HOST-PATHOGEN INTERACTIONS

PhoQ is an unsaturated fatty acid receptor that fine-tunes *Salmonella* pathogenic traits

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The *Salmonella enterica* PhoP/PhoQ two-component signaling system coordinates the spatiotemporal expression of key virulence factors that confer pathogenic traits. Through biochemical and structural analyses, we found that the sensor histidine kinase PhoQ acted as a receptor for long-chain unsaturated fatty acids (LCUFAs), which induced a conformational change in the periplasmic domain of the PhoQ protein. This resulted in the repression of PhoQ autokinase activity, leading to inhibition of the expression of PhoP/PhoQ-dependent genes. Recognition of the LCUFA linoleic acid (LA) by PhoQ was not stereospecific because positional and geometrical isomers of LA equally inhibited PhoQ autophosphorylation, which was conserved in multiple *S. enterica* serovars. Because orally acquired *Salmonella* encounters conjugated LA (CLA), a product of the metabolic conversion of LA by microbiota, in the human intestine, we tested how short-term oral administration of CLA affected gut colonization and systemic dissemination in a mouse model of *Salmonella*-induced colitis. Compared to untreated mice, CLA-treated mice showed increased gut colonization by wild-type *Salmonella*, as well as increased dissemination to the spleen. In contrast, the inability of the *phoP* strain to disseminate systemically remained unchanged by CLA treatment. Together, our results reveal that, by inhibiting PhoQ, environmental LCUFAs fine-tune the fate of *Salmonella* during infection. These findings may aid in the design of new anti-*Salmonella* therapies.

INTRODUCTION

Nontyphoidal *Salmonella* infections result most frequently in gastroenteritis, which ranges from self-limiting to severe disease that can progress to invasive, life-threatening infections. The increase in prevalence and the acquisition of antimicrobial resistance makes *Salmonella enterica* an emerging threat to public health, being one of the leading causes of foodborne illness across the world. The global human health impact of nontyphoidal *Salmonella* alone has been estimated to be 93.8 million illnesses, 80.3 million of which correspond to foodborne disease and account for 155,000 deaths each year worldwide (*1*).

The expression of more than 60 genes that encode *Salmonella* virulence traits is controlled directly or indirectly by the PhoP/PhoQ signal transduction system (*1*). PhoP/PhoQ is an orthodox two-component system (TCS) composed of PhoQ, a transmembrane sensor with both histidine kinase and aspartate phosphatase activities, and PhoP, PhoQ's cognate cytoplasmic response regulator. Stimulation of PhoQ causes autophosphorylation of a histidine residue, after which the phosphoryl group is transferred to PhoP, thus activating its transcriptional regulatory activity. In the absence of activating stimuli, PhoQ dephosphorylates PhoP to attenuate signaling (*1*, *2*). It has been shown that the PhoQ protein can detect a multiplicity of environmental conditions such as acidic pH, cationic antimicrobial peptides, magnesium availability, periplasmic oxidative conditions, and changes in osmotic strength (*3*–*7*). The detection of these environmental cues is crucial for the pathogen to reprogram gene expression accordingly and to adapt, persist, multiply, and survive throughout its life cycle outside of and within host niches.

The PhoP/PhoQ system can control the remodeling of surface lipopolysaccharides (LPSs) by modulating the average chain length of the O antigen portion of LPS molecules and by stimulating chemical modifications that change the overall charge of the lipid A portion of LPS molecules. These changes are important for *Salmonella* to alter the properties of its outer membrane as a barrier to antimicrobial compounds and also to decrease recognition by the innate immune system (*8*, *9*). PhoP/PhoQ also plays a major role in the complex network that promotes the timely expression of both *Salmonella* pathogenicity island 1 (SPI-1) genes, which enable *Salmonella* internalization in nonphagocytic cells, and of SPI-2 genes, which modify the *Salmonella*-containing vacuole and define *Salmonella* intravacuolar survival (*10*–*12*).

Our previous work established that long-chain unsaturated fatty acids (LCUFAs) can inhibit *Salmonella* PhoP/PhoQ activity by reducing the autophosphorylation activity of the sensor PhoQ (*13*). Accumulation of LCUFAs in the periplasm of mutant *Salmonella* strains that have defects in the cytoplasmic transport and/or metabolism of LCUFAs also repressed the PhoP-dependent regulon. This led us to conjecture that LCUFAs might act as ligands for the periplasmic sensory domain of PhoQ (*13*).

Once inside the mammalian host through oral acquisition, *Salmonella* would encounter LCUFAs along the gastrointestinal tract. LCUFAs are components of bile (*14*) and are synthesized de novo or generated by the intestinal microbiota through the enzymatic conversion of dietary fatty acids (*15*). Linoleic acid (LA; *cis*,*cis*-9,12-octadecadienoic acid), one of the most abundant LCUFAs in the Western diet (*16*), can be transformed into conjugated LA (CLA), a mixture of positional and geometric conjugated isomers of LA, by biohydrogenating bacteria that inhabit the gut lumen, such as *Bifidobacterium*, *Roseburia*, or *Lactobacillus* strains (*15*, *17*). Whether and how LCUFAs act as signaling molecules in the PhoP/PhoQ

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system and their function as cues in the host environment remain unclear.

Here, we elucidated the mechanism of action of LCUFAs in the inhibition of *Salmonella* PhoQ activity. We demonstrated that the interaction of LCUFAs with PhoQ structurally altered the periplasmic domain of PhoQ, which led to the inhibition of the sensor's autokinase activity, a decrease in PhoP activation, and a reduction in the expression of PhoP-activated genes. Our results indicated that the action of LCUFAs on the PhoP/PhoQ TCS is conserved among different nontyphoidal *Salmonella* serovars. The recognition of LCUFAs by PhoQ was not stereospecific, and we identified specific amino acid residues of PhoQ that are involved in binding to LCUFAs and conformational changes in PhoQ induced by LCUFAs. In a mouse model of *Salmonella*-induced colitis, LCUFAs reduced PhoQ autokinase activity and PhoP-dependent gene expression and enhanced *Salmonella* gut colonization and dissemination to the spleen, supporting a physiological role for LCUFAs' PhoQ-mediated signaling during *Salmonella* infection.

RESULTS

Stereoselectivity and specificity of LCUFAs for PhoQ inhibition and the effect of fresh bile on signaling

To examine the stereoselectivity of LCUFAs as signals that repress PhoP/PhoQ activity, we tested positional and geometrical isomers of LA (*cis*,*cis*-18:2) for their ability to affect expression of PhoP target genes in *S.* Typhimurium 14028s. β-Galactosidase activity assays were performed using representative PhoP-activated genes (*pagC*, *virK*, *pipD*, *pagK*, *pcgM*, and *pcgF*) fused to *lacZ*. Bacteria were grown in PhoP/PhoQ-activating conditions (media alone), in PhoP/PhoQ-repressing conditions (media supplemented with $MgCl_2$) (*5*, *13*), and in the presence or absence of fatty acids. Dose-response inhibition of the representative PhoP-activated *pagC* reporter expression did not differ between *cis*,*cis*-18:2 (LA), *trans*,*trans*-18:2, or the *cis*,*trans*-18:2 isomers (CLA) (Fig. 1A). Decreased expression of five additional PhoP-activated reporters confirmed the inhibitory action of 18:2 isomers over the PhoP-regulon (Fig. 1B). The well-known repressive action of millimolar concentrations of extracellular Mg^{2+} on PhoP-activated genes was also tested as a control (Fig. 1B). None of the 18:2 isomers tested altered the expression of *cpxP* (*18*) or *tppB* (*19*), used as reporters for the activity of the PhoP/PhoQ-unrelated TCSs CpxA/CpxR and EnvZ/OmpR, respectively (Fig. 1, C and D). This last result also verified the specificity of these LCUFAs as PhoQ signals and the selectivity of PhoQ as a sensor for LCUFAs among structurally related bacterial histidine kinases. The inhibition of *pagC* expression by the action of 18:2 isomers was also observed in other *S. enterica* serovar strains (*S.* Typhimurium STM23, *S.* Enteritidis PT4, and *S.* Dublin SDu5), indicating that the repressive action of LCUFAs on PhoP/PhoQ activity was not limited to the serovar Typhimurium 14028s strain (Fig. 2).

Within the gastrointestinal tract, *Salmonella* encounter the components of bile, which includes a mixture of LCUFAs (*20*). The addition of crude bile extracts from mice to *Salmonella* growth medium decreased expression of the *virK* reporter in a dose-dependent manner (fig. S1A) but did not alter EnvZ/OmpR-dependent *tppB* expression (fig. S1B), thus reproducing the effect of LCUFAs on the expression of PhoP-regulated genes. This suggests that host bile would likely contribute to physiological signaling through the PhoP/PhoQ TCS, similarly to the signaling action of bile LCUFAs on the transcriptional activator ToxT, which controls virulence gene expression in *Vibrio cholerae* (*21*).

We previously established that LA modulates the expression of the PhoP regulon by inhibiting the autophosphorylation activity of the sensor protein PhoQ (*13*). To verify that 18:2 isomers other than LA also exerted their action through the inhibition of PhoQ autokinase activity, we isolated PhoQ-enriched membrane vesicles derived from a *S.* Typhimurium *phoQ* strain that expresses PhoQ from a plasmid and determined PhoQ autophosphorylation activity in the presence of [γ-^{32}P]ATP (adenosine 5′-triphosphate) (*13*). The presence of *trans*,*trans*-18:2 or CLA in the *Salmonella* culture medium inhibited PhoQ autokinase activity in a dose-dependent manner, similarly to LA (Fig. 3). Therefore, independently of their stereochemical configuration, 18:2 fatty acids inhibited PhoP/PhoQ signaling by repressing PhoQ autophosphorylation.

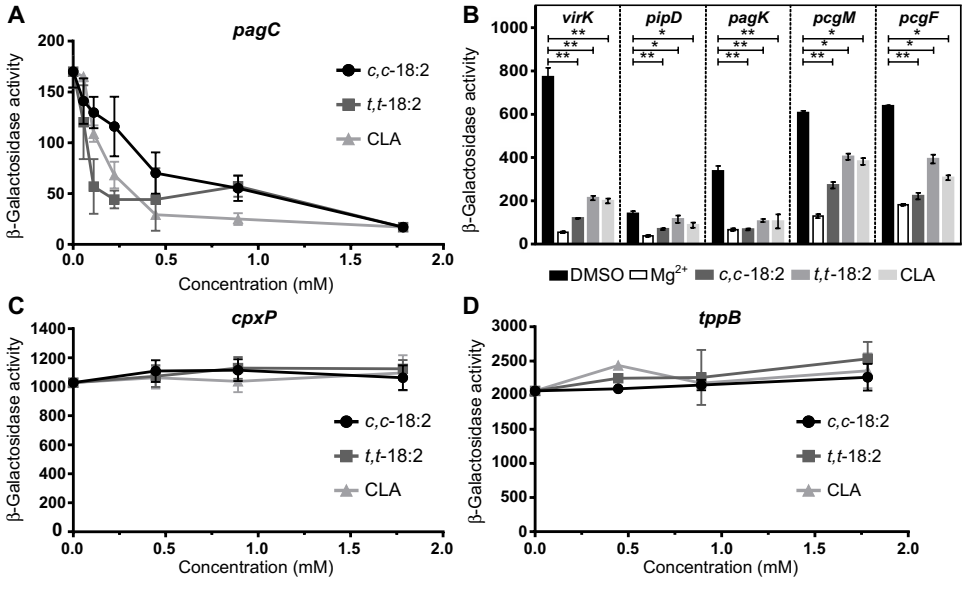

Fig. 1. LCUFAs repress PhoP-activated genes in *S.* Typhimurium 14028s. (**A**) Quantification of β-galactosidase activity in *S.* Typhimurium 14028s cells carrying the *pagC*::*lacZ* transgene in the presence of the indicated concentrations of *cis*,*cis*-9,12-octadecadienoic acid (*c*,*c*-18:2), *trans*,*trans*-9,12-octadecadienoic acid (*t*,*t*-18:2), or *cis*,*trans*-9,12-octadecadienoic acid (CLA). (**B**) Quantification of β-galactosidase activity in *S.* Typhimurium 14028s cells carrying the *virK*::*lacZ*, *pipD*::*lacZ*, *pagK*::*lacZ*, *pcgM*::*lacZ*, or *pcgF*::*lacZ* transgene in the presence of vehicle (DMSO), 10 mM $MgCl_2$ (Mg^{2+}), or the indicated unsaturated fatty acids at 1.8 mM. (**C** and **D**) Quantification of β-galactosidase activity in *S.* Typhimurium 14028s cells carrying *cpxP*::*lacZ* (C) or *tppB*::*lacZ* (D) in the presence of the indicated concentrations of *c*,*c*-18:2, *t*,*t*-18:2, or CLA. All results are the average of $n = 4$ independent assays performed in duplicate. Error bars indicate SD. All groups were compared with Mann-Whitney test. $^*P < 0.05$ and $^{**}P < 0.01$.

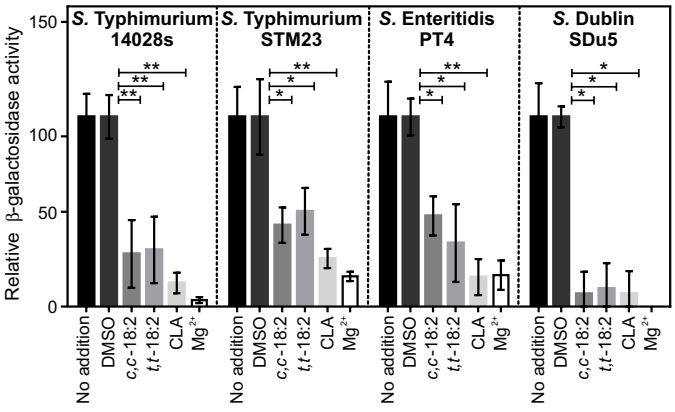

Fig. 2. LCUFAs inhibit PhoP-dependent gene expression in multiple *Salmonella* strains. Quantification of β-galactosidase activity in the indicated *Salmonella* strains carrying the *pagC*::*lacZ* transgene in the presence of vehicle (DMSO), 1.8 mM unsaturated fatty acids (*c,c*-18:2, *t,t*-18:2, and CLA), or 10 mM $MgCl_2$ (Mg^{2+}). All results are the average of $n = 3$ independent assays performed in duplicate. Error bars indicate SD. Values obtained for all groups were compared with Mann-Whitney test. $^*P < 0.05$ and $^{**}P < 0.01$. Nontreated or DMSO-treated samples were normalized to 100%.

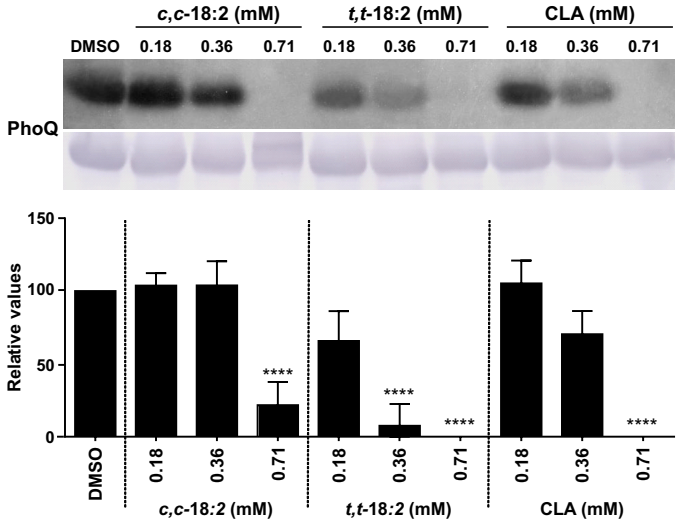

Fig. 3. Effect of *cis*, *trans*, and conjugated stereoisomers of LA on PhoQ activity. Autokinase activity of PhoQ in bacterial cell membrane preparations incubated with increasing concentrations of *c,c*-18:2, *t,t*-18:2, or CLA. The autophosphorylation reactions were analyzed by autoradiography (**top**), immunodetection (**middle**), and densitometry (**bottom**). Blots and autoradiographs are representative results from $n = 3$ independent experiments. Densitometry data represent three independent experiments. Error bars indicate SD. Ratios of the quantification of phosphorylated against total PhoQ from all groups were compared with Tukey's post hoc test. $^{****}P < 0.0001$.

Interaction of LCUFAs with the sensor domain of PhoQ

To explore the mechanism by which LCUFAs exert their action on PhoQ, resulting in the repression of the sensor's autokinase activity, we purified the periplasmic sensor domain of PhoQ, PhoQp, which encompasses amino acids 44 to 148, by optimization of a previously reported protocol [(*22*) and Materials and Methods]. Thermal shift assays were performed to investigate the stability of PhoQp in the presence of LA or the saturated fatty acid octadecanoic acid (18:0) in the range of 0 to 0.15 mM. The addition of LA, but not 18:0, resulted in a dose-dependent reduction of the melting temperature (Tm) value, indicative of PhoQp structure destabilization (Fig. 4A). These results also allowed us to calculate the binding affinity [association constant (K_a)] of LA to PhoQp (Fig. 4A). Consistent with these results, the LA isomers *trans*,*trans*-18:2 and CLA also caused a decrease in PhoQp Tm values at 0.8 mM (Fig. 4B). In contrast, Mg^{2+} addition elicited a dose-dependent increase in Tm values, indicative of PhoQp stabilization (Fig. 4C). These results were in agreement with the demonstrated independent effects determined for LCUFAs (repressors of PhoQ autokinase activity) or Mg^{2+} (an inducer of PhoQ phosphatase activity) over the modulation of PhoQ action (*2*, *13*).

We also followed the temperature destabilization process of PhoQp in the absence or presence of LA by nuclear magnetic resonance (NMR). We collected a series of ^{1}H-NMR spectra of PhoQp as a function of temperature and followed several isolated methyl resonances. Whereas most of them behaved in a similar way, the methyl peak at 0.63 parts per million (ppm) showed a different change in chemical shift at 45° and 50°C in the presence of LA compared to the absence of fatty acids (Fig. 4D and fig. S2). This observation not only reinforced the thermal shift assays results but also showed that the effect of unsaturated fatty acids on the stability of PhoQp was due to local interactions of the fatty acids with specific regions of the protein.

To determine the specific residues of PhoQ that interact with LCUFAs, we collected a series of ^{1}H-^{15}N heteronuclear single-quantum coherence (HSQC) NMR spectra of PhoQp in the presence of 0.8 mM (4 equivalents) LA or CLA. We analyzed the chemical shift perturbation (CSP) of PhoQp by acquisition of ^{1}H-^{15}N HSQC spectra of the ^{15}N-labeled protein using resonance assignments provided by K. G. Hicks (*23*). NMR experiments were performed in buffer 10 mM tris-HCl (pH 8.0) to match the conditions used in our previous in vitro assays (fig. S3). Of the 120 signals assigned on PhoQp at pH 3.5, 20 were broadened beyond detection at pH 6.5 and about 90 remained in the spectrum at pH 8.0; the remaining was presumably absent because of faster proton exchange at higher pH and the absence of divalent cations (Fig. 5A; as reported, addition of divalent cations yielded the PhoQp spectrum with about 20 additional peaks) (*23*, *24*). We could transfer assignments to 80 signals in our conditions and found that both LA and CLA caused CSPs >0.025 ppm in the spectrum. The addition of 4 equivalents of LA resulted in shifts on 12 assigned signals, whereas the addition of 4 equivalents of CLA resulted in shifts on 14 assigned signals. In contrast, no changes in chemical shifts were observed in the presence of 18:0 (Fig. 5, B and C). Together, these results reveal that LA and geometrical and positional isomers, chosen as representative LCUFAs, imposed a conformational change on the periplasmic sensor domain of PhoQ. These findings show that LCUFAs act as input signals for PhoQ and likely account for the molecular mechanism that underlies their actions as inhibitors of the PhoP/PhoQ TCS activity. In addition, it is worth pointing out that although both LCUFAs and millimolar Mg^{2+}, by acting as PhoQ signals, result in inhibition of the PhoP/PhoQ phosphorelay, the analysis comparing the PhoQp NMR spectra when LA, Mg^{2+}, or the two signal molecules together were added to the assay clearly shows that distinct and differentially located amino acids were perturbed by each separate ligand (fig.S4, A and B). These findings are also consistent with LA inhibiting the PhoP/PhoQ phosphorelay by reducing PhoQ autokinase activity and Mg^{2+} inhibiting the phosphorelay by stimulating PhoQ phosphatase activity.

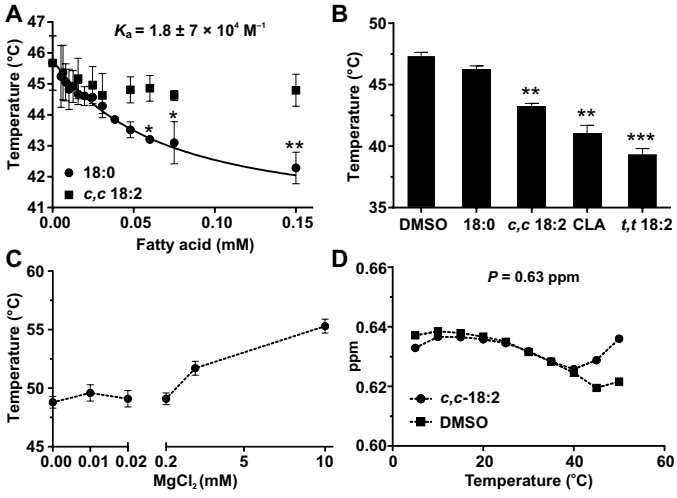

Fig. 4. Effects of *cis*, *trans*, and conjugated stereoisomers of LA on the thermal stability of PhoQp. (**A**) Thermal shift assay of the purified periplasmic domain of PhoQ (PhoQp) with increasing concentrations of LA (*c,c*-18:2) or the saturated fatty acid octadecanoic acid (18:0). The melting temperature (Tm) was calculated in a reaction medium containing PhoQp and the indicated concentrations of the corresponding fatty acid, and the affinity constant K_a was calculated. (**B**) Tm of PhoQp in the presence of vehicle (DMSO) or 4 equivalents of 18:0, *c,c*-18:2, CLA, or *t,t*-18:2. (**C**) Tm of PhoQp in the presence of the indicated concentrations of $MgCl_2$. Data shown in (A) to (C) represent results from $n = 3$ independent experiments. Error bars indicate SD. All groups were compared with Kruskal-Wallis test. *$P < 0.05$, ** $P < 0.01$, and ***$P < 0.001$. (**D**) Chemical shift of the methyl peak at 0.63 ppm in ^{1}H spectra of PhoQp with DMSO (vehicle) or 4 equivalents of *c,c*-18:2 upon increasing temperature. Results represent $n = 3$ independent assays.

The effect of oral administration of CLA on *Salmonella* invasion in vivo

Mice are intrinsically resistant to *Salmonella* serovar Typhimurium enterocolitis. However, streptomycin-pretreated mice have proven to be a robust model for serovar Typhimurium colitis (*25*, *26*). We chose this animal model because we were interested in determining the effect of a short-term dietary supplementation with LCUFAs on *Salmonella* virulence, taking into consideration that the effect of these fatty acids would be more pronounced during the intestinal phase of the infection (*27*).

To examine the effect of LCUFAs on *Salmonella* infection, we determined bacterial loads in the cecum content of streptomycin-pretreated mice that were orally infected with either wild-type or *phoP* mutant *S.* Typhimurium 14028s strains and treated orally with either CLA or phosphate-buffered saline (PBS) daily. We also determined bacterial loads in spleens at 24 and 72 hours post-infection (hpi). CLA-enriched formulations are commercially available as human dietary supplements (*28*) and are compatible with performing in vivo assays in mice that require large quantities of the compound. We first verified that CLA dietary supplement (ds-CLA; fig. S5A, inset) exerted the expected dose-dependent inhibition over PhoP-activated genes and that it concomitantly repressed PhoQ autokinase activity (fig. S5, A and B).

At 24 hpi, cecal bacterial loads [colony-forming unit (CFU)/g] showed no difference between ds-CLA– or PBS-treated mice infected with either the wild-type or the *phoP* strain, whereas bacterial counts in spleens were below the level of detection irrespective of treatment. At 72 hpi, ds-CLA–treated mice showed increased bacterial loads for the wild-type strain in cecum compared to PBS-treated mice (Fig. 6A). Irrespective of treatment, the *phoP* strain also showed moderately increased loads in cecum when compared to wild-type strain in PBS-treated mice. The fact that CLA-treatment did not affect the *phoP* strain as it did with the wild-type strain indicated that the effect could not be attributed to the higher availability of fatty acids as *Salmonella* nutrients.

In parallel, at 72 hours, the wild-type strain reached significantly higher loads in spleens of CLA-treated mice when compared to PBS-treated mice (Fig. 6B). Consistent with the reported intra-macrophage survival impairment and inability to reach the spleen of the *phoP* mutant (*29*, *30*), bacterial loads of the *phoP* strain were significantly lower than the wild-type strain in the spleens of either PBS-treated or ds-CLA–treated mice (Fig. 6B).

DISCUSSION

It has been well established that PhoQ functions as a remarkable bacterial receptor able to integrate multiple extra- and intracellular environmental signals along different steps of the *Salmonella* infection process. Input signals include Mg^{2+} limitation, which globally induces the PhoP regulon; sublethal concentrations of cationic antimicrobial peptides and mild acidic pH, both of which activate the expression of subsets of PhoP-regulated genes; and a decrease in the oxidizing activity of the periplasm, which has also been shown to stimulate PhoP/PhoQ through the DsbA-MgrB pathway in *Escherichia coli* (*3*–*7*, *13*, *31*). Our previous work demonstrated that LCUFAs also exert a repressive action on the PhoP/PhoQ TCS by inhibiting PhoQ autokinase activity (*13*). In this work, by thermal shift and NMR assays, we showed that the interaction with LCUFAs induced a conformational change in the periplasmic domain of PhoQ. The NMR spectra revealed that LCUFAs affected residues located within the β3 and β4 strands and the β3-β4 connecting loop of the PhoQp structure. These regions are distal from the acidic patch that connects the α5 and β6 strands, adjacent to the transmembrane domains, and the target for Mg^{+2} action (*23*). We previously demonstrated that the simultaneous presence of Mg^{2+} and LA results in an additive repression of PhoP-regulated genes, ruling out a potential competitive effect between these two PhoQ signals (*13*). These results are also in agreement with our observation that LCUFAs induce a conformation that represses PhoQ autophosphorylation activity, whereas high Mg^{2+} concentrations have been demonstrated to enhance PhoQ phosphatase activity toward phosphorylated PhoP (*2*). This is also in agreement with the results by Hicks *et al.* (*23*), who concluded that the PhoQ conformational status in acidic pH or under conditions of limited Mg^{2+} is different from that induced by cationic antimicrobial peptides and postulated that distinct conformational states exist for each of the unique PhoQ-activating and PhoQ-repressing stimuli.

During the infection of mammalian hosts, *Salmonella* physiologically encounters LCUFAs within biliary ducts and in the complex content of the gut, which is conditioned by the composition of food combined with metabolically active microbiota. We here showed that fresh bile and 18:2 isomers, including CLA, exerted inhibitory action on the PhoP/PhoQ-dependent regulon by specific and selective inactivation of PhoQ autokinase activity. This effect of LCUFAs was observed not only in *Salmonella* Typhimurium but also in other *S. enterica* serovar strains. Using the streptomycin treatment model of *Salmonella* infection in mice, we found that short-term oral

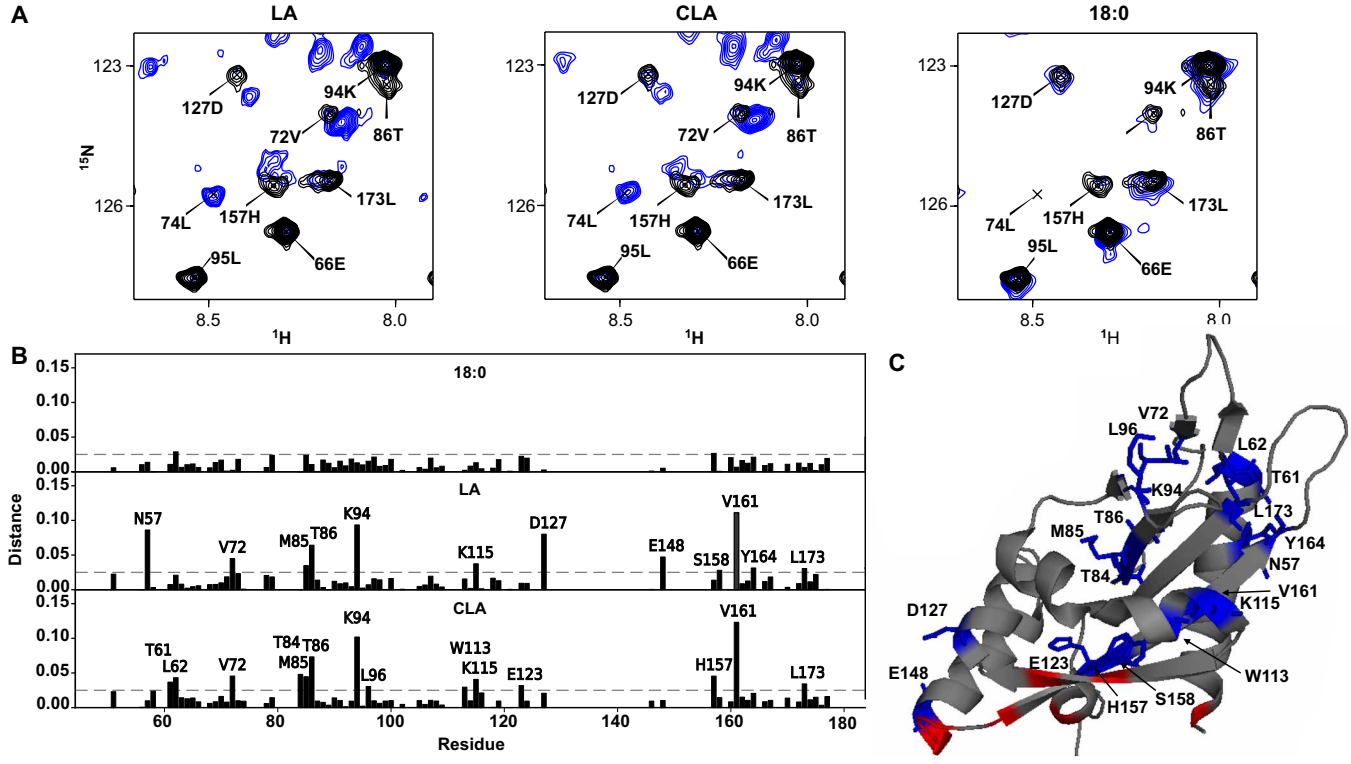

Fig. 5. NMR characterization of the interaction between PhoQp and fatty acids. (**A**) Partial ^{1}H-^{15}N HSQC spectra of PhoQp in the presence of vehicle (DMSO) or 4 equivalents of octadecanoic acid (18:0), *cis*,*cis*-9,12-octadecadienoic acid (LA), or *cis*,*trans*-9,12-octadecadienoic acid (CLA). ^{1}H-^{15}N HSQC spectra of PhoQp with vehicle (DMSO) are shown in fig. S3. (**B**) Plots of chemical shift perturbations (CSPs) for individual residues caused by the addition of 4 equivalents of 18:0, LA, or CLA. Residues that experienced CSPs >0.025 ppm in the presence of LA and CLA are indicated in each plot. (**C**) CSPs for individual residues caused by the addition of LA and CLA mapped onto the crystal structure of PhoQp (Protein Data Bank 1YAX). LA- and CLA-sensitive residues are shown in blue, and negatively charged residues that bind divalent cations (*24*) are shown in red.

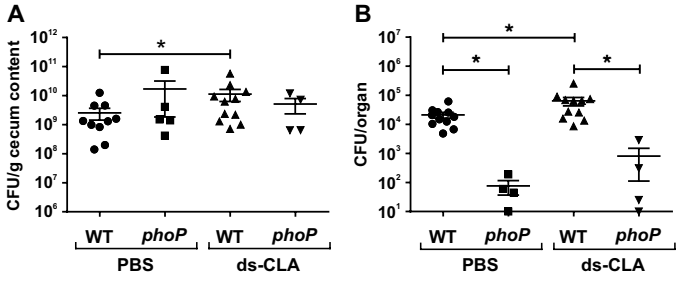

Fig. 6. Effect of dietary CLA in a mouse model of salmonellosis. C57BL/6 mice were infected with ~1 × 10^{8} CFU of wild-type or *phoP* mutant *S.* Typhimurium 14028 and then treated daily with PBS or 50 mg of CLA dietary supplement (ds-CLA). (**A**) Bacterial counts in cecum at 72 hpi. (**B**) Bacterial counts in spleens at 72 hpi. *P < 0.05, Mann Whitney test. Data shown are means and SEs from n = 11 animals per group for the wild-type (WT) strain and n = 5 animals per group for the *phoP* mutant strain.

administration of CLA provoked a mild but consistent increase in the capacity of wild-type *Salmonella* to colonize the cecum, which was also observed for the *phoP* mutant strain. This correlated with statistically significantly enhanced colonization of the spleen by wild-type *Salmonella* in CLA-treated mice. These results strongly suggested that LCUFA-mediated inhibition of PhoP-regulated gene expression during the intestinal phase of the infection provided *Salmonella* a modest advantage in the capacity to colonize the cecum, which was accompanied by a greater ability to reach the spleen, indicating increased survival inside macrophages. This reconciles with the fact that repression of the PhoP/PhoQ TCS favors the expression of SPI-1 genes in early invasion steps (*10*, *32*, *33*). Because the effects of LCUFA signaling on PhoP/PhoQ are reversible (*13*), LCUFA-mediated repression in the gastrointestinal tract should not lead to PhoP/PhoQ inhibition within *Salmonella* intracellular niches. These observations are in agreement with our in vitro LCUFA-mediated PhoP/PhoQ repression results and allow us to conclude that the interaction of CLA with orally acquired *Salmonella* would suffice for the wild-type strain to behave similarly to the *phoP* strain during the early steps of infection.

It has been established that *phoP* mutant strains fail to cause disease in susceptible hosts. PhoP/PhoQ-mediated host adaptation of lipid A promotes bacterial survival by lowering host cytokine and chemokine production. The *phoP* mutant strains are unable to either carry out the bacterial surface modifications that reduce the immunostimulatory capacity of their LPS or decrease the expression of critical antigens, such as the flagellar structural component FliC, which is recognized by Toll-like receptors in epithelial and phagocytic cells and by specific receptors in T cells (*34*). The failure of *phoP* strains to repress FliC triggers protective immune responses in the host, thus contributing their inability to cause disease. After oral infection, *phoP* strains can colonize Peyer's patches in the gut but are attenuated for virulence because they fail to spread to the liver and spleen (*29*). Given that it has been reported that *Salmonella* benefits from

the inflammatory response generated at the gut (*35*, *36*), we can conjecture that in ds-CLA–treated mice, an increased inflammatory response would provide wild-type *Salmonella* a colonization advantage. *Salmonella* could then dock to intestinal cells and inject the type three secretion system 1 effector proteins, which are encoded by SPI-1 genes, thus promoting its internalization. Once inside epithelial or phagocytic cells, PhoP-mediated activation of SPI-2 genes is required for *Salmonella* survival. Irrespective of the extracellular concentration of LCUFAs, the *phoP* strain would not be able to properly modulate the expression of SPI-1 and SPI-2 genes inside host cells (*33*, *37*). Within host cells, only the wild-type strain would be able to reprogram its PhoP/PhoQ-dependent expression pattern to survive inside phagocytic cells and disseminate to other organs, such as the spleen.

Although LCUFA content in the gastrointestinal tract is highly variable depending on host diet, microbiota composition, and health status (*38*–*40*), *Salmonella* can also be exposed to LCUFAs present in bile. *Salmonella* can colonize the hepatobiliary tract, and an association between the presence of *Salmonella* in bile or gallstones and chronic carriage of the bacterium has been reported (*41*, *42*). We have shown that fresh bile also represses the PhoP-regulated genes by the inhibition of PhoQ autokinase activity, similarly to purified LA or CLA. In bile from patients with cholelithiasis, Tsuchiya *et al.* (*43*) reported concentrations of individual free LCUFAs in the range of 0.23 to 2.0 mM, which is in agreement with the concentrations that inhibited PhoP/PhoQ activity in this study.

Several lines of evidence indicate that dietary CLA supplementation can provoke inflammatory and immunological host responses and that probiotic CLA-producing bacteria can ameliorate the impact of enteric pathogens (*44*–*47*). However, these effects have been shown to depend on numerous variables such as the relative contribution of each LA isomer, the animal model, or the particular *Lactobacillus* probiotic strain, and no clear consensus on a beneficial role for CLA has yet emerged [comprehensively reviewed in (*47*)]. Together, our results indicate that LCUFA-mediated inhibition of the PhoP/PhoQ system is beneficial for *Salmonella* during the early phases of infection in mice, favoring intestinal colonization of the gut, which, in turn, results in dissemination to internal organs.

In sum, we elucidated the structural basis of the inhibitory action of LCUFAs on the PhoP/PhoQ TCS as signals that are detected by PhoQ and conformationally alter the sensor protein, resulting in the inhibition of its autokinase activity. This, in turn, provokes the repression of PhoP-activated genes, shaping the niche-dependent expression of *Salmonella* virulence traits. The mechanism that underlies LCUFAs as relevant in vivo environmental cues sensed by PhoQ sheds new light on the complex processes that determine the outcome of *Salmonella*-associated disease. In addition, this knowledge will be a useful tool for the rational design of specifically tailored antivirulence compounds targeting PhoQ and developing new therapies to fight *Salmonella* infections.

MATERIALS AND METHODS

Chemicals and reagents

Nitrocellulose membranes were from Amersham Biosciences. [γ-^{32}P] ATP (3000 μCi/mmol) was obtained from PerkinElmer Life Sciences. The oligonucleotides were purchased from Invitrogen (listed in table S1). SYPRO Orange protein dye was from Thermo Fisher Scientific. All fatty acids (99% purity) were purchased from Sigma-Aldrich: octadecanoic acid (18:0), *cis*,*cis*-9,12-LA (18:2Δ6), *trans*,*trans*-9,12-octadecadienoic acid (18:2Δ6), and CLAs (a mixture of *cis* and *trans*-9,11- and -10,12-octadecadienoic acids, ≤1% LA). Absence of Mg^{2+} in the fatty acid stocks was verified by atomic absorption spectrometry. Octadecanoic acid was diluted in dimethyl sulfoxide (DMSO) to a final stock concentration of 60 mM, whereas unsaturated fatty acids were diluted in DMSO to a final stock concentration of 356 mM. ds-CLA was obtained from Natufarma (Argentina), and ds-CLA composition is detailed in fig. S5.

Bacterial strains, culture, and growth conditions

Bacterial strains used in this work are listed in table S2. Bacteria were grown at 37°C in lysogeny broth (LB) with shaking, with the addition of 0.7 mM isopropyl-β-D-thiogalactopyranoside (IPTG) when appropriate and/or the addition of fatty acids or bile at the concentrations indicated in each assay. Ampicillin was used at a final concentration of 100 μg·ml^{-1}, and streptomycin was used at 50 μg·ml^{-1}. For mouse infection experiments, bacteria were grown overnight (ON) at 200 rpm at 37°C in LB containing streptomycin (50 μg·ml^{-1}). Then, ON cultures were diluted 1:20 in the same medium plus 0.3 M NaCl and subcultured for 4 hours, with shaking at 100 rpm. Bacteria were then centrifuged and resuspended in cold PBS (~1 × 10^8 CFUs per 100 μl).

Genetic and molecular biology techniques

Plasmids used in this work are listed in table S3, and all constructs were verified by DNA sequencing. DNA was introduced into bacterial strains by electroporation using a Bio-Rad *E. coli* Pulser electroporator following the manufacturer's recommendations. *Salmonella* strains carrying *lacZ* reporter fusion to promoters on the chromosome were carried out using Lambda Red–mediated recombination in strain 14028s (*48*). For animal studies, strains were made streptomycin resistant by P22 phage transduction of the *aadA* gene from the streptomycin-resistant serovar Typhimurium strain SL1344 (www.sanger.ac.uk/Projects/Microbes/). The resulting transduced strains were able to grow in streptomycin (500 μg·ml^{-1}), although they were routinely grown in this antibiotic at 50 μg·ml^{-1}. In addition, growth curves were performed to verify that genetic manipulation did not affect the growth properties of the original isolates.

Effect of LCUFAs on *Salmonella* growth

The potential inhibitory effect of fatty acids was evaluated by growing *Salmonella* Typhimurium wild-type strain in LB in sterile 96-well microtiter plates, as described in (*13*, *43*). Fatty acids were tested in serial twofold dilutions from 0 to 2 mg·ml^{-1}. DMSO (vehicle of fatty acids) did not exceed a final concentration of 0.5% (v/v), tested to be not detrimental for bacterial growth. The concentrations used in these experiments did not alter the pH of the medium. No inhibition of bacterial growth was detected within the concentration range of fatty acids assayed.

β-Galactosidase activity assays

For β-galactosidase activity assays, bacteria were grown overnight with shaking at 37°C in LB with 10 mM $MgCl_2$ and/or each commercial fatty acid or bile at the final concentration indicated in each experiment. Ampicillin was used at a final concentration of 100 μg·ml^{-1} in the bacterial growth medium when appropriate. β-Galactosidase activity was determined, as described in (*49*).

Preparation of membranes enriched in PhoQ

Membranes to test the autokinase activity of the sensor PhoQ were prepared as described in (*13*, *23*). Briefly, overnight culture of *S.* Typhimurium strain PB4663 was used to inoculate LB containing the corresponding fatty acid at the final concentration indicated in each experiment and grown at 37°C to exponential phase [$A_{630\ nm}$ (absorbance at 630 nm) = 0.6]. Protein expression was induced by the addition of 0.7 mM IPTG for an additional 3 hours with shaking. Cells were collected and fractioned as detailed in (*13*). Last, the membranes were resuspended in 25 mM tris-HCl (pH 8.0) and 50 mM KCl. All procedures were carried out at 4°C. Protein concentration was determined by the bicinchoninic acid assay (Sigma-Aldrich) using bovine serum albumin as a standard.

Autokinase activity assays and immunodetection

For the autokinase activity assay, procedure was performed as described in (*13*). Briefly, membranes (50 μg of total protein) harboring PhoQ were incubated for 10 min at 37°C in a 30-μl reaction mixture containing [γ-^{32}P]ATP (0.16 μCi·μl^{-1}; PerkinElmer Life Sciences). Reactions were started by the addition of the reaction mixture and stopped by the addition of 6 μl of 5× SDS–polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer. All reactions were analyzed by SDS-PAGE (12% polyacrylamide), transferred to nitrocellulose, and then subjected to autoradiography or immunoblotted for the cytoplasmic domain of PhoQ, $PhoQ_{Cyt}$ (*13*). Phosphorylation and amounts of PhoQ in each condition assayed were densitometrically scanned using ImageJ software (*50*), and the ratio (phosphorylated against total PhoQ protein) was calculated taking the values obtained with LB + 0.05% (v/v) DMSO as 100%.

Expression and purification of the sensor domain of PhoQ (PhoQp)

The periplasmic sensor domain of the *S.* Typhimurium PhoQ protein (residues 45 to 190 preceded by an N-terminal Met; PhoQp) was purified from *E. coli* strain BL21(DE3) as described in (*22*) with a few modifications. Expression of PhoQp was achieved by addition of 0.7 mM IPTG to induce the DE3-encoded T7 RNA polymerase. For ^{15}N labeling and NMR experiments, cells were grown in M9 minimal medium supplemented with $^{15}NH_4Cl$ (1 g·ml^{-1}; Cambridge Isotope Laboratories). Cells were pelleted, resuspended in 10 mM tris-HCl (pH 8.0), and subjected to sonication. Cell lysate was combined with ammonium sulfate (55 and 90% saturation) to the supernatant. Fraction containing PhoQp was passed through an HiTrap desalting column (GE Healthcare) and purified by size exclusion chromatography (Superdex 75 column, GE Healthcare) in 100 mM NaCl and 10 mM tris-HCl (pH 8.0). PhoQp fraction was loaded onto a MonoQ HR 5/5 column and eluted using a linear gradient program with 0.02 to 1 M NaCl in 10 mM tris-HCl (pH 8.0). Fraction containing PhoQ was dialyzed and concentrated in 10 mM tris-HCl (pH 8.0) and 50 mM NaCl and kept at −80°C until further use.

Thermal shift assays

Thermal shift assays were performed using a real-time polymerase chain reaction thermal cycler (Mastercycler ep realplex, Eppendorf). Each 20 μl of standard assay contained 0.02 mM protein and 2.0 μl of the corresponding fatty acid from a commercial source at the final concentration indicated in each experiment, in a buffer containing 10 mM tris-HCl (pH 8.0), 150 mM NaCl, and SYPRO Orange at 10×. All experiments were performed in triplicate. Samples were heat-denatured from 20° to 80°C at a ramp rate of 1°C·min^{-1}. The protein unfolding curves were monitored by detecting changes in SYPRO Orange fluorescence. The fluorescence intensity was plotted as a function of temperature, and the initial and final slopes were fit to a linear function. The corrected fluorescence curves were used to obtain Tm by fitting the data the following equation

$$y = \mathrm{LL} + \frac{(\mathrm{UL} - \mathrm{LL})}{1 + \exp\left(\frac{\mathrm{Tm} - x}{a}\right)}$$

where LL and UL are the values of minimum and maximum intensities, respectively, and *a* denotes the slope of the curve at Tm (*51*).

The association constant of LA to PhoQp was estimated on the basis of the effect of LA on the Tm of PhoQp. Six data points (three replicas of two independent experiments) were averaged for each LA concentration used. The Tm data were then fit to the following equation

$$\mathrm{Tm} = \mathrm{Tm},0 + \mathrm{Amp} \times \left(\frac{\mathrm{LA} + \mathrm{PhoQp} + K_a^{-1} - \sqrt{\left((\mathrm{LA} + \mathrm{PhoQp} + K_a^{-1}\right)^2 - 4 \times \mathrm{LA} \times \mathrm{PhoQp}}}{2} \right)$$

where LA and PhoQp are the values of each concentration, Tm,0 is the Tm in the absence of LA, and Amp is the amplitude of the change in Tm induced by LA.

NMR spectroscopy

Protein–fatty acid interaction experiments were conducted on a 700-MHz Bruker NMR spectrometer at 298 K. ^{1}H one-dimensional spectra were acquired to follow protein destabilization of PhoQp at increasing temperature and referenced to sodium trimethylsilylpropanesulfonate standard. All spectra were processed with NMRPipe or TopSpin 3.5 and analyzed with CcpNmr. Fatty acid titrations were conducted in 10 mM tris-HCl (pH 8.0), 20 mM NaCl, and 10% D_2O, and 10 mM $MgCl_2$ was added when required. Titrations were performed by addition of the indicated fatty acid to a 0.2 mM protein sample. At each step a ^{1}H-^{15}N SOFAST-HMQC spectrum (*52*) was acquired. Backbone ^{1}H, ^{13}C, and ^{15}N resonance assignments of PhoQp were provided by K. G. Hicks (*23*) and were translated to each experimental condition by tracking chemical shifts through the titration series. Resonances that experienced a CSP greater than 0.025 ppm and/or that broadened beyond detection were considered statistically significantly affected. CSPs were calculated using the formula $((\Delta^1H)^2 + 0.15 \times (\Delta^{15}N)^2)^{1/2}$. Resonances that did not meet these criteria were considered unaffected.

Animal experiments

Animal experiments were performed as described in (*27*), with slight modifications as follows. Groups of 11 (for the wild-type strain) and 5 (for the *phoP* mutant) of 8- to 10-week-old female C57BL/6 mice (provided by the National Division of Veterinary Laboratories, Uruguay) were treated with 50 mg (72 μl) of ds-CLA or the same volume of PBS per oral gavage at 24-hour intervals. Animals were supplied with water and food ad libitum, except that 3 hours before oral gavage of ds-CLA, streptomycin, and *Salmonella*, food and water were withdrawn. Twenty-four hours before infection, mice were

pretreated with 25 mg of streptomycin per oral gavage. Two days after the first ds-CLA administration, animals were infected with ~1×10^8 CFUs of the indicated bacterial strain per oral gavage. Serial dilutions of the bacterial suspension used to infect animals were plated in LB-agar to exactly quantitate the CFUs inoculated. Treatment with ds-CLA (or PBS) at 24-hour intervals was maintained for the whole duration of the experiment. For bacterial enumeration in organs, at the indicated times after infection, mice were euthanized by cervical dislocation and the cecum content and the spleens were aseptically removed, weighted, homogenized, and resuspended in PBS for subsequent dilution and plating in *Salmonella-Shigella* agar plates containing streptomycin (50 $\mu g \cdot ml^{-1}$) (ceca) or LB-agar plates (spleens). Mice fresh bile was extracted, collected, and pooled from gallbladders of control mice and added to bacterial growth LB medium, as indicated (fig. S1). Experiments with animals were performed according to Uruguay guidelines for animal experimentation that meet the International Guiding Principles for Biomedical Research Involving Animals, and all protocols were approved by the University of the Republic Ethical Committee for Animal Experimentation, Uruguay.

Statistical analysis

After outlier removal, statistical analysis was performed using one-way analysis of variance (ANOVA). The normality and homogeneity of variance assumptions were validated using Shapiro-Wilk's and Levene's tests, respectively. Multiple comparison of means was computed with Tukey's post hoc test. For nonparametric analysis, the Kruskal-Wallis test and Dunn's test for multiple comparisons were used. In all cases, the overall statistical significance level was 0.05. For analysis of differences in the bacterial counts in cecal content and spleen, the Mann-Whitney test was used, considering $P < 0.05$ (two tailed) to be statistically significant.

SUPPLEMENTARY MATERIALS

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Fig. S1. Effect of fresh bile on PhoP-regulated genes.
Fig. S2. ^{1}H-NMR characterization of the interaction between PhoQp and LA.
Fig. S3. ^{1}H-^{15}N HSQC spectra of PhoQp.
Fig. S4. ^{1}H-^{15}N HSQC PhoQp spectra with ligands.
Fig. S5. Effect of dietary CLA on the activity of the PhoP/PhoQ TCS.
Table S1. Oligonucleotides.
Table S2. Bacterial strains.
Table S3. Plasmids.
References (*53*–*60*)

View/request a protocol for this paper from *Bio-protocol*.

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PhoQ is an unsaturated fatty acid receptor that fine-tunes *Salmonella* pathogenic traits

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How *Salmonella* senses the gut

Salmonella enterica commonly causes gastroenteritis, but it can also disseminate from the gut to cause invasive infections and bacteremia. The two-component system comprising the sensor histidine kinase PhoQ and the cytoplasmic response regulator PhoP controls the expression of many *Salmonella* virulence genes. Carabajal *et al*. found that long-chain unsaturated fatty acids (LCUFAs), such as those found in the gut and in bile, directly inhibited the autokinase activity of PhoQ and reduced the expression of PhoP-activated genes in various *S. enterica* serovars. Oral administration of conjugated linoleic acid (CLA) enhanced both gut colonization and dissemination of *Salmonella* to the spleen. Thus, the detection of LCUFAs by PhoQ enables *Salmonella* to modify its virulence gene expression program for optimal colonization of the gut niche.