Supporting Information

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SI Materials and Methods

Strains and Plasmids. All strains and plasmids used in this study are listed on Table S1. Strains were grown aerobically in LB at 37 °C. Recombinant DNA and molecular biology techniques were performed as previously described (1). All oligonucleotides used are listed in Table S2.

Construction of the Isogenic Mutants. Construction of an isogenic nonpolar *Salmonella* Typhimurium SL1344 *virulence and stress-related periplasmic protein* (*visP*) and *lpxO* single mutants, *visP*/*lpxO* double KO, and enterohemorragic *Escherichia coli* (EHEC) Δ*visP* (CP171) were all achieved using λ-red mutagenesis (2). The *visP* mutant (CGM300) was complemented with the *visP* gene cloned into pBADMycHisA (*KpnI* and *EcoRI*, 20 copies per cell; Invitrogen) vector, generating strain CGM301. The *lpxO* mutant (CGM302) was complemented with the *lpxO* gene cloned into pBAD33 (*KpnI* and *XbaI*) vector (3) (15 copies per cell), generating strain CGM303. The *visP*/*lpxO* mutant (CGM304) was double complemented with pBADMycHisA and pBAD33 constructions, generating strain CGM305.

Microarray Analysis. To obtain a more global understanding of alterations in a *qseC* mutant strain, a microarray study compared the *qseC* mutant with WT strains in LB broth. Therefore, they were diluted 1:100 and grown to OD_{600} of 1.0. Cells were added to the RNAprotect (Qiagen), and RNA was extracted using Qiagen RNA purification kits. cDNA was synthesized as previously described (4). The amino allyl dUTP provided a group onto which Cy3 or Cy5 was to be coupled. Equal amounts of differentially labeled cDNA (Cy5/Cy3) from control samples were hybridized to the *Salmonella* cDNA array, scanned with a Packard Biosciences ScanArray 5000, and quantified with DigitalGENOME (MolecularWare) spot-finding software at the Institute of Systems Biology (Seattle, WA) as previously described (4). Total RNA from three biologically independent replicates was analyzed. Genes showing differential expression from at least two of three replicates were considered for additional analyses. The expression levels (induction/repression) were determined by averaging fold change across all three biological replicates. Any gene with a significant mean expression change that was greater than or equal to two induced/repressed was considered differentially expressed as illustrated on Fig. S1. The Gene Expression Omnibus database accession number for the microarray results reported here is GSE38353.

Quantitative Real-Time RT-PCR. Overnight cultures were grown aerobically in LB at 250 rpm to late-exponential growth phase (OD_{600} of 1.0) for the in vitro assays. RNA from three biological samples was extracted using the RiboPure–Bacteria RNA isolation kit (Ambion–Invitrogen) following the manufacturer's guidelines. The primers used in the real-time assays were designed using Primer Express v1.5 (Applied Biosystems–Invitrogen). Briefly, diluted extracted RNA was mixed with validated primers (Table S2), RNase inhibitor, and reverse transcriptase (Applied Biosystems–Invitrogen) (5). The mix was used in a one-step reaction using an ABI 7500 sequence detection system. Data were collected using ABI Sequence Detection 1.2 software, normalized to endogenous *rpoA* levels, and analyzed using the comparative critical threshold method. Analyzed data were presented as fold changes over WT levels. The Student's unpaired *t* test was used to determine statistical significance. P value ≤ 0.05 was considered significant (5).

Macrophage Infection. J774 murine macrophage-like cells were infected with opsonized *S.* Typhimurium with normal mouse serum at 37 °C for 15 min and washed. These macrophage-like cells were infected using a multiplicity of infection of 100:1 for 30-min bacteria/cell interaction at 37 °C at 5% CO_2. These cells were treated with 40 μg/mL gentamycin for 1 h to kill extracellular bacteria and lysed with 1% Triton-X. Bacteria were diluted and plated in LB plates for CFU determination (6–8).

HeLa Invasion and Adhesion Assays. Epithelial HeLa cells were infected with *S.* Typhimurium at a multiplicity of infection of 100:1 for 90 min at 37 °C 5% CO_2 as previously described. These cells were treated with 40 μg/mL gentamycin for 1 h to kill extracellular bacteria and lysed with 1% Triton-X. Bacteria were diluted and plated in LB plates for CFU determination (7–10).

Docking Assays. Docking modeling was performed according to www.dockingserver.com instructions (11–14). Docking calculations were carried out using DockingServer. Gasteiger partial charges were added to the ligand atoms. Nonpolar hydrogen atoms were merged, and rotatable bonds were defined. Docking calculations were carried out on NAG.pdb, *N*-acetylmuramic acid.pdb, and prior modeled VisP structure. Essential hydrogen atoms, Kollman united atom type charges, and solvation parameters were added with the aid of AutoDock tools (13). Affinity (grid) maps of angstrom grid points and 0.375-Å spacing were generated using the Autogrid program (15). AutoDock parameter set- and distance-dependent dielectric functions were used in the calculation of the van der Waals and electrostatic terms, respectively. Docking simulations were performed using the Lamarckian genetic algorithm and the Solis & Wets local search method (7). Initial position, orientation, and torsions of the ligand molecules were set randomly. All rotatable torsions were released during docking. Each docking experiment was derived from 100 different runs that were set to terminate after a maximum of 2,500,000 energy evaluations. The population size was set to 150. During the search, a translational step of 0.2 Å and quaternion and torsion steps of five were applied (16, 17). Parameters details are listed in Dataset S1.

Structure Prediction of *Salmonella* VisP. A structure for the bacterial oligonucleotide/oligosaccharide-binding fold (BOF) -containing fragment of *Salmonella* VisP was predicted using MODELER v9.10 (15, 18). Briefly, residues 44–128 of *Salmonella* VisP and residues 44–128 of *E. coli* YgiW were first aligned. The known structure of the YgiW fragment (Protein Data Bank ID code 1NNX) was then used as a template to make five independent structure predictions of VisP using the automodel function of MODELER. There was no variation between the five predictions (Fig. 2*D*).

Protein Purification. LB medium (100 mL or 1 L) was inoculated from overnight growth at 1:100 and grown at 37 °C to an OD_{600} of 0.6. The cultures were then induced with 0.2% arabinose and grown for 3–4 h at 37 °C. The whole-cell lysates were extracted from culture growth of an OD_{600} of 1.0. Briefly, culture was pelleted (14,000 rpm for 5 min at 4 °C), resuspended in 20 μL lysis buffer/culture (50 mM Tris·HCl, pH 7.5, 100 mM NaCl, 5% glycerol, 1 mM DTT, 100 mM phenylmethylsulfonyl fluoride), subjected to lysozyme addition to a final concentration of 300 μg/mL, incubated at 4 °C for 2.5 h, and DNase I/-treated for 30 min at 4 °C; cell debris was then pelleted (14,000 rpm for 10 min at 4 °C), and supernatant containing whole-cell protein was removed. The PeriPreps kit (PeriPlasting Kit; EPICENTRE Biotechnologies) was used to

isolate VisP from periplasmic fractions using the manufacturer's instructions. The respective His-tagged protein fractions were centrifuged and loaded onto an Ni^{2+}-nitrilotriacetic acid (NTA) agarose gravity column (Qiagen). The column was washed with lysis buffer (50 mM phosphate buffer, pH 8, 1 M NaCl) and wash buffer (50 mM phosphate buffer, pH 8, 1 M NaCl, 50 mM imidazole), and protein fractions were eluted with elution buffer (50 mM phosphate buffer, pH 8, 1 M NaCl, 250 mM imidazole). The Flag-tagged protein fractions were isolated using immunoprecipitation by ANTI-FLAG M2 affinity gel (Sigma) according to the manufacturer's directions. All of the fractions containing purified protein were confirmed by SDS/PAGE and concentrated for further use.

Far Western. Same equimolar amounts of purified His-tagged protein were separated on a 12% SDS gel, transferred, and blocked with 3% milk in PBS containing 0.05% Tween. Replicate purified fractions were then probed with whole-cell lysates of the *ΔvisP/lpxO* double mutant or the double mutant overexpressing either Flag-tagged LpxO or His-tagged VisP. As another (negative) control, a replicate membrane was left unprobed by the whole-cell lysate. All membranes were then probed with either anti-His or anti-Flag primary antibodies and incubated with a secondary antibody. ECL reagent (GE) was added, and membranes were exposed to film to detect interacting proteins.

In Vivo Coimmunoprecipitation. LpxO-Flag and VisP-His tagged were both coexpressed in vivo in the *ΔvisP/lpxO* double mutant background. These proteins were purified by NTA agarose gravity column (Qiagen). Unbound fraction was the flow-through collected after NTA agarose gravity columns passage. And bound fraction was the imidazole-eluted one from the same columns. Using VisP-His tagged as bait and LpxO-Flag tagged as prey. All membranes were then probed with either anti-His or anti-Flag primary antibodies and incubated with a secondary antibody. ECL reagent (GE) was added, and membranes were exposed to film to detect interacting proteins.

Peptidoglycan Binding Assays. Insoluble peptidoglycan from *Bacillus subtilis* and cellulose conjugated to agarose (Sigma) were pelleted and washed in WashBuffer (25 mM Mes, pH 6.0, 25 mM NaCl); 10 μg protein were added to 50 μg peptidoglycan in a final volume of 200 μL. Reactions were incubated for 2 h at 4 °C, and then, the peptidoglycan was pelleted at 6,000 × *g* for 5 min and washed two times in WashBuffer. Bound protein was eluted by boiling the peptidoglycanin in 5× SDS/PAGE buffer (10% glycerol, 5% β-mercaptoethanol, 2% SDS, 62.5 mM Tris·HCl, 0.003% bromophenol blue, pH 6.8) and resolved by SDS/PAGE through 15% gels (19). Gels were transferred and detected by Western blotting with anti-His, and measurement of binding affinity was performed in triplicates.

Stress Response Assays. The resistance assays were performed in the presence of hydrogen peroxide and cadmium chloride (survival test) adapted from previous studies (20). Overnight cultures grown for 16 h in LB were regrown to midlog phase in LB (OD_{600} of 1.0), and either (*i*) 1 mL each culture was incubated with H_2O_2 at a final concentration of 34 mmol L^{-1} for 15 min without shaking or (*ii*) 1 mL each culture was incubated with a final concentration of 4 mg mL^{-1} $CdCl_2$ for 40 min without shaking. The acid resistance assay was also modified (21). From overnight, cultures were grown to OD_{600} of 1.0 in LB, and the cultures were diluted 40-fold in PBS (pH 7.2 to determine the initial number of cells) or LB (pH 2.5 and 5.0); the cells, both at pH 2.5 and 5.0, were incubated for 60 min without shaking. The percentage of cells surviving the stresses was calculated as the number of CFUs per milliliter remaining after each stress divided by the initial CFU per milliliter. All of the assays were tested in triplicates (22).

Polymyxin B Sensitivity Assay. The sensitivity assays were performed after overnight growth of the samples using LB broth containing 2.5 μg mL^{-1} polymyxin B (Sigma) and extra grown for 1 h at 37 °C with aeration. Serial dilutions were made in PBS and plated on selective agar, and survival percentages were calculated relative to initial input and normalized to WT survival (6–8).

Site Direct Mutagenesis. VisP site-directed mutagenesis was used to introduce the required mutations into the plasmids (Table S2) using the QuikChange II Site Directed Mutagenesis kit (Stratagene) following the manufacturer's recommendations.

Isolation of Labeled Lipid-A. Cultures were grown overnight at 37 °C and 200 rpm followed by a dilution to an OD_{600} of 0.05 in 7 mL LB medium. Cells were labeled with 2.5 μCi mL^{-1} ^{32}P (Amersham) immediately after dilution. Cells were harvested at OD_{600} of 1.0 by centrifugation, and pellets were washed in 1× PBS solution, pH 7.4. Isolation of ^{32}P–lipid-A was carried out by mild acid hydrolysis (23). A total count of 10,000 cpm/lane ^{32}P–lipid-A was analyzed by TLC in a solvent system of chloroform, pyridine, 88% (wt/wt) formic acid, and water (50:50:16:5, vol/vol). The TLC plate was dried and exposed to a Phosphoimager screen overnight followed by a visualization of lipid-A species using a Phosphoimager (PMI; Bio-Rad) and Quantity One software.

MS. Lipid-A was isolated from 25-mL cultures for analysis by MS as previously described (24). Briefly, cultures were grown overnight at 37 °C at 200 rpm followed by a dilution to an OD_{600} of 0.05 in 25 mL LB medium. Cells were harvested at OD_{600} of 1.0 by centrifugation, and pellets were washed in 1× PBS solution, pH 7.4. Isolation of lipid-A was carried out by mild acid hydrolysis. Lipid-A samples were washed one time with 12 mL 0.02 M HCl in 80% ethanol and one time with 70% ethanol to remove SDS and then dried under a stream of nitrogen. Lipid samples were analyzed using an MALDI-TOF/TOF (ABI 4700 Proteomics Analyzer) mass spectrometer in the negative ion linear mode as previously described (10).

Fluorescent Actin Staining Assay. Fluorescent actin staining was performed as previously described (25). Overnight cultures were grown aerobically in LB at 37 °C, diluted 1:100, and used to infect confluent monolayers of HeLa cells grown on glass coverslips at 37 °C and 5% CO_2. Cell infections were allowed to progress for 6 h at 37 °C and 5% CO_2. At 6 h, the coverslips were washed, fixed, permeabilized with 0.2% Triton X-100, and treated with FITC-labeled phalloidin to visualize actin accumulation; propidium iodide was added to stain bacteria. Samples were visualized by immunofluorescence with a Zeiss Axiovert microscope. The entire field of at least six coverslips from each strain was examined, and images of attaching and effacing (AE) lesions were taken and processed using ImageJ.

Colitis Model and Systemic Infections with *S.* Typhimurium. Mice (BALB/c, 7- to 9-wk old, female) were infected orally for the colitis model with a dose of 1 × 10^8 CFU *S.* Typhimurium SL1344 and respective isogenic mutants after a 24-h streptomycin pretreatment as previously described (26). Feces were collected 1 and 2 d postinfection, and serial dilutions were plated for CFU enumeration (six mice were used per group, repeated three times to ensure reproducibility); food and water were provided ad libitum. The systemic infections were also performed with mice (BALB/c, 7- to 9-wk old, female) infected using an i.p. route and a dose of 1 × 10^6 CFU *E. coli* K-12 strain DH5α (K-12 was used as a negative infectivity control to ensure that there were no issues with organ perforations during i.p. injection and that mice death was not caused by endotoxic effects) and predetermined lethal doses of 1 × 10^6 CFU *S.* Typhimurium SL1344 and respective isogenic

mutants. Mice were returned to their cages and monitored daily for signs of morbidity (anorexia, rapid shallow breathing, scruffy fur, decreased muscle tone, and lethargy) and death. After 20 d postinfection, the remaining animals were euthanized by CO_2 asphyxiation (eight mice were used per group, repeated three times to ensure reproducibility). The systemic organ colonization was performed as previously described (6); after 20 h i.p. infection, the mice were euthanized to remove the spleens and livers. Those organs were harvested, homogenized, and plated on LB agar plates for bacterial cell counts to determine tissue colonization (five mice were used per group, repeated three times to ensure reproducibility) (6, 27).

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Fig. S1. YgiW is part of the QseC regulon. (*A*) Validation by quantitative RT-PCR of *ygiW*(*visP*) and *qseC* expression levels with *ΔqseC* and *ΔvisP* strains, respectively. (*B*) Genomic organization of *qseBC* and *ygiV*/*visP* operon. (*C*) YgiV and VisP conserved domains based on Pfam domains databases. **P* < 0.01, **not statistically changed.

A

B

Fig. S2. VisP is a BOF protein. (*A*) VisP protein sequence multiple alignment in different bacterial species and strains. (*B*) Multiple alignment of BOF family members, distribution in different bacterial species, and respective gastrointestinal entries numbers of BOF members (1). Ap, *Actinobacillus pleuropneumoniae*; Av, *Azotobacter vinelandii*; Bp, *Bordetella parapertussis*; Ec, *E. coli*; Ha, *Hafnia alvei*; Hi, *Haemophilus influenzae*; Pa, *Pseudomonas aeruginosa*; Pl, *Photorhabdus luminescens*; Pm, *Pasteurella multocida*; Pp, *Pseudomonas putida*; Se, *S. enterica*; So, *Shewanella oneidensis*; St, *S. enterica* serovar Typhimurium; Vc, *Vibrio cholerae*; Vp, *Vibrio parahaemolyticus*; Vv, *Vibrio vulnificus*; Yp, *Yersinia pestis*.

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Fig. S3. VisP-His–tagged full length (pBADMycHisA) induced with 0.2% arabinose isolated from periplasmic purification kit (Epicentre Biotechnologies).

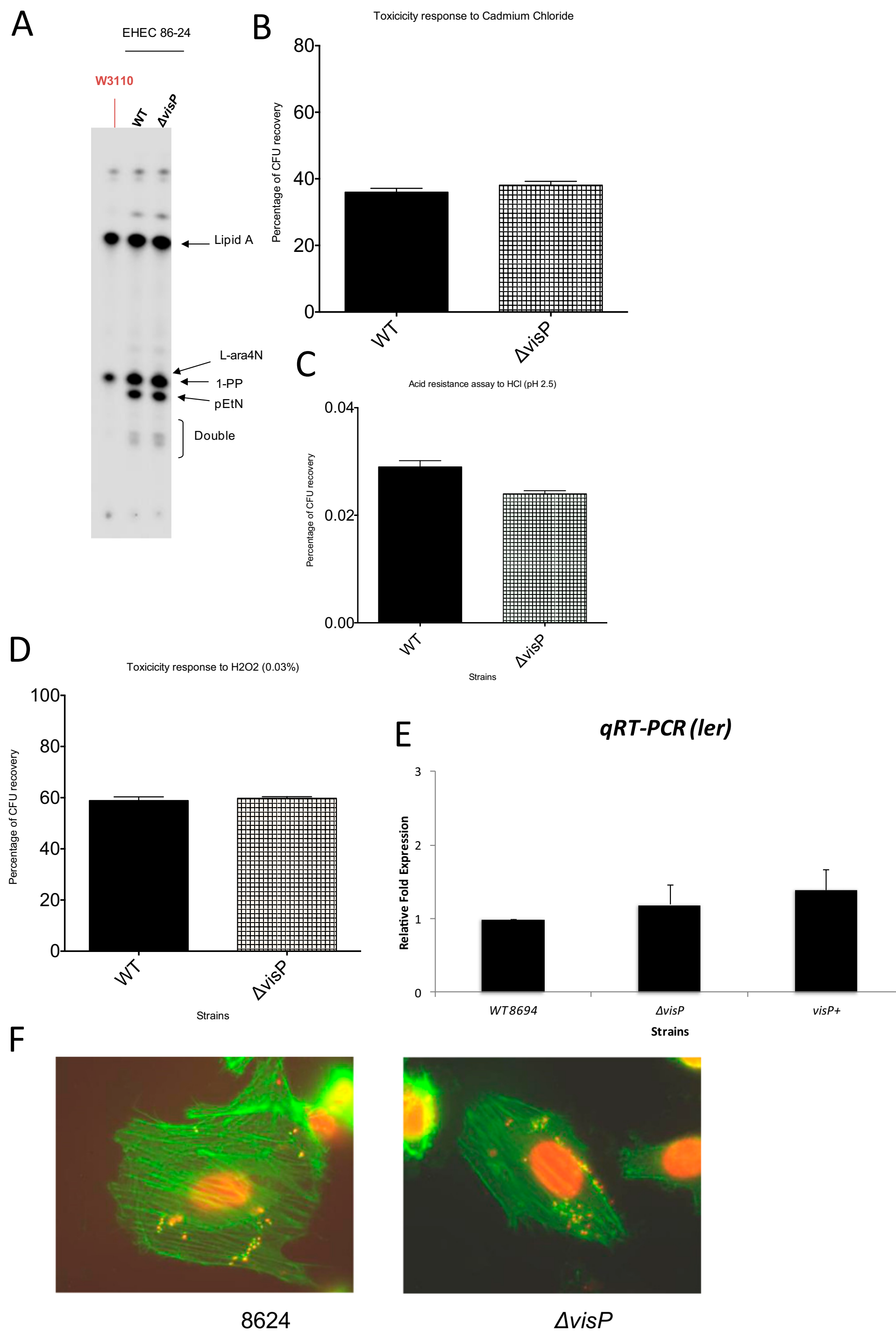

Fig. S4. Enterohemorragic *E. coli* (EHEC) VisP-dependent lipid-A profile, stress response, and virulence. (*A*) Isolation and separation lipid-A (LB medium) as previously performed. W3110 strain was used as control to visualize lipid-A [1,4 biphosphorylated and 1-diphosphate species (1-PP)]. (*B*) Cadmium chloride stress. (*C*) Acid resistance stress. (*D*) Hydrogen peroxide stress. (*E*) Quantitative RT-PCR of the *ler* gene (encoding the activator of the genes necessary for lesion formation on epithelial cells). (*F*) Fluorescent actin staining assay in HeLa cells with EHEC. Bacteria and HeLa nuclei stained in red, and the actin filaments and pedestals of HeLa stained in green. (Magnification: 1,000×.) EHEC forms these lesions of enterocytes to promote diarrhea in the human intestine. There are no differences in these lesions between WT and the *visP* EHEC mutant.

Fig. S5. (Continued)

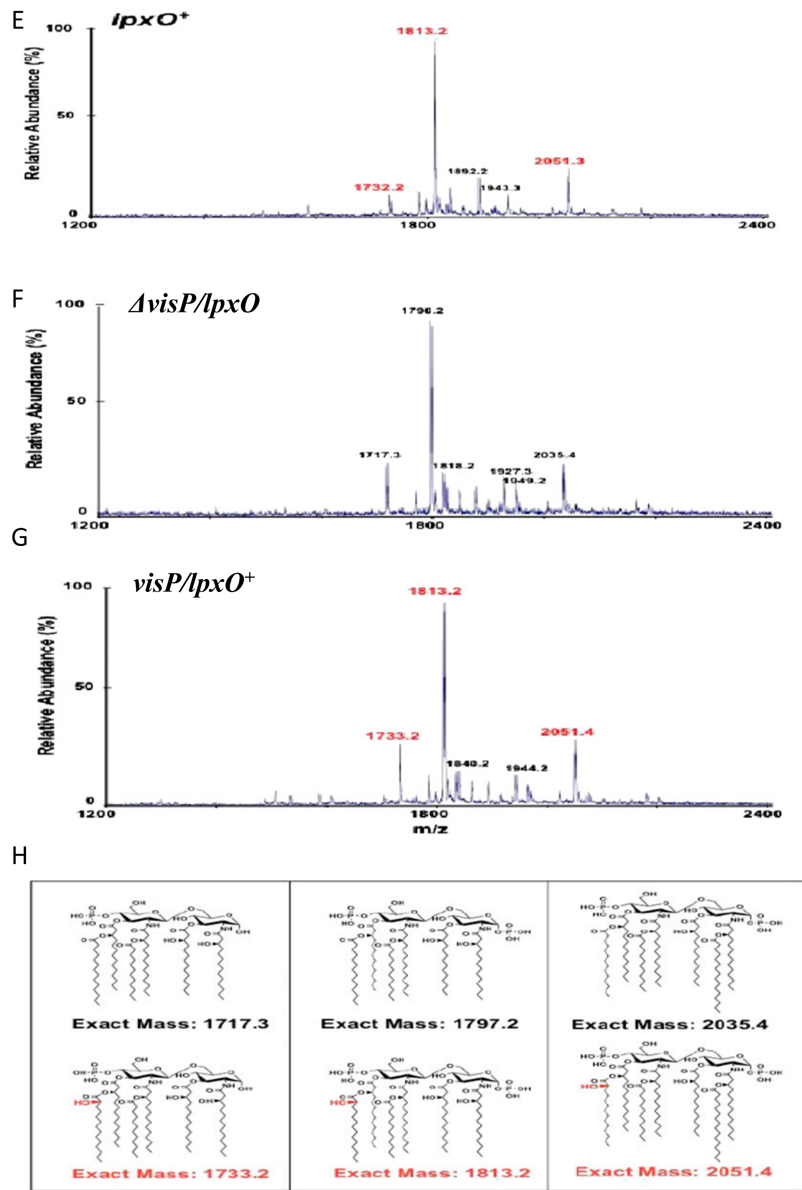

Fig. S5. MS analysis of lipid-A isolates from *S. enterica* serovar Typhimurium. (*A*) WT strain. (*B*) *ΔvisP* strain. (*C*) *visP*+ strain. (*D*) *ΔlpxO* strain. (*E*) *lpxO*+ strain. (*F*) *ΔvisP/lpxO* strain. (*G*) *visP/lpxO*+ strain. (*H*) Lipid-A species structures and their respective exact masses.

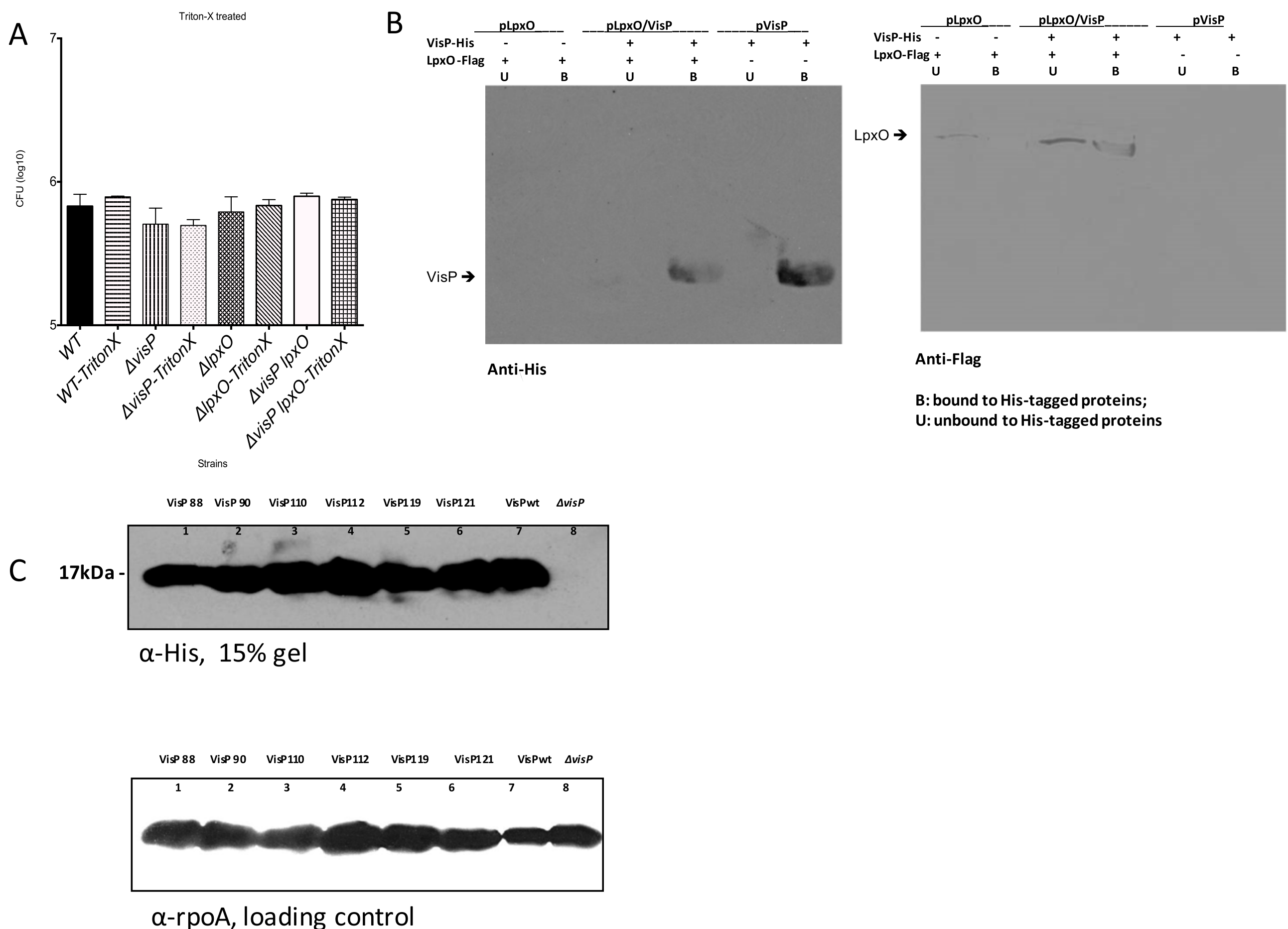

Fig. S6. (*A*) Direct triton-X effect control in the *Salmonella* strains used here. (*B*) Coimmunoprecipitation of LpxO-Flag tagged and VisP-His tagged, The Westerns were probed with either anti-His (*Left*) or anti-Flag (*Right*) antisera. (*C*) VisP point mutants and $VisP_{WT}$ protein expression control.

Table S1. Strains and plasmids used in this study

Strain or plasmid	Relevant genotype	Source
SL1344	*S. enterica* serovar Typhimurium prototype	1
86–24	EHEC O157:H7 prototype	2
CGM220	*qseC* mutant λ-red generate	3
CGM300	*visP* mutant λ-red generate	This study
CGM301	*visP* complemented strain (in *KpnI* and *EcoRI* pBADMycHisA)	This study
CGM302	*lpxO* mutant λ-red generate	This study
CGM303	*lpxO* complemented strain (in *KpnI* and *XbaI* pBAD33)	This study
CGM304	*visP* and *lpxO* double mutant λ-red generate	This study
CP171	EHEC *visP(Z2099)* double mutant λ-red generate	This study
CGM305	*visP* (pBADMycHisA) and *lpxO* (pBAD33) complemented	This study
TOP10	*F-mcrA D(mrr-hsdRMS-mcrBC) f80lacZDM15 DlacX74 deoR recA1 araD139 D(ara-leu)7697 galU galK rpsL (StrR) endA1 nupG*	Invitrogen
DH5α	*supE44 lacU169* (80 *lacZ* M15) *hsdR17*	Stratagene
pBADMycHisA	Cloning vector and C-terminal Myc-His expression vector	Invitrogen
pBAD33	Cloning vector	Invitrogen
TOPO PCR Blunt	PCR blunt cloning vector with topoisomerase	Invitrogen
pKD3	λ-Red template plasmid	4
pKD46	λ-Red helper plasmid (recombinase)	4
pKD20	λ-Red resolvase plasmid	5

1. McDonald IK, Thornton JM (1994) Satisfying hydrogen bonding potential in proteins. *J Mol Biol* 238(5):777–793.
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4. Walters M, Sperandio V (2006) Autoinducer 3 and epinephrine signaling in the kinetics of locus of enterocyte effacement gene expression in enterohemorrhagic Escherichia coli. *Infect Immun* 74(10):5445–5455.
5. Richardson AR, et al. (2011) Multiple targets of nitric oxide in the tricarboxylic acid cycle of Salmonella enterica serovar typhimurium. *Cell Host Microbe* 10(1):33–43.

Table S2. Oligonucleotides used in this study

Primer name	Sequence
visP-F	GGTACCGGAAAAGTAATCATGAAA
visP-R	GAATTCGCCGGATTCACTTTACGAAT
lpxO-F	GGTACCATGTTCGCAGCAATCATTATCGGT
lpxO-(3XFlag)R	TCTAGATTATTTATCATCATCATCTTTATAATCTATATCGTGATCTTTATAATCTCAGAGGAGGCTGAAAAGGATTAT
visP-F λ-red	AAGGGAAAAGTAATCATGAAAAAATTAGCTGCAATCGTTGCGTGTAGGCTGGGAGCTGCTTC
visP-R λ-red	TTACGGATTCACTTTACGAATCTGTTTTACGTCGATTTCGACATATGAATATCCTCCTTA
lpxO-F λ-red	TTAGAAGGACAGTGACGTTATGTTCGCAGCAATCATTATCGGGTGTAGGCTGGGAGCTGCTTC
lpxO-R λ-red	TCAGAGGAGGCTGAAAAGGATTATCGCGGCAAAAATAGCAGCCATATGAATATCCTCCTTA
qseC-F RT	TGGATAATGCGATTCGCTACAGT
qseC-R RT	GCGGGCATGTAAGGTAACGT
visP-F RT	TCCAGTCTTTATCCACTTCGCC
visP-R RT	TGCGACCGGTACTATCAATGTG
ler-EHEC-F RT	CGACCAGGTCTGCCCTTCT
ler-EHEC-R RT	GCGCGGAACTCATCGAAA
VisP-D88A-F	CGGTACTATCAATGTGGCCATTGACCACAAGCGCT
VisP-D88A-R	AGCGCTTGTGGTCAATGGCCACATTGATAGTACCG
VisP-D90A-F	AGCGCTTGTGGTCAATGGCCACATTGATAGTACCG
VisP-D90A-R	CGTTCCAGCGCTTGTGGGCAATGTCCACATTGATA
VisP-E110A-F	CGTTGAAATTCAGGGCGCAGTGGATAAAGACTGGA
VisP-E110A-R	TCCAGTCTTTATCCACTGCGCCCTGAATTTCAACG
VisP-D112A-F	AATTCAGGGCGAAGTGGCTAAAGACTGGAACTCTG
VisP-D112A-R	CAGAGTTCCAGTCTTTAGCCACTTCGCCCTGAATT
VisP-E119A-F	TAAAGACTGGAACTCTGTCGCAATCGACGTAAAACAGATTC
VisP-E119A-R	GAATCTGTTTTACGTCGATTGCGACAGAGTTCCAGTCTTTA
VisP-D121A-F	CTGGAACTCTGTCGAAATCGCCGTAAAACAGATTCGTAAAG
VisP-D121A-R	CTTTACGAATCTGTTTTACGGCGATTTCGACAGAGTTCCAG

Other Supporting Information Files

Dataset S1 (PDF)