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Unsaturated Long Chain Free Fatty Acids Are Input Signals of the *Salmonella enterica* PhoP/PhoQ Regulatory System*[S]

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Background: The PhoP/PhoQ system governs crucial *Salmonella typhimurium* pathogenic traits.
Results: A screening of natural compounds showed that long chain fatty acids present in the bacterial growth medium down-regulate the PhoP/PhoQ-dependent regulon.
Conclusion: Long chain unsaturated fatty acids specifically inhibit PhoQ autokinase activity.
Significance: The novel PhoQ input signal reveals a new scenario for the control of *Salmonella* virulence, providing a rationale for future antibacterial strategies.

The *Salmonella enterica* serovar Typhimurium PhoP/PhoQ system has largely been studied as a paradigmatic two-component regulatory system not only to dissect structural and functional aspects of signal transduction in bacteria but also to gain knowledge about the versatile devices that have evolved allowing a pathogenic bacterium to adjust to or counteract environmental stressful conditions along its life cycle. Mg^{2+} limitation, acidic pH, and the presence of cationic antimicrobial peptides have been identified as cues that the sensor protein PhoQ can monitor to reprogram *Salmonella* gene expression to cope with extra- or intracellular challenging conditions. In this work, we show for the first time that long chain unsaturated free fatty acids (LCUFAs) present in *Salmonella* growth medium are signals specifically detected by PhoQ. We demonstrate that LCUFAs inhibit PhoQ autokinase activity, turning off the expression of the PhoP-dependent regulon. We also show that LCUFAs exert their action independently of their cellular uptake and metabolic utilization by means of the β-oxidative pathway. Our findings put forth the complexity of input signals that can converge to finely tune the activity of the PhoP/PhoQ system. In addition, they provide a new potential biochemical platform for the development of antibacterial strategies to fight against *Salmonella* infections.

The *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium) PhoP/PhoQ system consists of an orthodox two-component regulatory system, comprising PhoQ, a transmembrane sensor with bifunctional (histidine kinase/phosphatase) activity, and PhoP, a cytoplasmic response regulator (1). This two-component regulatory system serves as a master regulator of *S.* Typhimurium virulence. The regulon governed by the *S.* Typhimurium PhoP/PhoQ two-component regulatory system includes genes that are critical for Mg^{2+} homeostasis (2) and those that provoke modifications of the LPS, which determine bacterial susceptibility to cationic antimicrobial peptides (2–5). The PhoP/PhoQ system is also involved in the bacterial entry mechanism into the host cell, modulating the expression of the injectisome and of translocated effectors (6–8). Once inside the cell, PhoP-modulated genes contribute to define the intracellular survival of *Salmonella*, being implicated in the intravacuolar proliferation capacity of the bacteria and in the traffic divergence of the *Salmonella*-containing vacuole from the canonical endocytic pathway (9–11).

We have shown previously that the environmental availability of Mg^{2+} signals on PhoQ and controls the switch between the kinase and the phosphatase activity of the sensor protein, modulating PhoP phosphorylation status and, thereby, the transcriptional activity of this response regulator (1, 12). In addition to Mg^{2+} limitation, which has been demonstrated to globally modulate the PhoP regulon (1, 2, 12–14), sublethal concentrations of cationic antimicrobial peptides and mild acidic pH activate the expression of subsets of PhoP-regulated genes (14–18). More recently, Lippa *et al.* (19) have shown that a decrease in the oxidizing activity of the periplasm can also stimulate PhoP/PhoQ through a DsbA/MgrB-dependent pathway. The multiplicity of signals that are able to act as an input of the PhoP/PhoQ system mirrors the intricate interaction of *Salmonella* with changing ambient conditions that this pathogen has to traverse through its life cycle.

Taking into consideration the relevant role of the PhoP/PhoQ system in the adaptive capacity of *S.* Typhimurium to either host or non-host environmental challenges and the fact that the two-component regulatory system can only be found in prokaryotes, low eukaryotes, and plants, PhoP/PhoQ consti-

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tutes an ideal target to search for new compounds that would prevent and/or control *Salmonella* virulence in the mammalian host.

Plant extracts are sources of compounds with exceptional chemical diversity that constantly give rise to novel therapeutic agents (20, 21). In this work, we carried out a screening of extracts from native plant species to identify naturally occurring molecules that would modulate PhoP/PhoQ activity. This search led us to determine that long chain unsaturated fatty acids (LCUFAs)[4] constitute specific input signals that down-regulate the PhoP/PhoQ system activity. We demonstrate that this repression is due to the inhibition of PhoQ autophosphorylation capacity. Exogenously provided long chain fatty acids are transported across the bacterial membrane and converted to acyl-CoA derivatives that are substrates either for β-oxidation or for the synthesis of membrane phospholipids. Therefore, we examined whether the incorporation of the LCUFAs into these metabolic pathways was responsible for PhoQ autokinase repression. By the analysis of the effect of LCUFAs on mutant strains that are blocked in these metabolic routes, we demonstrate that neither esterification into membrane phospholipids nor degradation by the β-oxidative path is a prerequisite for LCUFAs to exert their repressive action on PhoQ activity. Our results indicate that free unsaturated fatty acids constitute specific signals detected by PhoQ, which provoke the inhibition of the sensor autokinase activity. In turn, this effect is transmitted downstream, resulting in the inactivation of the regulator PhoP with a concomitant down-regulation of PhoP-activated gene expression.

The fact that exogenously provided LCUFAs are able to specifically signal on PhoQ not only widens the array of potential environmental scenarios that *Salmonella* is capable of perceiving *in vivo* in order to orchestrate the expression of virulence phenotypes by means of the PhoP/PhoQ system, but also provides a rationale for the future design of new specifically targeted antibacterial strategies.

EXPERIMENTAL PROCEDURES

Chemicals and Reagents—Nitrocellulose membranes were from Amersham Biosciences. [γ-^{32}P]ATP (3,000 μCi/mmol) and [1-^{14}C]linoleic acid (58.2 mCi/mmol) were obtained from PerkinElmer Life Sciences. TLC silica gel 60 F_{254} plates were purchased from Merck. Column chromatography silica gel (150-Å pore, 35–75-μm particles) was purchased from Analtech. The oligonucleotides were purchased from Sigma. All fatty acids (99% purity) were purchased from Sigma: palmitic (16:0), palmitoleic (16:1Δ^9), stearic (18:0), oleic (18:1Δ^9), linoleic (18:2Δ^6), and γ-linolenic acid (18:3Δ^6). The purity of unsaturated fatty acids was also checked by ^{1}H NMR analysis. ^{1}H NMR spectra were recorded on a Bruker Avance II at 300 MHz in $CDCl_3$ (7.26 ppm), in the presence of TMS (0.00 ppm) as the internal standard. The absence of Mg^{2+} in the fatty acid stocks was verified by atomic absorption spectrometry. Saturated fatty acids (palmitic and stearic acids) were diluted in 10% tergitol to a final stock concentration of 50 mg ml^{-1}, whereas unsaturated fatty acids (palmitoleic, oleic, linoleic, and linolenic) were diluted in absolute ethanol to a final stock concentration of 100 mg ml^{-1}.

The critical micelle concentration (CMC) for oleic, strearic, palmitic, and linoleic sodium salts was calculated to be in the 1.8–3.5 mM range (22, 23). Although the exact CMC values under our experimental conditions have not been determined, from these orientative data, the concentrations used in the assays reported here are below the respective CMC (0.5 mg/ml corresponds to concentrations of ≤1.8 mM for the fatty acids used in this work).

Plant Material—Plants were collected in Pergamino, Buenos Aires Province, Argentina, during November 2005. Voucher specimens were deposited at the Universidad Nacional de Rosario Herbarium. Collected plants were *Urtica urens* L. (Utricaceae) (ID MO010), *Lamium amplexicaule* L. (Laminaceae) (ID MO001), *Brassica rapa* L. (Brasicaceae) (ID MO003), and *Sida rhombifolia* L. (Malvaceae) (ID MO030).

Crude Extracts—A sample (200 g) of dried and powdered material (entire plant) was refluxed 3 times with 4 liters of methanol for 45 min. The extracts were combined after filtration, and the solvent was eliminated under reduced pressure by rotary evaporation.

Bacterial Strains, Cell Culture, and Growth Conditions—Bacterial strains used in this work are listed in supplemental Table S1. Bacteria were grown at 37 °C in Luria-Bertani (LB) broth with shaking, with the addition of 0.7 mM isopropyl-β-D-thiogalactopyranoside when appropriate, and/or the addition of fatty acids at the concentrations indicated in each assay. Ampicillin was used at a final concentration of 100 μg ml^{-1}.

Assays to Evaluate Potential Inhibitory Action of the Compounds under Study on Salmonella Growth—The potential inhibitory effect of plant extracts or fatty acids was evaluated by growing *Salmonella* Typhimurium wild-type strain in LB broth. The assay was carried out in sterile 96-well microtiter plates. Plant extracts in methanol or fatty acids (dissolved in ethanol for unsaturated or tergitol for saturated fatty acids) were diluted to give serial 2-fold dilutions that were added to LB, resulting in concentrations ranging from 0 to 6 mg ml^{-1} (for plant extracts) and from 0 to 2 mg ml^{-1} (for fatty acids). Neither the concentration range of plant extracts nor that of the fatty acids used in these experiments altered the pH of the medium. The final concentration of methanol, ethanol, or tergitol in the assays did not exceed 12, 4, or 4%, respectively, which was not detrimental for bacterial growth. Ampicillin was included as a positive control at 50 μg ml^{-1}. The plates were incubated, for 18 h with agitation at 37 °C, and optical density was measured at 630 nm ($A_{630\ nm}$), with the BioTek Synergy 2 multimode microplate reader. Inhibition of bacterial growth by each compound was evaluated by monitoring growth as detected by $A_{630\ nm}$. No inhibition of bacterial growth was detected within the concentration range of either fatty acids or plant extracts assayed in this work.

Bacterial Growth Curves—Overnight cultures of *S.* Typhimurium wild-type, *fadL*, *fadD*, or *phoPQ* strains were diluted 1:100 in a sterile 96-well microplate (Greiner Bio-one) with fresh LB medium with or without the addition of commercial

[4] The abbreviations used are: LCUFA, long chain unsaturated free fatty acid; CMC, critical micelle concentration; X-gal, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; HEX, hexane; DCM, dichloromethane.

linoleic acid at a final concentration of 0.5 mg ml^{-1}, and the optical density at 600 nm was measured every 60 min with the BioTek Synergy 2 multimode microplate reader. Samples were processed by duplicates, and proper blank controls were used in each microplate.

Genetic and Molecular Biology Techniques—Plasmids used in this work are listed in supplemental Table S1. Plasmid DNA was introduced into bacterial strains by electroporation using a Bio-Rad apparatus as recommended by the manufacturer. Deletion of various genes and concomitant insertion of an antibiotic resistance cassette were carried out using λ Red-mediated recombination (24, 25) in strain LB5010 (26). The mutations resulting from this procedure were transferred to the *S.* Typhimurium wild-type background by P22 transduction. Phage P22-mediated transductions were performed as described (27). To introduce the *lacZ* reporter gene, antibiotic resistance cassettes were removed using the temperature-sensitive plasmid pCP20 carrying the FLP recombinase (28). pCE36 was used to introduce the transcriptional *lacZ* fusion as described previously (25). The oligonucleotides used to perform these procedures were purchased from Sigma. Their sequences are listed in supplemental Table S2. Double mutant strains were generated by P22 transductions from previously obtained single mutant strains and selected by the adequate combination of antibiotic resistances, as listed in supplemental Table S1. Other conventional recombinant DNA techniques were performed according to standard protocols (29). Polymerase chain reaction-derived constructions and site-directed mutagenesis were all confirmed by PCR assays and DNA sequence analysis.

RNA Purification—Total RNA was extracted from mid-exponential phase cultures grown in LB medium at 37 °C. Briefly, 8 ml of ice-cold 5% water-saturated phenol (pH 5.5) in ethanol was added to 50-ml cultures to stop RNA degradation. Cells were centrifuged at 5,500 × *g* for 5 min at 4 °C; resuspended in 5 ml of 0.5 mg ml^{-1} lysozyme-Tris-HCl (10 mM), EDTA (1 mM), pH 8.0, 1% SDS; mixed; and placed in a water bath at 64 °C for 2 min. After incubation, 5.5 ml of 1 M sodium acetate, pH 5.2, was added. The sample was extracted twice with an equal volume of water-saturated phenol, pH 5.5, and incubated at 64 °C for 6 min. The aqueous layer was extracted with an equal volume of chloroform and precipitated with ethanol. RNA was resuspended in water, treated with RQ1 RNase-free DNase (Promega), and subjected to a final step of RNA cleanup using an RNeasy kit (Qiagen) according to the manufacturer's protocol. DNA contamination in RNA preparations was assessed by performing a control PCR prior to RT-PCR analysis.

Semiquantitative RT-PCR—cDNA synthesis was performed using random hexamers, 2 μg of total RNA, and 1 unit of SuperScript II RnaseH2 reverse transcriptase (Invitrogen). Five microliters of a 1:10 dilution of cDNA was used as the template for DNA amplification in RT-PCR (20 μl), using primers PhoP-NTR and PhoP-NTF (supplemental Table S2). A primer set for the 16 S rRNA was used as a control to confirm that equal amounts of total RNA were used in each reaction mixture. The amplified fragment was 186 bp. The number of cycles was adjusted according to the level of expression of mRNA to ensure that the comparison was performed in the linear range of the amplification.

TLC Bioautography—The inhibitory activity of the assayed compounds on PhoP/PhoQ-dependent reporter gene expression was studied by TLC bioautography, a technique that combines chromatography separation with *in situ* activity determination. The amount of extract loaded on the TLC plate was 250 μg of extract/band. After elution with a mixture of methanol/dichloromethane (94:6), the solvents were removed under an air current, and the plate was subjected to autographic analysis. Chromatograms were placed in sterile Petri dishes with covers. TLC plates were overlaid with soft agar medium (LB with 0.6% agar) containing an inoculum of the reporter bacterial cell suspension from an overnight culture at a final concentration of 50×10^5 cfu ml^{-1}, 0.33 mg ml^{-1} X-gal, and the addition of the adequate antibiotic when required. After solidification of the medium, the TLC plates were incubated overnight at 37 °C for 18 h. Inhibition of the reporter gene expression was detected as light blue or white areas on a blue background given by the β-galactosidase activity-mediated cleavage of the X-gal substrate. Concomitantly, growth inhibition could also be detected by the presence of a transparent halo in contrast of the turbid appearance of the LB-agar layer that denoted bacterial growth.

Bioguided Fractionation—26 g of crude extract was dissolved in 1.0 liters of MeOH/H_2O (9:1) and extracted with hexane (2 × 500 ml). The MeOH was evaporated from the aqueous phase under reduced pressure, and H_2O was added to a final volume of 200 ml. The resulting solution was extracted with dichloromethane (3 × 200 ml). The organic phases (hexane and dichloromethane) were dried with anhydrous Na_2SO_4, and the solvents were evaporated at reduced pressure to give the corresponding hexane (HEX) and dichloromethane (DCM) fractions. The DCM fraction was chromatographed on silica gel (Analtech) with a dichloromethane/methanol gradient (99:1 to 90:10). The active fraction was chromatographed onto a TLC silica gel 60 F_{254} preparative plate (Merck) with dichloromethane/methanol (95:5). The active fraction was analyzed by NMR and GC-MS.

To rule out a potential repressive action on the PhoP/PhoQ system activity due to the presence of Mg^{2+}, the content of this divalent cation was measured in *L. amplexicaule* extracts by flame photometry. The Mg^{2+} content was determined to be 23.0, 4.4, and 0.8 μM for a 4.0 mg/ml solution of the *L. amplexicaule* methanolic crude extract and DCM and HEX fractions, respectively.

Mass Spectrometry and NMR Analysis—^{1}H NMR spectra were recorded on a Bruker Avance II at 300 MHz in $CDCl_3$, in the presence of TMS (0.00 ppm) as the internal standard. GC-MS was performed using a PerkinElmer Life Sciences Autosystem XL gas chromatograph coupled to a TurboMass mass spectrometer. The column was as follows: SE-30, 25 m × 0.22 mm inner diameter (Scientific Glass Engineering, Ringwood, Victoria, Australia). Prior to analysis, the fatty acids present in the samples were derivatized to methyl esters according to the procedure described by Lepage and Roy (30). Briefly, a solution of the samples in HCl-containing methanolic medium was heated at 80 °C for 150 min. The solution was diluted with

6% Na_2CO_3, and the derivatives were then extracted with hexane.

β-Galactosidase Activity Assays—For the β-galactosidase activity assays, bacteria were grown overnight with shaking at 37 °C in LB with 5 mM $MgCl_2$, *L. amplexicaule* extract fraction, and/or each commercial fatty acid at the final concentration indicated in each experiment. In the case of strains harboring a plasmid vector, isopropyl-β-D-thiogalactopyranoside was added at a final concentration of 0.7 mM. Ampicillin was used at 100 μg ml^{-1} final concentration in the bacterial growth medium when appropriate. β-Galactosidase activity was determined as described (31).

Preparation of Membranes Enriched in PhoQ or EnvZ—Membranes to test the autokinase activity of the sensors PhoQ or EnvZ were prepared as described previously (1). Briefly, overnight cultures of *S.* Typhimurium strains PB4663, PB10359, and PB9079 were used to inoculate LB containing 100 μg ml^{-1} ampicillin and the corresponding fatty acid from a commercial source at the final concentration indicated in each experiment. These were then grown at 37 °C to logarithmic phase ($A_{630\,nm}$ = 0.6), and protein expression was induced by the addition of 0.7 mM isopropyl-β-D-thiogalactopyranoside for an additional 3 h with shaking. Cells were collected, washed twice with 10 mM Tris-HCl (pH 8.0), and resuspended in a solution containing 20 mM Tris-HCl (pH 8.0), 20% sucrose, 5 mM EDTA, and 150 μg ml^{-1} lysozyme. After a 40-min incubation at 4 °C, 20 mM $MgCl_2$ was added, and cells were centrifuged for 20 min at 9,000 × *g*. The pellet was resuspended in ice-cold 10 mM Tris-HCl (pH 8.0) and subjected to sonication. Nonruptured spheroplasts were removed by a brief centrifugation at 5,500 × *g*, and the membrane fraction was recovered after a 40-min centrifugation at 21,000 × *g*. The membranes were washed subsequently with 10 mM Tris-HCl (pH 8.0), 2 M KCl, 10 mM Tris-HCl, 5 mM EDTA, and 10 mM Tris-HCl by 40-min centrifugation rounds at 21,000 × *g*. Each time, the supernatant was discarded. Finally, the membranes were resuspended in 25 mM Tris-HCl (pH 8.0), 50 mM KCl. All procedures were carried out at 4 °C. Protein concentration was determined by the bicinchoninic acid assay (Sigma) using bovine serum albumin as a standard.

Autokinase Activity Assays and Immunodetection Analysis—For the autokinase assay, membranes (50 μg of total protein) harboring PhoQ or EnvZ were incubated for 10 min at 37 °C in a 30-μl reaction mixture containing 25 mM Tris-HCl (pH 8.0), 50 mM KCl, 1 mM $MgCl_2$, 50 μM nonlabeled ATP, and 0.16 μCi μl^{-1} [γ-^{32}P]ATP (PerkinElmer Life Sciences). Reactions were started by the addition of the reaction mixture and stopped by the addition of 6 μl of 5× SDS-PAGE sample buffer (2.5% β-mercaptoethanol, 9% glycerol, 10% SDS, 600 mM Tris-HCl (pH 6.8), 0.006% bromphenol blue). The amount of radiolabeled PhoQ or EnvZ increased proportionally to the amount of membrane protein used in the range from 0.16 to 2.6 μg μl^{-1}; this indicated that PhoQ was not in excess at 1.67 μg μl^{-1}, the membrane concentration used in the autophosphorylation assay. Samples were heated for 3 min at 65 °C. All reactions were analyzed by SDS-PAGE (12% polyacrylamide), transferred to nitrocellulose, and then subjected to Western blot or autoradiography analysis. Western blot membranes were incubated with rabbit anti-$PhoQ_{Cyt}$ or anti-$EnvZ_{cyt}$ polyclonal antibodies, obtained as described (1), and developed by incubation with protein A conjugated with phosphatase, coupled to a chromogenic reaction using nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate as substrates. Autoradiographies and Western blot membranes were densitometrically scanned using ImageJ software (32) to perform quantitative determinations.

Analysis of Lipids by TLC—Overnight cultures of *S.* Typhimurium wild-type, *fadL*, and *fadD* strains were diluted 1:100 in fresh LB medium containing commercial linoleic acid at a final concentration of 0.5 mg ml^{-1} and 0.075 μCi ml^{-1} [1-^{14}C]linoleic acid. Cells were grown at 37 °C for 5 h with shaking. Then cells were harvested by centrifugation at 3,000 × *g* for 10 min, washed three times with phosphate-buffered saline (PBS, pH 7.4), and resuspended in 200 μl of the same buffer. Lipids were extracted over a weekend by the method of Bligh and Dyer (33) and spotted onto a TLC silica gel 60 F_{254} plate (Merck). Lipids were separated using a solvent system consisting of diethyl ether/petroleum ether/acetic acid (70:30:2, v/v/v). The TLC plates were exposed overnight to a Storage Phospho Screener screen, and product formation was detected and analyzed using an Amersham Biosciences Storm Imaging System equipped with Storm Scanner Control. Total lipids were developed with 10% $CuSO_4$ in 8% H_3PO_4 as a charring reagent. Densitometry was calculated for the major spots observed by TLC.

Statistical Analysis—Statistical analysis of all quantitative data shown was performed using one-way analysis of variance and the Holm-Sidak test with an overall significance level of 0.05.

RESULTS

Plant Extracts with PhoP/PhoQ System Inhibitory Action—We performed a systematic screen from four plant extracts (please see "Plant Material" under *Experimental Procedures*) in a search for compounds that could modulate the activity of the PhoP/PhoQ system in *Salmonella*. The methanolic total extract of each plant species was first screened by a dot agar overlay bioautography assay. Briefly, the crude methanolic extract was spotted onto a TLC plate and analyzed for activity without development of the plate. Inhibitory activity was detected by overlaying the plate with an aqueous solution of soft agar containing X-gal and a homogeneous suspension of the *S.* Typhimurium strain that harbors a reporter transcriptional *lacZ* fusion to *virK*, a previously characterized PhoP-activated gene (12, 34). Those extracts that produced a light blue or white halo (intact X-gal substrate) over the blue background of the hydrolyzed/oxidized X-gal were further analyzed (see "Experimental Procedures" for details). Among tested extracts, the *L. amplexicaule*-derived extract showed the largest inhibition zone.[5] With this *L. amplexicaule* extract, we also performed β-galactosidase activity assays in liquid medium to quantitatively assess the inhibitory action on the expression of the PhoP-dependent reporters. We tested the activity of seven different PhoP-activated genes with transcriptional fusions to *lacZ* when bacteria were grown in LB (PhoP/PhoQ-activating condition) or in LB supplemented with 5 mM $MgCl_2$ (PhoP/PhoQ-repressing condition) or a 1 or 4 mg ml^{-1} concentration

[5] M. O. Salazar *et al.*, unpublished results.

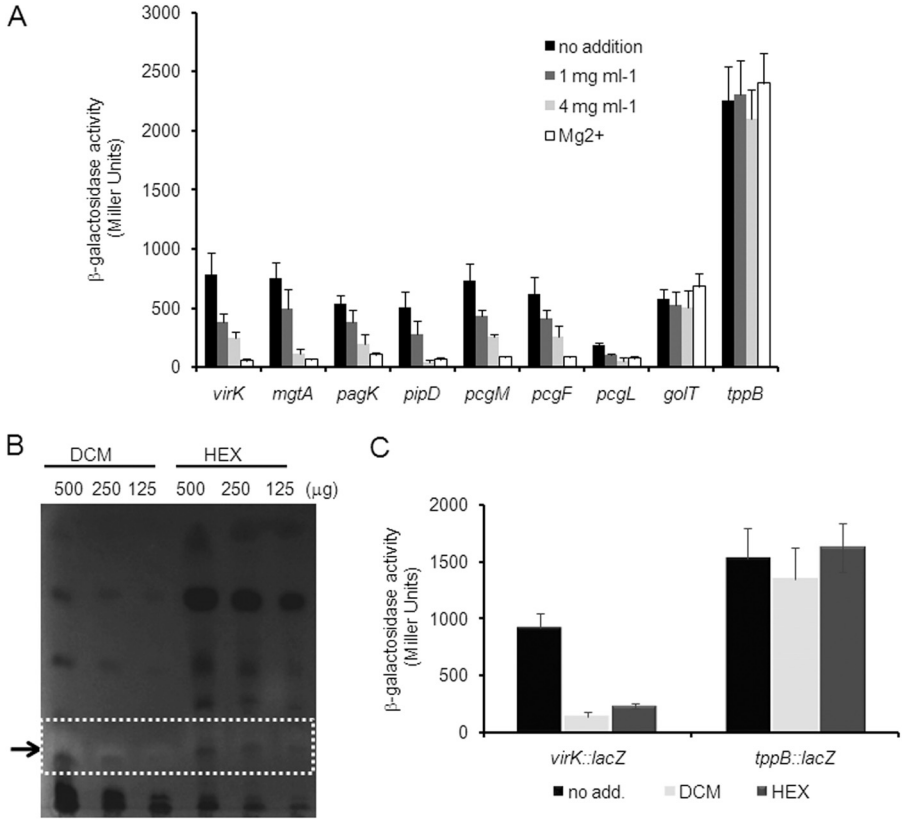

FIGURE 1. **Inhibitory action of *L. amplexicaule* plant extract on the expression of *S.* Typhimurium PhoP-activated genes.** *A*, β-galactosidase activity from *lacZ* transcriptional fusions to seven different PhoP-activated genes (*virK*, *mgtA*, *pagK*, *pipD*, *pcgM*, *pcgF*, and *pcgL*) and two PhoP-unrelated control reporters (*tppB* and *golT*). Cells were grown overnight either in LB (PhoP-activating condition), in LB plus the indicated concentration of the *L. amplexicaule* methanolic extract, or in LB supplemented with 5 mM $MgCl_2$ (PhoP-repressing condition). *B*, 500, 250, or 125 μg of DCM or HEX fractions of *L. amplexicaule* was loaded onto a TLC plate, and the separation was carried out with dichloromethane/methanol (95:5). LB-agar with a suspension of the reporter *virK*::*lacZ* strain was layered over the chromatogram as described under "Experimental Procedures." The *arrow* points at the area where *clear halos* (intact X-gal) over the *colored* hydrolyzed X-gal background were detected in each *lane*. *C*, β-galactosidase activity from *virK*::*lacZ* was carried out as in *A* using LB added with a 4 mg ml^{-1} concentration of the *L. amplexicaule* DCM or HEX fractions, as indicated. β-Galactosidase activity was measured as described under "Experimental Procedures." Results are the average of three independent assays performed in duplicate, and *error bars* correspond to S.D.

of the *L. amplexicaule* methanolic extract. We also tested the PhoP/PhoQ-unrelated transcriptional reporters *tppB*::*lacZ* and *golT*::*lacZ* as controls; *tppB* is a two-component OmpR/EnvZ-controlled gene, whereas *golT* is the transcriptional regulator of an operon involved in *Salmonella* resistance to transition metals (35–37). As shown in Fig. 1*A*, the expression of all PhoP-activated genes assayed was down-regulated in those cultures where the semipurified fraction of *L. amplexicaule* was added to LB, compared with LB with no additions, whereas *golT* or *tppB* gene expression was unaffected, showing that the observed inhibitory effect was specific for the genes that belong to the PhoP/PhoQ regulon.

Next, increasing polarity solvents (hexane and dichloromethane) were used to fraction *L. amplexicaule* dry extract. Both the HEX and DCM fractions showed inhibitory activity by either bioautographic or liquid β-galactosidase activity assays (Fig. 2, *B* and *C*, respectively). The DMC fraction was subjected to sequential bioactivity-guided purification by chromatographic steps (silica gel column followed by preparative TLC; see "Experimental Procedures") to obtain a semipurified bioactive fraction. We excluded bactericidal activity and verified that subinhibitory concentrations of the plant extracts were used (see "Experimental Procedures"). The composition of this bioactive fraction recovered from the preparative TLC was simultaneously determined by GC-MS and by ^{1}H NMR analysis (supplemental Figs. S1 and S2). Both analyses revealed that the fraction was mainly composed of fatty acids of 16–30-carbon length chain, 16 and 18-carbon species being the most abundant constituents.

To examine whether long chain fatty acids were responsible for the observed effect, the action of single unsaturated fatty acids with chain lengths of 16 and 18 carbons (C16 and C18) from commercial source was tested. C16 or C18 unsaturated fatty acids added to the bacterial LB growth medium down-regulated the expression of *virK* (Fig. 2*A*), whereas they did not exert a repressive effect on the expression of *tppB* (Fig. 2*B*). The four LCUFAs assayed down-regulated *virK* expression when a concentration higher than 0.25 mg ml^{-1} was added to LB. To explore whether a fatty acid moiety was enough to repress PhoP-activated genes, equivalent concentrations of palmitic (C16:0) or stearic (C18:0) saturated fatty acids added to LB were tested. As shown in Fig. 2, *C* and *D*, the saturated fatty acids did not alter either *virK* or *tppB* expression levels compared with LB alone, indicating that LCUFAs exert a specific inhibitory effect on the PhoP/PhoQ system activity. To further substantiate this observation, we also assayed the action of linoleic acid as a representative LCUFA on *ppiA* expression, used as reporter of the activity of the CpxA/CpxR two-component system (38).

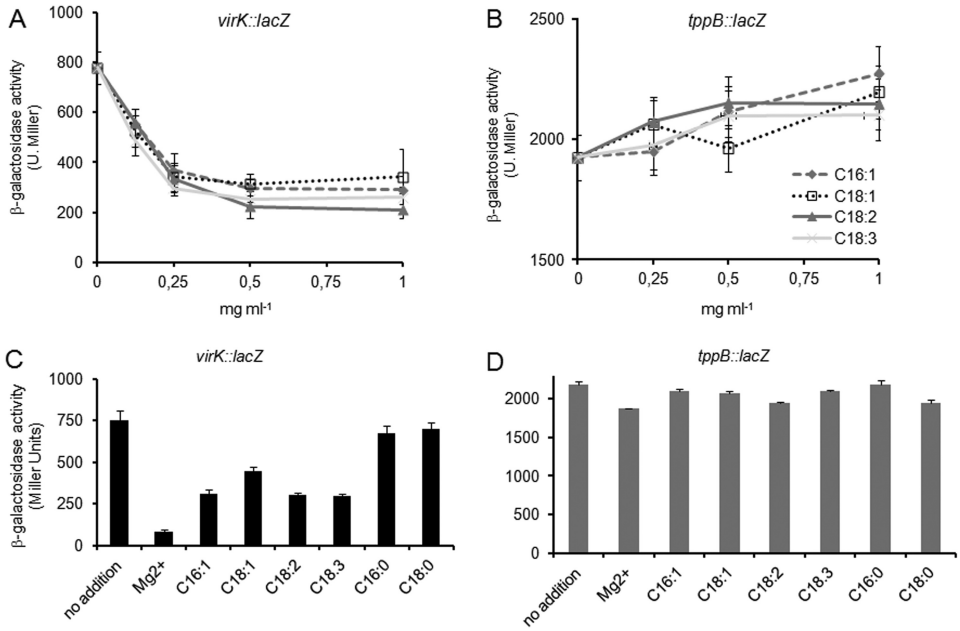

FIGURE 2. **LCUFAs repress the expression of *S.* Typhimurium PhoP-activated genes.** β-Galactosidase activity from *virK*::*lacZ* (*A*) or *tppB*::lacZ (*B*) transcriptional fusions was measured in cells grown overnight in LB with the indicated final concentration of the corresponding unsaturated fatty acid. β-Galactosidase activity from *virK*::*lacZ* (*C*) and *tppB*::*lacZ* (*D*) transcriptional fusions was measured in cells grown overnight in LB with no addition or supplemented with the indicated fatty acid (0.5 mg ml^{-1}) or with 5 mM $MgCl_2$. LCUFAs are named as follows: palmitoleic acid (*C16:1*), oleic acid (*C18:1*), linoleic acid (*C18:2*), and γ-linolenic acid (*C18:3*). β-Galactosidase activity was measured as described under "Experimental Procedures." Results are the average of three independent assays performed in duplicate, and *error bars* correspond to S.D.

As shown in supplemental Fig. S3, the expression of *ppiA* was modulated by pH and by ethanol (which was also used as the C18:2-solubilizing agent), previously described signals detected by the Cpx system (38, 39), but it was not affected by the action of the fatty acid in the bacterial growth medium. Additionally, as shown in Fig. 3*A*, PhoP-activated genes other than *virK* were down-regulated by linoleic acid (C18:2), confirming the specific action of the LCUFAs on the PhoP-regulon. (The well known repressing action of extracellular millimolar concentrations of Mg^{2+} on PhoP-activated genes was tested as a control.). We also assayed the combined action of Mg^{2+} and C18:2. The results shown in Fig. 3*B* indicate that, when simultaneously present in the *Salmonella* growth medium, Mg^{2+} and C18:2 are able to exert a repressive action that is higher than that observed when each inhibitor is separately tested, whereas no synergic effect is observed. This result led us to conjecture that LCUFAs might have a different target site of action on the PhoP/PhoQ system from that of divalent cations.

We also measured the transcriptional activity of the PhoP reporter gene from bacteria grown in LB plus C18:2 or from these preconditioned bacteria after the fatty acid has been removed by replacing the growth medium by fresh LB. As shown in supplemental Fig. S4*A*, the repression of *virK* expression could be relieved, provided the LCUFA was removed from the growth medium, indicating that the inhibitory action of LCUFAs on the PhoP/PhoQ system activity is reversible. The reversibility of the repression exerted by Mg^{2+} and the null effect on *tppB* expression levels obtained in all tested conditions are also shown as controls (supplemental Fig. S4, *A* and *B*).

The Transcriptional Activity of phoPQ Is Down-regulated by the Action of C18:2—Taking into account that *phoPQ* expression is subjected to autoregulation as part of the PhoP/PhoQ regulon (40), the concerted down-regulation in the expression of PhoP-activated genes allowed us to predict that the transcriptional activity of *phoP* would be turned off when bacteria are grown in the presence of LCUFAs. As shown in Fig. 3*C*, when bacteria were grown in LB with added C18:2, *phoP* transcription levels assessed by semiquantitative RT-PCR decreased an estimated 40% compared with those obtained in LB with no additions. The repressing action of Mg^{2+} on *phoP* expression (75% repression) and the transcriptional levels of 16 S rRNA, which were unaffected in the three conditions assayed, were also analyzed as controls (Fig. 3*C*).

LCUFAs Specifically Inhibit PhoQ Autokinase Activity—To define the PhoP/PhoQ transduction mechanism step that was affected by LCUFAs, we first examined the action of these compounds on PhoQ autokinase activity. With this aim, we isolated PhoQ-enriched membrane vesicles derived from the *S.* Typhimurim *phoQ*$^{-}$ strain that expresses PhoQ from the pUHE-21–2::*phoQ* plasmid, as we described previously (1) (also see "Experimental Procedures"). This assay system proved to preserve a PhoQ functional protein in its native bacterial membrane environment, avoiding conformational or functional artifacts that would result from the solubilization of the protein with detergents and reconstitution in artificial proteoliposomes. The *Salmonella*-derived vesicles were prepared from bacteria grown in LB with or without the addition of the indicated concentration of LCUFA, as described under "Experimental Procedures."

To determine PhoQ autophosphorylation activity, vesicles were incubated with a [γ-^{32}P]ATP-containing reaction medium, and samples were analyzed by SDS-PAGE followed by autoradiography, as described previously (1). As shown in Fig. 4*A*, PhoQ autophosphorylation was inhibited in a dose-dependent man-

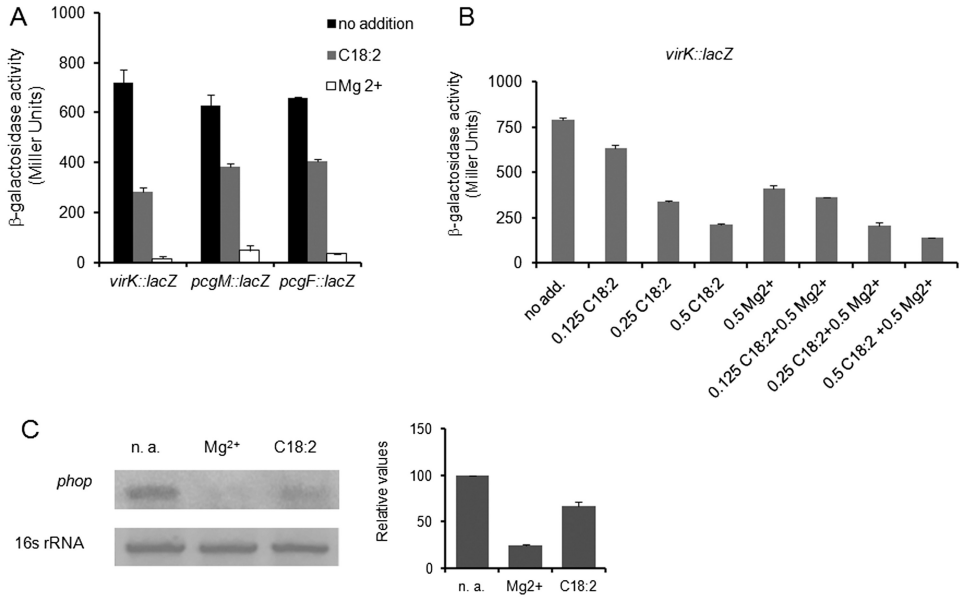

FIGURE 3. **LCUFAs repress PhoP-PhoQ regulon genes.** *A*, β-galactosidase activity from *virK*::*lacZ*, *pcgM*::*lacZ*, or *pcgF*::*lacZ* transcriptional fusions was measured in cells grown overnight in LB with no addition or supplemented with 0.5 mg ml^{-1} linoleic acid (*C18:2*) or with 5 mM $MgCl_2$. *B*, β-galactosidase activity from *virK*::*lacZ* transcriptional fusion was measured in cells grown overnight in LB with no addition, with the addition of 0.5 mM $MgCl_2$, or supplemented with 0.125, 0.25, or 0.5 mg ml^{-1} linoleic acid (*C18:2*) alone or in combination with 0.5 mM $MgCl_2$, as indicated. *C*, *phoP* mRNA expression determined by semiquantitative RT-PCR from *S.* Typhimurium wild-type strain grown to midexponential phase in LB with no additions (*n.a.*) or with the addition of either 0.5 mg ml^{-1} linoleic acid (*C18:2*) or 5 mM $MgCl_2$, as indicated. The RT-PCR products amplified from *phoP* and 16 S rRNA mRNAs isolated from bacteria grown to stationary phase were resolved by agarose gel electrophoresis. The plot shows the average and S.D. of the ratio obtained for *phoP* relative to the 16 S rRNA values obtained by densitometry from three independent experiments.

ner when bacteria were grown in LB containing increasing concentrations of C18:2. Inhibition of the PhoQ autokinase activity was detected when a concentration as low as 50 μg ml^{-1} C18:2 was used. Moreover, and consistent with the above shown β-galactosidase activity assays, the inhibitory action on PhoQ autokinase activity was observed for LCUFAs but not when saturated fatty acids of equivalent carbon chain lengths were tested (Fig. 4*B*). When EnvZ (an orthodox *Salmonella* histidine kinase sensor of the two-component family that has analogous structural and functional properties to PhoQ (41, 42)) autokinase activity was examined, following the same protocol employed for PhoQ, no detectable effect of saturated or unsaturated fatty acids on the autokinase activity of this sensor protein was observed (Fig. 4*C*), indicating that LCUFAs act selectively on the PhoQ sensor autophosphorylation activity. These last results also allow us to discard a potential nonspecific inhibitory action of the LCUFAs on the activity of the conserved catalytic domain of histidine kinases that would prevent autophosphorylation in the presence of ATP. Additionally, we verified that the addition of C18:2 in the autokinase reaction medium did not exert any effect on PhoQ autophosphorylation capacity (supplemental Fig. S5). The action of equivalent final concentrations of the fatty acid solvents employed added to the bacterial growth medium, tergitol or ethanol, was also tested to verify that they exerted no action on the autokinase activity of the sensor proteins (Fig. 4, *B* and *C*, *lanes T* and *E*, respectively). To exclude a potential action of fatty acids on PhoQ or EnvZ expression, we simultaneously monitored the protein level attained by each sensor by Western blot, and the ratio between the levels of labeled *versus* immunodetected bands was calculated by densitometry; the plot with the calculated ratios is shown *below* each autophosphorylation assay image in all autokinase assays.

We have demonstrated previously that millimolar concentrations of Mg^{2+} repress the PhoP/PhoQ system activity by inducing the phosphatase activity of PhoQ (1). The results shown in Fig. 4*D* corroborate our previous results demonstrating that the addition of increasing concentrations of Mg^{2+} to the *Salmonella* growth medium, which are inhibitory to the expression of PhoP-dependent genes, does not affect the autophosphorylation levels of PhoQ and also indicate that the addition of the cation in combination with C18:2 to the growth medium does not interfere with the repression exerted by C18:2 on PhoQ autophosphorylation levels. This last result allows us to conclude that the inhibitory effect exerted by LCUFAs when present in the *Salmonella* growth medium targets PhoQ autokinase capacity.

The Inhibitory Action of LCUFAs on the PhoP/PhoQ System Does Not Require a Functional β-Oxidative Metabolic Pathway—In either *Escherichia coli* or *Salmonella enterica*, exogenously provided long chain fatty acids are incorporated by the fatty acid β-oxidative metabolic route to finally render acetyl-CoA (43, 44). Lesley and Waldburger (45) have shown previously that acetyl-CoA exerts an inhibitory effect on the autokinase activity of PhoQ. To explore whether the increase in the intracellular concentration of acetyl-CoA or of an intermediate metabolite upon *S.* Typhimurium growth in LCUFAs was responsible for the observed PhoQ autokinase down-regulatory effect, we generated *Salmonella* mutant strains harboring deletions in *fadL*, *fadD*, or *fadBA*. These genes code for the proteins responsible for critical steps along the fatty acid β-oxidative pathway: transport across the envelope, activation to an acyl-CoA derivative, and iterative dehydrogenation cycles of the fatty acid, respectively (46). *fadL*, *fadD*, and *fadBA* are all part of the FadR regulon. FadR is a transcriptional regulator that plays

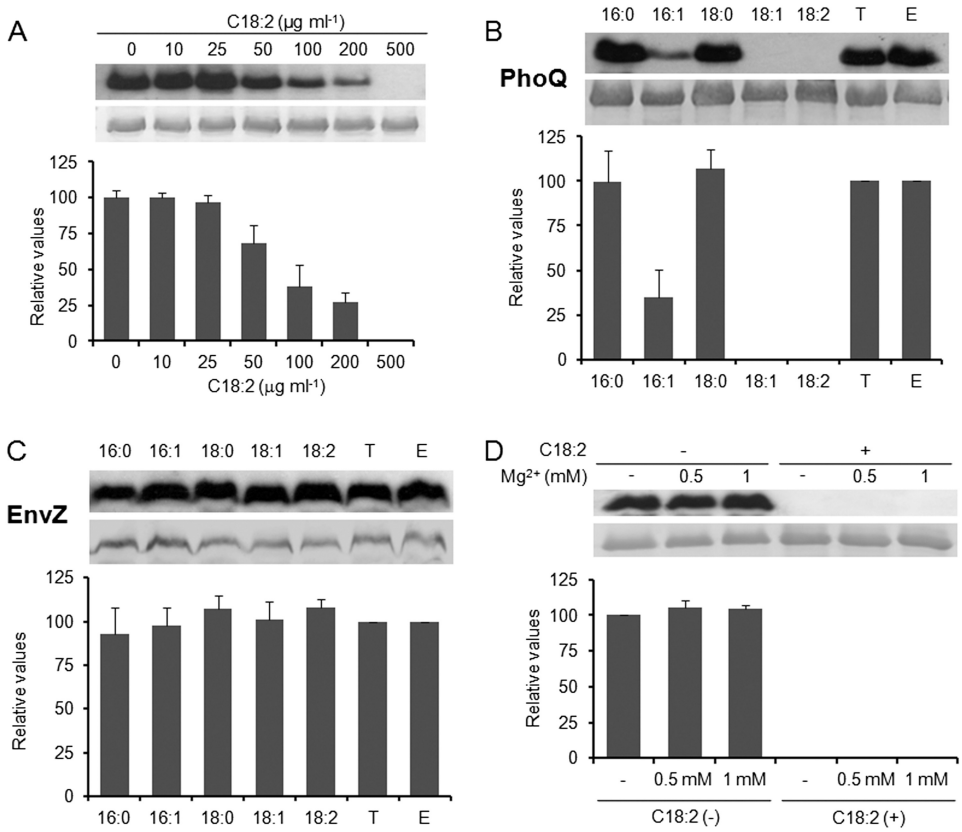

FIGURE 4. **The autokinase activity of PhoQ is the target of the inhibition by LCUFAs.** Increasing concentrations of linoleic acid (*C18:2*) as indicated (*A*), 0.5 mg ml^{-1} saturated or unsaturated fatty acids as indicated (*B* and *C*), or 0.5 mg ml^{-1} linoleic acid plus the indicated concentration of $MgCl_2$ (*D*) were added to the *Salmonella* growth medium. Membranes obtained by cell fractionation, harboring PhoQ or EnvZ, were incubated for 10 min at 37 °C in a reaction medium containing [γ-^{32}P]ATP, as described under "Experimental Procedures." The autophosphorylation reactions were analyzed by SDS-PAGE (12% polyacrylamide) and transferred to nitrocellulose, followed by autoradiography (*top*) or by immunodetection analysis developed with anti-$PhoQ_{Cyt}$ or anti-$EnvZ_{Cyt}$ polyclonal antibodies, respectively (*middle*). The phosphorylation levels and the protein expression levels of PhoQ or EnvZ in each condition assayed were determined by densitometry (as described under "Experimental Procedures"), and the ratio (labeled against immunodetected) was plotted (*bottom*), taking the values obtained with LB alone or with the addition of tergitol or ethanol as 100%, when either saturated or unsaturated fatty acids were used, respectively. Fatty acids are indicated as follows: palmitic acid (*16:0*), palmitoleic acid (*16:1*), stearic acid (*18:0*), oleic acid (*18:1*), linoleic acid (*18:2*), tergitol (*T*), and ethanol (*E*). Data shown represent results from three independent experiments. *Error bars*, S.D.

a dual role in fatty acid metabolism (47). Upon interaction with a fatty acyl-CoA, FadR derepresses the genes involved in β-oxidation, including *fadL*, *fadD*, and *fadBA*, whereas it activates the expression of fatty acid biosynthesis key enzymes (46). Therefore, to corroborate the metabolic response of the *S.* Typhimurium strain under analysis in the presence of LCUFAs, we generated *lacZ*-fused transcriptional reporters of the aforementioned *fad* genes. By β-galactosidase activity assays, we demonstrated that, as predicted, *fadBA* expression was induced when C18:2 was added to the bacterial growth medium (Fig. 5). *fadL-lacZ* or *fadD-lacZ* reporter constructs retain their respective promoter regions intact, whereas coding regions were removed and replaced by *lacZ*. These gene deletions block the entrance or the CoA activation of the fatty acid, abrogating its conversion to the corresponding acyl-CoA derivative. Consistently, the expression of *fadD* and *fadL* remained unchanged in the presence of fatty acids compared with LB with no additions (Fig. 5). We also examined the transcriptional activity of the *fad* genes in a wild-type *versus* a *phoP* background to exclude a potential regulatory effect by the PhoP/PhoQ system on their expression. As shown in Fig. 5, the expression of the *fad* genes was unaffected in the *phoP* mutant strain when compared with the otherwise isogenic wild-type strain grown

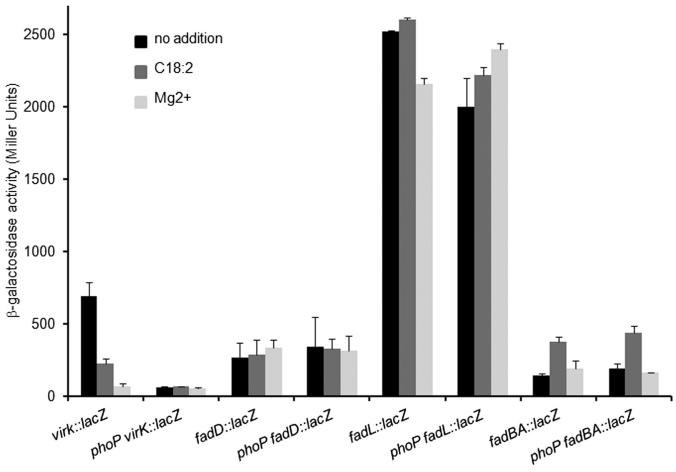

FIGURE 5. **Expression profile of β-oxidation pathway genes in the presence of LCUFAs.** β-Galactosidase activity of *virK*::*lacZ*, *fadD*::*lacZ*, *fadL*::*lacZ*, or *fadBA*::*lacZ* transcriptional fusions, under wild-type and *phoP* genetic backgrounds, was measured from cells grown overnight in LB supplemented with 5 mM $MgCl_2$ (PhoP-repressing condition) or with the addition of 0.5 mg ml^{-1} linoleic acid (*C18:2*), as indicated. Results are the average of three independent assays performed in duplicate, and *error bars* correspond to S.D.

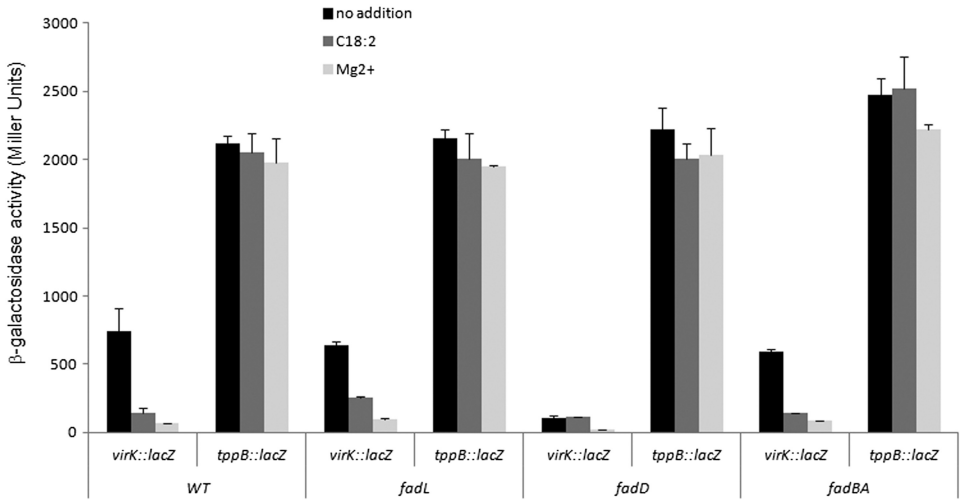

FIGURE 6. **The integrity of the β-oxidation pathway is not required for the LCUFA-mediated inhibition of PhoP/PhoQ activity.** β-Galactosidase activity of *virK*::*lacZ* and *tppB*::*lacZ* transcriptional fusions under wild-type, *fadL*, *fadD*, or *fadBA* genetic backgrounds was measured from cells grown overnight in LB supplemented with 5 mM Mg^{2+} or 0.5 mg ml^{-1} linoleic acid (*C18:2*), as indicated. Results are the average of three independent assays performed in duplicate, and *error bars* correspond to S.D.

in LB either with or without the addition of C18:2 or Mg^{2+}. Moreover, we verified that the addition of C18:2 to the LB medium did not significantly alter the growth pattern of *phoPQ* or *fad* mutant strains, compared with the wild-type strain (supplemental Fig. S6, *A* and *B*).

In the *fadL* or *fadBA* genetic backgrounds, C18:2 added to the bacterial growth medium rendered inhibition of *virK* expression levels equivalent to those observed in the wild-type strain (Fig. 6). *virK* transcriptional inhibition was observed when either wild-type or *fad* strains were grown in LB + Mg^{2+}, whereas the transcriptional activity of the *tppB*::*lacZ* reporter was unaltered in the three *fad* backgrounds in all conditions tested (Fig. 6). However, in the *fadD* strain, an estimated 7-fold reduction in the basal levels of *virK* expression was observed (Fig. 6, LB with no addition), precluding an accurate evaluation of the effect of the unsaturated fatty acid. Identical results were obtained when other PhoP-activated genes were assayed in the same *fad* backgrounds (not shown). Despite this last observation, which will be further examined, our results indicate that the inhibition of PhoP-activated genes by C18:2 does not require the integrity of the unsaturated fatty acid β-oxidative pathway.

Taken together, these results show that LCUFAs present in the bacterial growth medium exert a repressive action on the PhoP/PhoQ system activity that is independent of the metabolic degradative fate of these compounds in the cell and rule out the potential involvement of an accumulation of acetyl-CoA or of an intermediate metabolite of the β-oxidative pathway as responsible for the observed inhibition of PhoP-activated gene expression by LCUFAs.

As previously mentioned, FadD is required to activate the translocated exogenous unsaturated fatty acids into CoA-conjugated thioesthers. These acyl-CoA compounds can be either degraded by the β-oxidative path or used as fatty acid donors for esterification into membrane phospholipids by the PlsB/PslC-dependent pathway (48). In consequence, the observed reduction in *virK* expression in a *fadD* mutant strain can conceivably be attributed to lower levels of PhoQ protein expression, to a reduced PhoQ autokinase activity due to a change in the phospholipid composition of the membrane where the sensor is anchored, or to the accumulation of LCUFAs incorporated from the growth medium that cannot be vectorially transported to the cytoplasm and therefore inhibit *phoQ* expression by the *phoPQ* autoregulatory loop.

To explore this issue, we performed the autokinase activity assay using PhoQ-containing vesicles obtained from a *fadD* mutant strain (*fadD*/pUHE21–2::*phoQ*) grown in LB with or without the addition of C18:2. In this assay, because PhoQ expression was driven from the pUHE21-2lacI^{q} promoter, PhoQ expression is independent of the PhoP-modulated autoregulatory mechanism. As shown in Fig. 7, a reduction of PhoQ autokinase activity levels can be detected in the *fadD* mutant when compared with the wild-type strain in LB with no additions. As tested by simultaneous PhoQ immunodetection, this decrease correlates with a reduction in PhoQ steady-state levels in the *fadD* strain relative to the wild-type strain (Fig. 7, *bottom*). This result might be due to a deficient assembly or to increased degradation of PhoQ in this genetic background and contributes to explain the low basal transcriptional expression of *virK* observed for the *fadD* mutant strain in the above shown β-galactosidase activity assays. Nevertheless, we observed undetectable PhoQ autophosphorylation levels in the samples obtained either from the wild type or from the *fadD* strain when C18:2 was present in the bacterial growth medium, relative to those obtained from bacteria grown in LB. Together, our results clearly show that exogenously provided free LCUFAs exert a repressive action on PhoQ autokinase activity, irrespective of their metabolic fate in the bacterial cell.

DISCUSSION

The PhoP/PhoQ regulatory system is central to the pathogenic properties of *Salmonella* Typhimurium. The fact that PhoP/PhoQ belongs to the so-called two-component family signal transduction systems, which are absent in mammalian organisms, makes it an ideal target for the development of anti-

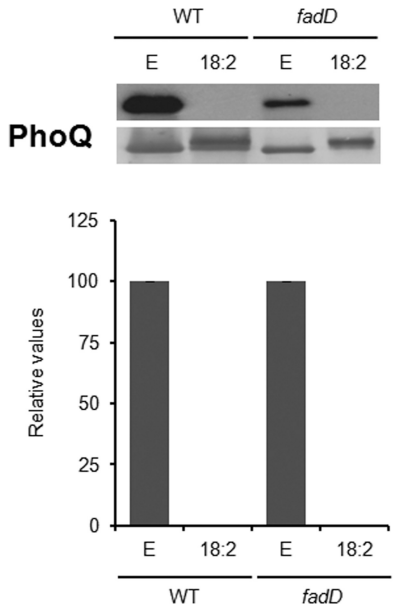

FIGURE 7. **The inhibition of the autokinase activity of PhoQ by LCUFAs is independent of *fadD*.** 0.5 mg ml^{-1} linoleic acid (*18:2*) was added to the *Salmonella* wild-type or *fadD* strains growth medium. Membranes obtained by cell fractionation, harboring PhoQ, were incubated for 10 min at 37 °C in a reaction medium containing [γ-^{32}P]ATP, as described under "Experimental Procedures." The autophosphorylation reactions were analyzed by SDS-PAGE (12% polyacrylamide) and transferred to nitrocellulose, followed by autoradiography (*top*) or immunodetection with anti-PhoQ$_{Cyt}$ polyclonal antibodies (*middle*). The phosphorylation levels and the protein expression levels of PhoQ in each condition assayed were determined by densitometry and the ratio (labeled against immunodetected) was plotted (*bottom*); the value obtained with the addition of ethanol was taken as 100%. *18:2*, linoleic acid; *E*, ethanol. Data shown represent results from three independent experiments.

microbial strategies. In this work, while searching for compounds of natural sources that could modulate the activity of *Salmonella* PhoP/PhoQ signal transduction system, we found that LCUFAs could reversibly down-regulate PhoP/PhoQ activity. The action of these fatty acids was detected when bacteria were grown in the presence of a subinhibitory concentration of either *L. amplexicaule*-derived extracts that contain LCUFAs in their composition or C16 or C18 chain length unsaturated fatty acids from a commercial source.

We demonstrated that free LCUFAs specifically repress the PhoP/PhoQ system because they down-regulated the expression of both the *phoPQ* operon and PhoP-activated genes at the transcriptional level, whereas these compounds exerted no effect on genes controlled by other signal transduction mechanisms. Moreover, we showed that equivalent concentrations of saturated fatty acids of the same carbon chain length as the assayed LCUFAs did not alter the expression of PhoP-dependent genes.

The transcriptional inhibition of *phoP*-dependent genes could be attributable to a down-regulation of the activated status of the transcriptional regulator PhoP, which is dictated by the PhoQ sensor activity. When the autophosphorylation ability of PhoQ-harboring vesicles obtained from bacteria grown in LB containing concentrations of linoleic acid (C18:2) that were non inhibitory for bacterial growth was measured, we found that the sensor protein autokinase activity was severely repressed, in a dose-dependent manner. This repression was replicated when other LCUFAs were assayed, whereas no effect was detected when saturated fatty acids of equal chain length were tested. No fatty acid-dependent effect was observed when the functionally and structurally related sensor EnvZ autokinase activity was assayed, ruling out a potential broad action of these compounds on the catalytic domain of Class I histidine kinases (as classified in Ref. 49). The absence of effect also observed when the activity of transcriptional reporters dependent on either the EnvZ/OmpR or CpxA/CpxR system was determined contributed to reinforce this notion. Additionally, these results also show that the *in vivo* or *in vitro* inhibition of the PhoP/PhoQ system by LCUFAs is unlikely to be the result of nonspecific detergent effects on these sensor proteins. Besides, saturated fatty acids with similar CMC values showed no inhibitory action, and the repressive action of C18:2 could be detected when concentrations as low as 50 μg ml^{-1} were assayed (Fig. 4*A*), a concentration far below the estimated CMC for this fatty acid in aqueous solution.

We have previously demonstrated that Mg^{2+} extracellularly supplied to the *Salmonella* growth medium specifically induces PhoQ phosphatase activity while exerting no effect on PhoQ autokinase activity (1). When the transcriptional expression levels of a representative PhoP-activated gene from bacteria grown in the simultaneous presence of C18:2 and Mg^{2+} were measured, we found that they exerted stronger repression compared with the values obtained when the action of each single factor was analyzed, suggesting that their inhibitory actions were independent. In support of this, we herein showed that this divalent cation did not interfere with the repressive action of C18:2 on PhoQ autophosphorylation levels. Although we cannot rule out the possibility that fatty acids somehow simultaneously induce PhoQ phosphatase capacity, our results clearly indicate that Mg^{2+} and C18:2 exert a differential signaling action on the PhoQ sensor protein and that both signals can act in concert to down-regulate the expression of the PhoP-dependent regulon.

The average 50% LCUFA-mediated repression obtained for PhoP-dependent gene transcription levels did not entirely correlate with the strongest inhibition (≥95%) obtained from the *in vitro* PhoQ autokinase activity assays. Whereas the *in vitro* autokinase assay only detects PhoQ autophosphorylation capacity, the level of expression from a given PhoP-regulated gene reflects the global transduction process. Phosphodonors other than PhoQ, such as acetyl-phosphate or other histidine kinases as a result of cross-talk, could be responsible for PhoP phosphorylation levels (50). In contrast, irrespective of the source of phospho-PhoP, when PhoQ phosphatase activity is triggered by high extracellular Mg^{2+}, 75–85% inhibition can be detected in the expression of PhoP-activated genes, supporting the notion that LCUFAs and Mg^{2+} differentially target PhoQ catalytic activities.

Exogenously supplied fatty acids can be incorporated and metabolized by *Salmonella* through the fatty acid β-oxidative pathway. We hypothesized that the repressing effect of LCUFAs on the PhoP/PhoQ system could be the result of an increased intracellular concentration of a β-oxidation intermediate metabolite, of acetyl-CoA (the β-oxidation end product), or of the increased incorporation of unsaturated fatty acids in membrane phospholipids. Therefore, we tested the action of

C18:2 when the function of key components of the *Salmonella* fatty acid β-oxidation pathway (FadL (transmembrane transporter that mediates the lateral passage of fatty acids to the bacterial periplasm (51)), FadD (which couples translocation to import of the LCFA into the cytoplasm by its activation to a CoA thioester), and FadBA (acyl-CoA dehydrogenase complex)) has been abrogated by mutagenesis of their respective encoding genes, *fadL*, *fadD*, and *fadBA*.

First, the possibility of a potential cross-regulatory action of PhoP over the expression of the *fad* genes assayed was discarded. We here demonstrate that either the presence of the FadL long chain fatty acid membrane translocator or the FadBA downstream dehydrogenase complex is dispensable for LCUFAs to exert their repressive action on the PhoP/PhoQ system. Even when in a *fadL* strain translocation across the membrane would conceivably be bypassed by passive diffusion or by transbilayer flip-flop of the LCUFAs at slow rates (52), FadD activity is still necessary to convert the transported LCUFA to its activated CoA-derivative, which can be used as a membrane phospholipid acyl donor. In the *fadD* genetic background, and simultaneously circumventing a possible effect on the autoregulatory control of PhoQ expression, we showed that PhoQ autokinase activity was repressed by the action of C18:2. Therefore, we can rule out that the PhoQ inhibitory action of exogenously supplied LCUFAs is due to a FadD-mediated change in the phospholipid composition of the bacterial envelope membranes that would perturb the catalytic activity of membrane-anchored PhoQ protein. These findings also indicate that the basal down-regulation in the PhoP-regulated gene expression observed in the *fadD* mutant strain (Fig. 6) is attributable to a reduction in PhoQ autokinase activity caused by accumulation of LCUFAs together with a decrease in PhoQ protein levels due to *phoPQ* autoregulation combined with diminished PhoQ or stability in the *fadD* background.

As mentioned before, a *fadD* mutant strain is unable to channel fatty acids into the β-oxidative or the phospholipid synthesis pathways (with the sole exception of the 2-acylglycerophosphoethanolamine acyltransferase/acyl carrier protein synthetase pathway, which can preferentially incorporate saturated fatty acids at very low rates (53, 54)). In consequence, when LCFUAS are provided in the bacterial growth medium, it is reasonable to speculate that the interaction of PhoQ with accumulated LCUFAs would be responsible for the induction of a conformational change that switches off the autokinase activity of the sensor protein. Accordingly, it has been shown recently that either *E. coli* or *Sinorhizobium melilotti fadD* mutant strains accumulate unsaturated free fatty acids, and it was proposed that *fadD* is also required for utilization of endogenous free fatty acids that could have been released as a result of membrane lipids remodeling (55). To back this conjecture in our system, we compared the esterified *versus* the free fatty acid levels of *Salmonella* wild-type, *fadD*, and *fadL* strains when they were grown in the presence of labeled [^{14}C]C18:2 by TLC analysis. As shown in supplemental Fig. S7, free fatty acids represent 33% of the total label incorporated into the bacterial cell in the *fadL* and 93% in the *fadD* mutants in comparison with 3% in the wild-type strain. This result supports the notion that free LCUFA accumulation either by a blockage in the vectorial transport to the bacterial cytoplasm or by an exogenous oversupply of the metabolic capacity of the cell would be the active species that act as signals repressing the autokinase activity of PhoQ. Because no obvious recognition motif for free unsaturated fatty acids can be found by *in silico* predictive analysis of PhoQ amino acid sequence or from its structural features, further work is under way in our laboratory to determine the biochemical basis of the LCUFA-PhoQ interaction.

In this context, it is worth highlighting that in *Vibrio cholerae*, the causative agent of cholera, an acute intestinal infection, a virulence mechanism involving detection of unsaturated free fatty acids has been described. Chatterjee *et al.* (56) have shown that unsaturated fatty acids isolated from bile at a concentration of 0.03% are able to inhibit the expression of virulence factors, whereas they enhance *V. cholerae* motility. Later, the work of Lowden *et al.* (57) demonstrated that direct binding of unsaturated free fatty acids (including oleic and linoleic acids that repress the transcription of the *tcp* toxin-co-regulated pilus and of the *ctx* cholera toxin-encoding genes, at a concentration of 0.02%) to ToxT provokes a conformational alteration of this virulence master regulator by decreasing its ability to bind DNA. More recently, Antunes *et al.* (58) have found that small hydrophobic molecules present in freshly extracted bovine bile were able to repress PhoP activity, although they could not assess the chemical identity of the compound. In addition, Goldberg *et al.* (59) have shown that the presence of a polar residue in the second transmembrane helix of PhoQ, which would stabilize a structural water-filled cavity in the sensor protein, is essential for the ability of PhoQ to switch from kinase- to phosphatase-dominant conformations. They showed that the replacement of hydrophilic to hydrophobic residues in transmembrane domains abrogated PhoQ kinase without affecting its phosphatase capacity. It is tempting to speculate that the hydrophobic environment generated by accumulation of LCUFAs could provoke an analogous impact on PhoQ conformation.

Finally, free LCUFAs are present in environmental niches that *Salmonella* has to traverse along the infection process in the mammalian host. LCUFAs can be found in the digestive tract, forming part of bile components, in the ruminal content and in the mammalian intestine due to degradation of fatty acid-containing nutrients by lipolytic enzymes. Few reports provide accurate information about free LCUFA content in mammalian fluids, and most of them are expressed as percentage relative to total fatty acids (60–62). However, from these data, it is clear that free LCUFAs levels in these animal contents vary depending on the metabolic, dietary, and health status of the organism. *Salmonella* can be found colonizing the hepatobiliary tract, and the relevance of the association between the presence of *Salmonella* in bile or gallstones and the bacterial chronic carriage has been established previously (63–66). Chaterjee *et al.* (56) reported that linoleic acid represents 14%, whereas oleic acid represents 23%, of total fatty acids present in ox bile. In bile of cholelithiasis patients, Tsuchiya *et al.* (67) reported concentrations of individual free LCUFAs in the 0.23–2.0 mM range, which is in agreement with PhoP/PhoQ activity inhibitory values shown in this work.

Together with our results, these previous data allow us to speculate that the presence of LCUFAs, such as linoleic acid, might function as a signal that, in combination with divalent cations, aids *Salmonella* in distinguishing between extracellular and intracellular environments. In response to these signals, the PhoP/PhoQ system will remain turned off. Conversely, once inside the intravacuolar ambient of the host cell, the absence of repressing cues together with triggering conditions, such as acidic pH and the presence of cationic peptides, will turn on the system. This will allow *Salmonella* to induce the expression of virulence factors that counteract the defense mechanisms of the infected cell, favoring bacterial survival and dissemination.

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SUPPLEMENTAL DATA

Unsaturated long-chain free fatty acids are input signals of the *Salmonella enterica* PhoP/PhoQ regulatory system

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Running Title: Unsaturated fatty acids repress *Salmonella* PhoQ autokinase activity

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Key words: PhoP/PhoQ input signal; *Salmonella*; unsaturated fatty acids; two-component system

Table S1

Strain or plasmid	Stock name	Genotype or property	Source or reference
***S.* Typhimurium**			
ATCC 14028s	14028s	wild-type	ATCC
phoPQ	PB2069	*phoPQ*	(1)
virK::lacZ	EG9532	*pcgG9283::*Mud*J*	(2)
virK::lacZ phoP	EG9534	pcgG9283::MudJ *phoP7953::Tn10*	(2)
*mgtA::*lacZ	EG9521	*mgtA9226*::Mud*J*	(2)
*pagK::*lacZ	PB3111	*pbgF*::Mud*J*	(3)
pipD::lacZ	EG9341	*pipD*::Mud*J*	(4)
pcgM::lacZ	PB3118	*pcgM*::Mud*J*	(3)
pcgF::lacZ	EG9530	*pcgF9281::*Mud*J*	(2)
pcgL::lacZ	EG9331	*pcgL*::Mud*J*	(4)
golT	PB6083	14028s with pPB1223	(5)
tppB::lacZ	PB3062	*tppB*::Mud*I*	(6)
virK::lacZ phoQ [pUHE21-2::*phoQ*]	PB4663	*pcgG9283::*Mud*J* *phoQ*::Tn10 with pEG9050	This work
virK::lacZ [pUHE21-2::*envZ*]	PB9079	*pcgG9283::*Mud*J* with pPB1134	This work
fadL::lacZ	PB8714	*fadL::lacZ*	This work
fadL::lacZ phoP	PB9060	*phoP7953::Tn10* *fadL*::*lacZ*	This work
fadD::lacZ	PB8196	*fadD*::*lacZ*	This work

fadD::lacZ phoP	PB8239	*phoP7953::Tn10 fadD*::*lacZ*	This work
fadBA::lacZ	PB7963	*fadBA*::lac*Z*	This work
fadBA::lacZ phoP	PB8241	*phoP7953::Tn10 fadAB*::lac*Z*	This work
fadL	PB8575	Δ*fadL*	This work
fadL virK::lacZ	PB8601	Δ*fadL pcgG9283::*Mud*J*	This work
fadL tppB::lacZ	PB10453	Δ*fadL tppB::*Mud*I*	This work
fadD	PB8099	Δ*fadD*	This work
fadD virK::lacZ	PB8176	Δ*fadD pcgG9283::*Mud*J*	This work
fadD tppB::lacZ	PB8049	Δ*fadD tppB::*Mud*I*	This work
fadBA virK::lacZ	PB7983	Δ*fadBA pcgG9283::*Mud*J*	This work
fadBA tppB::lacZ	PB8264	Δ*fadBA tppB*::Mud*I*	This work
phoQ	MS5996s	*phoQ*5996::Tn10	(7)
virK::lacZ phoQ fadD	PB10357	*pcgG*9283::Mud*J* phoQ5996::Tn10 Δ*fadD*	P22-mediated transduction from MS5996 to PB8176
virK::lacZ phoQ fadD [pUHE21-2::*phoQ*]	PB10359	*pcgG*9283::Mud*J* phoQ5996::Tn10 Δ*fadD* with pEG9050	This work
ppiA::lacZ		*ppiA::lacZ*	Soncini, *et al.* (unpublished)
ppiA::lacZ cpxR		*ppiA::lacZ cpxR*	Soncini, *et al.* (unpublished)

	Plasmids		
pUHE21-2	pUHE21-2*lacI*^{q}	rep$_{pMB1}$ Ap^r *lacI*^{q}	(4)
pP*golTS*	pPB1223	pMC1871::P*golTS*	(8)
pUHE21-2::*phoQ*	pEG9050	rep$_{pMB1}$ Ap^r *lacI*^{q} *phoQ*$^+$	(9)
pUHE21-2::*envZ*	pPB1134	rep$_{pMB1}$ Ap^r *lacI*^{q} *envZ*$^+$	This work

Table S2

Primers	**5' -> 3' sequence**
FadAB P1	TGCGCTGCAATGCGAGTTATTTAGGGGATATTATCTTTGATG TAGGCTGGAGCTGCTTCG
FadAB P2	CTGGTACGACCAGATCACTTTGTGGATTCAGGAGACTGACCA TATGAATATCCTCCTTA
FadD P1	CGCCGGCTTAACCGGCGTCTCTCGTTAGCTGACTTAACGCTG TAGGCTGGAGCTGCTTCG
FadD P2	GGTTGTGATGACGACGAACACTCATTTTAGAGGTGATGCACA TATGAATATCCTCCTTA
FadL P1	AGCATGTAACATAGTTTGTATAAAAATAATCAATGAGGTTTG TAGGCTGGAGCTGCTTCG
FadL P2	GAGCGCAATGCTCACCTTTTTGATCCGTTTCAGAAAGAGACA TATGAATATCCTCCTTA
FwDELFadL	ACGCGCTTTTTCGGTTAACG
FwDELFadD	GGCCGGGCTAACCAGTCGGTA
FwDELFadAB	CAAGACAGTCCAACAGATCTG
EnvZ Fw #310	CGGGATCCGATGAGGCGAATGGCG
EnvZ Rv #292	ACGACAAAAGAGGCATAAGCTTGGG
phoP-NTR1	TAAGGAAAGGCCGTC
phoP NTF	GAGGATCCATATGATGCGCGTACTGG

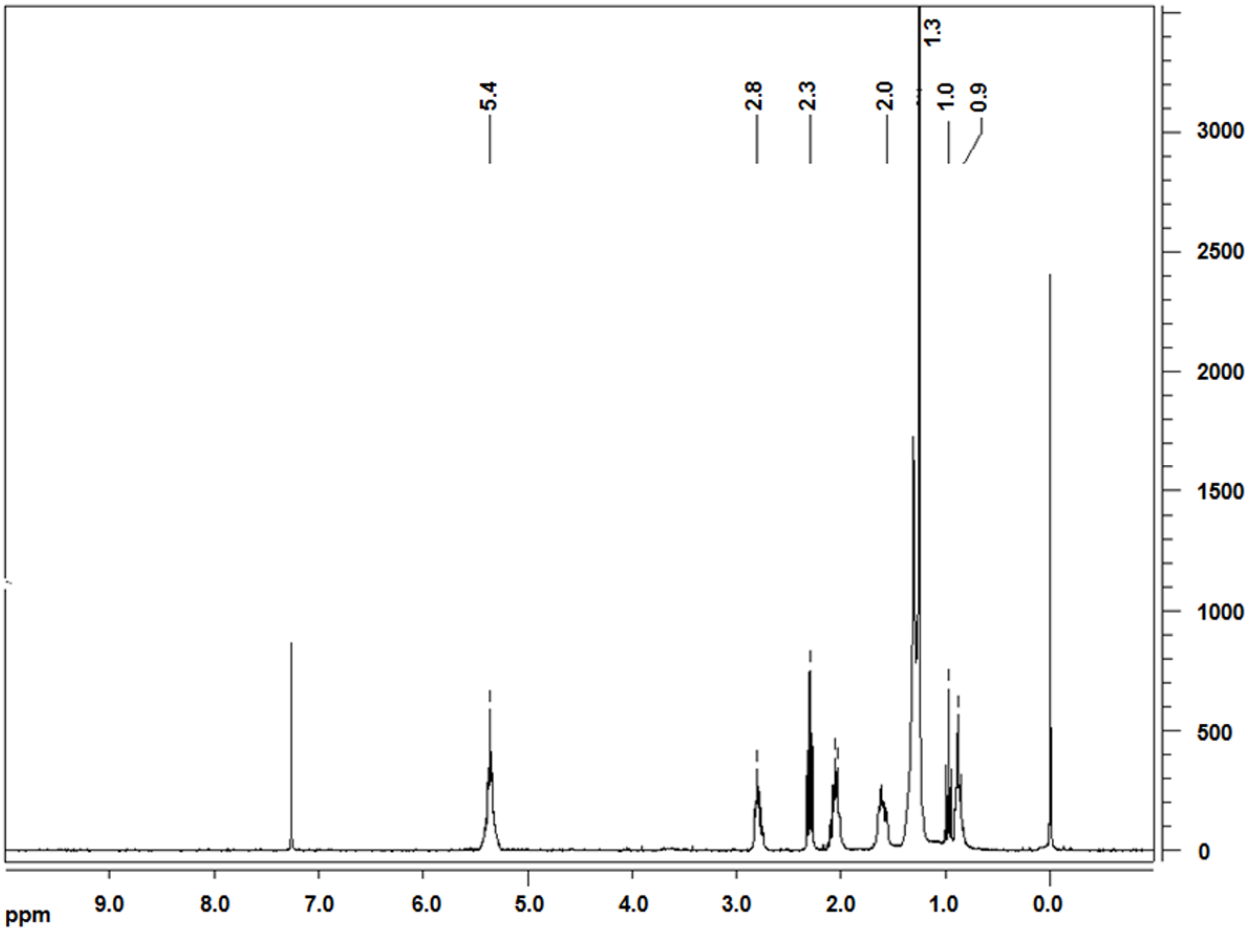
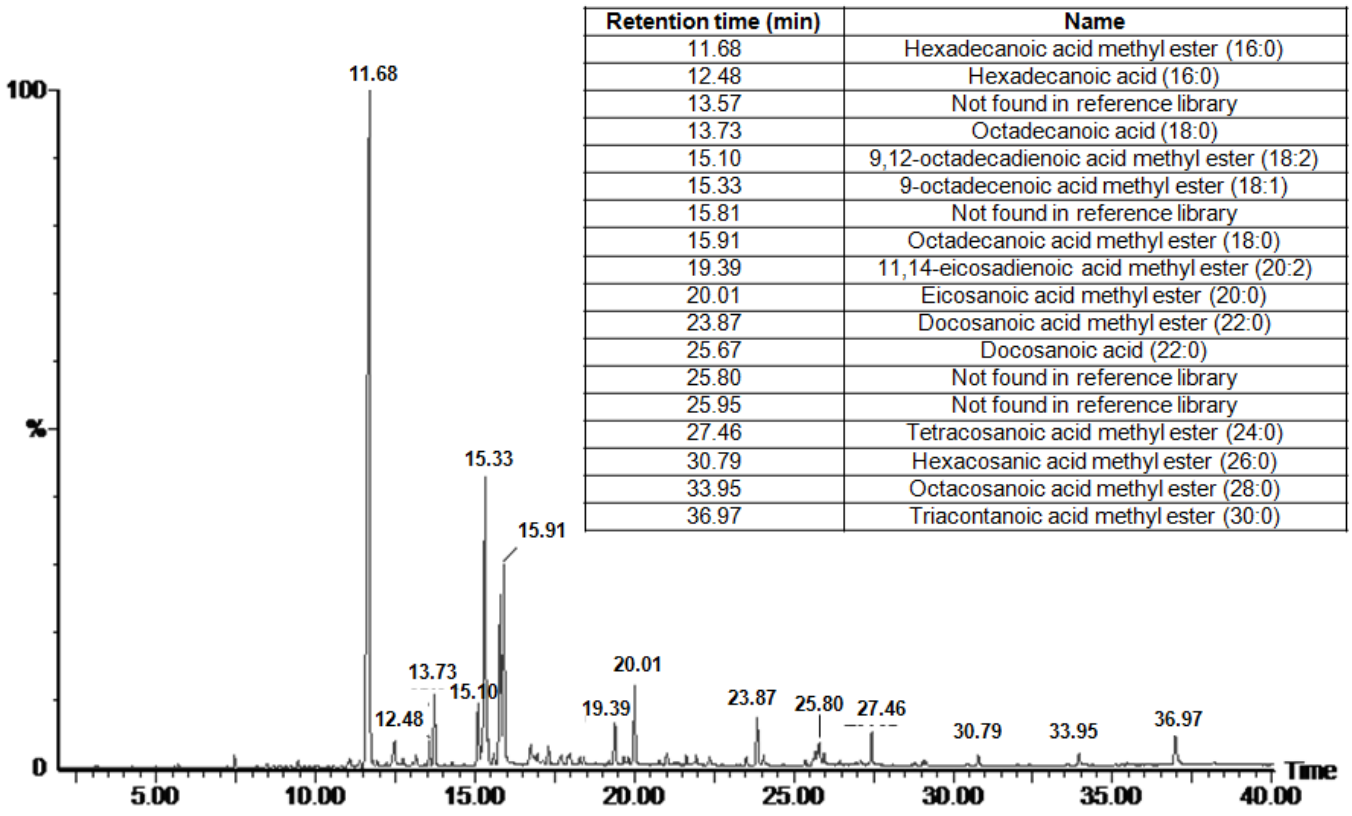


FIGURE S1. ***^{1}H RMN spectrum of the bioactive TLC fraction of Lamium amplexicaule.*** The spectrum was recorded from 20 mg of sample dissolved in $CDCl_3$ (500 uL), chemical shifts are expressed in ppm.



Retention time (min)	Name
11.68	Hexadecanoic acid methyl ester (16:0)
12.48	Hexadecanoic acid (16:0)
13.57	Not found in reference library
13.73	Octadecanoic acid (18:0)
15.10	9,12-octadecadienoic acid methyl ester (18:2)
15.33	9-octadecenoic acid methyl ester (18:1)
15.81	Not found in reference library
15.91	Octadecanoic acid methyl ester (18:0)
19.39	11,14-eicosadienoic acid methyl ester (20:2)
20.01	Eicosanoic acid methyl ester (20:0)
23.87	Docosanoic acid methyl ester (22:0)
25.67	Docosanoic acid (22:0)
25.80	Not found in reference library
25.95	Not found in reference library
27.46	Tetracosanoic acid methyl ester (24:0)
30.79	Hexacosanic acid methyl ester (26:0)
33.95	Octacosanoic acid methyl ester (28:0)
36.97	Triacontanoic acid methyl ester (30:0)

FIGURE S2. ***GC-MS chromatogram of the bioactive TLC fraction of Lamium amplexicaule.*** The sample was derivatized to methyl esters as described in Experimental procedures. Retention times are expressed in minutes. The table shows the correlation of different peaks with different fatty acids and methyl esters derivates.

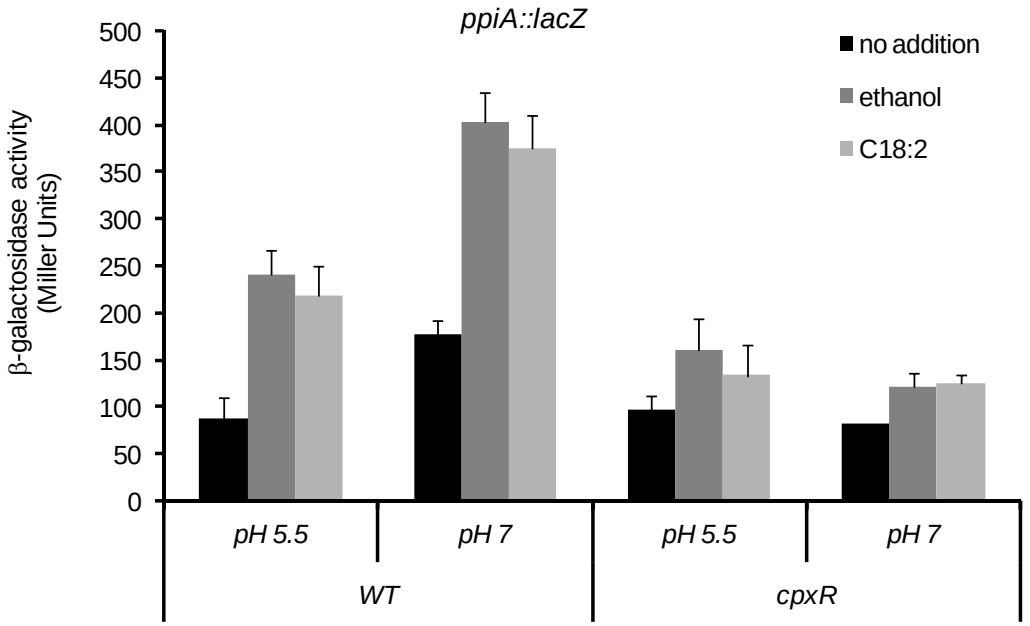


FIGURE S3. ***The expression of ppiA is not affected by linoleic acid.*** β-galactosidase activity from *ppiA*::*lacZ* transcriptional fusion was measured in cells grown overnight in LB (buffered with 100 mM MES-NaOH pH 5.5 or 7.0), with no addition or supplemented with ethanol 0.5% (E) or 0.5 mg ml^{-1} of linoleic acid (18:2). β-galactosidase activity was measured as described under Experimental Procedures. Results are average of three independent assays performed in duplicate, and error bars correspond to standard deviations.

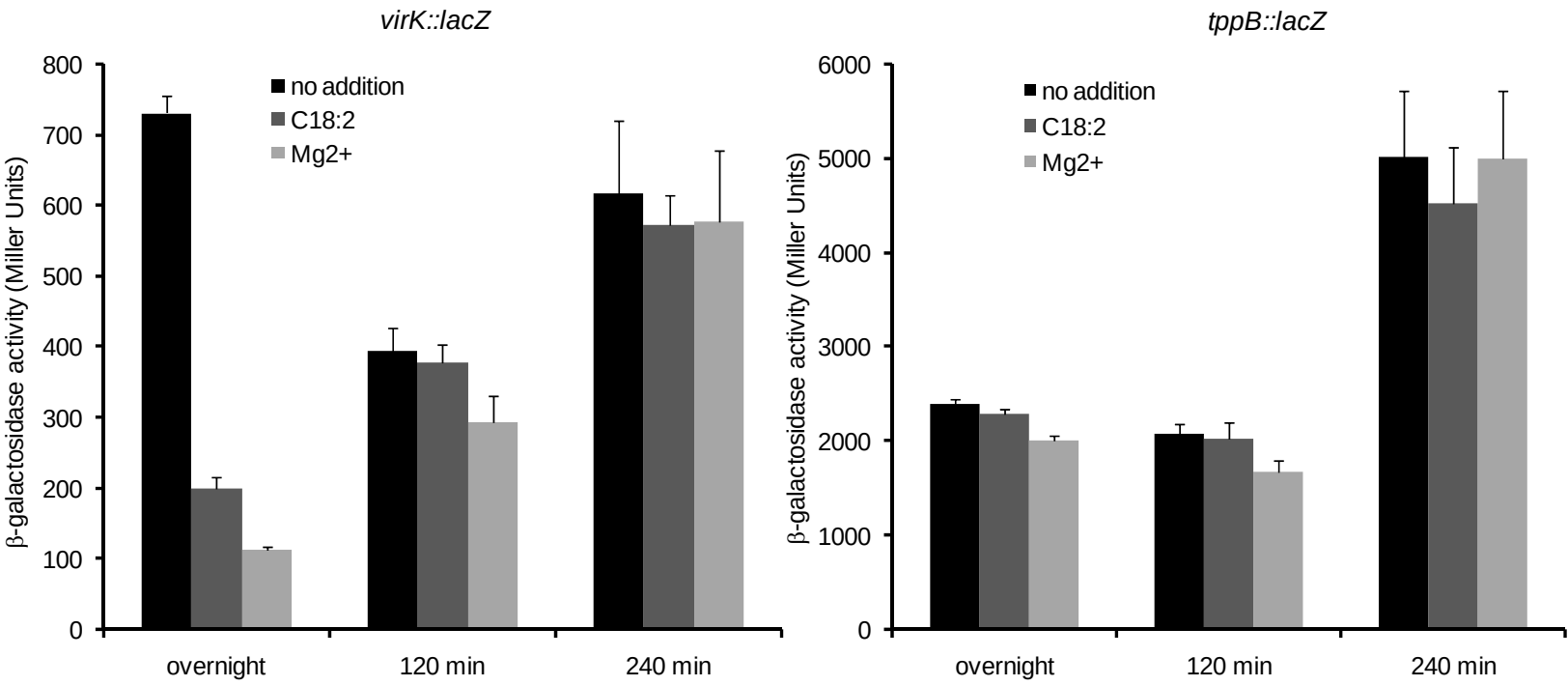


FIGURE S4. ***Reversible inhibition of linoleic acid over the expression of S. Typhimurium PhoP-activated genes***. β-galactosidase activity from (A) *virK*::*lacZ* or (B) *tppB*::*lacZ* transcriptional fusions was measured in cells grown overnight in LB with no addition or supplemented with 0.5 mg ml^{-1} linoleic acid or with 5 mM $MgCl_2$. Then, cells from each condition were diluted $^{1}/_{100}$ in fresh LB, and β-galactosidase activity was measured after the indicated times, as described under Experimental Procedures. Results are average of three independent assays performed in duplicate, and error bars correspond to standard deviations.

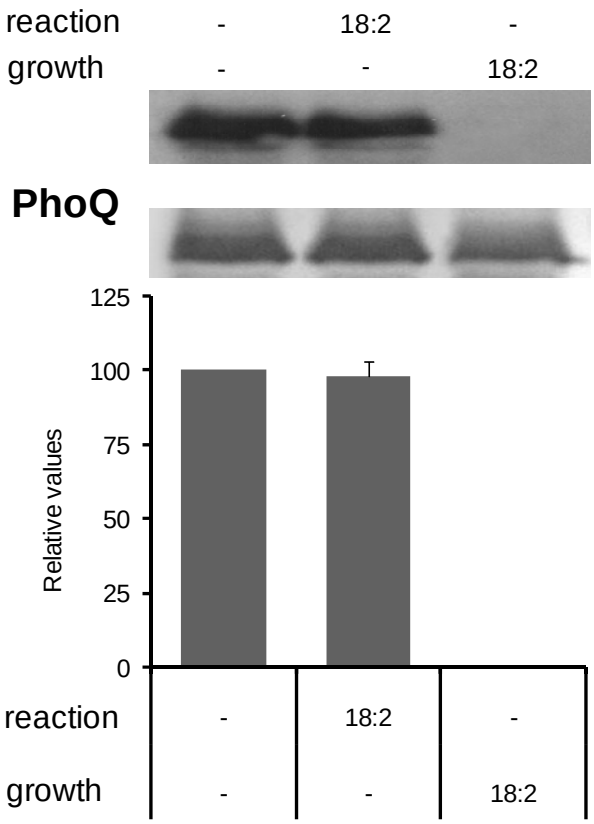


FIGURE S5. ***The autokinase activity of PhoQ is not affected by linoleic acid present in the reaction medium.*** *S.* Typhimurium cells were grown in LB without addition (-) or with 0.5 mg ml^{-1} linoleic acid (18:2). Membranes harboring PhoQ obtained by cell fractionation were incubated 10 min at 37°C in a reaction medium containing [γ-^{32}P]ATP alone (-) or with the addition of linoleic acid (18:2) at a final concentration of 0.5 mg ml^{-1} when indicated, as described under Experimental Procedures. The autophosphorylation reactions were analyzed by SDS-PAGE (12% polyacrylamide) and transferred to nitrocellulose, followed by autoradiography (upper image) or immunodetection with anti-$PhoQ_{Cyt}$ polyclonal antibodies (middle image). The phosphorylation levels and the protein expression levels of PhoQ in each condition assayed were determined by densitometry and the ratios were plotted (bottom). The value obtained without additions to the growth and reaction media was taken as 100%. Data shown represents results from three independent experiments.

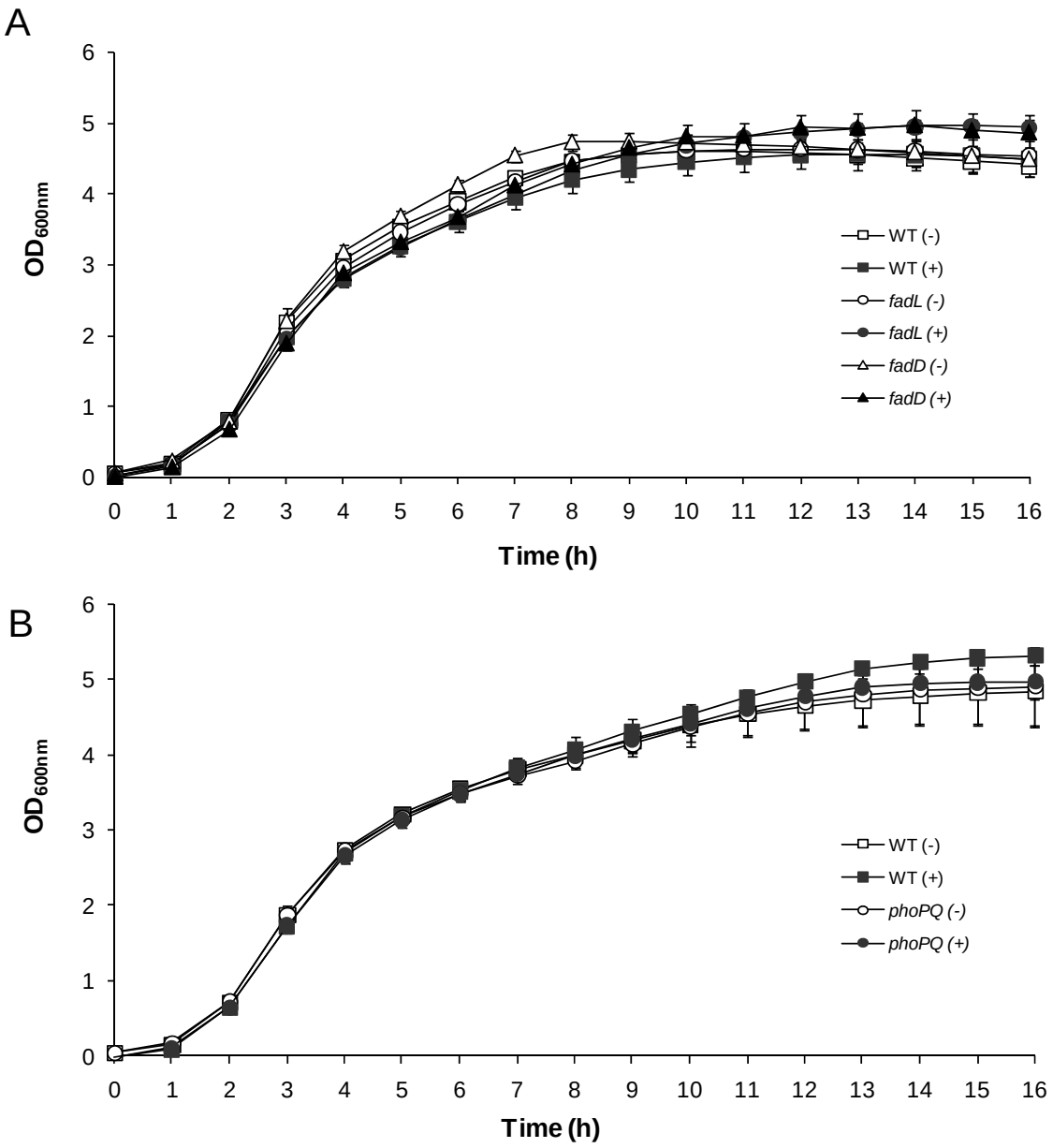


FIGURE S6. ***Growth pattern of Salmonella strains in the presence of linoleic acid***. *S. typhimurium* wild type (wt), *fadL* and *fadD* strains (A), or wild-type (wt) and *phoPQ* strains (B) were grown in LB without (-) or with addition (+) of 0.5 mg ml^{-1} of C18:2 (linoleic acid), and the OD_{630} was measured at the indicated time points. Results are average of two independent assays performed in duplicate, and error bars correspond to standard deviations.

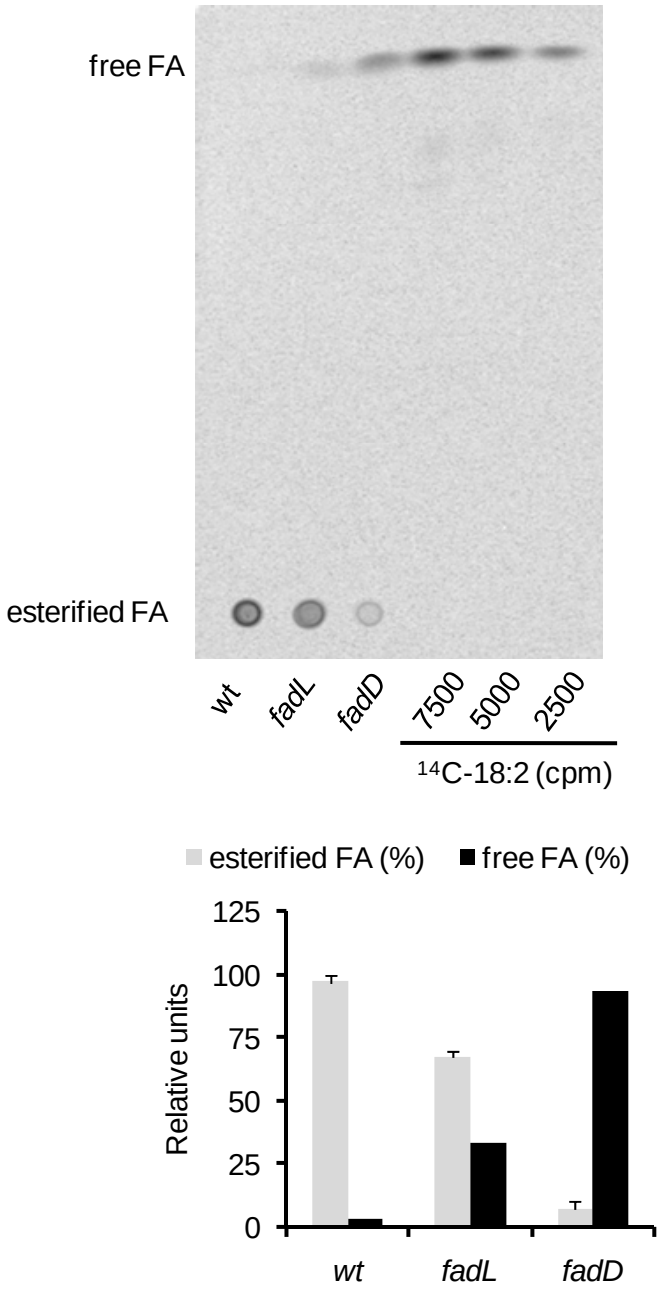


FIGURE S7. ***Detection of fatty acids distribution in Salmonella grown in radiolabeled linoleic acid.*** Thin-layer chromatography was performed with lipids extracted from cultures of the indicated *Salmonella* strains grown in the presence of labeled ^{14}C-18:2, as described in Experimental procedures. Samples were spotted onto a TLC Silica gel 60 F_{254} plate (Merck), and lipids were separated using a solvent system consisting of diethyl ether: petroleum ether: acetic acid (70/30/2, v/v/v); 7500, 5000 and 2500 counts per minute (cpm) of labeled ^{14}C-18:2 were also spotted as controls of the assay (upper figure). Densitometry was calculated for the major spots observed by TLC. The percentage of free and esterified fatty acid relative to the total lipid content was plotted for each strain (lower figure). Results are average of two independent assays, and error bars correspond to standard deviations.

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