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# PhoPQ-Mediated Regulation Produces a More Robust Permeability Barrier in the Outer Membrane of *Salmonella enterica* Serovar Typhimurium▽

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**The PhoPQ two-component system of *Salmonella enterica* serovar Typhimurium produces a remodeling of the lipid A domain of the lipopolysaccharide, including the PagP-catalyzed addition of palmitoyl residue, the PmrAB-regulated addition of the cationic sugar 4-aminoarabinose and phosphoethanolamine, and the LpxO-catalyzed addition of a 2-OH group onto one of the fatty acids. By using the diffusion rates of the dyes ethidium, Nile red, and eosin Y across the outer membrane, as well as the susceptibility of cells to large, lipophilic agents, we evaluated the function of this membrane as a permeability barrier. We found that the remodeling process in PhoP-constitutive strains produces an outer membrane that serves as a very effective permeability barrier in an environment that is poor in divalent cations or that contains cationic peptides, whereas its absence in *phoP* null mutants produces an outer membrane severely compromised in its barrier function under these conditions. Removing combinations of the lipid A-remodeling functions from a PhoP-constitutive strain showed that the known modification reactions explain a major part of the PhoPQ-regulated changes in permeability. We believe that the increased barrier property of the remodeled bilayer is important in making the pathogen more resistant to the stresses that it encounters in the host, including attack by the cationic antimicrobial peptides. On the other hand, drug-induced killing assays suggest that the outer membrane containing unmodified lipid A may serve as a better barrier in the presence of high concentrations (e.g., 5 mM) of $Mg^{2+}$.**

Cells of gram-negative bacteria are surrounded by the outer membrane (OM), which functions primarily as a permeability barrier (35). Large, hydrophilic compounds are excluded by the narrow porin channels, and lipophilic compounds cross the unusual, asymmetric bilayer of this membrane only slowly. Our early analysis by using steroids as probes (37) showed that the diffusion across the OM bilayer is nearly 2 orders of magnitude slower than the diffusion across a typical phospholipid bilayer, such as the phospholipid bilayer that exists in the inner, cytoplasmic membrane. A recent study (11) suggested that this estimate likely needs to be adjusted somewhat downward. Nevertheless, the OM bilayer clearly functions as a formidable permeation barrier, because perturbing this bilayer results in a striking sensitization of *Escherichia coli* and *Salmonella enterica* serovar Typhimurium to various lipophilic inhibitors (44).

One major factor that contributes to this barrier property is presumably the asymmetric structure of the OM bilayer, whose outer leaflet is composed nearly entirely of lipopolysaccharides (LPS) (25). (In fact, very low permeability was found in symmetric LPS bilayers assembled in the laboratory [40].) According to the lattice model for diffusion in liquids, a statistical average of rapidly fluctuating variable distances creates transient holes within the bilayer into which solutes can migrate (41). Thus, an effective membrane barrier requires components that interact strongly with their neighbors, so that such transient holes are less likely to form. Indeed, strong lateral interactions presumably occur between neighboring LPS molecules that carry multiple negative charges, through the bridging effect of divalent cations (and possibly through hydrogen bonding) (35). A corollary of this model is that when the stabilizing divalent cations are removed, the OM becomes unstable and its permeability to lipophilic solutes increases. Indeed, in response to low divalent cation concentrations, *S. enterica* serovar Typhimurium remodels its OM extensively through signaling by the PhoPQ two-component system (21).

The remodeling includes the increased transcription of genes involved in LPS modification, such as *pagP*, *lpxO*, and a group of genes regulated by the *pmrAB* system (21, 38). (The product of another PhoPQ-regulated gene, *pagL*, is inactive in *S. enterica* serovar Typhimurium until its latency is released under specific conditions [27, 38].) This modification appears to be essential for the survival of the pathogen in the host, as shown by the fact that compromising the PhoPQ system results in the loss of virulence (12). There are several likely mechanisms that are involved in the contribution of LPS modification to virulence. For example, the PagP-catalyzed palmitoylation of lipid A greatly decreases the capacity of LPS to activate the innate immune response through Toll-like receptor 4 (28). Similarly, both this palmitoylation reaction and the *pmrAB*-regulated addition of the cationic 4-aminoarabinose group and phosphoethanolamine (34, 38) increase the resistance of *Sal-*

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TABLE 1. Bacterial strains and plasmid used

| Strain or plasmid | Properties | Derivation or reference[a] |
|---|---|---|
| Strains | | |
| CS093 | Wild-type *S. enterica* serovar Typhimurium | ATCC 14028 |
| CS015 | CS093 *phoP102*::Tn*10*d-*cam* | 21 |
| CS022 | CS093 *pho-24* (PhoP constitutive) | 21 |
| CS401 | CS093 *phoN2 zxx-6251*::Tn*10*d-*cam rpsL* (Str$^r$) | 22 |
| CS491 | CS022 *rpsL* | Transduction of *rpsL* from CS401 |
| TG241 | CS491 *lpxO* | — |
| WLP97 | CS491 *pagP* | — |
| TG273 | CS491 *lpxO pagP* | — |
| TG275 | CS491 *pagP lpxO pmrA*::Tn*10*d-*tet* | Transduction of *pmrA* from CS338 (= JSG421) |
| CS338 | Also JSG421; *pmrA*::Tn*10*d-*tet* | 22 |
| HN1138 | CS093 *tolC*::*kan* | Transposon mutagenesis |
| HN1139 | CS015 *tolC*::*kan* | Transduction of *tolC* from HN1138 |
| HN1140 | CS022 *tolC*::*kan* | Transduction of *tolC* from HN1138 |
| HN1141 | CS491 *tolC*::*kan* | Transduction of *tolC* from HN1138 |
| HN1142 | TG241 *tolC*::*kan* | Transduction of *tolC* from HN1138 |
| HN1143 | WLP97 *tolC*::*kan* | Transduction of *tolC* from HN1138 |
| HN1144 | TG273 *tolC*::*kan* | Transduction of *tolC* from HN1138 |
| HN1145 | TG275 *tolC*::*kan* | Transduction of *tolC* from HN1138 |
| HN1147 | CS491 *phoP102*::Tn*10*d-*cam tolC*::*kan* | Transduction of *phoP* from CS015 into HN1141 |
| HN1148 | CS022 *pmrA*::Tn*10*d-*tet tolC*::*kan rpsL* | Successive transductions of *pmrA* and *rpsL* into HN1140 |
| D21 | *E. coli* K-12 unaltered for LPS | *E. coli* Genetics Stock Center |
| D21f2 | "Deep rough" mutant lacking the R core entirely | *E. coli* Genetics Stock Center |
| Plasmid | | |
| pKAS32 | Amp$^r$ Str$^s$ (*rpsL*$^+$); low-copy-number allelic exchange vector | 39 |

[a] —, in-frame deletions were introduced by using pKAS32.

*monella* cells to cationic antimicrobial peptides (18, 22). Yet the effect of this PhoPQ-regulated remodeling on the most fundamental property of the OM bilayer, its low fluidity and consequently its effective barrier function, has not been examined so far.

Thus, we asked if OM permeability is altered in a series of isogenic strains altered in the PhoPQ-mediated modification of LPS. Our results show that the PhoPQ signaling system contributes to the assembly of a tightly organized OM with very low permeability in divalent cation-poor environments. Further, a significant portion, if not all, of this beneficial alteration of the OM property appears to be caused by the known PhoPQ-regulated modification of lipid A structure.

## MATERIALS AND METHODS

**Bacterial strains.** The strains used, all of which except D21 and D21f2 are derivatives of *S. enterica* serovar Typhimurium CS093 (= ATCC 14028), are listed in Table 1. For comparisons between *phoP* null and PhoP-constitutive strains, strains containing *phoP102*::Tn*10*d-*cam* and *pho-24* (20), respectively, were used. The *tolC* mutants used were constructed by transduction of the *tolC*::*kan* allele described below into an isogenic series of strains, and one series contained in addition the identical *rpsL* allele.

HN1138 was created by Li Zhang by transposon mutagenesis of CS093 with an EZ-TN <Kan-2>Tnp transposon kit (Epicenter Biotechnologies, Madison, WI) following screening for clones hypersusceptible to carbonyl cyanide *meta*-chlorophenylhydrazone (CCCP). The location of the transposon was determined to be within the *tolC* gene by sequencing from the inserted sequence. The *tolC*::*kan* alleles were then transduced into various strains with P22HT*int*, and the presence of the inactivated gene in the transductant was confirmed by PCR.

CS491 was constructed by P22HT*int* phage-mediated transduction of the *rpsL* (Str$^r$) marker, while TG275 was made by transduction of *pmrA*::Tn*10*d-*tet*. *lpxO* and *pagP* mutants were made by allelic replacement. Briefly, genomic regions containing either *lpxO* or *pagP* were deleted in frame, cloned into the pKAS32 suicide vector (39), and transferred by conjugation from *E. coli* SM10λpir into streptomycin-resistant *S. enterica* serovar Typhimurium strain CS491. The resulting isolates that were resistant to ampicillin but sensitive to streptomycin were integrants that contained chromosomal insertions of pKAS32-Δ*lpxO* and pKAS32-Δ*pagP*, respectively. Plasmid DNA sequences were resolved from the chromosome by plating the integrants onto Luria-Bertani (LB)-streptomycin agar and by screening for the isolates that were also ampicillin resistant. Resulting chromosomal deletions of *lpxO* and *pagP* were confirmed by PCR amplification of the flanking regions on the bacterial chromosome.

**Media used.** Strains were grown in many cases in LB broth (10 g Bacto tryptone per liter, 10 g Bacto yeast extract per liter, 5 g NaCl per liter) or on LB agar with supplements as indicated below. LB media with 1 mM EDTA were used to produce divalent cation-poor growth conditions. Similarly, M9 minimal medium (1) was modified by removing $MgSO_4$ and $CaCl_2$ and by substituting $(NH_4)_2SO_4$ (1.32 g/liter) for $NH_4Cl$ in order to provide a source of sulfur. This medium was designated modified M9 medium, to which divalent cations were added as indicated below.

**Assay of fluorescent dye influx.** Ethidium bromide was used in most cases as the fluorescent probe for permeability of the OM bilayer. (Influx of dyes was used in the past as an indicator of the function [or more correctly lack of function] of efflux pumps [31] but has not been used to measure the OM permeability in a quantitative manner.) Cells were grown overnight in LB broth without shaking. One milliliter of the culture was diluted into 19 ml of fresh LB broth, and the culture was grown with shaking at 37°C until the optical density at 600 nm ($OD_{600}$) reached a value between 1.8 and 2.4. Ten-milliliter portions were harvested by centrifugation at room temperature, and the cells were washed twice with 50 mM potassium phosphate buffer (pH 7) by centrifugation at room temperature. Cells were resuspended in 0.5 ml of the same buffer, and the optical density was determined after 20-fold dilution. The amount of cells corresponding to 0.4 $OD_{600}$ unit was added to the same potassium phosphate buffer (final volume, 2 ml) containing, in some experiments, $MgCl_2$ and/or CCCP at concentrations indicated in Results. After addition of ethidium bromide (final concentration, 6 μM; added from a 2 mM stock solution by using a Gilson positive displacement micropipette for precision) to the mixture, the fluorescence of the ethidium-nucleic acid complex generated by the influx of ethidium into cells was determined at room temperature by using a Shimadzu RF6301 spectrofluorometer with excitation and emission wavelengths of 545 and 600 nm, respectively. The widths of the slits were 5 and 10 nm, respectively. Each experiment was repeated at least three times; we found that the relative behavior of various strains was quite reproducible (exceptions are described below), although the absolute rates of dye entry varied somewhat from experiment to experiment. In several experiments, a neutral dye, Nile red (Molecular Probes), or an acidic dye,

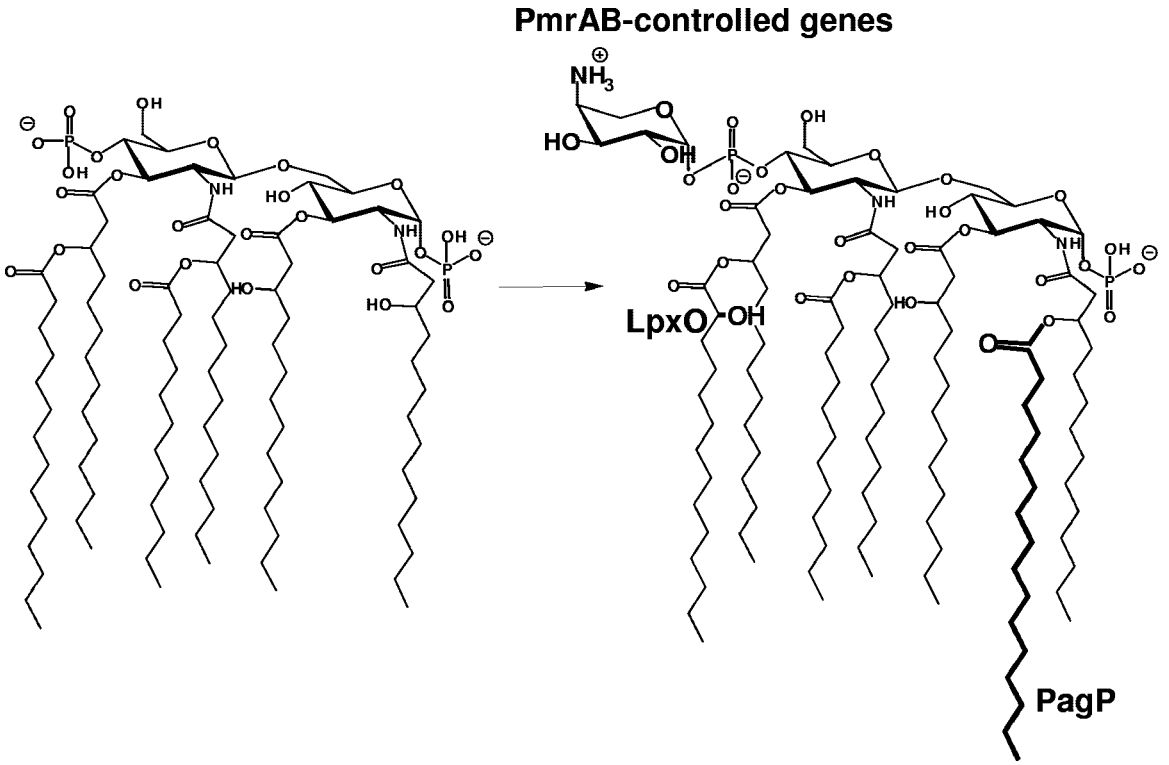


FIG. 1. Modification of lipid A structure by the PhoPQ regulatory system. The modified structures are indicated by bold type and thick lines.

eosin Y (Sigma), was used as the probe. The details are described in the legend to Fig. 4.

**MIC determination.** MICs were determined with 96-well microtiter plates, using a standard twofold broth microdilution method. LB broth was the medium, unless specified otherwise. The final volume in each well was 100 μl. The size of the inoculum in all experiments was around 2,000 cells per well. Each experiment was repeated at least three times.

**Killing assays.** Early-exponential-phase cultures ($OD_{600}$, ~0.1) of various strains were diluted 100-fold into modified M9 medium (with 0.1 or 5 mM $MgCl_2$) containing the concentrations of erythromycin, novobiocin, or rifampin indicated below. After 3 h of incubation at 37°C with aeration by shaking, the suspensions were diluted and plated onto LB agar plates to assay for CFU. Experiments were repeated at least three times to confirm the reproducibility of data.

## RESULTS

**PhoP-constitutive signaling produces a more robust permeability barrier: studies with *tolC* mutants.** The PhoPQ two-component system regulates three known remodeling modifications of the lipid A moiety of LPS (21, 38), palmitoylation of the 3-hydroxyl group in the 3-OH-myristyl residue at the 2 position of the backbone (6), addition of 4-aminoarabinose (or phosphorylethanolamine [34, 38]) to the 4′ phosphate group, and addition of a 2-hydroxy group to the myristate residue at the 3′ position (16) (Fig. 1). In this study, we routinely compared strains with a *phoP* null allele (*phoP102*, with a Tn*10*d-*cam* insertion), which results in lipid A with none of the modifications mentioned above (21), with strains containing the *pho-24* mutation (a missense mutation in the *phoQ* sensor gene that locks the PhoP protein in the activated state [21]), which results in the PhoP-constitutive phenotype with lipid A with all of the modifications (21).

To examine the OM permeability of these mutant strains, intact bacterial cells were often incubated with ethidium bromide. Once ethidium traverses the OM, it crosses the much more fluid and permeable conventional bilayer of the cytoplasmic membrane very rapidly (as is true for any lipophilic solute [41]) and binds to nucleic acids in the cytosol, producing a fluorescence signal. Thus, one can determine the rate-limiting step, the flux across OM, from the rate of fluorescence increase. Ethidium, a large (about 12 Å across), hydrophobic dye with a delocalized positive charge, is unlikely to diffuse through the narrow porin channel (7 by 11 Å in *E. coli* OmpF [8]) (also see below). However, ethidium is a good substrate for the constitutive AcrAB-TolC multidrug efflux system (31), and it was necessary to disable this system for the OM permeability assay. We used an isogenic series of strains containing a null mutation in the *tolC* gene, coding for the OM channel component of this system (13). The *tolC* mutation also inactivates most other efflux systems in enteric bacteria that pump out dyes and drugs directly into the medium (30).

We compared a *phoP* null strain with an isogenic PhoP-constitutive strain in this manner. Resuspension of cells in divalent cation-free buffers is expected to destabilize the divalent cation-bridged LPS leaflet of the OM bilayer. When the ethidium entry rates in 50 mM phosphate buffer (pH 7.0) were compared for these two strains, the *phoP* null mutant showed higher permeability than the PhoP-constitutive strain in the

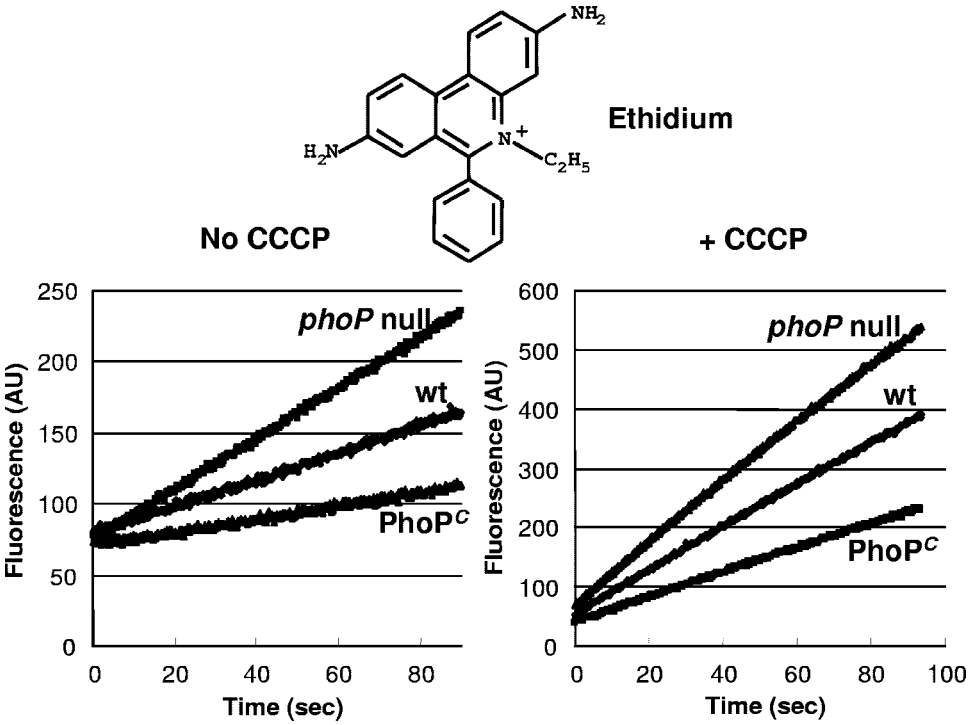


FIG. 2. Ethidium influx into *tolC* null mutants. HN1138 (*phoP*$^+$ *tolC*) (wt), HN1139 (*phoP* null, *tolC*), and HN1140 (PhoP constitutive [PhoP$^C$], *tolC*) were grown in LB broth with aeration by shaking, and cells were harvested at the beginning of the stationary phase (at an $OD_{600}$ between 2.3 and 2.4). Cells were washed and resuspended in 50 mM potassium phosphate buffer (pH 7.0), and the ethidium influx was assayed in the absence and presence of 50 μM CCCP. AU, arbitrary units.

absence of $Mg^{2+}$ (Fig. 2, left panel). Indeed, the OM permeability of the PhoP-constitutive strain was very low, and the difference between the rates for the *phoP* null mutant and the PhoP-constitutive strain was more than fourfold (Fig. 2, left panel). The isogenic *phoP*$^+$ *tolC* strain showed intermediate permeability, as expected from the fact that some of the PhoPQ-regulated modification reactions, especially the palmitoylation of lipid A, occur in the wild-type strain to a considerable extent (21). We used *tolC* strains in most of the subsequent experiments.

When cells from different growth phases were compared, the cells from the late exponential to early stationary phase, when the growth rate was decreasing, showed the largest difference in permeability between the PhoP-constitutive and *phoP* null mutants (results not shown); cells from this growth phase were used routinely in subsequent assays.

When the OM permeability was measured in the presence of different concentrations of the $Mg^{2+}$ ion (Fig. 3), the permeability was found to decrease very strongly in the *phoP* null mutant but only modestly in the PhoP-constitutive strain. These effects were presumably due to the increased bridging, by $Mg^{2+}$, of neighboring LPS molecules, especially the unmodified LPS with its more numerous negative charges. The experiment whose results are shown in Fig. 3 was repeated four times, and the results were reproducible except that in one experiment the two strains showed identical permeabilities at $Mg^{2+}$ concentrations of 5 mM or more. When the $Ca^{2+}$ ion was added to cells resuspended in 50 mM HEPES-NaOH buffer, it had an effect similar to that of $Mg^{2+}$ (not shown).

In order to show that the different OM permeabilities of the two strains were not limited to cationic dyes, such as ethidium, we also examined the diffusion of a neutral dye, Nile red, and an anionic dye, eosin Y. Nile red is practically nonfluorescent in water, and its fluorescence increases very strongly when it partitions into the fluid interior of the cytoplasmic membrane (17) (and possibly the inner leaflet of the OM); thus, the time-dependent increase in Nile red fluorescence in intact cells reflects its penetration rate across the impermeable outer leaf-

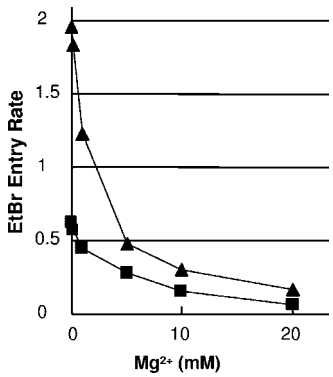


FIG. 3. Effect of $Mg^{2+}$ on the rates of entry of ethidium in *tolC* mutants. The initial rates of entry were measured with HN1140 (PhoP constitutive, *tolC*) (■) and HN1139 (*phoP* null, *tolC*) (▲) in potassium phosphate buffer (pH 7.0). The rates are expressed as the change in fluorescence intensity (in arbitrary units) per second. EtBr, ethidium bromide.

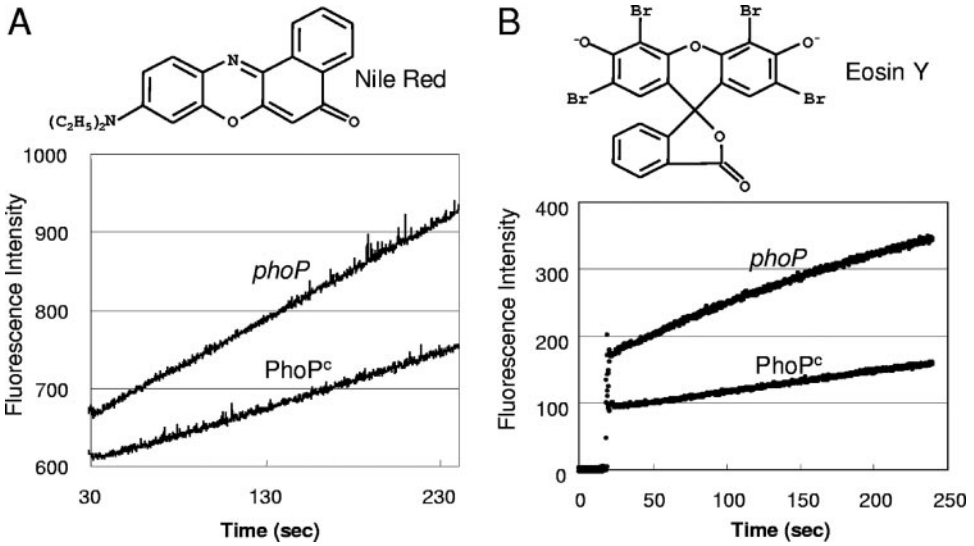


FIG. 4. Penetration of a neutral dye and an acidic dye across the OM of *tolC* mutants. Isogenic strains HN1139 (*phoP* null, *tolC*) and HN1140 (PhoP constitutive [PhoP$^c$], *tolC*) were grown and harvested as described in Materials and Methods. For the influx of a neutral dye, Nile red, the cells were resuspended in 50 mM potassium phosphate buffer (pH 7.0), and the dye was added to a final concentration of 2 μM at the beginning of the experiment. Fluorescence was monitored at 630 nm with excitation at 540 nm. For the assay of the penetration of an acidic dye, eosin Y, cells were washed and resuspended in 50 mM morpholineethanesulfonic acid (MES)-KOH buffer (pH 5.5) at a concentration of 40 $OD_{600}$ units/ml. Eosin Y (Na salt) was added to a concentration of 1 mM, and the mixtures were kept at 37°C for 2 h to allow for passive accumulation of this weakly acidic dye in the cytosol. For the assay, 5 μl of the mixture was added, at about 20 s, to 2 ml of 50 mM phosphate buffer (pH 7.0), and the fluorescence was recorded at 540 nm with excitation at 520 nm.

let of the OM. (As with any lipid-soluble probe, its partition into the lipid interior is positively correlated with the fluidity of lipids [41]; thus, the possibility of its partition into the LPS leaflet with very low fluidity can be ignored.) As shown in Fig. 4A, the PhoP-constitutive strain produced a stronger barrier for the influx of this neutral dye than the *phoP* null mutant.

Eosin Y does not bind to nucleic acids or membranes and thus did not show increased fluorescence upon entry into cells. Thus, we first produced a strong cytosolic accumulation of this weakly acidic dye by incubating cells at pH 5.5. Since the cytoplasmic pH of *E. coli* is maintained at 7.4 (26) and the more membrane-permeable species is likely to be the protonated, uncharged dye, this results in the sequestration of a high concentration of deprotonated, anionic species in the cytosol (as with any lipophilic, weak acid [26]), culminating in the concentration-dependent quenching of the accumulated dye. When the cells were diluted into a pH 7.0 buffer, there was a spontaneous efflux of the dye, followed by the dequenching of fluorescence. The rate of fluorescence increase was determined presumably by the rate-limiting step, the diffusion across OM, and was shown to be lower in the PhoP-constitutive strain than in the *phoP* mutant also with this acidic dye (Fig. 4B).

**More efficient barrier in PhoP-constitutive mutant is also confirmed by higher drug resistance in divalent cation-poor media.** As an additional approach to examine differences in OM bilayer permeability, we measured the resistance to large, lipophilic antimicrobial agents that are expected to cross the OM through the bilayer region. When a minimal medium (modified M9 medium) supplemented with 5 mM $MgCl_2$ was used, there was no significant difference in the MICs or the sizes of inhibition zones (in a disk diffusion assay) of erythromycin, cloxacillin, novobiocin, and fusidic acid between the isogenic PhoP-constitutive and *phoP* null strains (not shown). Also, the MICs of apramycin and gentamicin, aminoglycosides that are thought to cross the OM at least in part by disordering

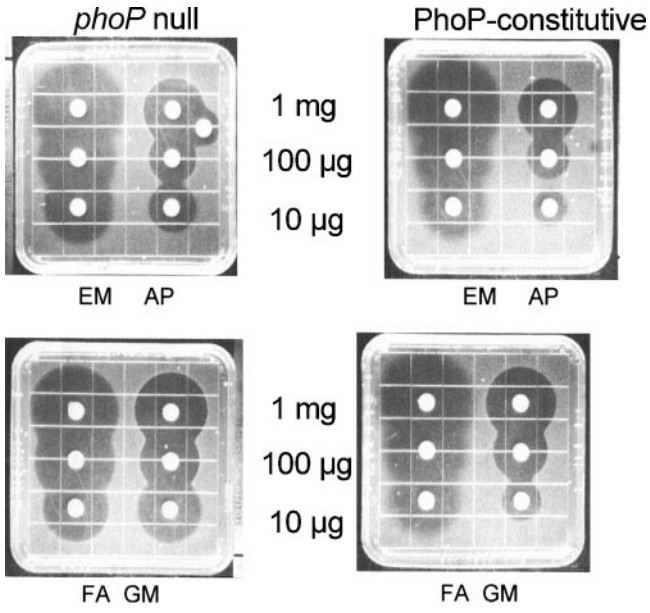


FIG. 5. Disk diffusion drug susceptibility assay. Either the *phoP* null strain HN1139 (left panels) or the PhoP-constitutive strain HN1140 (right panels) was spread on plates of modified M9 minimal medium containing 0.1 mM $MgCl_2$ and 0.4% glucose. Disks contained the indicated amounts of erythromycin (EM), apramycin (AP), fusidic acid (FA), and gentamicin (GM).

TABLE 2. MICs of lipophilic agents in LB and divalent cation-depleted LB (LB with 1 mM EDTA)

| Strain | Mutations present | MIC (μg/ml) of: | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Erythromycin | | Novobiocin | | Rifampin | | Fusidic acid | | Apramycin | |
| | | No EDTA | 1 mM EDTA | No EDTA | 1 mM EDTA | No EDTA | 1 mM EDTA | No EDTA | 1 mM EDTA | No EDTA | 1 mM EDTA |
| HN1139 | *phoP*::Tn*10*d-*cam tolC* | 4 | 0.06 | 0.5 | 0.016 | 4 | <0.004 | >2 | 0.008 | 16 | 8 |
| HN1140 | *pho-24 tolC* | 4 | 1 | 1 | 0.13 | >4 | 0.5 | 2 | 0.25 | 32 | 32 |
| HN1142 | *pho-24 lpxO tolC rpsL* | 4 | 0.5 | 1 | 0.13 | >4 | 0.5 | 2 | 0.25 | 32 | 32 |
| HN1143 | *pho-24 pagP tolC rpsL* | 4 | 0.5 | 1 | 0.13 | >4 | 0.25 | 2 | 0.13 | 32 | 32 |
| HN1147 | *pho-24 pmrA*::Tn*10*d-*tet tolC rpsL* | 4 | 0.5 | 1 | 0.13 | >4 | 0.5 | 2 | 0.25 | 32 | 32 |
| HN1144 | *pho-24 lpxO pagP tolC rpsL* | 4 | 0.5 | 1 | 0.13 | >4 | 0.25 | 2 | 0.13 | 32 | 32 |
| HN1148 | *pho-24 lpxO pagP pmrA*::Tn*10*d-*tet tolC rpsL* | 4 | 0.25 | 0.5 | 0.03 | >4 | 0.25 | 2 | 0.06 | 32 | 32 |

the LPS leaflet of the bilayer (23), remained similar for this pair of strains. However, when the $MgCl_2$ concentration was lowered to 0.1 mM, the *phoP* null mutant was clearly more susceptible to at least erythromycin, apramycin, and gentamicin in the disk diffusion assay (Fig. 5), although the susceptibilities to fusidic acid, novobiocin, and rifampin remained similar for the two strains. Similarly, in this assay the *phoP* null mutant was more susceptible than the PhoP-constitutive strain to the growth-inhibitory activity of bile salts such as cholate and deoxycholate. Interestingly, when the $Mg^{2+}$ concentration was increased to 5 mM, the susceptibility pattern of the two strains was reversed. The diameters of inhibition zones with 1 mg cholate, for example, were 20 and 13 mm with 0.1 mM $Mg^{2+}$ for the *phoP* null and PhoP-constitutive strains, respectively, but they were 17 and 22 mm, respectively, with 5 mM $Mg^{2+}$.

Another convenient assay medium was LB medium containing 1 mM EDTA. The MICs in this medium are shown in Table 2. The data show that the MICs of erythromycin, novobiocin, rifampin, fusidic acid, and apramycin for the PhoP-constitutive *pho-24* strain were 4 to 128 times higher than those for the isogenic *phoP* null mutant. In contrast, in unmodified LB medium, the difference between the strains was at most twofold, which is usually considered insignificant in a twofold serial dilution assay such as this one.

Killing of the *phoP* null and PhoP-constitutive strains in the presence of different concentrations of antibiotics was examined in both low-$Mg^{2+}$ (0.1 mM) and high-$Mg^{2+}$ (5 mM) minimal media. As expected from the results of other experiments, in the low-$Mg^{2+}$ medium the PhoP-constitutive strain resisted the killing action of erythromycin, novobiocin, and rifampin significantly better than the *phoP* null strain (Fig. 6). In contrast, in the high-$Mg^{2+}$ medium the order was reversed, and the *phoP* null strain survived better than the PhoP-constitutive strain in the presence of any of the antibiotics tested (Fig. 6). Although this result appears to be different from the result of the ethidium influx assay (Fig. 3), where the *phoP* null strain remained slightly more permeable than the PhoP-constitutive strain even with 5 mM $Mg^{2+}$, the differences were small and not always reproducible in the ethidium assay, and early-stationary-phase cells were used in that assay, in contrast to the early-exponential-phase cells used in the killing assay.

Because the PhoPQ system may respond to the presence of cationic peptides in phagosomes (4) rather than to the paucity of divalent cations, MICs were determined in unmodified LB medium containing various concentrations of polymyxin B nonapeptide (PMBN), a cationic peptide that perturbs the OM bilayer without the killing action of unmodified polymyxin (44). As shown in Table 3, in the absence of PMBN, the PhoP-constitutive strain and the *phoP* null mutant had identical

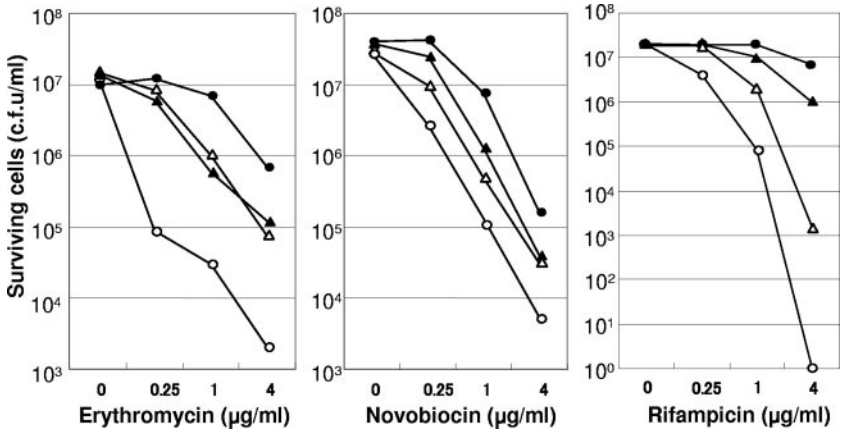


FIG. 6. Killing rates in the modified M9 medium. Early-exponential-phase cultures of HN1139 and HN1140 were diluted 100-fold into modified M9 medium containing the indicated concentrations of erythromycin, novobiocin, or rifampin. The numbers of surviving cells after 3 h of incubation were determined by plating onto LB agar plates. Circles, HN1139 (*phoP* null, *tolC*); triangles, HN1140 (PhoP constitutive, *tolC*); open symbols, M9 medium containing 0.1 mM $MgCl_2$; filled symbols, M9 medium containing 5 mM $MgCl_2$.

TABLE 3. Rifampin MICs in the presence of PMBN

| Strain | Genotype | MIC (μg/ml) in the presence of: | | | | |
|---|---|---|---|---|---|---|
| | | No PMBN | 0.01 μg/ml PMBN | 0.1 μg/ml PMBN | 1 μg/ml PMBN | 10 μg/ml PMBN |
| CS093 | Wild type | 16 | 16 | 8 | 1 | 0.06 |
| CS015 | *phoP*::Tn*10*d-*cam* | 8 | 8 | 4 | 0.5 | 0.016 |
| CS022 | *pho-24* (PhoP constitutive) | 8 | 8 | 8 | 2 | 0.13 |
| TG275 | *pho-24* (PhoP constitutive) *lpxO pagP pmrA*::Tn*10*d-*tet rpsL* | 8 | 8 | 8 | 1 | 0.06 |

rifampin MICs. However, in the presence of 10 μg/ml PMBN, the former strain was eight times more resistant to rifampin than the latter strain.

**Contribution of PhoPQ-regulated lipid A modification reactions.** The PhoPQ two-component system is known to produce at least three alterations in the lipid A structure (21), as shown in Fig. 1 (also see reference 15 for alterations within phagosomes). The genes regulated by the PmrAB system add a cationic sugar, 4-aminoarabinose (20), to the 4′ phosphate group on the nonreducing glucosamine residue; they are also needed (34) for addition of phosphoethanolamine to lipid A (38). PagP catalyzes the addition of a relatively long-chain fatty acid residue, the palmitoyl group, as a 3-substituent on the 3-OH-myristyl group at the 2 position of the reducing glucosamine residue (6). There is also hydroxylation of the 2 position of the myristoyl residue at the 3′ position of the nonreducing glucosamine residue, catalyzed by the dioxygenase LpxO (16, 21). We therefore tried to determine if these modification reactions played a role in the conversion of the leakier OM bilayer of the *phoP* null mutant into the more effective barrier in the PhoP-constitutive strain. To this end, we carried out a permeability assay with various PhoP-constitutive *tolC* mutants containing additional mutations in an $Mg^{2+}$-free buffer (Fig. 7). In this experiment, the presence of all three mutations (*pmrA*, *pagP*, and *lpxO*) made the PhoP-constitutive strain almost as permeable as the *phoP* null strain, indicating that the known PhoP-dependent lipid A modifications explain most of the alterations in the passive permeability of the OM bilayer. In contrast, elimination of any one, or even two, of the three modification reactions did not alter the OM permeability very much (Fig. 7). It was not possible to test all the possible combinations of mutations in one experiment, and the data shown in Fig. 7 might give the impression that *pmrA* had an exceptionally strong effect on permeability. However, in separate experiments (not shown), the *pmrA pho-24* double mutant was shown to have a barrier nearly as strong as that of the *pagP pho-24* or *lpxO pho-24* double mutant. Thus, it appears that the cooperation of all three mechanisms is needed to produce a robust barrier in the divalent cation-poor environment. Caution should be used in interpreting these data, however, because some of these mutations produce secondary effects. For example, *pmrA* mutation is known to release the latency of PagL, which catalyzes the hydrolysis of a 3-O-linked fatty acid residue from lipid A (27).

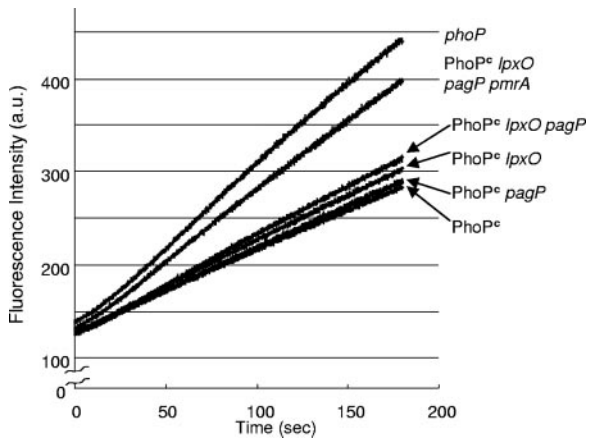


FIG. 7. Ethidium influx into PhoP-constitutive strains carrying additional mutations in lipid A remodeling genes. Ethidium entry was measured as described in Materials and Methods. The strains used were an isogenic series, all containing an *rpsL* mutation and the same *tolC* null mutation, and included HN1147 (*phoP* null), HN1145 (PhoP constitutive [PhoP$^c$], *lpxO pagP pmrA*), HN1144 (PhoP constitutive, *lpxO pagP*), HN1142 (PhoP constitutive, *lpxO*), HN1143 (PhoP constitutive, *pagP*), and HN1140 (PhoP constitutive). a.u., arbitrary units.

We noted that the OM permeability of the triple-defective PhoP-constitutive mutant still did not exactly match that of the *phoP* null mutant (Fig. 7). A similar result, indicating that elimination of all three known mechanisms of lipid A modification did not produce full permeabilization of OM, was also obtained in drug susceptibility assays (Tables 2 and 3). Of particular interest is the observation (Table 2) that inactivation of the three known lipid A modification genes did not produce any decrease in the MIC of apramycin, suggesting that other PhoPQ-mediated OM alterations play a predominant role in its penetration.

**Studies of the OM permeability barrier with CCCP-treated cells.** We wanted to use a different approach to confirm the results obtained with *tolC* strains in order to exclude the possibility that the results were affected by secondary effects of *tolC* mutation. Since most of the drug/dye efflux pumps in *E. coli* are energized by the proton motive force (30) (the exception is MacB, which is not involved in the extrusion of dyes [29]), we inactivated the efflux process through deenergization with the proton conductor CCCP. Ethidium essentially did not enter salmonella cells in the absence of CCCP, but the rate of net entry was greatly increased in the presence of CCCP (Fig. 8), presumably because of inactivation of the efflux process. However, the entry rate did not reach a clear plateau even at 200 μM CCCP (Fig. 8). This was unexpected because the assay of the proton motive force, using as an indicator the accumulation of a substrate of an ion gradient-coupled transporter, [$^{14}$C]proline (26), showed that more than 95% of the proton

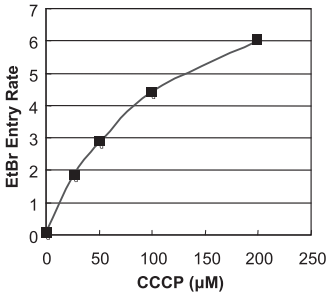


FIG. 8. Rate of entry of ethidium into CS093 cells with various concentrations of CCCP. Wild-type *S. enterica* serovar Typhimurium strain CS093 was grown in LB broth with aeration by shaking and was harvested when the $OD_{600}$ reached 1.8. The influx of ethidium into cells, which generates fluorescence due to binding of the dye with nucleic acids, was measured as described in Materials and Methods, in 50 mM potassium phosphate buffer (pH 7.0). Cells were preincubated with the indicated concentrations of CCCP for 5 min, before addition of 6 μM (final concentration) ethidium. The ordinate indicates the entry rate of ethidium expressed as the change in the fluorescence emission intensity (in arbitrary units) per second. EtBr, ethidium bromide.

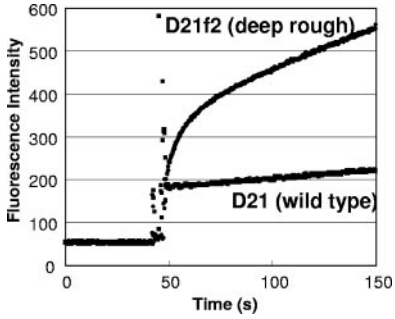


FIG. 10. Rate of entry of ethidium into wild-type (D21) and deep rough LPS mutant (D21f2) cells of *E. coli* K-12 in the presence of 50 μM CCCP. Cells were grown in LB broth with aeration by shaking and were harvested when the $OD_{600}$ reached 1.8. The influx of ethidium into cells, which generates fluorescence due to binding of the dye with nucleic acids, was measured as described in Materials and Methods, in 50 mM potassium phosphate buffer (pH 7.0) containing 5 mM $MgCl_2$. Cells were preincubated with CCCP for 5 min before addition of 2 μM (final concentration) ethidium at 50 s.

motive force was dissipated with 25 μM CCCP (not shown). Since 25 μM CCCP was probably sufficient, we routinely used either 50 or 100 μM in our assay.

When the entry rates of ethidium in the presence of 100 μM CCCP were compared for CS022 (PhoP-constitutive mutant) and the isogenic *phoP* null mutant CS015, the former strain was shown to have a more efficient barrier against permeation of the dye under these conditions (Fig. 9), thus confirming the data obtained with *tolC* mutants in the absence of CCCP (Fig. 2, left panel). When 2 mM $Mg^{2+}$ was added to the washing and resuspension buffer, the ethidium entry rates decreased significantly for both strains (Fig. 9). The difference between the two strains almost disappeared when $Mg^{2+}$ was present (Fig. 9);

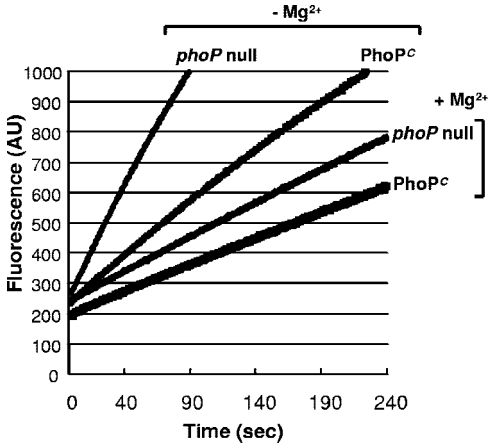


FIG. 9. Influx of ethidium into CS015 (*phoP* null) and CS022 (PhoP-constitutive [$PhoP^c$]) mutant cells. The strains were grown in LB broth with shaking, and the cells were harvested when the $OD_{600}$ reached 1.8; then the cells were washed and resuspended in 50 mM potassium phosphate buffer with or without 2 mM $MgCl_2$. The ethidium influx was assayed in the same buffer with 100 μM CCCP, as described in Materials and Methods. AU, arbitrary units.

these results are also similar to those obtained with *tolC* strains in the absence of CCCP (Fig. 2, left panel).

When the assay with the *tolC* strains was done in the presence of 50 μM CCCP, the *phoP* null mutant remained more permeable than the PhoP-constitutive strain (Fig. 2, right panel). However, with both strains the OM permeability was much higher than that in the absence of CCCP (note the difference in ordinate scales in the two panels of Fig. 2). The increase in permeability occurred even though the efflux pumps should have been completely inactive due to the *tolC* null mutation. These results therefore indicate that CCCP increased OM permeability by an additional mechanism, presumably by partitioning into the OM bilayer, and explain why the permeability kept increasing even at high CCCP concentrations beyond that required for complete collapse of the proton motive force (Fig. 8).

**Ethidium influx assay measures the permeability of the LPS/phospholipid bilayer domain of OM.** Mutant strains producing deep rough LPS are hypersusceptible to various dyes (2), a result suggesting that a dye, ethidium, traverses the OM largely through the LPS-containing bilayer domain. Nevertheless, we confirmed experimentally that the OM bilayer is the main site of ethidium penetration as follows. (i) When the ethidium permeation rates for a deep rough mutant and its isogenic *E. coli* K-12 parent, both deenergized for active efflux with CCCP, were compared, the former, with its compromised LPS/phospholipid bilayer (35), showed far greater permeability than the latter (Fig. 10), in spite of the fact that the former contained fewer porin molecules (3). (ii) No decrease in ethidium permeability was seen in a porin-deficient (*ompF*::Tn*5* Δ*ompC*) strain of *E. coli* K-12 compared with its wild-type parent (not shown).

## DISCUSSION

A major conclusion from our study is that the PhoPQ-regulated remodeling of the OM improves its barrier function (Fig. 2 to 7 and 9; Tables 2 and 3). In the comparison of *tolC* strains in an $Mg^{2+}$-free buffer, the PhoPQ-activated modifica-

tions resulted in a more-than-fourfold increase in the barrier function against the penetration of a cationic dye, ethidium, in comparison with the *phoP* null mutant, in which the modification was absent (Fig. 2, left panel). Because we are dealing with the nonspecific penetration of a lipophilic probe presumably through transient holes in the lattice of bilayer lipids (41), we can expect that our observations with ethidium would apply to other lipophilic probes. Indeed, in a divalent cation-poor environment, the OM of the PhoP-constitutive strain was also less permeable to a neutral dye, Nile red, and an acidic dye, eosin Y (Fig. 4), as well as to bile salts and some antibiotics that include basic (e.g., erythromycin), acidic (e.g., novobiocin), and zwitterionic (e.g., rifampin) compounds (see Results). The increased permeability of the bilayer in the *phoP* mutant, however, could be prevented nearly completely by the presence of millimolar concentrations of $Mg^{2+}$ (Fig. 2 and 3). We noted that the "leakiness" of the *phoP* OM does not result from gross rupture of the OM bilayer, since the mutant remains resistant to large, hydrophilic agents, such as bacitracin (results not shown). Rather, the different degrees of ethidium permeability seen here seem to reflect the tightness of interaction between neighboring LPS (and OM protein) molecules, which is needed for the production of a bilayer leaflet with unusually low fluidity and therefore with low passive permeability to lipophilic probes.

What is the biological significance of the more robust permeability barrier? Obviously, when the salmonella cells are in a divalent cation-poor environment, this would help them survive by preventing the entry of noxious compounds. This may be significant in an environment such as natural waters. This was also thought to be the case for phagosomes, where the divalent cation concentration (measured by using the expression of a PhoPQ-regulated gene as a sensor) was reported to be in the micromolar range (14). The PhoPQ system, however, responds to the presence of cationic peptides (4, 5, 7). Indeed, a recent study with nanoparticles showed that the $Mg^{2+}$ concentration within phagosomes is about 1 mM (32). Nevertheless, at 1 mM $Mg^{2+}$ cells with a remodeled OM bilayer are significantly more resistant to the permeation of exogenous compounds than cells without such a modification (Fig. 3), and this may be advantageous for the pathogen because it slows the influx of antimicrobial compounds generated by the host cells, certainly including cationic, lipophilic antimicrobial peptides and perhaps NO. The higher resistance of a PhoP-constitutive strain to polymyxin B, a polycationic lipopeptide antibiotic, has been known for some time (18, 22, 36). Beyond the actual permeation rates of noxious compounds, the permeability values of the bilayer are also an indicator of the robustness of the membrane structure, because permeation of solutes involves local stretching and deformation of the bilayer lattice as the solute molecule inserts into it.

The simplest explanation for the more effective barrier property of the PhoP-constitutive OM is based on the known modification of the lipid A (22), the bilayer-forming part of LPS. Thus, in unmodified lipid A, the acidic phosphate (and pyrophosphate) residues of the neighboring molecules are bound together by the bridging action of divalent cations. When some of these cations are removed, either in a divalent cation-poor environment or by competition from polycations such as cationic antimicrobial peptides, the structure becomes unstable. Addition of the positively charged compound 4-aminoarabinose (and the zwitterionic phosphoethanolamine whose positive charge presumably occupies a strategically important location) through the action of PmrAB-activated genes would decrease the electrostatic repulsion between the neighboring, polyanionic, lipid A molecules and would stabilize the bilayer. Similarly, addition of one more hydrocarbon chain by the palmitoyl transferase PagP, which is stimulated by the translocation of phospholipids into the outer leaflet of OM (24), would also stabilize the bilayer by increasing the hydrophobic interaction within the bilayer. Finally, LpxO adds a 2-hydroxyl group in a fatty acid chain. One of us has argued, on the basis of the structural similarity between lipid A and sphingolipids, that this may increase the H bonding between neighboring LPS molecules, thereby resulting in bilayer stabilization (35).

When all of the genes coding for these lipid A remodeling reactions were inactivated, the OM permeability of the PhoP-constitutive strain increased to nearly the level seen in *phoP* null mutants (Fig. 7). These results show that a major part of the PhoPQ-regulated modification of OM permeability involves modification of the lipid bilayer. However, in many assays, even the triple deletion brought the permeability not quite up to the level of the permeability of the *phoP* null mutant strain (Fig. 7 and Tables 2 and 3). This is not surprising because other reactions regulated by PhoP, such as modification of the proximal part of the LPS core oligosaccharide domain (42), the length of the O chain in LPS (9), or the induction/repression of OM proteins (19), are expected to contribute to the production of an effective barrier.

There are precedents for a structural defect in lipid A producing an unstable, presumably leaky bilayer; thus, *S. enterica* serovar Typhimurium strains defective in MsbB (also called LpxM), which catalyzes the addition of myristate onto the 3-OH group of the 3-hydroxymyristoyl residue at the 3′ position of lipid A, suffer from poor growth, especially in divalent cation-poor media (33). Although the OM permeability of these strains has not been measured, it seems very likely that the strains have the compromised barrier property, as they are hypersensitive to bile salts, which typically diffuse across the OM bilayer. However, the direct cause of the phenotypes of these mutants is still not clear, as *msbB* mutants are defective in the addition of 4-aminoarabinose (34, 43).

Finally, if the bilayer remodeled under PhoPQ regulation has such a robust barrier effect under various conditions, one may wonder why salmonella cells do not always carry out the remodeling reactions. Perhaps a clue comes from the observation that the unmodified bilayer of the *phoP* null mutant behaves better than the remodeled bilayer in the presence of 5 mM $Mg^{2+}$, at least under certain conditions (Fig. 6). A similar observation was also made with the disk inhibition assay using cholate (see Results). In the presence of such a high concentration of divalent cations, their bridging function may produce a tighter interaction between the neighboring lipid A molecules and, hence, a less permeable bilayer. It should be noted that our body fluids contain several millimolar levels of both $Mg^{2+}$ and $Ca^{2+}$ (10).

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