to LpxT, inhibiting its activity. (**B**) Model for activation of the PhoP/PhoQ and PmrA/PmrB two-component systems and regulation by the PhoP and PmrA proteins. Transcription of PhoP-activated genes is promoted when the sensor PhoQ experiences low cytoplasmic pH (*31*), low periplasmic Mg^{2+}, or the presence of particular antimicrobial peptides in the periplasm. Transcription of PmrA-activated genes is promoted when the sensor PmrB experiences Fe^{3+}, Al^{3+}, or a mildly acidic pH in the periplasm (*4*). PmrA is also activated in low Mg^{2+} by the PhoP-activated *pmrD* gene product. Phosphorylated PhoP promotes transcription of the *pmrD* and *lpxT* genes. Phosphorylated PmrA promotes transcription of the *pbgP*, *pmrC*, *pmrR*, and *ugd* genes. The *pbgP*, *ugd*, and *pmrC* genes encode enzymes that compete with LpxT for the modification of the lipid A 1-phosphate. The lipid A modifications catalyzed by PbgP, Ugd, and PmrC reduce the negative charge of lipid A. (**C**) Schematic of the lipid A structure over time after bacteria encounter low Mg^{2+} or a mildly acidic pH. Under low Mg^{2+}, *lpxT* is activated by PhoP within 5 min, resulting in 1-PP lipid A. The additional phosphate group at the 1-phosphate prevents the addition of pEtN at this position. By 30 min, lipid A is preferentially modified at the 4′-phosphate with L-Ara4N. PmrR later inhibits LpxT, thereby preventing the incorporation of a phosphate at the 1 position. pEtN is not detected at later times despite inhibition of LpxT by PmrR, suggesting that L-Ara4N–modified lipid A is not a good substrate for PmrC. In a mildly acidic pH, transcription of *lpxT* gene occurs after that of genes encoding Ugd, PbgP, PmrC, and PmrR, resulting in lipid A modified with both L-Ara4N and pEtN.

distinct expression kinetics of the genes that encode lipid A–modifying proteins when the inducing signals act on PmrB versus PhoQ. Unexpectedly, the strongest reduction in lipid A negative charge requires a prior increase in its negative charge. Our data also suggest that environmental signals control a broad array of bacterial cellular structures by altering the abundance of undecaprenyl phosphate, an essential

lipid molecule that transports sugar substrates for incorporation into various cell envelope structures.

RESULTS

PhoP promotes transcription of the *lpxT* gene in low Mg^{2+}

The PhoP/PhoQ and PmrA/PmrB two-component systems are the major activators of lipid A modifications in Gram-negative bacteria (*2*). Lipid A modifications that increase the resistance of *Salmonella* to polymyxin B reduce the negative charge of lipid A; however, all the lipid A–modifying proteins encoded by genes that are activated by PhoP or PmrA, or both, that have been described in the literature either reduce or do not alter the negative charge of lipid A (*2*). Therefore, we hypothesized that PhoP or PmrA, or both, may repress *lpxT* expression because the LpxT protein increases lipid A's negative charge (*14*). Unexpectedly, we found that the abundance of *lpxT* mRNA in wild-type *Salmonella* was 10 times higher after growth in low Mg^{2+} (10 μM) than in high Mg^{2+} (10 mM) (Fig. 2A), which are inducing and repressing conditions, respectively, for both the PhoP/PhoQ (*24*) and PmrA/PmrB systems (Fig. 1B) (*4*). In agreement with the mRNA data, the abundance of the LpxT protein also increased in low Mg^{2+} compared to high Mg^{2+} (fig. S1). Transcriptional activation of the *lpxT* gene in low Mg^{2+} was not observed in a *phoP* single mutant or a *phoP pmrA* double mutant; by contrast, it was normal in a *pmrA* single mutant (Fig. 2A). These results indicate that low Mg^{2+} promoted *lpxT* transcription in a *phoP*-dependent but *pmrA*-independent manner.

PhoP binding to the *lpxT* promoter is required for *lpxT* transcriptional activation

According to *Salmonella* genome annotations, the *lpxT* start codon is located 35 nucleotides (nt) downstream of the *yeiR* stop codon (Fig. 2B). The short intergenic distance between *yeiR* and *lpxT* suggested that these genes form an operon and that PhoP promotes transcription of both *yeiR* and *lpxT*. However, *yeiR* mRNA abundance was not altered upon a switch from high to low Mg^{2+} medium (fig. S2). These results argue that separate promoters transcribe the *Salmonella yeiR* and *lpxT* genes under PhoP-inducing conditions, as proposed for the *E. coli* homologs of these genes (*10*).

We mapped the *lpxT* TSS to a G residue 25 nt upstream of the *lpxT* start codon in wild-type *Salmonella* grown in low Mg^{2+} (Fig. 2C). No transcript was detected in cultures of wild-type *Salmonella* grown in high Mg^{2+} or in a *phoP* mutant grown in low Mg^{2+} (Fig. 2C). These results agree with the *lpxT* expression behavior of a different *Salmonella* strain grown under a different PhoP-inducing condition (*26*). Moreover, they support the notion that wild-type *Salmonella* activates *lpxT* transcription during growth in low Mg^{2+} in a *phoP*-dependent manner and independently of transcription of the *yeiR* gene.

We identified a putative PhoP binding site within the *yeiR* coding region (Fig. 2B). This site is a bona fide PhoP binding site because DNase I footprinting analysis demonstrated that phosphorylated PhoP, which is the active form of the PhoP protein (*27*), protects nucleotides −47 to −19 relative to the TSS, overlapping the predicted PhoP binding site (Fig. 2D) (*28*). In agreement with the notion that PhoP activates *lpxT* transcription directly (that is, by binding to the *lpxT* promoter), substitution of 3 nt of the PhoP binding site markedly decreased binding of the PhoP protein to a DNA fragment harboring the *lpxT* promoter (Fig. 2E). Furthermore, an engineered strain with the 3-nt substitutions in the chromosomal copy of the PhoP binding site in the *lpxT* promoter failed to increase the abundance of *lpxT* mRNA in low Mg^{2+} (Fig. 2F), behaving like the *phoP* null mutant (Fig. 2F). A separate engineered strain with a different 3-nt substitution in the PhoP binding site of the *lpxT* promoter ($lpxT^{A}$) also failed to increase *lpxT* mRNA amounts in low Mg^{2+} (fig. S3).

Collectively, the results described in this section establish that PhoP directly promotes *lpxT* transcription when *Salmonella* experiences low Mg^{2+}. This result is paradoxical because low Mg^{2+} also promotes expression of the *pmrR* gene, which encodes an LpxT inhibitor, through the stabilization of the PmrA protein by the PhoP transcriptional target PmrD (Fig. 1B) (*12*). Next, we explored the phenotypic consequences of PhoP promoting the transcription of both *lpxT* and *pmrR* by examining the kinetics with which the mRNAs corresponding to lipid A phosphate–modifying genes are produced, as well as their impact on the resulting lipid A profiles.

The *lpxT* gene is transcribed before other lipid A phosphate–modifying genes when the inducing signal is low Mg^{2+}

We analyzed the abundance of *lpxT*, *pbgP*, *pmrC*, *pmrR*, and *ugd* transcripts at different times after wild-type *Salmonella* was switched from noninducing (high Mg^{2+}) to inducing (low Mg^{2+}) conditions. The *lpxT* transcript reached a maximum within 5 min of the shift and then decreased, eventually reaching preinduction values by 120 min (Fig. 3). This behavior differed from the expression of the *pmrR*, *pbgP*, *pmrC*, and *ugd* genes, which peaked between 20 and 60 min and retained >50% of the maximum values at 120 min (Fig. 3).

We reasoned that *lpxT* transcription precedes that of *pbgP*, *pmrC*, *pmrR*, and *ugd* in wild-type *Salmonella* experiencing low Mg^{2+} because PhoP activates the *lpxT* promoter directly (Fig. 2) but acts on the *pbgP*, *pmrC*, *pmrR*, and *ugd* genes indirectly by increasing transcription from the *pmrD* promoter, resulting in synthesis of the PmrD protein, the protector of phosphorylated PmrA (Fig. 1B) (*18*). In support of this notion, *pmrD* mRNA amounts increased at the same time as those of *lpxT*, preceding the increase in *pbgP*, *pmrC*, *pmrR*, and *ugd* mRNAs (Fig. 3). Thus, *lpxT* is transcribed before other genes that encode proteins that target the same lipid A position and before the LpxT inhibitor–encoding gene *pmrR*.

PhoP-dependent transcription of *lpxT* favors lipid A modification with L-Ara4N during growth in low Mg^{2+}

The rapid and transient *lpxT* expression exhibited by *Salmonella* experiencing low Mg^{2+} (Fig. 3) resulted in a rapid and transient presence of two phosphates at position 1 of lipid A (1-PP), the addition of which is catalyzed by LpxT (*14*). We detected the presence of radiolabeled 1-PP lipid A within 10 min of switching wild-type *Salmonella* from high to low Mg^{2+} (Fig. 4A) in the presence of radiolabeled phosphate. The abundance of 1-PP lipid A then decreased, and 1-PP lipid A was no longer observed by 1 hour (fig. S4).

The L-Ara4N modification was first detected at 20 min after the switch to low Mg^{2+} (Fig. 4A). By 1 hour, the lipid A population contained combinations of L-Ara4N with other modifications including 2-OH myristation or palmitation, but not pEtN (fig. S4). Thus, the switch to low Mg^{2+} resulted in the transient production of 1-PP lipid A, which decreased in abundance and then disappeared as the L-Ara4N modification was detected. These results suggest that the transient increase in 1-PP lipid A taking place shortly after the switch to low Mg^{2+} favors further modification with L-Ara4N over modification with pEtN.

If the transient increase in 1-PP lipid A mediated by LpxT is required for the subsequent lipid A decoration with L-Ara4N and no

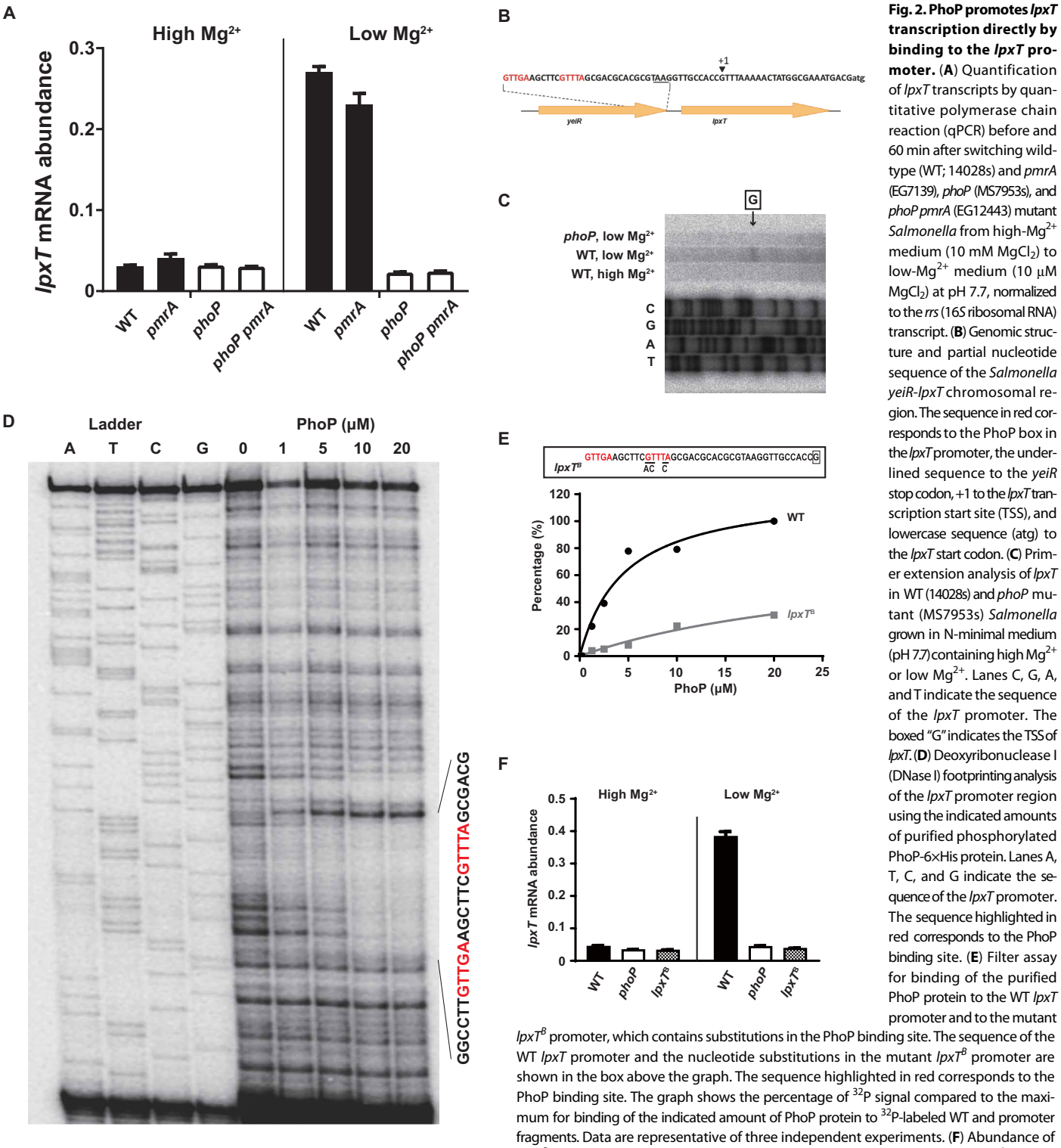

Fig. 2. PhoP promotes *lpxT* transcription directly by binding to the *lpxT* promoter. (**A**) Quantification of *lpxT* transcripts by quantitative polymerase chain reaction (qPCR) before and 60 min after switching wild-type (WT; 14028s) and *pmrA* (EG7139), *phoP* (MS7953s), and *phoP pmrA* (EG12443) mutant *Salmonella* from high-Mg^{2+} medium (10 mM $MgCl_2$) to low-Mg^{2+} medium (10 μM $MgCl_2$) at pH 7.7, normalized to the *rrs* (16*S* ribosomal RNA) transcript. (**B**) Genomic structure and partial nucleotide sequence of the *Salmonella yeiR-lpxT* chromosomal region. The sequence in red corresponds to the PhoP box in the *lpxT* promoter, the underlined sequence to the *yeiR* stop codon, +1 to the *lpxT* transcription start site (TSS), and lowercase sequence (atg) to the *lpxT* start codon. (**C**) Primer extension analysis of *lpxT* in WT (14028s) and *phoP* mutant (MS7953s) *Salmonella* grown in N-minimal medium (pH 7.7) containing high Mg^{2+} or low Mg^{2+}. Lanes C, G, A, and T indicate the sequence of the *lpxT* promoter. The boxed "G" indicates the TSS of *lpxT*. (**D**) Deoxyribonuclease I (DNase I) footprinting analysis of the *lpxT* promoter region using the indicated amounts of purified phosphorylated PhoP-6xHis protein. Lanes A, T, C, and G indicate the sequence of the *lpxT* promoter. The sequence highlighted in red corresponds to the PhoP binding site. (**E**) Filter assay for binding of the purified PhoP protein to the WT *lpxT* promoter and to the mutant $lpxT^B$ promoter, which contains substitutions in the PhoP binding site. The sequence of the WT *lpxT* promoter and the nucleotide substitutions in the mutant $lpxT^B$ promoter are shown in the box above the graph. The sequence highlighted in red corresponds to the PhoP binding site. The graph shows the percentage of ^{32}P signal compared to the maximum for binding of the indicated amount of PhoP protein to ^{32}P-labeled WT and promoter fragments. Data are representative of three independent experiments. (**F**) Abundance of *lpxT* transcript before and 60 min after switching WT (14028s), *phoP* (MS7953s), and $lpxT^B$ (XH16) *Salmonella* from N-minimal medium (pH 7.7) containing high Mg^{2+} to low Mg^{2+}, normalized to the *rrs* transcript. Mean and SD of three independent experiments are shown.

pEtN, an *lpxT* mutant should have both L-Ara4N and pEtN in its lipid A. As predicted, the lipid A of the *lpxT* mutant was undermodified with the L-Ara4N but hypermodified with various double modifications of L-Ara4N and pEtN (Fig. 4B). The lipid A identities were verified by mass spectrometry (table S1). Moreover, a *pbgP* single mutant had little pEtN in its lipid A (Fig. 4C) despite its inability to produce L-Ara4N (*8*). By contrast, pEtN was the dominant modification in the lipid A of the isogenic *pbgP lpxT* double mutant (Fig. 4C), reinforcing

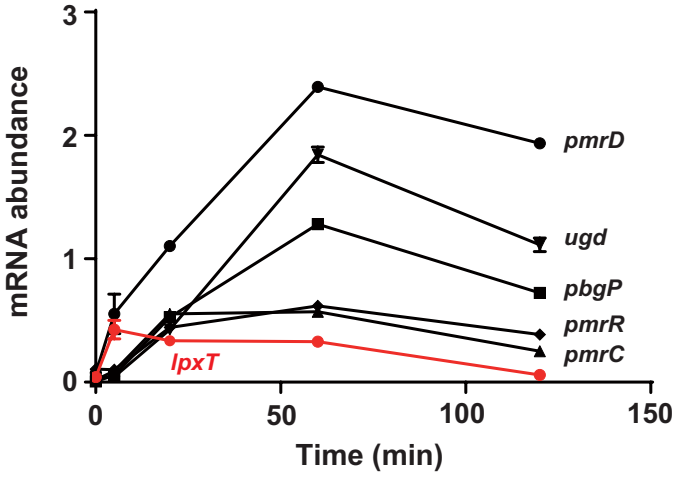

Fig. 3. The *lpxT* gene is expressed earlier relative to other lipid A–modifying genes in low Mg^{2+}. Quantification of the indicated transcripts at different times after switching WT *Salmonella* (14028s) from high Mg^{2+} to low Mg^{2+} at pH 7.7, normalized to the *rrs* transcript. Data are means ± SD of three independent experiments.

the notion that the addition of a phosphate group to lipid A by LpxT hinders the incorporation of pEtN into lipid A even in organisms that cannot make L-Ara4N. In agreement with this notion, wild-type and *pmrC* mutant *Salmonella* exhibited similar lipid A profiles during growth in low Mg^{2+} (fig. S5). Furthermore, disruption of both *pmrA* and *lpxT* abolished all L-Ara4N, pEtN, and 1-PP modifications (fig. S6). Cumulatively, these results indicate that LpxT promotes modification of lipid A with a single L-Ara4N over pEtN or double modification with both L-Ara4N and pEtN.

Because the *lpxT* gene is transcribed even in a *phoP* mutant (Fig. 2A), albeit to a lesser extent than in wild-type *Salmonella*, we wondered whether the increased *lpxT* transcription promoted in low Mg^{2+} by the PhoP protein was responsible for the L-Ara4N–modified lipid A (Fig. 4). Thus, we examined the lipid A profile of the *lpxT* promoter mutant ($lpxT^B$) that is refractory to PhoP activation (Fig. 2F). The use of this mutant avoids potentially confusing effects resulting from the pleiotropic effects of *phoP* inactivation, such as the inability to activate the PmrA protein in low Mg^{2+} (Fig. 1B) (*18*).

The $lpxT^B$ promoter mutant harbored less lipid A species with L-Ara4N and more lipid A species doubly modified with L-Ara4N and pEtN compared to the wild-type strain (Fig. 4B). This phenotype is specific to the PhoP-inducing condition of low Mg^{2+} because the $lpxT^B$ mutant

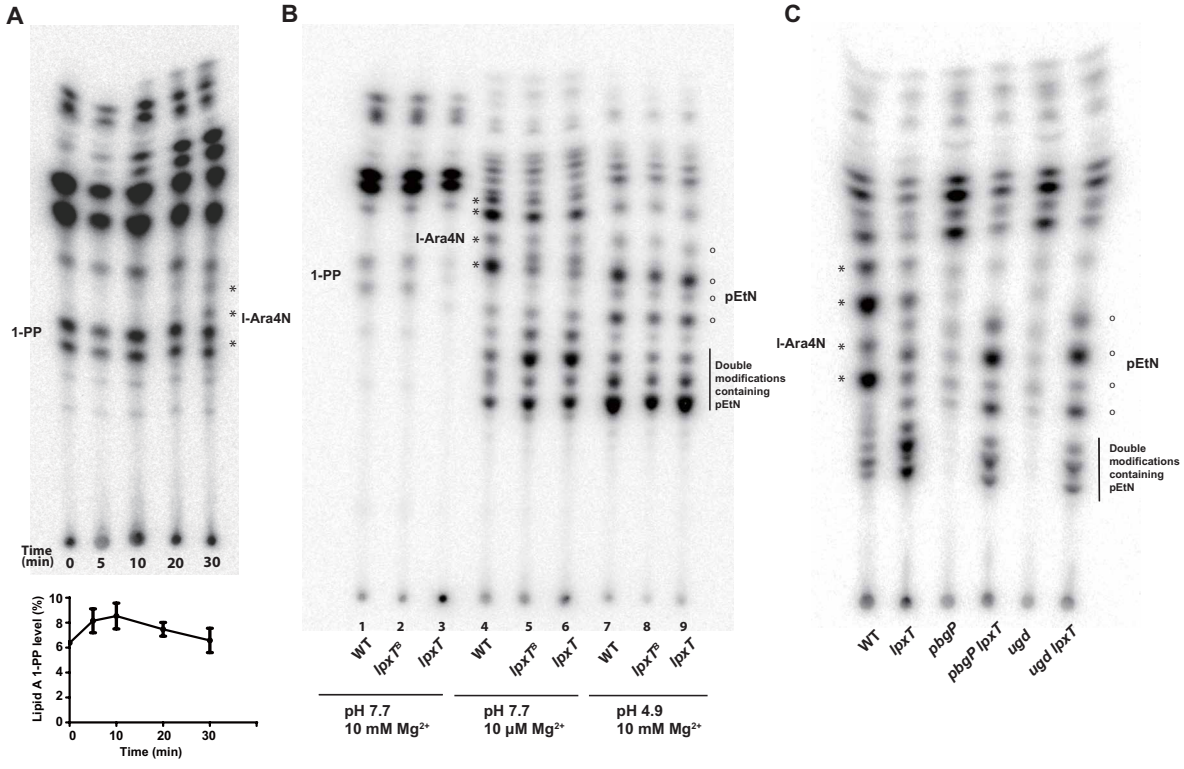

Fig. 4. PhoP-dependent *lpxT* expression promotes lipid A modification with L-Ara4N in low Mg^{2+} but not in a mildly acidic pH. (**A**) Autoradiogram of a thin-layer chromatography (TLC) plate showing lipid A from WT *Salmonella* (14028s) at different times after they were switched from N-minimal medium (pH 7.7) containing high Mg^{2+} to low Mg^{2+} in the presence of radiolabeled phosphate. Data are representative of three independent experiments. The graph below the autoradiogram shows the quantification of lipid A 1-PP from the image, representing mean and SD (normalized to the total signal of each lane). (**B**) TLC showing lipid A from WT (14028s), $lpxT^B$ (XH16), and *lpxT* (DC72) *Salmonella* grown in N-minimal medium (pH 7.7) containing high Mg^{2+}, N-minimal medium (pH 7.7) containing low Mg^{2+}, or N-minimal medium (pH 4.9) containing high Mg^{2+} in the presence of radiolabeled phosphate. (**C**) TLC showing lipid A from WT (14028s), *lpxT* (DC72), *pbgP* (EG9241), *pbgP lpxT* (XH246), *ugd* (EG17898), and *ugd lpxT* (XH251) *Salmonella* grown in N-minimal medium (pH 7.7) containing high Mg^{2+} or low Mg^{2+}. Data are representative of two independent experiments. The 1-PP band shows different mobility in each of the images because the samples in each panel were run for different amounts of time. Asterisks (*) indicate positions of lipid A containing L-Ara4N, and open circles (o) indicate positions of lipid A containing pEtN.

retained normal 1-PP modification after growth under the noninducing high Mg^{2+} condition (Fig. 4B). The latter result indicated that the *lpxT* gene itself was functioning properly in the *lpxT* promoter mutant ($lpxT^B$). That is, the inability of the *lpxT* mutant promoter to respond to PhoP did not compromise *lpxT* expression under noninducing conditions for PhoP. Moreover, when grown in low Mg^{2+}, the *pmrC* single mutant and the *pmrC* $lpxT^B$ double mutant displayed the same lipid A profile containing L-Ara4N and no pEtN (fig. S5). These results established that PhoP activation of *lpxT* transcription is required for normal lipid A modification with L-Ara4N.

Similar transcription timing of *lpxT* and other lipid A modification genes results in lipid A with pEtN during growth in a mildly acidic pH

A mildly acidic pH activates both PmrB and PhoQ, resulting in transcription of PmrA- and PhoP-activated genes, respectively (Fig. 1B) (*17*, *29*, *30*). However, these sensors differ in that PmrB responds to a mildly acidic pH through protonation of periplasmic histidine residues (*17*), whereas PhoQ does so through its cytoplasmic region through a mechanism that does not involve histidine residues (*31*). In addition, full activation of the PhoP/PhoQ system under a mildly acidic pH requires the PhoP-activated gene *ugtL*, which encodes a protein that stimulates PhoQ autophosphorylation (*30*).

We reasoned that the kinetics with which a mildly acidic pH induces PmrA- and PhoP-activated genes might differ from that taking place in low Mg^{2+} because low Mg^{2+} activation requires the PhoP-dependent transcription of the *pmrD* gene (*18*), which specifies a protein that protects phosphorylated PmrA from dephosphorylation by its cognate sensor PmrB (*21*), and results in transcription of genes directly activated by PmrA. By contrast, PmrB-mediated activation of PmrA by a mildly acidic pH does not require PhoP or PmrD (Fig. 1B). That is, when the inducing signal is a mildly acidic pH, the PhoP-dependent *lpxT* transcription may not precede that of PmrA-activated genes, as observed in low Mg^{2+}.

When wild-type *Salmonella* was shifted from noninducing conditions to medium with a mildly acidic pH, the mRNA amounts of the PmrA-activated genes *pbgP*, *pmrC*, and *pmrR* reached a maximum within 5 min (Fig. 5A). By contrast, *lpxT* mRNA amounts reached a maximum at 10 min after the shift (Fig. 5A), raising the possibility that PmrR may be available to readily inhibit LpxT activity when the inducing condition is a mildly acidic pH. If true, the lipid A profile of wild-type *Salmonella* grown in a mildly acidic pH should no longer display the *lpxT*-dependent accumulation of L-Ara4N observed when the inducing signal was low Mg^{2+} (Fig. 4). As predicted, wild-type *Salmonella*, the *lpxT* promoter mutant, and the *lpxT* deletion mutant produced identical lipid A profiles with an abundance of pEtN-modified lipid A when grown in a mildly acidic pH (Fig. 4B). This result indicates that LpxT is not actively adding phosphate to lipid A when the inducing signal is a mildly acidic pH, resulting in a lipid A modified with both pEtN and L-Ara4N.

A mildly acidic pH promotes greater polymyxin B resistance than does low Mg^{2+}

Genetic experiments previously revealed that *Salmonella* mutants defective in the *pbgP* or *ugd* genes, which fail to modify lipid A with L-Ara4N, are 100 times more sensitive to polymyxin B than are wild-type *Salmonella* after growth in low Mg^{2+} (*9*). By contrast, a *pmrC* mutant, which is defective in lipid A modification with pEtN, displayed marginally increased polymyxin B sensitivity compared to wild-type *Salmonella* (*9*). We have recapitulated these results (fig. S7) and determined that the *lpxT* null mutant was slightly more resistant to polymyxin B than was wild-type *Salmonella* (fig. S7).

We hypothesized that growth in low Mg^{2+} enhanced polymyxin B resistance compared to growth in a mildly acidic pH because the lipid A of wild-type *Salmonella* grown in low Mg^{2+} was modified with L-Ara4N and no pEtN (Fig. 4B) and also because inactivation of the genes required for lipid A modification with L-Ara4N decrease polymyxin B resistance much more than does inactivation of the *pmrC* gene (fig. S7). Unexpectedly, survival of wild-type *Salmonella* exposed to polymxyin B was seven times higher after growth in a mildly acidic pH than it was in low Mg^{2+} (Fig. 5B). As predicted from the results

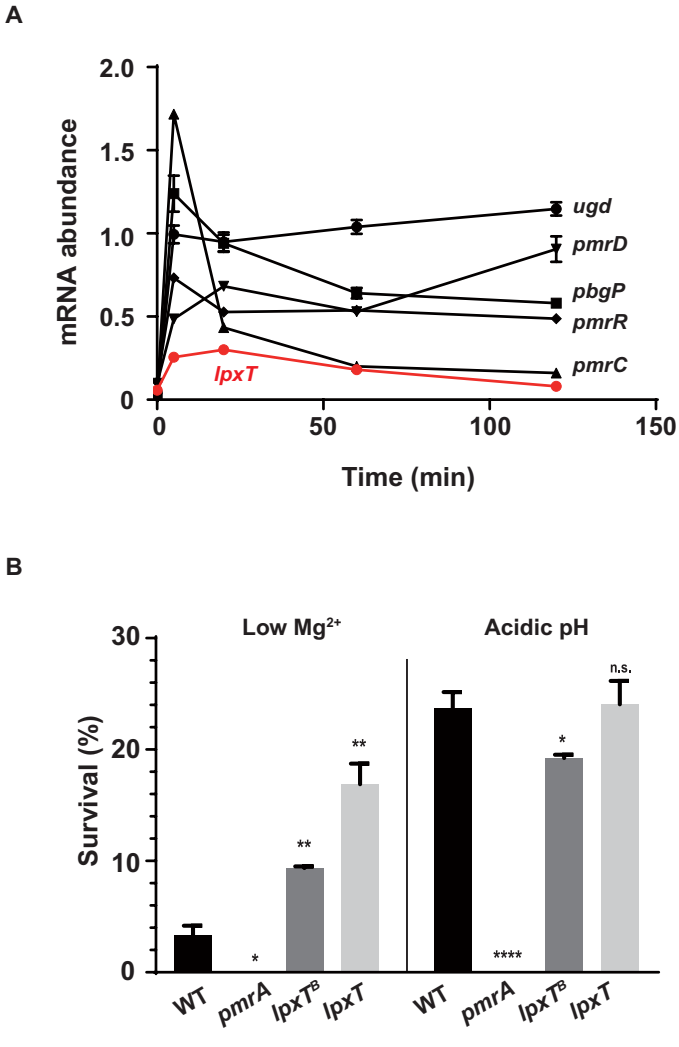

Fig. 5. Effects of low Mg^{2+}, acidic pH, and PhoP-dependent transcription of the *lpxT* gene on polymyxin B resistance. (**A**) Quantification of the indicated transcripts at different times after switching WT *Salmonella* (14028s) from high Mg^{2+} at pH 7.7 to high Mg^{2+} at pH 4.9, normalized to the *rrs* transcript. Data are means ± SD of three independent experiments. (**B**) Percent survival of WT (14028s), *pmrA* (EG7139), $lpxT^B$ (XH16), and *lpxT* (DC72) *Salmonella* exposed to polymyxin B after growth in N-minimal medium (pH 7.7) with low Mg^{2+} or that (pH 4.9) with high Mg^{2+} (acidic pH). Survival values were calculated as relative to the original inoculum. Mean and SD of three independent experiments are shown. Unpaired Student's *t* tests were performed comparing mutant strains with the WT under the same growth condition. *$P < 0.05$, **$P < 0.01$, and ****$P < 0.0001$; n.s. indicates $P > 0.05$ (not significant).

presented in the previous section (Fig. 4, B and C), the *lpxT* promoter mutant and the *lpxT* null mutant exhibited similar polymyxin B resistance as did the wild-type strain when the inducing condition was a mildly acidic pH (Fig. 5B). By contrast, the *lpxT* promoter mutant was more resistant to polymxyin B than was the wild-type strain, and the *lpxT* null mutant was more resistant than the *lpxT* promoter mutant when the inducing condition was low Mg^{2+} (Fig. 5B). Control experiments demonstrated that the *pmrA* mutant is several orders of magnitude more sensitive than the wild-type strain regardless of the inducing condition (Fig. 5B), in agreement with previous results (*18*). In summary, *Salmonella* survival to polymxyin B is higher when its lipid A is modified with both L-Ara4N and pEtN compared to modification with only L-Ara4N (Fig. 6).

PhoP-dependent activation of *lpxT* is conserved in a subset of Gram-negative species

A wide range of Gram-negative species have the ability to modify their lipid A phosphates. However, the specific chemical modification and the regulation of the genes responsible for such modifications vary among species. For example, *Yersinia pestis* differs from *Salmonella* in lacking a *pmrC* gene and in its *pbgP* promoter being directly activated by both the PhoP and PmrA proteins (*32*). In *P. aeruginosa*, the LpxT enzyme can transfer an additional phosphate to lipid A (*33*), and the *pmrC* gene is activated by the ColR/ColS two-component system but not by PmrA/PmrB (*34*).

To determine whether the PhoP-mediated activation of *lpxT* transcription identified in *Salmonella* (Fig. 2) is conserved in other Gram-negative species, we examined bacterial genomes for sequences related to the *Salmonella lpxT*, *pbgP*, *phoP*, and *pmrC* genes, as well as for sequences resembling a PhoP binding site in the *lpxT* promoter (table S2). *Citrobacter freundii*, *K. pneumoniae*, and *E. coli* harbor a predicted PhoP binding site upstream of the *lpxT* coding region, which is highly conserved with the one in the *Salmonella lpxT* promoter (Fig. 6A). This suggests that *lpxT* transcription is promoted in a *phoP*-dependent manner in these species as well. In addition, a 10-nt stretch located four bases upstream of the *lpxT* TSS is fully conserved in the four species, suggesting that it is the binding site for an as-yet-unidentified regulatory molecule that controls *lpxT* expression. By contrast, *Yersinia* and *Pseudomonas*, species in which the *pmrC* gene is either absent or not regulated by the PmrA/PmrB system, lack sequences resembling a PhoP binding site in their *lpxT* promoter regions. This analysis suggests a correlation between PhoP activation of *lpxT* transcription and the presence of a PmrA-activated *pmrC* gene.

We determined that *lpxT* mRNA abundances were threefold higher in wild-type *E. coli* grown in low Mg^{2+} than in those grown in high Mg^{2+} (Fig. 6B). By contrast, an *E. coli phoP* null mutant displayed similarly low amounts of *lpxT* mRNA under both inducing and repressing conditions (Fig. 6B). Although the expression behavior of the *E. coli lpxT* gene was qualitatively similar to that of the *Salmonella lpxT* homolog, the threefold induction exhibited by the *E. coli lpxT* gene was much lower (Fig. 6B) than the 10-fold increase exhibited by the *Salmonella lpxT* gene (Fig. 2A) under identical inducing conditions. Given that the *E. coli* PmrD protein is defective in activating the PmrA protein (*35*), the lower induction of the *lpxT* gene of *E. coli* might reflect the decreased need to activate the *lpxT* gene ahead of *pmrR* when the inducing condition is low Mg^{2+}.

DISCUSSION

We have established that differences in the expression kinetics of lipid A–modifying genes are responsible for the distinct lipid A profiles exhibited by *Salmonella* experiencing different inducing conditions for these genes. That is, both low Mg^{2+} and a mildly acidic pH activate the PhoP/PhoQ and PmrA/PmrB systems, causing transcription of their target genes (Figs. 2 and 5). However, these two inducing conditions give rise to different lipid A modifications because transcription of the *lpxT* gene precedes that of other lipid A modification genes including *pbgP*, *pmrC*, and *ugd*, as well as *pmrR*, which encodes an inhibitor of LpxT when the inducing signal is low Mg^{2+} but not when it is a mildly acidic pH (Figs. 2 and 5). PhoP-activated *lpxT* transcription is necessary for lipid A modification with L-Ara4N and no pEtN because preventing this activation results in a lipid A modified with both L-Ara4N and pEtN (Fig. 4B). Given that LpxT is responsible for the incorporation of an additional phosphate into lipid A (*14*, *36*), our results indicate that, paradoxically, a decrease in lipid A negative charge resulting from covalent lipid A modification with L-Ara4N requires a previous lipid A modification that increases its negative charge.

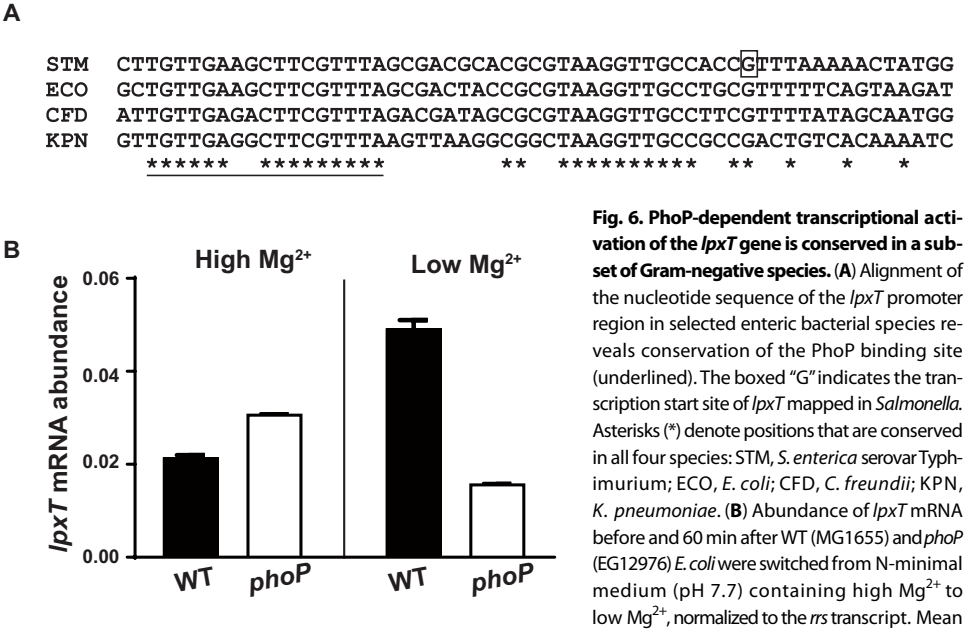

Fig. 6. PhoP-dependent transcriptional activation of the *lpxT* gene is conserved in a subset of Gram-negative species. (**A**) Alignment of the nucleotide sequence of the *lpxT* promoter region in selected enteric bacterial species reveals conservation of the PhoP binding site (underlined). The boxed "G" indicates the transcription start site of *lpxT* mapped in *Salmonella*. Asterisks (*) denote positions that are conserved in all four species: STM, *S. enterica* serovar Typhimurium; ECO, *E. coli*; CFD, *C. freundii*; KPN, *K. pneumoniae*. (**B**) Abundance of *lpxT* mRNA before and 60 min after WT (MG1655) and *phoP* (EG12976) *E. coli* were switched from N-minimal medium (pH 7.7) containing high Mg^{2+} to low Mg^{2+}, normalized to the *rrs* transcript. Mean and SD of three independent experiments are shown.

The expression kinetics of lipid A phosphate modification genes determines the distinct chemical modifications of the lipid A phosphates

When *Salmonella* experiences low Mg^{2+}, the PhoP/PhoQ system is activated before the PmrA/PmrB system because, under this inducing condition, PmrA activation is dependent on the PhoP-activated *pmrD*

gene (Fig. 1B) (*18*). By being directly activated by the PhoP protein (Fig. 2), *lpxT* is expressed before the lipid A–modifying genes that are directly controlled by the PmrA protein (Fig. 3), most critically *pmrR*, which specifies an LpxT inhibitor (*12*). Therefore, PhoP-dependent *lpxT* expression induced in low Mg^{2+} results in 1-PP lipid A (Fig. 4A), which favors further modification of lipid A phosphates with L-Ara4N and the exclusion of pEtN (Fig. 4B).

By contrast, when the activation signal is a mildly acidic pH, PmrA/PmrB activation does not lag behind that of PhoP/PhoQ (Fig. 1B). This is because PmrB detects a mildly acidic pH in the periplasm (*17*) and does not depend on the PmrD protein to activate PmrA. In addition, PhoQ responds to mild acidification through its cytoplasmic domain (*31*). Thus, the early *pmrR* expression taking place under a mildly acidic pH, which results in the production of the PmrR protein, is anticipated to decrease LpxT activity, resulting in a lipid A with both L-Ara4N and pEtN modifications (Fig. 4B). That is, the *lpxT* gene is expressed in a mildly acidic pH (Fig. 5A), but the LpxT protein is inhibited by PmrR (*12*).

In agreement with the model presented above (Fig. 1B), mutants lacking the *lpxT* gene or with nucleotide substitutions in the *lpxT* promoter that render it refractory to activation by PhoP behave like wild-type *Salmonella* in a mildly acidic pH but show different behaviors when the inducing signal is low Mg^{2+} (Figs. 4B and 5B). Our findings raise the possibility of an analogous mechanism being responsible for lipid A deacylation by the PhoP-activated PagL protein occurring only when modification with L-Ara4N and pEtN is prevented (*37*).

Transcription kinetics governs critical cellular processes

Our results highlight the fundamental role that gene expression timing plays in physiological responses to stress conditions. Low Mg^{2+} and a mildly acidic pH promote different lipid A profiles (Fig. 4B) despite stimulating the expression of the same set of lipid A modification genes (Figs. 2 and 5). This is not the only example of expression timing, rather than simply the expression or nonexpression of specific genes at steady-state, dictating phenotypic outcomes. For example, a surge in the transcription of *phoP* is necessary to jump-start *Salmonella*'s virulence program (*20*). This surge requires PhoP to positively feedback on its own transcription because a strain constitutively expressing PhoP is attenuated for virulence despite achieving the same steady-state abundance of PhoP-activated transcripts (*20*). Likewise, the increased amounts of phosphorylated PhoP generated after several hours in low Mg^{2+} enable *Salmonella* to transcribe PhoP-dependent genes that require high amounts of active PhoP protein (*38*). That is, the increased amounts of phosphorylated PhoP in this case depend on the Mg^{2+} importer MgtA removing Mg^{2+} away from the periplasmic Mg^{2+}-sensing domain of the sensor PhoQ (*38*). Because transcription elongation into the *mgtA* coding regions is triggered in low cytoplasmic Mg^{2+} (*39*), expression of a subset of PhoP-activated genes takes place only when the bacterium experiences the signals promoting *mgtA* expression. Furthermore, the stimulation of PmrB by Fe^{3+} activates PmrA to different extents at early and late times due to a negative feedback loop that hinders Fe^{3+} access to PmrB (*12*). These examples illustrate how cellular behaviors in response to particular environmental conditions change over time even when the condition triggering the response does not change.

Transcript abundance does not always reflect the activity of the corresponding protein

mRNA abundance is often used as proxy for the amounts and activities of the corresponding proteins. This is because mRNA determinations are straightforward across a genome, whereas activity assays are not available for the vast majority of gene products. Our data demonstrate two examples in which the presence of particular mRNAs is not accompanied by the activity of the specified gene products. First, when the *lpxT* gene was transcribed in response to a mildly acidic pH (Fig. 5A), the LpxT-mediated modification of lipid A was not observed (Fig. 4B) presumably because of the presence of the LpxT inhibitor PmrR (*12*). Likewise, the *pmrC* gene was highly induced in low Mg^{2+} (Fig. 3), but the lipid A did not contain detectable pEtN (Fig. 4B) due to preceding PhoP-dependent *lpxT* transcription (Fig. 3) favoring the incorporation of L-Ara4N over the incorporation of pEtN (Fig. 4B).

Environmental control of LpxT expression affects multiple pathways

As discussed above, *Salmonella* favors lipid A modified with L-Ara4N under low Mg^{2+} and with both L-Ara4N and pEtN when experiencing a mildly acidic pH (Fig. 1C). The fact that the lipid A from *Salmonella* harvested from inside macrophages contains both L-Ara4N and pEtN (*15*) is in agreement with three notions: first, that the macrophage phagosome containing *Salmonella* is mildly acidic (*40*); second, that an acidic pH is critical to activate the PhoP/PhoQ system inside macrophages (*40*); and third, that an acidic pH is the signal promoting expression of lipid A phosphate–modifying genes inside macrophages (*15*).

Wild-type *Salmonella* grown in a mildly acidic pH is seven times more resistant to polymyxin B than when grown in low Mg^{2+} (Fig. 5B). Because polymyxins are not used to treat *Salmonella* infections, our findings raise the possibility of the lipid A modification with both L-Ara4N and pEtN, which is favored under a mildly acidic pH, mediating resistance to host-derived products, decreasing recognition by the host immune system, playing a yet-to-be identified function, or a combination of these roles. We note that a *pmrA* null mutant, which is unable to modify lipid A with either L-Ara4N or pEtN (*2*), is hypervirulent in cultured macrophages and in mice inoculated through the intraperitoneal route (*41*).

Finally, the LpxT protein removes the distal phosphate from its substrate—undecaprenyl diphosphate (C_{55}-PP)—and produces C_{55}-P, which is an essential carrier that shuffles sugar moieties across the inner membrane for various biosynthetic pathways (*14*, *42*). The identification of environmental conditions and regulatory factors that control *lpxT* gene expression raises the possibility of the essential C_{55}-P metabolism being affected by the environmental conditions encountered by bacteria. This hypothesis is supported by the observation that the PhoP and PmrA proteins have been implicated in controlling the expression of the *ybjG* gene, which, like *lpxT*, encodes a C_{55}-PP phosphatase that is required for recycling C_{55}-P (*43*) in *E. coli*.

MATERIALS AND METHODS

Bacterial strains, plasmids, primers, and growth conditions

S. enterica serovar Typhimurium strains were derived from the wild-type strain 14028s. Unless otherwise stated, bacteria were grown at 37°C in LB broth or in N-minimal medium (pH 7.7) (*44*) supplemented with 0.1% casamino acids, 38 mM glycerol, and 10 mM or 10 μM of $MgCl_2$. When necessary, antibiotics were added at the following final concentrations: ampicillin (50 μg/ml), chloramphenicol (20 μg/ml), kanamycin (50 μg/ml), and tetracycline (10 μg/ml). P22 transduction of *Salmonella* strains was performed as described (*45*). *E. coli* DH5a

was used as a host for the preparation of plasmid DNA. Bacterial strains and plasmids used in this study are listed in table S3. Primers used in this study are listed in tables S4 and S5.

All experiments were carried out with wild-type *S. enterica* serovar Typhimurium strain 14028s, wild-type *E. coli* strains MG1655 or W3110, and mutant derivatives. LB (Becton, Dickinson and Co.), Super Optimal Broth (SOB) (Becton, Dickinson and Co.), or Super Optimal broth with Catabolite repression (SOC) (SOB supplemented with 20 mM glucose) media were used for cloning and strain construction, as noted. When necessary, ampicillin was used at 50 μg/ml, and tetracycline was used at 12.5 μg/ml. Fusaric acid (FA) plates were used for selection against tetracycline-resistant bacteria [LB agar (40 g/liter; Becton, Dickinson and Co.), $NaH_2PO_4{\cdot}H_2O$ (10 g/liter) (Sigma-Aldrich), chlortetracycline hydrochloride (60 mg/liter) (Sigma-Aldrich), FA (12 mg/liter) (Acros Organics), and 0.1 mM $ZnCl_2$ (Sigma-Aldrich)] (*46*). For experiments in which gene expression was measured, bacteria were grown in N-minimal medium (pH 7.7 or pH 4.9) (*47*) and the indicated concentration of $MgCl_2$. For time course experiments, cells were grown in N-minimal medium (pH 7.7; 10 mM Mg^{2+}) to log phase and then either washed with fresh N-minimal medium (pH 7.7; no Mg^{2+}) twice and resuspended with N-minimal medium (pH 7.7; 10 μM Mg^{2+}) or resuspended with N-minimal medium (pH 4.9; 10 mM Mg^{2+}) before being incubated for the indicated times. All incubations were carried out at 37°C with shaking at 250 rpm.

Strain construction

Strains harboring chromosomal point mutations in the *lpxT* promoter region were constructed by a method based on selection for loss of tetracycline resistance (*46*). First, the promoter region of *lpxT* was replaced with a tetracycline resistance (Tet^R) cassette. This was carried out by transforming wild-type *Salmonella* (14028s) expressing the phage λ Red recombinase machinery from plasmid pKD46 with a DNA fragment containing the Tet^R cassette from transposon Tn*10* flanked by ~60-nt regions of nucleotide sequence identity to the *lpxT* promoter region. The DNA fragment was generated using primers W3423 and W3424 for strain XH16 and primers W3427 and W3428 for strain XH15, with genomic DNA from MS7953s as a template. Electroporated cells were recovered in SOC medium at 30°C and plated on LB/Ap/Tet/plates and incubated at 30°C. The resulting strains were grown to mid-log phase in SOB/Ap/10 mM L-arabinose at 30°C and made electrocompetent by washing twice in ice-cold water and once in ice-cold 10% glycerol. Next, a DNA oligo corresponding to the *lpxT* promoter and containing the desired point mutation(s) was generated using two primers W3425 and W3426 that are reverse complement to each other (W3425 and W3426 for XH16 and W3430 and W3433 for XH15). The resulting product was transformed into electrocompetent cells. Electroporated cells were recovered in SOC at 37°C. Transformants were cured of pKD46 by plating on LB at 42°C and tested for loss of Ap and Tet resistance, and mutations were confirmed by sequencing PCR products generated from purified genomic DNA. To construct the strain specifying a C-terminally FLAG-tagged LpxT protein (XH201), the DNA fragment amplified from pKD3 using primers W4309/W4310 was introduced into wild-type *Salmonella* 14028s harboring plasmid pKD46.

When necessary, P22 phage transduction was used to move selectable markers. XH246 was generated by using a P22 lysate grown on strain EG9241 to infect strain DC74, which is a derivative of strain DC72 with the Km cassette removed by using plasmid pCP20 (*48*). XH248 was generated by infecting XH16 with a P22 lysate prepared on strain EG9460. XH251 was generated by infecting DC72 with a P22 lysate prepared on strain EG17898.

RNA isolation and complementary DNA synthesis

A bacterial pellet was isolated from 1 ml of liquid culture and placed immediately on dry ice. The pellet was resuspended in 1 ml of 2:1 RNAprotect reagent (Qiagen)/water and incubated at room temperature for 5 min. After centrifugation at 5000 rpm for 10 min, the pellet was stored at −80°C. The sample was resuspended in 100 μl of lysozyme (10 mg/ml; Sigma-Aldrich) and incubated at room temperature for 2 hours with occasional vortexing. Total RNA was isolated using the RNeasy Mini kit (Qiagen) and the recommended on-column DNase I treatment, according to the manufacturer's instructions. For first-strand complementary DNA (cDNA) synthesis, 1 μg of purified RNA in 16 μl of ribonuclease (RNase)–free water was mixed with 4 μl of VILO Master Mix (Invitrogen) and incubated at 25°C for 10 min, 42°C for 60 min, and 85°C for 5 min. The resulting cDNA was diluted by adding 100 μl of water before qRT-PCR.

qRT-PCR

For quantification of cDNA abundance, 2 μl of diluted cDNA was mixed with 7.5 μl of 2× SYBR Green Master Mix (Applied Biosystems) and 5.5 μl of primer mix (each primer diluted to 72.7 μM in water). Reactions were carried out in a 7500 Real-Time PCR machine (Applied Biosystems) using the manufacturer-provided software and settings. Transcript quantities were determined by fitting C_T (threshold cycle) values to a standard curve generated with *Salmonella* genomic DNA.

DNase I footprinting assay

DNase I foot printing assay was conducted as described (*41*). DNA fragments corresponding to the *lpxT* region were generated by PCR using primer W3436 and ^{32}P-labeled primer W3435 with genomic DNA from strain 14028s as a template. Unincorporated [γ-^{32}P]–adenosine 5′-triphosphate was removed by using G-50 microcolumns (GE Healthcare). A total of 2×10^4 cpm of labeled probe (~10 fmol), 200 ng of poly(deoxyinosinic-deoxycytidylic) (Sigma-Aldrich), and purified His-tagged PhoP (PhoP-6×His) (*49*) were mixed with binding buffer [20 mM Hepes (pH 8.0), 10 mM KCl, 2 mM $MgCl_2$, 0.1 mM EDTA, 0.1 mM dithiothreitol, bovine serum albumin (50 μg/ml), and 10% (v/v) glycerol] in a total volume of 20 μl and incubated at room temperature for 20 min. DNase I (Gibco) (0.01 U), 10 mM $CaCl_2$, and 10 mM $MgCl_2$ were added and incubated at room temperature for 3 min. Final concentrations of PhoP protein were 0, 1, 5, 10, and 20 μM. The reaction was stopped by the addition of 100 μl of a phenol chloroform solution, and the aqueous phase was precipitated with ethanol. The precipitate was dissolved in sequence-loading buffer and electrophoresed on a 6% acrylamide/7 M urea gel, together with a sequence ladder generated with the labeled primer by using the T7 Sequenase 2.0 DNA Sequencing kit (Amersham Biosciences), and the gels were dried and autoradiographed.

Filter-binding assay

Determination of binding efficiency of DNAs to the PhoP protein was carried out by the nitrocellulose-binding method (*50*). ^{32}P-radiolabeled DNAs were incubated with a series of dilutions of the PhoP protein under the same condition used in the electrophoretic mobility shift assay at room temperature for 20 min. Final concentrations of PhoP protein were 0, 1, 2, 5, 10, and 20 μM. The samples were filtered through 0.45-μm nitrocellulose filters (HAWP, Millipore) under vacuum, and

the filters were washed with 5 ml of the binding buffer, air-dried, and quantified using PhosphorImager (GE Healthcare).

Primer extension assay

Cultures were grown as indicated in the figure legend. Total RNA was extracted as described (*51*). cDNA synthesis was performed using 2 pmol of ^{32}P-labeled 14997 primer with 10 μg of total RNA and 1 U of SuperScript II RNase H2 reverse transcriptase (Life Technologies). The extension products were analyzed by electrophoresis on a 6% acrylamide/7 M urea gel and compared with sequence ladders initiated with the same ^{32}P-labeled primer that was used for primer extension.

Polymyxin B susceptibility assay

Antimicrobial peptide susceptibility assays were conducted as described (*7*), with modifications. Bacteria were grown in N-minimal medium with 10 μM Mg^{2+} (pH 7.7) or with 10 mM Mg^{2+} (pH 4.9) for 4 hours. Bacterial cells were then diluted 200× in phosphate-buffered saline (PBS), combined with 1:1 ratio with polymyxin B (final concentration of 5 μg/ml). After 1-hour incubation at 37°C with aeration, samples were serially diluted in PBS and plated on LB agar plates for enumeration. The percentage survival was calculated as follows: survival (%) = colony-forming units (CFU) of peptide-treated culture/CFU of no-peptide culture × 100.

Isolation of labeled lipid A

Cultures were diluted to an OD_{600} (optical density at 600 nm) of 0.05 in 5 ml of fresh medium and labeled with ^{32}Pi (2.5 μCi/ml; Amersham Biosciences) in N-minimal medium, as indicated. For the time course analysis, cells were first grown in 5 ml of N-minimal containing 10 mM Mg^{2+} to log phase, washed twice with N-minimal medium, and resuspended with 5 ml of N-minimal medium containing 10 μM Mg^{2+} and ^{32}Pi (2.5 μCi/ml) for the indicated time. Cells were harvested by centrifugation, and the isolation of ^{32}P-labeled lipid A was carried out by mild acid hydrolysis, as previously described (*8*, *52*). ^{32}P-lipid A species (~1000 cpm·per lane) were analyzed by TLC in a solvent system of chloroform, pyridine, 88% formic acid, and water (50:50:16:5, v/v) and visualized using a PhosphorImager (GE Healthcare).

Analysis of lipid A by mass spectrometry

Bacterial strains were grown as described earlier in 25 ml of medium for 4 hours. Lipid A was purified according to published protocols (*53*) and analyzed by the Keck Mass Spectrometry & Proteomics Resource at Yale University.

Western blot assay

Cells were grown as described above. Crude extracts were prepared in B-PER reagent (Pierce) with lysozyme (100 μg/ml) and EDTA-free protease inhibitor (Roche). Samples were loaded onto 4 to 12% NuPAGE gels (Life Technologies) and transferred to nitrocellulose membrane using the iBot machine (Life Technologies). Membranes were blocked with 3% skim milk solution at room temperature for 1 hour. Then, samples were analyzed using anti-FLAG or anti-GroEL antibodies. Rabbit anti-FLAG antibodies were used at 1:4000 dilution. Mouse anti-GroEL antibodies were used as control at 1:12,000 dilution. Secondary horseradish peroxidase–conjugated anti-rabbit or anti-mouse antiserum (GE Healthcare) was used at 1:4000 dilution. Blots were developed with the Amersham ECL Western Blotting Detection Reagents (GE Healthcare) or SuperSignal West Femto Chemiluminescent system (Pierce).

SUPPLEMENTARY MATERIALS

www.sciencesignaling.org/cgi/content/full/11/529/eaar7921/DC1

Fig. S1. LpxT protein amounts in wild-type *Salmonella*.
Fig. S2. *yeiR* and *lpxT* transcript abundances in wild-type *Salmonella* in low Mg^{2+}.
Fig. S3. Abundance of *lpxT* transcript in wild-type, *phoP*, and *lpxT* promoter mutant *Salmonella*.
Fig. S4. Lipid A profiles of wild-type *Salmonella* in low Mg^{2+}.
Fig. S5. Lipid A profiles of wild-type, *lpxT* promoter, *pmrC*, and double *pmrC lpxT* promoter mutant *Salmonella*.
Fig. S6. Lipid A profiles of wild-type, *lpxT*, *pmrA*, and *pmrA lpxT Salmonella*.
Fig. S7. Resistance of wild-type, *pmrA*, *pbgP*, *pmrC*, and *lpxT Salmonella* to polymyxin B.
Table S1. Lipid A profiles of isogenic *Salmonella* strains determined by mass spectrometry.
Table S2. Coexistence of lipid A–modifying genes in the genomes of seven Gram-negative bacterial species.
Table S3. Bacterial strains and plasmids used in this study.
Table S4. Primers used in the construction of strains and plasmids.
Table S5. Primers used in this study for the quantification of transcripts in *Salmonella*.

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Gene expression kinetics governs stimulus-specific decoration of the *Salmonella* outer membrane

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Same genes, different phenotypes

The lipopolysaccharide (LPS) coat of Gram-negative bacteria can be modified to evade the host immune response or confer resistance to antimicrobial compounds. Modifications that reduce the negative charge of the LPS component lipid A increase the resistance of *Salmonella* to the antibiotic polymyxin B and other cationic antimicrobial compounds. Hong *et al*. found that different stimuli elicited distinct lipid A modification profiles in *Salmonella enterica* serovar Typhimurium, although they promoted expression of the same set of genes encoding lipid A–modifying enzymes. Different stimuli caused the genes to be activated with different kinetics, thus altering the temporal order in which the lipid A–modifying enzymes were produced. This resulted in distinct lipid A profiles and different effects on resistance to polymyxin B. These findings illustrate how bacteria can use a limited set of enzymes to generate a range of adaptations to different stimuli.