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RNA chaperone activates *Salmonella* virulence program during infection

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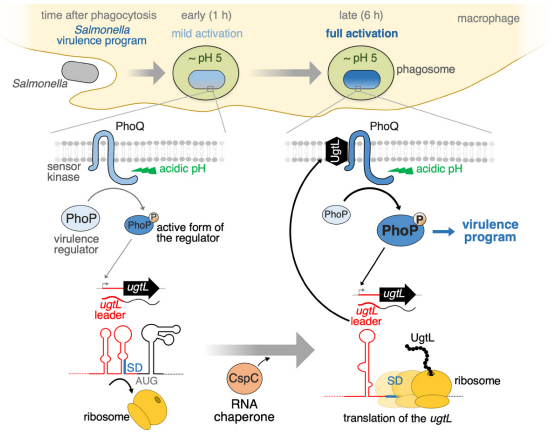
ABSTRACT

Organisms often harbor seemingly redundant proteins. In the bacterium *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium), the RNA chaperones CspC and CspE appear to play redundant virulence roles because a mutant lacking both chaperones is attenuated, whereas mutants lacking only one exhibit wild-type virulence. We now report that CspC—but not CspE—is necessary to activate the master virulence regulator PhoP when *S.* Typhimurium experiences mildly acidic pH, such as inside macrophages. This CspC-dependent PhoP activation is specific to mildly acidic pH because a *cspC* mutant behaves like wild-type *S.* Typhimurium under other PhoP-activating conditions. Moreover, it is mediated by *ugtL*, a virulence gene required for PhoP activation inside macrophages. Purified CspC promotes *ugtL* translation by disrupting a secondary structure in the *ugtL* mRNA that occludes *ugtL*'s ribosome binding site. Our findings demonstrate that proteins that are seemingly redundant actually confer distinct and critical functions to the lifestyle of an organism.

GRAPHICAL ABSTRACT



INTRODUCTION

Organismal evolution often results from a gene duplication event followed by the divergence of one copy of the duplicated gene (1–3). The resulting organism exhibits new abilities that, while related to those conferred by the original gene, expand the environments or conditions in which the organism can survive and proliferate. Prokaryotes and eukaryotes harbor a family of nucleic acid binding proteins with a domain originally identified in bacterial proteins designated cold shock proteins (Csps) (4) because the founding members of this family are expressed in response to cold shock (5,6). However, not all members of the Csp family are induced by cold shock. For example, the CspC and CspE proteins of the facultative intracellular pathogen *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium) are similarly expressed at 15°C and 37°C (7) and required for virulence in a warm-blooded host (8).

CspC and CspE appear to play redundant roles in *S.* Typhimurium virulence because a *cspC cspsE* double mutant is attenuated in Balb/C mice, whereas mutants lacking only one of the corresponding genes exhibit wild-type virulence (8). This notion is reinforced by the high (i.e. 84%) amino

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acid identity shared by these proteins, which also share, albeit not all, RNA targets (8,9). However, the apparent redundancy may reflect that CspC and CspE reciprocally control each other's expression, resulting in non-physiological overproduction of one protein when the gene for the other protein is deleted (10) and rescue of mutants lacking only one of the two genes. Moreover, if CspC and CspE were truly redundant, one of the two corresponding genes would have been lost or become a pseudogene during evolution. Therefore, we reasoned that there must be a condition in which only one protein—CspC or CspE—is needed. We have now established that *S.* Typhimurium requires CspC, but not CspE, to activate a critical virulence program inside macrophages and solved the mechanism by which CspC achieves this task.

The two-component system is a form of signal transduction present in all domains of life. Typically, a two-component systems consists of a sensor protein that responds to a specific signal(s) by modifying the phosphorylated state of a cognate partner protein that generates a response (11). In *S.* Typhimurium, the PhoP/PhoQ two-component system governs virulence (12–15). The sensor PhoQ responds to a mildly acidic pH (16,17), low periplasmic Mg^{2+} (18), certain antimicrobial peptides (19), increased osmolarity (20), and particular long chain fatty acids (21) by activating the transcriptional regulator PhoP. PhoQ's ability to detect a mildly acidic pH in the bacterial cytoplasm is critical for virulence in mice and for expression of PhoP-activated virulence genes inside macrophages (16,22) (Figure 1A). Preventing acidification of the phagosome harboring *S.* Typhimurium inhibits both bacterial replication (23,24) and transcription of PhoP-activated genes (25,26) inside macrophages.

PhoP activation in mildly acidic pH and inside macrophages requires the UgtL protein to promote PhoQ autophosphorylation (22), which enhances the fraction of active (i.e., phosphorylated) PhoP (PhoP-P) protein (27) (Figure 1A). It also needs the DNA binding protein SsrB to further transcription from the *phoP* and *ugtL* promoters (28), which are directly activated by PhoP-P (29,30).

We now report that activation of the master virulence regulator PhoP requires CspC but not CspE when *S.* Typhimurium experiences mildly acidic pH, such as inside macrophages. We determine that CspC activates PhoP by binding to the unusually long leader region of the *ugtL* transcript, which stimulates *ugtL* translation by disrupting a secondary structure that sequesters *ugtL*'s ribosome binding site. This activation is critical for virulence because a *cspC* null mutant reaches lower numbers in murine liver and spleen than the wild-type strain. Our findings reveal that organisms retain highly similar yet functionally singular proteins because these play critical and independent roles under specific conditions.

MATERIALS AND METHODS

Bacterial strains, plasmids, oligodeoxynucleotides and growth conditions

Bacterial strains and plasmids used in this study are listed in Supplementary Table S1. All *S. enterica* serovar Typhimurium strains were derived from the wild-type strain 14028s (31) and constructed by phage P22-mediated transductions as described (32). Bacteria were grown at 37°C in Luria-Bertani (LB) broth or N-minimal media (33) supplemented with 0.1% casamino acids, 38 mM glycerol, and the indicated pH (pH 7.6 or pH 4.9) and 1 mM of $MgCl_2$ unless specified. *Escherichia coli* DH5α was used as the host for preparation of plasmid DNA (34). To induce plasmid expression, isopropyl β-D-1-thiogalactopyranoside (IPTG) was added at the indicated concentrations (0.1-0.5 mM). When necessary to select for plasmid maintenance, appropriate antibiotics were added at the following final concentrations: ampicillin at 50 μg ml^{-1}, chloramphenicol at 20 μg ml^{-1}, kanamycin at 50 μg ml^{-1}, and tetracycline at 10 μg ml^{-1}. For the mRNA stability assay, rifampicin was used at 250 μg mg ml^{-1}. DNA oligonucleotides used in this study are listed in Supplementary Table S2.

Construction of chromosomal mutant strains

To generate *S.* Typhimurium strains deleted for the *cspC*, *yobF* or *cspE* genes, a *cat* cassette was amplified from pKD3 using primers 14972/14975, 15170/14973, or 15133/15134 and was introduced into the wild-type strain (14028s) harboring plasmid pKD46 (35).

To generate *S.* Typhimurium strains expressing the *ugtL* gene from the heterologous p$lac_{1\text{-}6}$ promoter, a *cat* cassette with plac_{1-6} was introduced upstream of the *ugtL* leader region at the specified locations, replacing the wild-type *ugtL* promoter. The *cat* fragment with the p$lac_{1\text{-}6}$ was amplified from plasmid pKD3 using primers 16655/16656 (–182), 16655/16688 (–171) or 16655/16658 (–12) then introduced into wild-type *S.* Typhimurium (14028s) harboring plasmid pKD46 (35). The *cat* cassette was removed using plasmid pCP20 (35).

To generate an *S.* Typhimurium strains with *ugtL-FLAG*, a *kan* cassette was amplified from pKD4 using primers 16686/16687 and was introduced into wild-type *S.* Typhimurium (14028s) harboring plasmid pKD46 (35). The *cat* cassette was removed using plasmid pCP20 (35).

To generate *S.* Typhimurium strain expressing $CspC^{H32R}$, a *tetAR* cassette was amplified using primers 15124/15125 and introduced into the *cspC* gene of wild-type *S.* Typhimurium (14028s). Then, the *tetAR* cassette was replaced by annealed primers of 15128/15129 to create a *S.* Typhimurium strain specifying the $CspC^{H32R}$ protein. The resulting strain lacking tetracycline resistance was purified on media containing fusaric acid (36), and the presence of the mutation was confirmed by sequencing the corresponding DNA.

To generate an *S.* Typhimurium strain with a substitution of the *STM14_1939* gene start codon (ATG) by a stop codon (TAA), a cassette with *cat* and P*rhaB-relE* was amplified from plasmid pSLC-242 (37) using primers W3781/W3782 and was introduced into the *STM14_1939* gene of wild-type *S.* Typhimurium (14028s) harboring plasmid pKD46 (35). Then, the *cat* p*rhaB-relE* cassette was replaced by annealed oligonucleotides W3783/W3784 to substitute ATG to TAA. The resulting strain was obtained following selection against RelE-mediated toxicity on media containing 0.2% rhamnose as described (37). The pres-

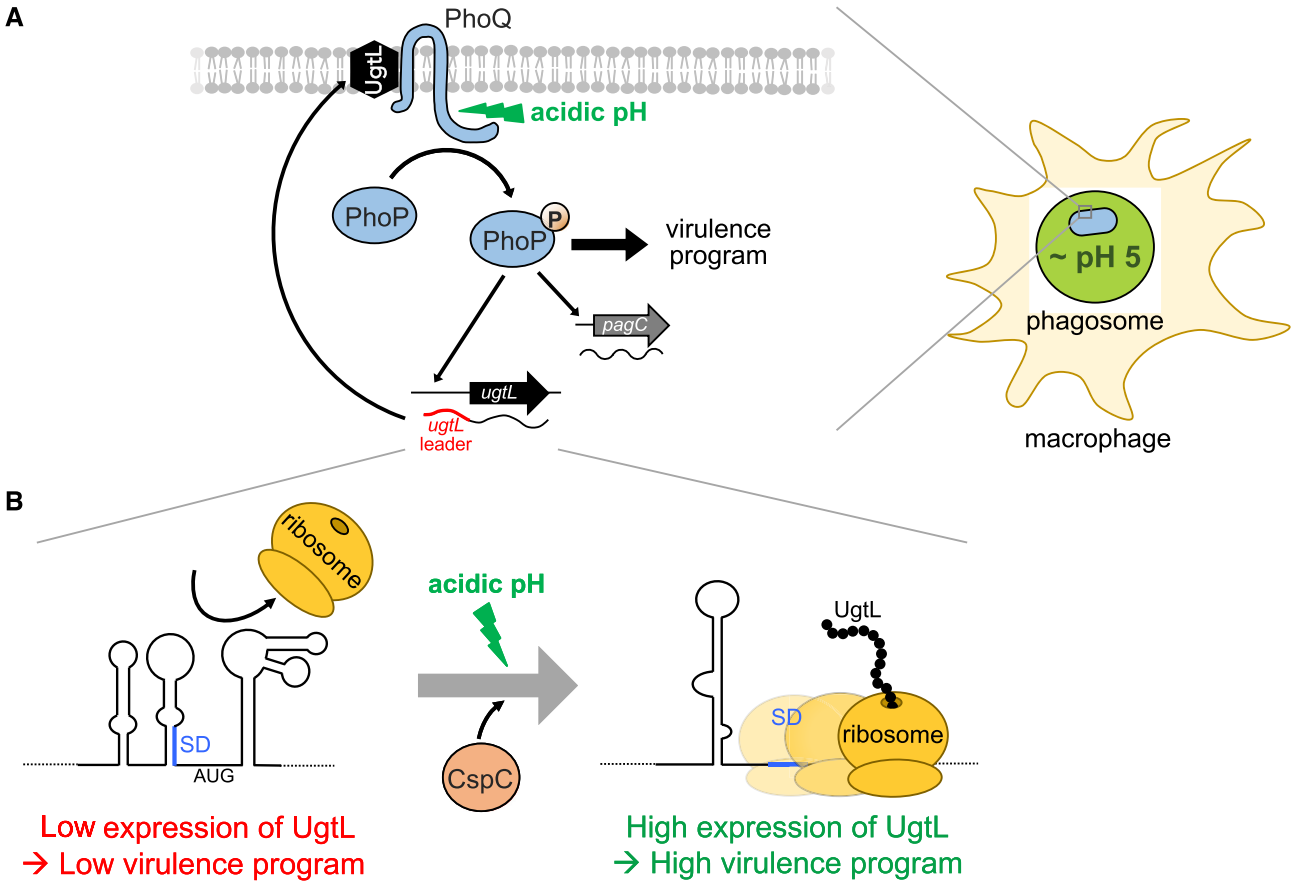

Figure 1. CspC activates mildly acidic pH-responsive virulence program by promoting *ugtL* translation. (**A**) *S.* Typhimurium PhoQ partially activates PhoP when experiencing mildly acidic pH inside a macrophage phagosome, thereby promoting transcription of PhoP-activated genes, including *ugtL* and *pagC*. (**B**) The *ugtL* transcript contains a 182 or 171 nt leader sequence that, in the absence of CspC, can adopt a secondary structure that inhibits translation of the *ugtL* gene by sequestering its ribosome binding site (Shine-Dalgarno – SD – sequence) (left panel). CspC binding to the *ugtL* leader liberates *ugtL*'s SD, allowing ribosome binding, resulting in UgtL synthesis (right panel). The produced UgtL protein enhances PhoP phosphorylation, thereby activating the PhoP-dependent virulence program.

ence of the engineered mutation was confirmed by DNA sequencing.

Construction of plasmids

Plasmid pCspC and pCspC^{H32R} was constructed as follows: the *cspC* gene was amplified from wild-type *S.* Typhimurium (14028s) or isogenic *cspC* (H32R) mutant (JC636) using primers 15113/14967, then introduced between the BamHI and HindIII sites of pUHE21-2*lacI*^{q} (29).

Plasmid pCspC-His6 was constructed as follows: the *cspC* gene was amplified from wild-type *S.* Typhimurium (14028s) using primers 15113/15841, then introduced between the BamHI and HindIII sites of pUHE21-2*lacI*^{q} (29).

Plasmid pYobF was constructed as follows: the *yobF* gene was amplified from wild-type *S.* Typhimurium (14028s) using primers 15137/15138, then introduced between the BamHI and HindIII sites of pUHE21-2*lacI*^{q} (29).

Plasmid pCspE was constructed as follows: the *cspE* gene was amplified from wild-type *S.* Typhimurium (14028s) using primers 18147/18148, then introduced between the BamHI and HindIII sites of pUHE21-2*lacI*^{q} (29).

Plasmid to generate Tn*5-tetAR* transposon (pMOD3-TetAR) was constructed as follows: the *tetAR* genes were amplified from *phoP S. enterica* (MS7953s) (12) using primers 14937/14938, then introduced between the *BamH*I and *Hind*III sites of pMOD-3 (Epicentre, TNP10623).

Genetic screening

The Tn*5-tetAR* transposon was amplified using ME Plus 9 primer set (Epicentre, TNP10623) and pMOD3-TetAR as template DNA. Transposome was prepared using PCR products and transposase (Epicentre, TNP92110) according to the manufacturer's instruction. The resulting transposome was introduced into the recipient strain by electroporation, and transformants were selected on LB agar plates containing tetracycline. Transformants were washed three times with N-minimal media with 1 mM of Mg^{2+} at pH 7.6 and grown in the same media for 3 h. Aliquots of the resulting culture were then plated on N-minimal agar with 1 mM of Mg^{2+} at pH 4.9 supplemented with 60 μg/ml X-gal. When necessary, tetracycline was added to induce transcription from the *tetA* promoter. Selected clones were purified on LB agar plates containing tetracycline, and the pheno-

type was further confirmed. The site of Tn*5-tetAR* cassette insertion was identified by DNA sequencing according to the manufacturer's instructions. Briefly, genomic DNA extracted from selected mutants with DNeasy Blood & Tissue Kits (Qiagen) were digested with PstI or SphI, ligated, then transformed into *E. coli* EC100D pir^+ strain (Epicentre, ECP09500). The resulting self-ligated plasmids were extracted and used for DNA sequencing using primers 15715 or 15716.

Measuring β-galactosidase activity

β-Galactosidase activity was determined as follows. Bacteria were collected and resuspended in 1 ml of Z-buffer [60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$, 50 mM β-mercaptoethanol, pH 7.0], then 40 μl chloroform and 20 μl SDS (0.1%) were added. After 5 min incubation at room temperature, 100 μl of samples were transferred to a 96-well plate and 100 μl of 1.2 mM 2-nitrophenyl β-D-galactopyranoside (ONPG, Sigma-Aldrich). Absorbance at 420 nm for each reaction was determined every 20 s for 20–30 min. β-Galactosidase activity is shown as the rate of ONPG conversion (V_{max}) divided by the OD_{600} values of the samples.

lac fusions to PhoP-activated genes

lac transcriptional fusions to the PhoP-activated *pcgL* and *mgtC* genes were generated upon insertion of the Mud*J* transposon in the corresponding genes (18,38). Mud*J* contains stop codons in all three reading frames prior to the *trpB-trpA*(truncated)-*lacZ* genes (39), allowing the generation of transcriptional (but not translational) fusions. By contrast, the *ugtL-lac* fusion was constructed using a HindIII digested fragment from a plasmid containing Mud*J* (40). DNA sequencing analysis of the *ugtL-lac* fusion using primers 16327/14895 demonstrated that the portion of Mud*J* DNA containing stop codons is no longer present and that the truncated *trpB* gene from the HidIII site in Mud*J* is fused in frame to the *ugtL* gene (Supplementary Figure S1). Because *trpB* and *trpA* genes are translationally coupled (41), the phenotypic behavior of the *ugtL-lac* fusion is that of a translational fusion.

Measuring gene expression by GFP assay

Fluorescence and OD_{600} values were measured by using multidetector, VICTOR3 (PerkinElmer). The measured values of GFP expression were divided by 1000 times the OD_{600} values.

Quantitative RT-PCR (qRT-PCR)

Total RNA was isolated using RNeasy Kit (Qiagen) according to the manufacturer's instructions. The purified RNA was quantified using a Nanodrop machine (NanoDrop Technologies). Complementary DNA (cDNA) was synthesized using High Capacity RNA-to cDNA Master Mix (Applied Biosystems). The mRNA amount of the *pmrD*, *phoP*, *mgtC*, *pgtE*, *ugtL* and *cspC* genes was determined by quantification of cDNA using Fast SYBR Green PCR Master Mix (Applied Biosystems) and appropriate primers (*phoP*: 4489/4490; *pmrD*: 4491/4492; *mgtC*: 6962/6963; *pgtE*: 7109/7111; *ugtL*: 7295/7302; *ugtL* coding region: 16038/16039; *cspC*: 14982/14983) and monitored using a QuantStudio 6 machine (Applied Biosystems). Data were normalized to the levels of 16S ribosomal RNA amplified with primers 3203 and 3204.

Culture of macrophage cells

The murine-derived macrophage-like cell line J774A.1 was cultured in Dulbecco modified Eagle medium (DMEM; Life Technologies) supplemented with 10% FBS (Life Technologies) at 37°C under 5% CO_2.

Determination of bacterial mRNA abundance inside macrophages

J774A.1 macrophages were seeded in 6-well tissue culture plates with 2×10^6 cells per well one day before infection with *S.* Typhimurium. Confluent monolayers were inoculated with bacterial cells that had been grown overnight in LB broth, washed with PBS and resuspended in 0.1 ml of prewarmed DMEM at a multiplicity of infection of 10. Following 30 min incubation, the wells were washed three times with prewarmed PBS to remove extracellular bacteria and then incubated with prewarmed medium supplemented with 100 mg ml^{-1} gentamicin for 1 h to kill extracellular bacteria. Next, wells were washed three times with PBS and then incubated with prewarmed medium supplemented with 10 mg ml^{-1} gentamicin. At the desired times, samples were harvested using TRIzol™ reagent (Invitrogen) solution. Total RNA was isolated and cDNA was synthesized as described above. mRNA amounts were determined by qRT-PCR as described above.

Western blot analysis

Bacterial cells were grown as described and crude extracts were prepared in B-PER bacterial protein extraction reagent (Pierce) with 100 μg ml^{-1} lysozyme and EDTA-free protease inhibitor (Roche). Samples were separated in 4–12% NuPAGE gels (Life Technologies). Then, samples were analyzed by Western blotting using antibodies recognizing FLAG (Sigma; 1:2,000), PhoP (1:2,000), GroEL (Abcam; 1:5,000), or AtpB (Abcam; 1:5,000). Secondary horseradish peroxidase-conjugated antisera recognizing rabbit or mouse antibodies (GE healthcare) were used at 1:5,000 dilution. Blots were developed with the Amersham ECL Western Blotting Detection Reagents (GE Healthcare) or SuperSignal West Femto Chemiluminescent system (Pierce), and visualized using LAS-4000 (Fuji Film). The density of protein bands was determined by quantification using ImageJ software version 1.52 (NIH).

In vivo detection of phosphorylated PhoP

Whole-cell extracts were prepared as described (42) and normalized by OD_{600}. The samples were electrophoresed on 12.5% polyacrylamide gels containing acrylamide-Phos-tag™ ligand (Phos-tag™ Consortium) as described by the

manufacturer (gels were copolymerized with 50 μM Phos-tag™ acrylamide and 100 μM $MnCl_2$) with standard running buffer [0.4% (w/v) SDS, 25 mM Tris, 192 mM glycine] at 4°C under 20 mAmp for 3.5 h, transferred to nitrocellulose membranes, and analyzed by immunoblotting using polyclonal rabbit antibodies recognizing PhoP (1:2000) and polyclonal mouse antibodies recognizing AtpB (1:5,000). Secondary horseradish peroxidase-conjugated antisera recognizing rabbit and mouse antibodies (GE healthcare) were used at 1:5000 dilution. The blots were developed with the Amersham ECL Western Blotting Detection Reagents (GE Healthcare) or SuperSignal West Femto Chemiluminescent system (Pierce), and were visualized using LAS-4000 (Fuji Film). The density of protein bands was determined by quantification using ImageJ software version 1.52 (NIH).

Purification of CspC proteins

To purify proteins, *E. coli* BL21 (DE3) strains harboring plasmids expressing His_6-tagged wild-type or variant CspC proteins were grown in LB at 37°C for 3 h and 0.7 mM of IPTG was added to induce gene expression and further incubated at 30°C for 3 h. Cells were collected and washed twice with a solution containing 50 mM Tris–HCl (pH 8.0) and 150 mM NaCl. Washed cells were resuspended in solution A [50 mM Tris–HCl (pH 8.0), 150 mM NaCl] containing 150 μg ml^{-1} lysozyme, 1 mM $MgCl_2$, DNase I (Promega) and EDTA-free protease inhibitor cocktail (Roche), and incubated at 4°C for 30 min. Cells were broken using Cell Disruptor (Constant Systems Ltd). After adding imidazole to 20 mM as final concentration, cell debris was removed by centrifugation (12 000×*g*, 30 min) and the supernatant was applied to Ni-Nta agarose (Qiagen) column. The column was washed with solution A containing 25 mM imidazole, and proteins were eluted with solution A containing 100–300 mM imidazole and dialyzed with the same solution without imidazole.

Electrophoretic mobility shift assay

A DNA template containing a T7 promoter sequence followed by the sequence of the *ugtL* gene -corresponding to positions –171 to + 66 region relative to the *ugtL* ATG start codon was generated by PCR using primers W3583/W3505. *In vitro* transcription was then performed to generate the *ugtL* transcript using the Megascript T7 Transcription Kit (Ambion), which was subsequently 5′ end-labeled as described (43). Electrophoretic mobility shift assay was performed using 4 pmol of 5′ end-labeled *ugtL* mRNA with increasing concentrations of purified CspC or $CspC^{H32R}$ proteins in 10 μl reactions as described (8) with some modifications. Briefly, 5′ end-labeled *ugtL* mRNA was denatured 1 min at 95°C and then cooled for 5 min on ice. Yeast RNA (Ambion) was then added to the reaction (0.1 mg/ml) and the volume completed to 5 μl with binding buffer (10 mM Tris–HCl pH 8, 1 mM EDTA, 50 mM KCl, 7.4% glycerol). 5 μl of either CspC or $CspC^{H32R}$ protein corresponding to each final concentration was added to the RNA and the binding reactions were incubated for 20 min on ice. Samples were mixed with 3 μl of native loading buffer (50% (v/v) glycerol, 0.5× TBE, 0.2% (w/v) bromophenol blue) before loading on a pre-cooled native 6% polyacrylamide gel (Thermo Scientific). Gels were run in 0.5× TBE at 100 V for 3 h 45 min at 4°C. Gels were dried and analyzed using a phosphorimager (Typhoon FLA 9000, GE Healthcare).

Enzymatic probing of CspC interaction with *ugtL* mRNA

A DNA template containing a T7 promoter sequence followed by the sequence of the *ugtL* –171 to + 264 region (relative to *ugtL* ATG start codon) was generated by PCR using primers W3583/W4321. *In vitro* transcription was then performed to generate a *ugtL* transcript using the Megascript T7 Transcription Kit (Ambion), which was subsequently 5′ end-labeled as described (43). Enzymatic probing of CspC interaction with *ugtL* mRNA was performed using 4 pmol of 5′ end-labeled *ugtL* mRNA with increasing concentrations of purified CspC protein in 10 μl reactions as described (43) with some modifications. Briefly, 5′ end-labeled *ugtL* mRNA was denatured 1 min at 95°C and then cooled for 5 min on ice. Yeast RNA (Ambion) was then added to the reaction (0.1 mg/ml) and the volume completed to 5 μl with binding buffer (10 mM Tris–HCl pH 8, 1 mM EDTA, 50 mM KCl, 7.4% glycerol). 5 μl of CspC protein corresponding to each final concentration was added to the RNA and the binding reactions were incubated for 20 min on ice. Samples were then preincubated for 2 min at 37°C before the addition of 0.1 U of RNase T1 (Ambion). The T1 digestion reactions were incubated for 2 min at 37°C and then stopped with 88 μl of stop solution (50 mM Tris–Cl pH 8.0, 0.1% SDS) and 100 μl of phenol:chloroform 5:1 (Sigma). Samples were spun at 14 000 rpm (Eppendorf 5430R) for 10 min at 4°C, and the aqueous phase precipitated with 250 μl of ethanol for 1 h at –20°C. Pellets were resuspended in 5 μl of water and 15 μl of loading buffer II (95% formamide, 18 mM EDTA, 0.025% (w/v) SDS, 0.025% (w/v) xylene cyanol, 0.025% (w/v) bromophenol blue, Ambion). Alkaline and RNase T1 sequencing ladders were generated according to the manufacturer's directions (Ambion). Samples were denatured prior to loading (90°C, 1 min, then ice for 1 min), and separated by denaturing PAGE on 8% polyacrylamide/7 M urea sequencing gels under constant power (40 W). Gels were dried and analyzed using a phosphorimager (Typhoon FLA 9000, GE Healthcare).

In vitro transcription/translation assay

DNA templates for *in vitro* transcription and translation were generated using primers 17894 /17898 (full-length, -182 nt) or 17894 /17899 (leaderless, –12 nt) and genomic DNA of wild-type *S.* Typhimurium (14028s) or an isogenic strain mutated in the *ugtL* leader region (JC1718). PURExpress® *In Vitro* Protein Synthesis Kit (New England Biolabs, E6800) was used to synthesize UgtL protein from a DNA template. Purified CspC proteins (wild-type or variant) were added at 5 μM. Aliquots were taken at desired time points and the reaction was stopped by adding SDS loading dye, then the resulting mixtures were frozen in dry ice. After boiling for 5 min, samples were separated in

4–12% NuPAGE gels (Life Technologies) and proteins were detected by Western blot analysis.

Mouse infection and determination of bacterial number in mouse organs

Six-week-old female C3H/HeN mice were purchased from Charles River Laboratories. Three to five mice in each group were infected intraperitoneally with 0.1 ml of PBS containing ∼1 × 10^4 (for bacterial count in organs) or ∼4 × 10^4 (for mouse survival) *S.* Typhimurium that had been grown overnight in LB broth and resuspended and diluted in PBS. Mouse survival was monitored two to four times per day for sixteen days. Animals were housed in temperature- and humidity-controlled rooms and maintained on a 12 h light/12 h dark cycle. All procedures complied with regulations of the Institutional Animal Care and Use Committee of the Yale School of Medicine. At five days after infection, bacterial colonization in spleen and liver was determined as follows. Mice were euthanized using carbon dioxide, and the spleen and liver were removed aseptically. The organs were homogenized in 0.5 ml (spleen) or 1 ml (liver) of ice-cold PBS and serially diluted in PBS. Bacterial loads were determined by plating the diluents on LB agar media.

Quantification of protein bands from western blot images

The density of protein bands was determined by quantification using ImageJ software version 1.52 (NIH).

Nucleotide sequence comparisons

Upstream regions of the *ugtL* gene from different serovars of *S. enterica* and from *S. bongori* were aligned using Clustal Omega (EMBL-EBI); *S. enterica* subsp. *enterica* serovar Typhimurium (14028s), *S. bongori* (NCTC12419), *S. enterica* subsp. *diarizonae* (SA20044251), *S. enterica* subsp. *arizonae* (RKS2983), *S. enterica* subsp. *houtenae* (CFSAN000552), *S. enterica* subsp. *salamae* (RSE42), *S. enterica* subsp. *indica* (NCTC12420), *S. enterica* subsp. *enterica* serovar Parayphi A (ATCC11511), *S. enterica* subsp. *enterica* serovar Typhi (Ty2), *S. enterica* subsp. *enterica* serovar Dublin (ATCC39184), *S. enterica* subsp. *enterica* serovar Gallinarum (1984), *S. enterica* subsp. *enterica* serovar Paratyphi B (SPB7), *S. enterica* subsp. *enterica* serovar Cholerasuis (SC-B67), *S. enterica* subsp. *enterica* serovar Heidelberg (41578), and *S. enterica* subsp. *enterica* serovar Enteritidis (92-0392). The phylogenetic tree was made by the interactive Tree of Life software (v5) based on analysis of those regions using Clustal Omega.

Statistical analyses

Sample sizes (biological replicates) for each experimental group or condition are described in each figure legend. For comparisons of two groups, *t*-tests were applied. For comparisons of more than three groups, one-way ANOVA with Brown-Forsythe and Welch tests were applied. Mantel-Cox tests were performed for comparisons of mice survival. These analyses provide *P*-values for each comparison.

RESULTS

CspC promotes expression of the PhoP-activated virulence gene *ugtL* in mildly acidic pH

Activation of the *S.* Typhimurium PhoP/PhoQ system in mildly acidic pH requires the *ugtL* gene (22) and the *ugtL* transcriptional activator SsrB (28). Because an *ssrB* null mutant retains residual PhoP/PhoQ activation (28), we hypothesized that a factor activates PhoP in an SsrB-independent manner when *S.* Typhimurium experiences mildly acidic pH. Thus, we searched for the putative factor by screening a library of transposon-generated mutants in a strain harboring a *lac* fusion to the chromosomal copy of the *ugtL* gene (Supplementary Figure S1) (40) and lacking the *ssrB* gene (Figure 2A). We used a derivative of transposon Tn*5* that allows for both gene inactivation and increased expression of genes located adjacent to the site of transposon insertion. The latter property results from transcription originating from the promoters of the divergent *tetR* and *tetA* genes within the transposon (Figure 2B), which confer constitutive and inducible expression, respectively. We screened ∼15,000 transposon mutants, looking for blue colonies on N-minimal media (pH 4.6) agar plates containing X-Gal (60 μg/ml) (Figure 2A) because the *ssrB* mutant forms white colonies, whereas the isogenic *ssrB*$^+$ strain forms blue colonies on these plates. Then, we used phage P22 to transduce the transposon in candidate clones into the parental (*ssrB ugtL-lac*) strain and verified that the resulting transductants retained the phenotype of the original transposon-generated mutants (Figure 2C). The transposon in one mutant was located upstream of the coding region of *yobF* (Figure 2B), a gene forming an operon with the downstream *cspC* gene (Figure 2B).

We hypothesized that the increased *ugtL-lac* expression of the mutant is due to overexpression of the *yobF* and/or *cspC* genes because the transposon was oriented with the constitutive *tetR* promoter toward the *yobFcspC* operon (Figure 2B). Deletion of the *cspC* gene in an *ssrB*$^+$ background decreased *ugtL-lac* expression nearly as much as deletion of the *ssrB* gene (Figure 2C). The *cspC* defect was corrected by a plasmid with a heterologous promoter transcribing the *cspC* gene but not by an isogenic plasmid transcribing the *yobF* gene or the plasmid vector (Figure 2C and Supplementary Figure S2A). *ugtL* mRNA amounts were much lower in a *cspC* mutant carrying the plasmid vector than in the isogenic strain with a plasmid expressing the *cspC* gene from a heterologous promoter, which behaved like the wild-type strain (Figure 2D). (Although a strain lacking the *yobF* gene also exhibits reduced *ugtL* expression, this phenotype is due to polar effects on the downstream *cspC* gene because the *cspC*-expressing plasmid corrected *ugtL* expression, whereas an isogenic plasmid transcribing the *yobF* gene or the plasmid vector did not (Supplementary Figure S2A).)

The *cspC*-expressing plasmid rescued the *ssrB* mutant as well as the isogenic plasmid expressing the *ssrB* gene (Figure 2D). By contrast, the *ssrB*-expressing plasmid failed to rescue the *cspC* mutant, behaving like the vector control (Figure 2D). SsrB is not a transcriptional activator of

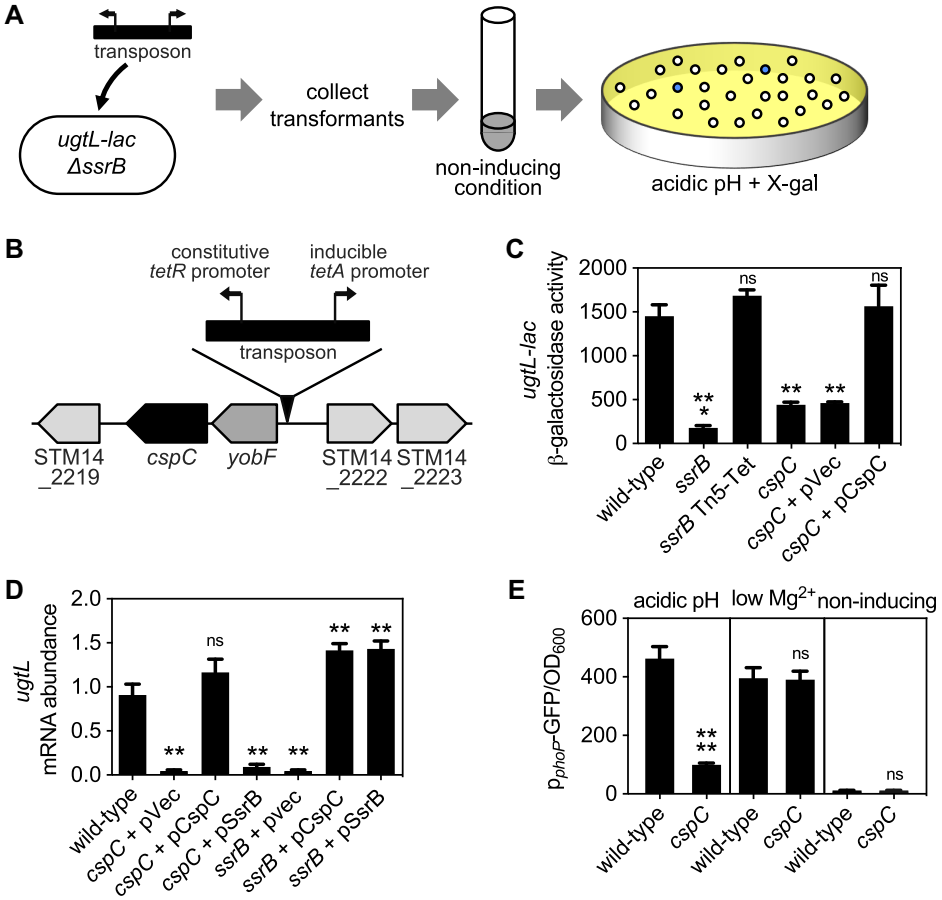

Figure 2. CspC promotes expression of PhoP-activated genes in mildly acidic pH. (**A**) Schematic of genetic screen to identify genes with increased expression of the PhoP activated *ugtL* gene in mildly acidic pH. A transposon was introduced into the *S.* Typhimurium *ssrB* mutant harboring a chromosomal *ugtL-lac* fusion (JC257). The resulting transposon-generated mutants were collected and grown in N-minimal media with 1 mM of Mg^{2+} at pH 7.6 (non-inducing) media, then plated on N-minimal media with 1 mM of Mg^{2+} at pH 4.9 (acidic pH) agar containing X-gal. (**B**) Schematics of the transposon and a chromosomal locus where integration of the transposon restored wild-type *ugtL* expression. (**C**) β-Galactosidase activity produced from isogenic *S.* Typhimurium strains with a chromosomal *ugtL-lac* fusion in wild-type (EG11250), *ssrB* (JC257), *ssrB* mutant containing transposon (JC485), and *cspC* (JC619) with an empty vector (pVec) or a plasmid expressing the *cspC* gene (pCspC) grown to mid-log phase in N-minimal media with 1 mM of Mg^{2+} at pH 4.9 supplemented with 0.2 mM IPTG. (**D**) mRNA abundance of the *ugtL* gene produced by isogenic wild-type (14028s), *cspC* (JC577), and *ssrB* (EG14411) *S.* Typhimurium strains with pVec, pCspC, or a plasmid expressing *ssrB* (pSsrB) grown to mid-log phase in N-minimal media with 1 mM of Mg^{2+} at pH 4.9 supplemented with 0.2 mM IPTG grown to mid-log phase in N-minimal media with 1 mM of Mg^{2+} at pH 4.9. (**E**) Fluorescence produced from a p*phoP-gfp* transcriptional fusion displayed by isogenic wild-type (14028s) and *cspC* (JC577) *S.* Typhimurium strains grown to mid-log phase in N-minimal media with 1 mM of Mg^{2+} at pH 4.9 (acidic pH), 10 μM Mg^{2+} at pH 7.6 (low Mg^{2+}), or 1 mM Mg^{2+} at pH 7.6 (non-inducing). The mean and SD from four independent experiments are shown ($n = 4$) (C–E). One-way ANOVA with Brown-Forsythe and Welch tests (wild-type versus others) (C and E) or two-tailed *t*-test (wild-type vs. the *cspC* mutant) (D) ns, not significant; $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$.

the *cspC* gene because wild-type and *ssrB* mutant strains harbored similar *cspC* mRNA amounts (Supplementary Figure S2B). The results in this section establish that CspC is necessary for *ugtL* expression when *S.* Typhimurium experiences mildly acidic pH, and that CspC overexpression bypasses a requirement for *ssrB*.

CspC promotes transcription of multiple PhoP-activated genes in mildly acidic pH

We reasoned that *cspC* inactivation should reduce expression of multiple PhoP-activated genes in addition to *ugtL* (Figure 2C) because UgtL is necessary to activate PhoP in mildly acidic pH (22). As proposed, fluorescence from a p_{phoP}-*gfp* fusion, in which the PhoP-activated *phoP* promoter drives transcription of a promoterless *gfp* gene (44), was lower in a *cspC* mutant than in the isogenic wild-type strain (Figure 2E). In addition, the mRNA abundances of the PhoP-activated genes *mgtC, pgtE,* and *ugtL* were also lower in a *cspC* mutant than in wild-type *S.* Typhimurium (Supplementary Figure S2C), indicating that the CspC effect is neither limited to a particular PhoP-activated gene nor to how gene expression is determined.

Critically, the CspC dependence of p_{phoP}-*gfp* expression is specific to mildly acidic pH because isogenic *cspC* strains exhibited similar fluorescence when grown in the PhoQ-activating condition low Mg^{2+}, or under non-activating conditions for PhoQ (Figure 2E). Taken together, these results indicate that CspC is required for expression of PhoP-activated genes in mildly acidic pH.

CspC promotes transcription of PhoP-activated genes in a PhoP- and PhoQ-dependent manner

Members of the Csp protein family bind both RNA and single stranded DNA (45), raising the possibility of CspC promoting transcription of PhoP-activated genes independently of the DNA binding protein PhoP. However, neither deletion of the *cspC* gene nor *cspC* expression from a heterologous promoter altered expression from the chromosomal *ugtL-lac* fusion in strains lacking the *phoP* gene (Figure 3A). By contrast, a plasmid that expressed *phoP* from a heterologous promoter rescued *ugtL-lac* expression in a *cspC phoP* double mutant, albeit not to the levels achieved in a *phoP* single mutant (Figure 3A). These results indicate that CspC promotes *ugtL* expression in a PhoP-dependent manner.

CspC furthers the active state of PhoP because the PhoP-P-to-PhoP ratio was larger in wild-type *S.* Typhimurium than in the *cspC* mutant (Figure 3B), and also because the ratio increased beyond the levels exhibited by the wild-type strain upon increasing the concentration of the inducer used to activate the heterologous promoter driving *cspC* transcription (Figure 3B). The increase in PhoP-P (Figure 3B) resulted in corresponding increases in the mRNA amounts of PhoP-activated genes, albeit not to the same extent (Figure 3C and Supplementary Figure S2C).

CspC furthers PhoP phosphorylation in a PhoQ-dependent manner because the *cspC*-expressing plasmid increased the PhoP-P-to-PhoP ratio in the *cspC* single mutant but not in a *cspC phoQ* double mutant, which had no detectable PhoP-P (Figure 3D). This is consistent with PhoQ being the only known PhoP phosphodonor and PhoP-P phosphatase (46,47). Furthermore, the *cspC*-expressing plasmid did not alter the PhoP-P-to-PhoP ratio in a *cspC phoP* phoQ* strain (Figure 3E), which lacks the *cspC* and *phoQ* genes and expresses the PhoP* variant that autophosphorylates from acetyl phosphate (46) (Figure 3F). These results indicate that CspC requires PhoQ to increase PhoP-P abundance.

In a *phoP*phoQ* strain, CspC increases expression specifically of *ugtL* among PhoP-activated genes

The *cspC*-expressing plasmid did not alter expression of the PhoP-activated *mgtC-lac* and *pcgL-lac* chromosomal gene fusions in a *phoP*phoQ* genetic background (Figure 3G), behaving like the vector control (Figure 3G). These results are in full concordance with the *cspC*-expressing plasmid not altering PhoP-P amounts in the *cspC phoP*phoQ* strain (Figure 3E). By contrast, the *cspC*-expressing plasmid did increase expression from the chromosomal *ugtL-lac* gene fusion in *phoP*phoQ* strain (Figure 3G), suggesting that CspC acts specifically on *ugtL*. In agreement with these results, an increase in *cspC* transcription from the *cspC*-expressing plasmid resulted in >100-fold increase in UgtL protein amounts in the *phoP*phoQ* strain (Figure 3H); by contrast, the vector control had no effect (Figure 3H).

PhoP activation by CspC is dependent on *ugtL*'s coding and leader regions

We reasoned that CspC favors PhoP phosphorylation via UgtL because UgtL is required for PhoQ autophosphorylation under mildly acidic pH (22) and also because *ugtL* expression is highly dependent on CspC (Figures 2D, 3C, 3G and 3H). As proposed, the *cspC*-expressing plasmid increased the PhoP-P to PhoP ratio in the *cspC* mutant (Figure 3B and Supplementary Figure S3), but not in the *ugtL* mutant (Supplementary Figure S3). By contrast, an isogenic plasmid harboring the *ugtL* coding region enhanced the PhoP-P to PhoP ratio in both *cspC* and *ugtL* single mutants (Supplementary Figure S3). These data reinforce the notion that CspC promotes PhoP-P phosphorylation by enhancing *ugtL* expression and indicate that *cspC* is no longer required to activate PhoP if the *ugtL* coding region is not preceded by the *ugtL* leader region.

The *ugtL* transcript harbors an unusually long leader (i.e. 182 and 171 nt long depending on the transcription start site) (Figure 4A), raising the possibility that it may be targeted by the RNA chaperone CspC (45). In agreement with this notion, deletion of the *cspC* gene no longer decreased PhoP-P amounts (Figure 4B) or fluorescence from p_{phoP}-*gfp* (Figure 4C) in a chromosomal mutant lacking 158 nt of the *ugtL* leader sequence (i.e. from –170 to –13 relative to the *ugtL* start codon) (designated 'leaderless' in Figure 4A). Moreover, mutants lacking most of the *ugtL* leader region displayed higher PhoP-P amounts (designated 'leaderless' in Figure 4B) and p_{phoP}-*gfp* expression (designated 'leaderless' in Figure 4C) than isogenic strains with the full-length *ugtL* leader. These results indicate that CspC activates PhoP via the *ugtL* leader and that sequences within the *ugtL* leader hinder UgtL expression.

Small open reading frames (ORFs) in leader mRNAs often exert regulatory functions on the associated coding regions (48,49). Although the *ugtL* leader harbors a small ORF designated *STM14_1939* (Figure 4A), deletion of the *cspC* gene decreased PhoP-P amounts to the same extent in a strain in which the putative start codon of *STM14_1939* (ATG) was replaced by a stop codon (TAA) in the *S.* Typhimurium chromosome (Figure 4B). This result argues that CspC furthers PhoP-P abundance independently of *STM14_1939* translation.

CspC increases UgtL protein amounts independently of the promoter that transcribes the *ugtL* gene

To further investigate how CspC increases UgtL amounts, we engineered a set of isogenic strains in which the chromosomal PhoP-dependent *ugtL* promoter was replaced by the heterologous $p_{lac1\text{-}6}$ promoter (50), which is constitutive and PhoP independent (51), so that transcription started 182, 171 or 12 nt upstream of the *ugtL* start codon (Figure 4D). We determined that the *cspC*-expressing plasmid increased UgtL abundance in the two strains harboring the full-length (i.e., 182 and 171 nt long) *ugtL* leader (Figure 4E), whereas the plasmid vector control had no effect (Figure 4E). By contrast, the *cspC*-expressing plasmid behaved like the vector control in the strain lacking most of the *ugtL*

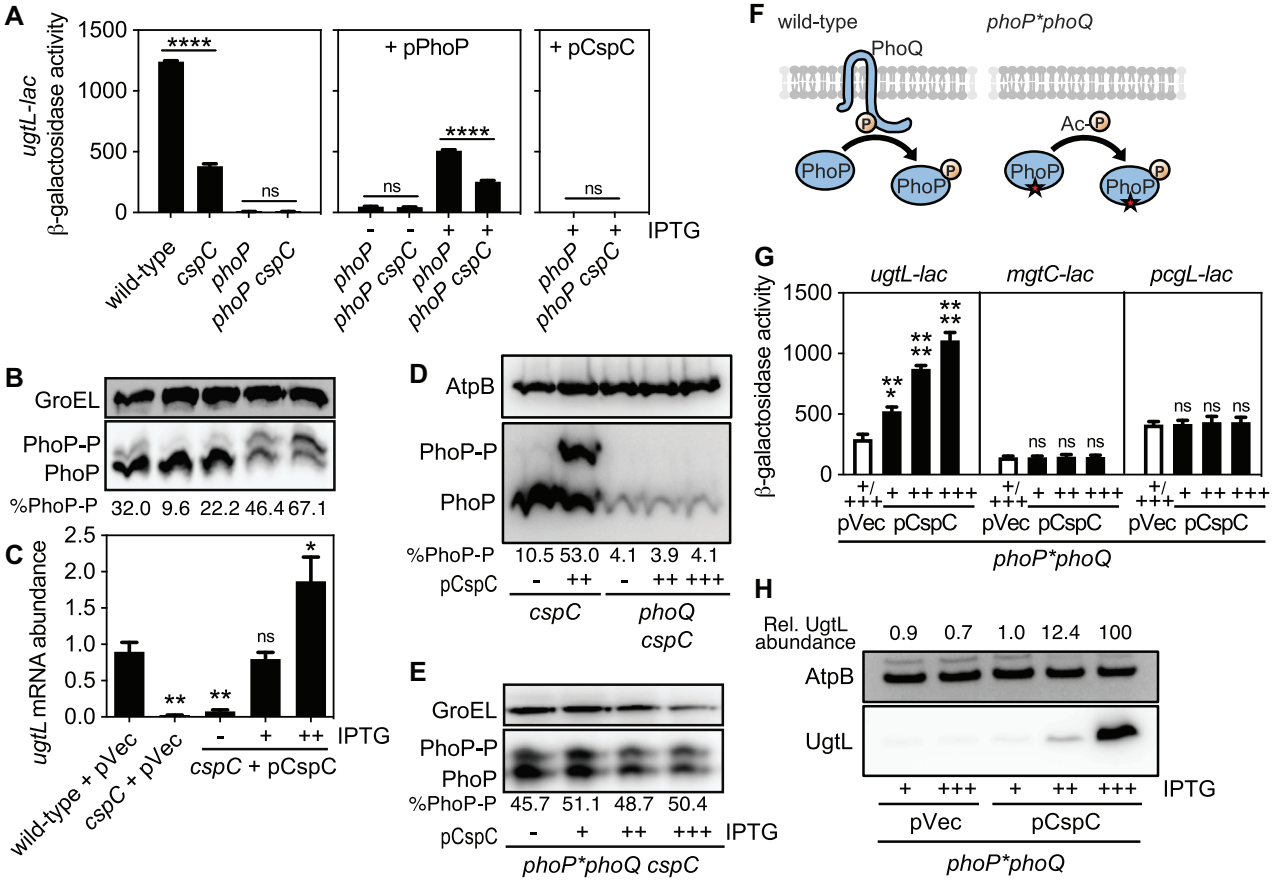

Figure 3. CspC activates PhoP in a PhoQ-dependent manner but increases UgtL protein abundance independently of PhoQ. (**A**) β-Galactosidase activity produced from a chromosomal *ugtL-lacZ* fusion in isogenic wild-type (EG11250), *cspC* (JC619), *phoP* (EG11251), and *phoP cspC* (JC669) *S.* Typhimurium strains with a plasmid expressing the *phoP* (pPhoP) or *cspC* (pCspC) genes grown to mid-log phase in N-minimal media with 1 mM of Mg^{2+} at pH 4.9 supplemented with 0.2 mM IPTG. The mean and SD from four independent experiments are shown ($n = 4$). Two-tailed *t*-test with $cspC^{+}$ versus $cspC^{-}$; ns, not significant; $^{****}P < 0.0001$. (B, D, E) Phos-tag Western blot analysis of crude extracts prepared from (**B**) isogenic wild-type (14028s) and *cspC* (JC577), (**D**) *cspC* (JC577), *cspC phoQ* (JC737) and (**E**) *phoP*phoQ cspC* (JC1210) *S.* Typhimurium strains with an empty vector (pVec) or pCspC grown to mid-log phase in N-minimal media with 1 mM of Mg^{2+} at pH 4.9 supplemented with 0.1 (+), 0.2 (++) or 0.5 (+++) mM IPTG using antibodies recognizing PhoP or the loading control GroEL or AtpB. Representatives of at least three independent experiments are shown. Numbers under the blots indicate % phosphorylated PhoP (PhoP-P). (**C**) mRNA abundance of the *ugtL* gene produced by the same strains used in (B). The mean and SD from four independent experiments are shown ($n = 4$). One-way ANOVA with Brown-Forsythe and Welch tests (wild-type versus others); ns, not significant; $^{*}P < 0.05$, $^{**}P < 0.01$. (**F**) Schematics of PhoP phosphorylation in wild-type (left) and *phoP*phoQ* (right) strains. PhoP phosphorylates from acetyl phosphate (Ac-P) in the absence of PhoQ in a *phoP*phoQ* strain. (**G**) β-Galactosidase activity produced from a chromosomal fusion of *ugtL-lac*, *mgtC-lac* and *pcgL-lac* harboring *phoP*phoQ S.* Typhimurium strains (JC667, EG10874 and EG10877, respectively) with pVec or pCspC grown to mid-log phase in N-minimal media with 1 mM of Mg^{2+} at pH 4.9 supplemented with 0.1 (+), 0.2 (++) or 0.5 (+++) mM IPTG. Strains with pVec showed similar expression in both 0.1 and 0.5 mM IPTG, thus merged in the same column (+/+++). The mean and SD from four independent experiments are shown ($n = 4$). One-way ANOVA with Brown-Forsythe and Welch tests (pVec versus others); ns, not significant; $^{***}P < 0.001$, $^{****}P < 0.0001$. (**H**) Western blot analysis of crude extracts prepared from *ugtL-FLAG phoP*phoQ S.* Typhimurium (JC1289) with pVec or pCspC grown to mid-log phase in N-minimal media with 1 mM of Mg^{2+} at pH 4.9 supplemented with 0.1 (+), 0.2 (++) or 0.5 (+++) mM IPTG using antibodies recognizing FLAG or the loading control AtpB. A representative of at least three independent experiments is shown. Numbers above the blot indicate relative amounts of UgtL protein normalized to the sample with 0.5 mM IPTG.

leader region (Figure 4D and 4E). These results indicate that CspC increases UgtL amounts via the *ugtL* leader and independently of the promoter driving *ugtL* transcription. How, then, does CspC further UgtL amounts by targeting the *ugtL* leader mRNA?

CspC stimulates *ugtL* translation by liberating *ugtL*'s ribosome binding site

We determined that the purified CspC protein alters the secondary structure of the *ugtL* leader mRNA. That is, treatment of the *ugtL* leader mRNA with RNase T1, which cleaves unpaired guanosine residues, revealed that CspC alters cleavage at multiple locations (Figure 5A). Taken together with computational analysis of the secondary structures potentially adopted by the *ugtL* leader mRNA, the T1 cleavage data indicate that regions –7 to –13 and + 3 to + 47 nt (relative to the *ugtL* start codon) are exposed in the presence of CspC, whereas regions –36 to –41 and –70 to –73 nt are protected (Figure 5A). Regions –7 to –13 and + 3 to + 47 nt can form a stem-loop that sequesters the ribosome binding site (Shine-Dalgarno sequence) of *ugtL* (Figure 5B), which exhibits perfect complementarity to the 3′ end of the 16S rRNA and is located at an

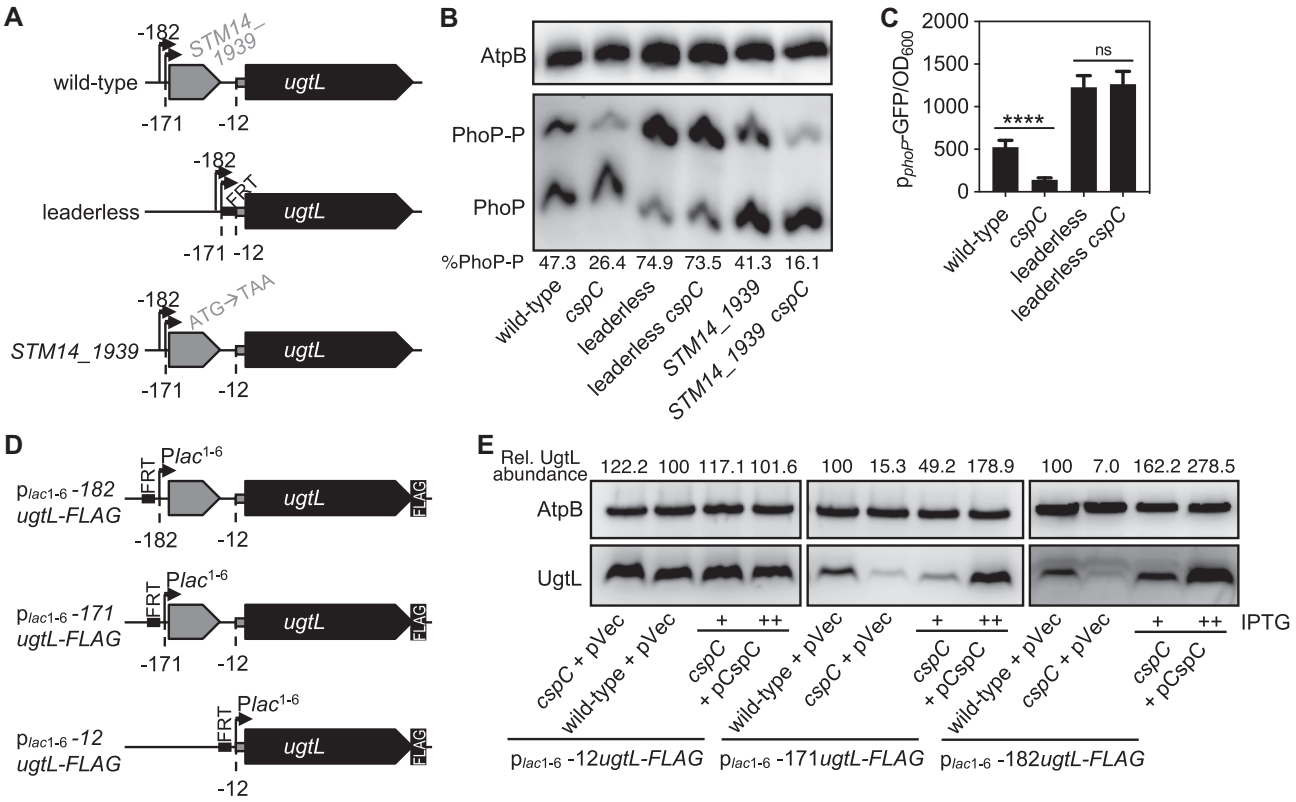

Figure 4. CspC enhances PhoP phosphorylation and UgtL abundance via the *ugtL* leader region. (**A** and **D**) Schematics of wild-type and variant *S.* Typhimurium *ugtL* promoters and leaders with the coding region (*ugtL* or *ugtL-FLAG*). Numbers indicate distance from the *ugtL* start codon. (**B**) Phos-tag Western blot analysis of crude extracts prepared from isogenic wild-type (14028s), *cspC* (JC577), leaderless (JC1237), leaderless *cspC* (JC1239), *STM14_1939* (HS1119) and *STM14_1939 cspC* (JC1267) *S.* Typhimurium strains grown to mid-log phase in N-minimal media with 1 mM of Mg^{2+} at pH 4.9 (acidic pH) using antibodies recognizing PhoP or the loading control AtpB. A representative of at least three independent experiments is shown. Numbers under the blots indicate % phosphorylated PhoP (PhoP-P). (**C**) Fluorescence from a P*phoP-gfp* transcriptional fusion displayed by isogenic wild-type (14028s), *cspC* (JC577), leaderless (JC1237), and leaderless *cspC* (JC1239) *S.* Typhimurium strains grown to mid-log phase in N-minimal media with 1 mM of Mg^{2+} at pH 4.9 (acidic pH). The mean and SD from four independent experiments are shown ($n = 4$). Two-tailed *t*-test with each mutant vs. wild-type; ns, not significant; ****$P < 0.0001$. (**E**) Western blot analysis of crude extracts prepared from isogenic *S.* Typhimurium strains with $p_{lac1\text{-}6}$ –12 *ugtL-FLAG* (JC1362), $p_{lac1\text{-}6}$ –12 *ugtL-FLAG cspC* (JC1363), $p_{lac1\text{-}6}$ –171 *ugtL-FLAG* (JC1364), $p_{lac1\text{-}6}$ –171 *ugtL-FLAG cspC* (JC1365), $p_{lac1\text{-}6}$ –182 *ugtL-FLAG* (JC1344), or $p_{lac1\text{-}6}$ –182 *ugtL-FLAG cspC* (JC1346) with an empty vector (pVec) or pCspC grown to mid-log phase in N-minimal media with 1 mM of Mg^{2+} at pH 4.9 using antibodies recognizing FLAG or the loading control AtpB. When indicated, IPTG were added at concentrations of 0.1 (+) or 0.2 (++) mM. Representatives of at least three independent experiments are shown. Numbers above the blot indicate relative amounts of UgtL protein normalized to isogenic wild-type.

optimal distance from the *ugtL* start codon. This analysis suggested that CspC enhances UgtL amounts by disrupting an mRNA secondary structure that hinders *ugtL* translation.

The purified wild-type CspC protein stimulated *ugtL* translation in an *in vitro* transcription/translation system with a template that includes the full-length *ugtL* leader and coding regions much more than the negative control (Figure 5D, left eight lanes), corresponding to the purified $CspC^{H32R}$ protein, a CspC variant defective in nucleotide-binding (Supplementary Figure S4A) (52). By contrast, a DNA template lacking most of the *ugtL* leader region produced similar UgtL amounts with CspC or $CspC^{H32R}$ (Supplementary Figure S4B). The latter *ugtL* template produced more UgtL protein than the one containing the full *ugtL* leader (Supplementary Figure S4B). This biochemical result, which is in agreement with our *in vivo* findings (Figure 4E), likely reflects that *ugtL*'s ribosome binding site is no longer sequestered by upstream sequences in the mutant lacking most of the *ugtL* leader region.

If CspC promotes *ugtL* translation by disrupting a secondary structure that sequesters *ugtL*'s ribosome binding site (Figure 5B), mutations in the *ugtL* leader region that hinder sequestration of the ribosome binding site should render *ugtL* translation CspC independent. We determined that similar UgtL amounts were produced from DNA templates with mutations antagonizing sequestration of *ugtL's* ribosome binding site (Figure 5C) upon addition of wild-type CspC or $CspC^{H32R}$ proteins (Figure 5D, right eight lanes). Moreover, these *ugtL* leader mutations render UgtL abundance *cspC* independent *in vivo* (Figure 5E).

The CspC stimulation of *ugtL* translation is necessary for wild-type PhoP-P amounts because the PhoP-P-to-PhoP ratio was higher in the *cspC* mutant harboring the wild-type *cspC*-expressing plasmid than in the wild-type strain harboring the vector control (Supplementary Figure S4C).

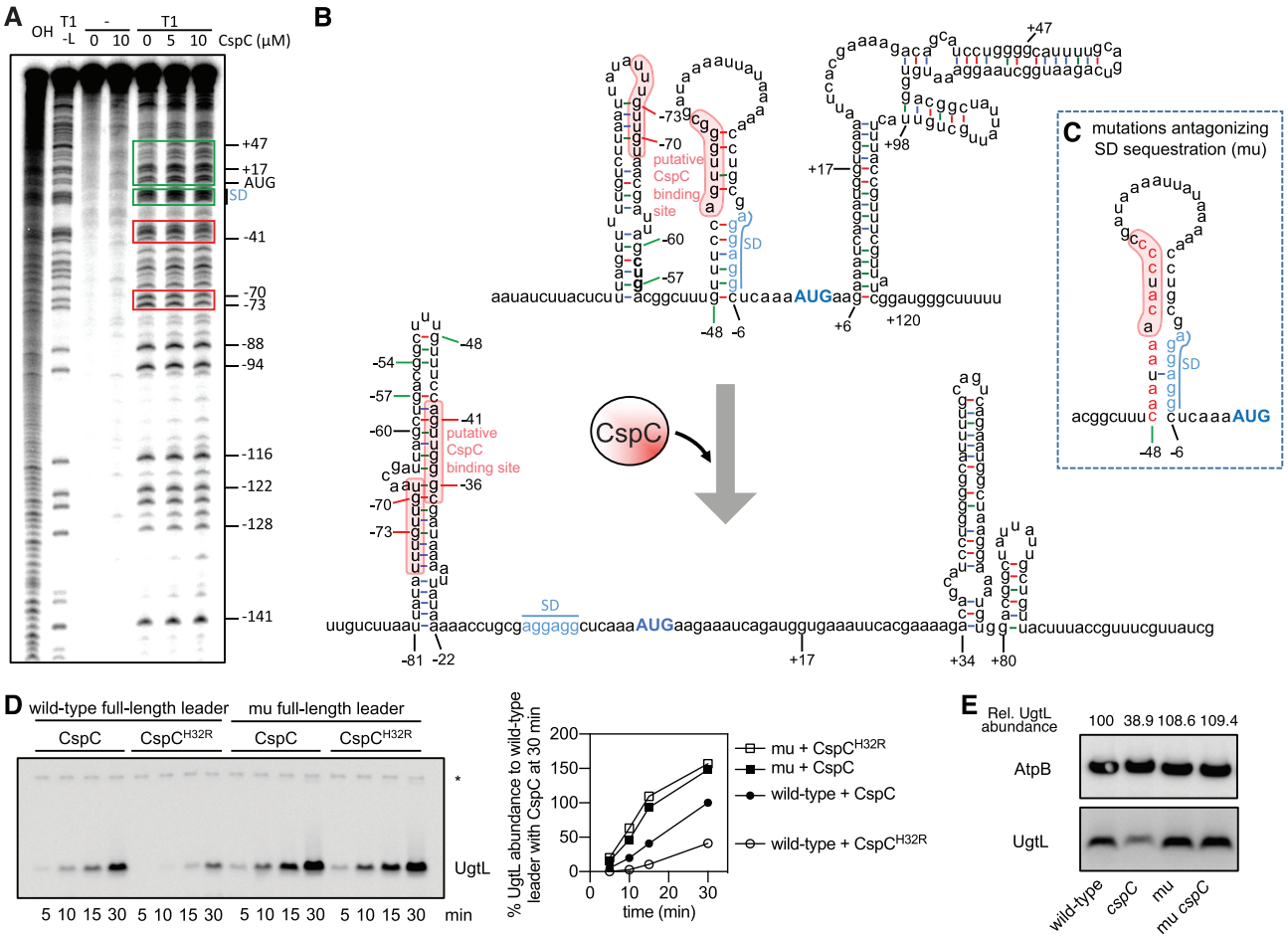

Figure 5. CspC promotes translation of the *ugtL* mRNA by binding to the *ugtL* leader. (**A**) RNA probing of CspC binding to the *ugtL* mRNA. ^{32}P-labeld *in vitro* synthesized *ugtL* mRNA (nucleotides –171 to +264 relative to *ugtL* AUG start codon; 4 nM) was incubated with purified CspC protein (0, 5 or 10 μM) before addition of RNase T1. Boxed bands indicate nucleotides that are protected (red) or deprotected (green) upon addition of increasing CspC concentrations. –, non-reacted controls; OH, alkaline ladder; T1-L, RNase T1 ladder (guanine residues). A representative of two independent experiments is shown. (**B**) Predicted alternative structures adopted by the *ugtL* 5′ leader region. In the absence of CspC, a stem-loop structure is predicted to sequester *ugtL*'s Shine-Dalgarno sequence (SD) (top panel). CspC binding promotes the formation of an alternative stem-loop structure liberating the SD sequence, thus allowing *ugtL* mRNA to be translated (bottom panel). Secondary structures of *ugtL* 5′-leader and coding regions were determined based on mfold predictions and the RNA probing data presented in (A). (**C**) Schematic of mutant *ugtL* leader region with nucleotide substitutions that antagonize SD sequestration (in red). (**D**) *In vitro* transcription/translation of the *ugtL-FLAG* gene from DNA fragment containing the wild-type or mutated (mu) full-length leader (182 nt) and coding region with wild-type or variant CspC proteins (mutations in the leader region are shown in (C)). 9 μM DNA template was incubated with 5.6 μM of purified CspC or CspCH^{32R} proteins. Synthesized UgtL-FLAG proteins at each time were analyzed by Western blot using antibodies recognizing FLAG. An asterisk indicates non-specific band. A representative of at least three independent experiments is shown. Relative amounts of UgtL protein are displayed in the right panel. (**E**) Western blot analysis of crude extracts prepared from isogenic *ugtL-FLAG* wild-type (JC1373), *cspC* (JC1729), mu (JC1728), mu *cspC* (JC1738) *S.* Typhimurium strains grown to mid-log phase in N-minimal media with 1 mM of Mg^{2+} at pH 4.9 using antibodies recognizing FLAG or the loading control AtpB. A representative of at least three independent experiments is shown. Numbers above the blot indicate relative amounts of UgtL protein normalized to isogenic wild-type.

Moreover, the *cspC* mutant harboring the plasmid expressing CspC^{H32R} showed a low PhoP-P-to-PhoP ratio, behaving like the *cspC* mutant carrying the plasmid vector (Supplementary Figure S4C).

We considered the additional possibility of CspC protecting the *ugtL* mRNA from degradation because CspC does so with some mRNAs in *E. coli* (9). However, *cspC* inactivation did not alter the stability of the *ugtL* mRNA when transcription was driven by the $p_{lac1\text{-}6}$ promoter and the full *ugtL* leader preceded the *ugtL* coding region (Supplementary Figure S5). (The use of the $p_{lac1\text{-}6}$ promoter allowed us to focus on effects taking place after transcription initiation.) The CspC protein does not appear to alter *ugtL* mRNA abundance in the *in vitro* transcription/translation system because similar UgtL protein amounts were synthesized when either the wild-type CspC protein or the CspC^{H32R} protein were added to reactions containing a DNA template harboring mutations antagonizing sequestration of *ugtL's* ribosome binding site (Figure 5C and 5D). The *ugtL* leader mutations that prevent sequestration of *ugtL's* ribosome binding site render UgtL protein abundance *cspC* independent *in vivo* (Figure 5E). Cumulatively, the results in this section indicate that CspC binding to the *ugtL* leader RNA promotes the formation of a structure that exposes the ribosome binding site, thereby enhancing *ugtL* translation.

CspC-dependent *ugtL* translation promotes expression of PhoP-activated genes inside macrophages

The CspC-dependent activation of the PhoP protein is critical when *S.* Typhimurium is inside macrophages because the mRNA abundances of the PhoP-activated *pmrD*, *ugtL*, and *pagC* genes were lower in the *cspC* mutant than in the isogenic wild-type strain (Figure 6A). This decrease was more prominent at 6 h than at 1 h post-infection (Figure 6A), reflecting that 6 h is a time at which the PhoP/PhoQ system is highly active (28,53,54). This activation is strictly dependent on CspC because the *cspE* mutant exhibited amounts of PhoP-activated mRNAs that were equal to or slightly higher than those produced by the wild-type strain (Figure 6A). In agreement with this notion, the highly reduced UgtL protein amounts displayed by a *phoP*phoQ* strain lacking both *cspC* and *cspE* and the $p_{lac1\text{-}6}$ promoter driving *ugtL* transcription in mildly acidic pH were corrected by a plasmid expressing *cspC* from a heterologous promoter but not by an isogenic plasmid expressing *cspE* (Supplementary Figure S6). Thus, PhoP activation taking place in mildly acidic phagosome is specifically dependent on CspC, not CspE.

An *S.* Typhimurium strain with mutations antagonizing sequestration of *ugtL*'s ribosome binding site (Figure 5C) rescued the *cspC* null mutant, producing amounts of PhoP-activated mRNAs inside macrophages that were equivalent to or slightly higher than those of the wild-type strain (Figure 6A). Taken together, the results in this section indicate that the CspC-dependent stimulation of *ugtL* translation is necessary for transcription of PhoP-activated genes inside macrophages.

CspC promotes *S.* Typhimurium colonization of the murine liver and spleen

It was reported that wild-type and *cspC S.* Typhimurium strains display similar virulence in Balb/C mice (8). This mouse strain lacks functional copies of *Slc11a1,* a gene that confers resistance to infection by multiple intracellular pathogens, including *S.* Typhimurium (55). Although *phoP* or *phoQ* mutants are attenuated in both $Slc11a1^{+/+}$ and $Slc11a1^{-/-}$ mice (12–16,22) (Supplementary Figure S7), mutants lacking certain PhoP-activated genes exhibit virulence defects only in $Slc11a1^{+/+}$ hosts (56,57). Thus, we reexamined *cspC*'s role in *S.* Typhimurium virulence by determining the number of bacteria in the liver and spleen of C3H/HeN mice, which are $Slc11a1^{+/+}$ (55), following intraperitoneal inoculation.

We recovered 140- and 21-fold fewer *cspC* mutant bacteria than wild-type *S.* Typhimurium from the livers and spleens, respectively (Figure 6B). In agreement with this notion, C3H/HeN mice inoculated intraperitoneally displayed slightly longer survival when inoculated with the *cspC* mutant than when infected with the wild-type strain (Supplementary Figure S7). Taken together, these data indicate that the CspC-dependent activation of the PhoP protein furthers *S.* Typhimurium virulence.

DISCUSSION

Organisms can harbor seemingly redundant genes – those causing subtle or undetectable consequences when singly deleted (1,2,8,58). Although the RNA chaperones CspC and CspE of *S.* Typhimurium share 84% amino acid identity, we have now established that CspC, but not CspE, activates the master virulence regulatory system PhoP/PhoQ in mildly acidic pH conditions (Figures 2E, 3B and 4B), such as inside a macrophage phagosome (Figure 6A), and that this activation is necessary for *S.* Typhimurium accumulation in host tissues (Figure 6B). CspC increases UgtL protein amounts (Figure 4E and 5E) by binding the leader region of the *ugtL* transcript (Figure 5A), which disrupts a secondary structure that sequesters *ugtL*'s ribosome binding site and enables *ugtL* translation (Figure 5 and Supplementary Figure S4A). CspC's action does not alter the stability of the *ugtL* transcript (Supplementary Figure S5), like the action of some small RNAs that impact translation without perturbing the stability of the corresponding mRNAs (71,72). The resulting UgtL protein (Figure 5E) stimulates PhoQ autophosphorylation (22) which, by increasing the PhoP-P-to-PhoP ratio (Figure 4B), promotes transcription of PhoP-activated genes (Figures 2DE, 6A and Supplementary Figure S2C). Our results explain why CspC is no longer required for transcription of PhoP-activated genes when PhoP is phosphorylated independently of PhoQ (Figure 3E) or when the portion of the *ugtL* leader that inhibits *ugtL* translation is absent (Figure 4AB), and why a *cspC cspE* mutant exhibits decreased expression of PhoP-activated genes (8).

CspC is necessary to activate the PhoP/PhoQ system in mildly acidic pH (Figure 2E) and inside macrophages (Figure 6A). By contrast, CspC is dispensable for PhoP/PhoQ activation in low Mg^{2+} (Figure 2E), a condition that activates PhoP/PhoQ as much as mildly acidic pH (16). Unlike the *cspC* mutant, the *cspE* mutant exhibited wild-type PhoP activation inside macrophages (Figure 6A). The physiological role of CspE remains enigmatic because one group implicated CspE in resistance to bile salts (59) and flagella-dependent motility (60), whereas another group reported these phenotypes only in the *cspC cspE* double mutant (8). The condition-dependent activity of the CspC and CspE proteins is reminiscent of the distinct roles that β-globins play during human development. That is, the three genes specifying human β-globins are expressed at different developmental stages: one in embryos, another one in fetuses, and the third one in neonates onwards (61). Critically, these hemoglobins show varying affinities for oxygen (62), indicating that their shared functions are optimized to the corresponding developmental stages.

Salmonella virulence requires activation of the PhoP/PhoQ system with the correct timing and extent because constitutive activation of the PhoP protein (63) or a failure to generate a surge of PhoP-P (64) attenuates *S.* Typhimurium virulence as much as inactivating the *phoP* gene. We have now established that this is due, in part, to the inhibition of *ugtL* translation by the *ugtL* leader, which enables *S.* Typhimurium to delay PhoP activation inside macrophages until the CspC protein exerts its effect on

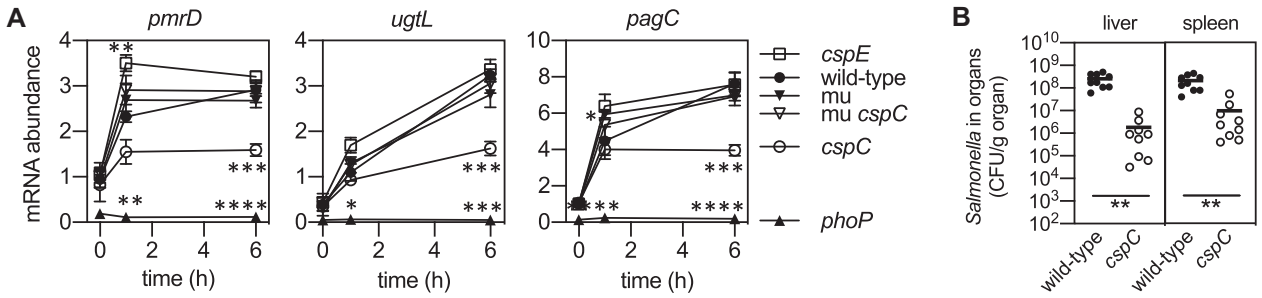

Figure 6. CspC is required for PhoP activation inside macrophages and *S.* Typhimurium colonization of murine liver and spleen. (**A**) mRNA abundance of the PhoP-activated *pmrD*, *ugtL*, and *pagC* genes in isogenic wild-type (14028s), *cspC* (JC577), *phoP* (MS7953s), *ugtL* leader mutation (mu; JC1718), *ugtL* leader mutation (mu *cspC*; JC1742), and *cspE* (JC640) *S.* Typhimurium strains harvested from the macrophage-like cell line J774A.1 at the indicated times. The mean and SD from three to five independent experiments are shown ($n = 3$–5). One-way ANOVA with Brown-Forsythe and Welch tests (wild-type versus others) were applied at each time point; no *, not significant, $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, $****P < 0.0001$. (**B**) Number of bacteria in the liver and spleen of C3H/HeN mice five days after intraperitoneal inoculation with $\sim 10^4$ colony forming units of isogenic wild-type (14028s) or *cspC* (JC577) *S.* Typhimurium ($n = 9$, pooled from two independent experiments). Circles indicate values from individual mouse and bars indicate the mean of each group. Two-tailed *t*-test with wild-type and the *cspC* mutant; $**P < 0.01$.

ugtL translation. Thus, PhoP-activated genes are expressed inside macrophages at earlier times and higher levels in an engineered strain with *ugtL* leader mutations that render *ugtL* translation *cspC* independent than in wild-type *S.* Typhimurium (Figure 6A).

The high degree of shared amino acid identity and the similar biochemical properties among Csps render them interchangeable for rescuing the cold-sensitivity phenotype of a *csp* quadruple mutant when overexpressed (65). However, Csps bind distinct, albeit partially overlapping, RNA ligands (8). That CspC binds to the *ugtL* mRNA better than CspE *in vivo* (8) provides a cogent explanation for PhoP activation inside macrophages being mediated by CspC, not CspE (Figure 6A).

CspC likely activates PhoP in pathogenic *Salmonella* serovars in addition to *S.* Typhimurium because the CspC amino acid sequence is 100% identical in all examined *Salmonella* isolates, and also because the *ugtL* leader regions bound by the CspC protein are highly conserved in *S. enterica* serovars that infect warm-blooded hosts but divergent in those associated with cold-blooded animals (Supplementary Figure S8).

That the virulence gene *ugtL* requires an RNA chaperone—CspC—for translation is in contrast to translation of the *prfA* gene of *Listeria monocytogenes* (66) and the *lcrF* gene from *Yersinia pestis* (67), which does not appear to require an RNA chaperone as an increase in temperature is sufficient to melt the corresponding RNA structures, thereby liberating sequestered ribosome binding sites. CspA appears to enhance its own translation under cold-shock conditions (68), but it remains unclear whether CspA does so directly because the effect was observed only with crudely purified ribosomes from cold-shock conditions relative to those purified from control conditions (68). For instance, the translation initiation factors 1 (IF1) and IF3 are found in ribosome extracts from cold-shock conditions and may increase translation of the *cspA* mRNA (68) because IF1 complements the growth of the *B. subtilis csp* mutant (69). Unlike the *Salmonella* CspC (Figure 5), Csps from *Thermotoga maritima*, *Bacillus subtilis*, and *B. caldolyticus* actually inhibit translation *in vitro* (70).

Finally, our paper shows that an RNA chaperone thought to be redundant plays a critical role in controlling virulence gene expression inside host cells. Thus, organisms retain highly similar proteins because these play singular and critical physiological roles in specific circumstances encountered during their lifestyles.

DATA AVAILABILITY

The data that support the findings of this study are available from the corresponding author on reasonable request.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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REFERENCES

1. Stephens,S.G. (1951) Possible significances of duplication in evolution. *Adv. Genet.*, **4**, 247–265.

2. Laruson,A.J., Yeaman,S. and Lotterhos,K.E. (2020) The importance of genetic redundancy in evolution. *Trends Ecol. Evol.*, **35**, 809–822.
3. Conant,G.C. and Wolfe,K.H. (2008) Turning a hobby into a job: how duplicated genes find new functions. *Nat. Rev. Genet.*, **9**, 938–950.
4. Heinemann,U. and Roske,Y. (2021) Cold-shock domains-abundance, structure, properties, and nucleic-acid binding. *Cancers (Basel)*, **13**, 190.
5. Goldstein,J., Pollitt,N.S. and Inouye,M. (1990) Major cold shock protein of *Escherichia coli*. *Proc. Natl. Acad. Sci. U.S.A.*, **87**, 283–287.
6. Al-Fageeh,M.B. and Smales,C.M. (2006) Control and regulation of the cellular responses to cold shock: the responses in yeast and mammalian systems. *Biochem. J.*, **397**, 247–259.
7. Kroger,C., Colgan,A., Srikumar,S., Handler,K., Sivasankaran,S.K., Hammarlof,D.L., Canals,R., Grissom,J.E., Conway,T., Hokamp,K. *et al.* (2013) An infection-relevant transcriptomic compendium for *Salmonella enterica* Serovar Typhimurium. *Cell Host Microbe*, **14**, 683–695.
8. Michaux,C., Holmqvist,E., Vasicek,E., Sharan,M., Barquist,L., Westermann,A.J., Gunn,J.S. and Vogel,J. (2017) RNA target profiles direct the discovery of virulence functions for the cold-shock proteins CspC and CspE. *Proc. Natl. Acad. Sci. U.S.A.*, **114**, 6824–6829.
9. Phadtare,S. and Inouye,M. (2001) Role of CspC and CspE in regulation of expression of RpoS and UspA, the stress response proteins in *Escherichia coli*. *J. Bacteriol.*, **183**, 1205–1214.
10. Shenhar,Y., Biran,D. and Ron,E.Z. (2012) Resistance to environmental stress requires the RNA chaperones CspC and CspE. *Environ. Microbiol. Rep.*, **4**, 532–539.
11. Stock,A.M., Robinson,V.L. and Goudreau,P.N. (2000) Two-component signal transduction. *Annu. Rev. Biochem.*, **69**, 183–215.
12. Fields,P.I., Groisman,E.A. and Heffron,F. (1989) A *Salmonella* locus that controls resistance to microbicidal proteins from phagocytic cells. *Science*, **243**, 1059–1062.
13. Groisman,E.A., Chiao,E., Lipps,C.J. and Heffron,F. (1989) *Salmonella* Typhimurium *phoP* virulence gene is a transcriptional regulator. *Proc. Natl. Acad. Sci. U.S.A.*, **86**, 7077–7081.
14. Miller,S.I., Kukral,A.M. and Mekalanos,J.J. (1989) A two-component regulatory system (*phoP phoQ*) controls *Salmonella* Typhimurium virulence. *Proc. Natl. Acad. Sci. U.S.A.*, **86**, 5054–5058.
15. Galan,J.E. and Curtiss,R. 3rd. (1989) Virulence and vaccine potential of *phoP* mutants of *Salmonella* Typhimurium. *Microb. Pathog.*, **6**, 433–443.
16. Choi,J. and Groisman,E.A. (2016) Acidic pH sensing in the bacterial cytoplasm is required for *Salmonella* virulence. *Mol. Microbiol.*, **101**, 1024–1038.
17. Prost,L.R., Daley,M.E., Le Sage,V., Bader,M.W., Le Moual,H., Klevit,R.E. and Miller,S.I. (2007) Activation of the bacterial sensor kinase PhoQ by acidic pH. *Mol. Cell*, **26**, 165–174.
18. Garcia Vescovi,E., Soncini,F.C. and Groisman,E.A. (1996) Mg^{2+} as an extracellular signal: environmental regulation of *Salmonella* virulence. *Cell*, **84**, 165–174.
19. Bader,M.W., Sanowar,S., Daley,M.E., Schneider,A.R., Cho,U., Xu,W., Klevit,R.E., Le Moual,H. and Miller,S.I. (2005) Recognition of antimicrobial peptides by a bacterial sensor kinase. *Cell*, **122**, 461–472.
20. Yuan,J., Jin,F., Glatter,T. and Sourjik,V. (2017) Osmosensing by the bacterial PhoQ/PhoP two-component system. *Proc. Natl. Acad. Sci. U.S.A.*, **114**, E10792–E10798.
21. Viarengo,G., Sciara,M.I., Salazar,M.O., Kieffer,P.M., Furlan,R.L. and Garcia Vescovi,E. (2013) Unsaturated long chain free fatty acids are input signals of the *Salmonella enterica* PhoP/PhoQ regulatory system. *J. Biol. Chem.*, **288**, 22346–22358.
22. Choi,J. and Groisman,E.A. (2017) Activation of master virulence regulator PhoP in acidic pH requires the *Salmonella*-specific protein UgtL. *Sci. Signal*, **10**, eaan6284.
23. Rathman,M., Sjaastad,M.D. and Falkow,S. (1996) Acidification of phagosomes containing *Salmonella* Typhimurium in murine macrophages. *Infect. Immun.*, **64**, 2765–2773.
24. Puiac,S., Negrea,A., Richter-Dahlfors,A., Plant,L. and Rhen,M. (2009) Omeprazole antagonizes virulence and inflammation in *Salmonella enterica*-infected RAW264.7 cells. *Antimicrob. Agents Chemother.*, **53**, 2402–2409.
25. Alpuche Aranda,C.M., Swanson,J.A., Loomis,W.P. and Miller,S.I. (1992) *Salmonella* Typhimurium activates virulence gene transcription within acidified macrophage phagosomes. *Proc. Natl. Acad. Sci. U.S.A.*, **89**, 10079–10083.
26. Martin-Orozco,N., Touret,N., Zaharik,M.L., Park,E., Kopelman,R., Miller,S., Finlay,B.B., Gros,P. and Grinstein,S. (2006) Visualization of vacuolar acidification-induced transcription of genes of pathogens inside macrophages. *Mol. Biol. Cell*, **17**, 498–510.
27. Shin,D. and Groisman,E.A. (2005) Signal-dependent binding of the response regulators PhoP and PmrA to their target promoters *in vivo*. *J. Biol. Chem.*, **280**, 4089–4094.
28. Choi,J. and Groisman,E.A. (2020) Horizontally acquired regulatory gene activates ancestral regulatory system to promote *Salmonella* virulence. *Nucleic Acids Res.*, **48**, 10832–10847.
29. Soncini,F.C., Vescovi,E.G. and Groisman,E.A. (1995) Transcriptional autoregulation of the *Salmonella* Typhimurium *phoPQ* operon. *J. Bacteriol.*, **177**, 4364–4371.
30. Perez,J.C. and Groisman,E.A. (2009) Transcription factor function and promoter architecture govern the evolution of bacterial regulons. *Proc. Natl. Acad. Sci. U.S.A.*, **106**, 4319–4324.
31. Fields,P.I., Swanson,R.V., Haidaris,C.G. and Heffron,F. (1986) Mutants of *Salmonella* Typhimurium that cannot survive within the macrophage are avirulent. *Proc. Natl. Acad. Sci. U.S.A.*, **83**, 5189–5193.
32. Davis,R.W., D.B. and Roth,J.R. (1980) In: *Advanced Bacterial Genetics*. Cold Spring Harbor Lab Press, NY.
33. Snavely,M.D., Miller,C.G. and Maguire,M.E. (1991) The *mgtB* Mg^{2+} transport locus of *Salmonella* Typhimurium encodes a P-type ATPase. *J. Biol. Chem.*, **266**, 815–823.
34. Hanahan,D. (1983) Studies on transformation of *Escherichia coli* with plasmids. *J. Mol. Biol.*, **166**, 557–580.
35. Datsenko,K.A. and Wanner,B.L. (2000) One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. *Proc. Natl. Acad. Sci. U.S.A.*, **97**, 6640–6645.
36. Maloy,S.R. and Nunn,W.D. (1981) Selection for loss of tetracycline resistance by *Escherichia coli*. *J. Bacteriol.*, **145**, 1110–1111.
37. Khetrapal,V., Mehershahi,K., Rafee,S., Chen,S., Lim,C.L. and Chen,S.L. (2015) A set of powerful negative selection systems for unmodified Enterobacteriaceae. *Nucleic Acids Res.*, **43**, e83.
38. Soncini,F.C., Garcia Vescovi,E., Solomon,F. and Groisman,E.A. (1996) Molecular basis of the magnesium deprivation response in *Salmonella* Typhimurium: identification of PhoP-regulated genes. *J. Bacteriol.*, **178**, 5092–5099.
39. Metcalf,W.W., Steed,P.M. and Wanner,B.L. (1990) Identification of phosphate starvation-inducible genes in *Escherichia coli* K-12 by DNA sequence analysis of *psi*::*lacZ*(Mu d1) transcriptional fusions. *J. Bacteriol.*, **172**, 3191–3200.
40. Hilbert,F., Garcia-del Portillo,F. and Groisman,E.A. (1999) A periplasmic D-alanyl-D-alanine dipeptidase in the gram-negative bacterium *Salmonella enterica*. *J. Bacteriol.*, **181**, 2158–2165.
41. Aksoy,S., Squires,C.L. and Squires,C. (1984) Translational coupling of the *trpB* and *trpA* genes in the *Escherichia coli* tryptophan operon. *J. Bacteriol.*, **157**, 363–367.
42. Barbieri,C.M. and Stock,A.M. (2008) Universally applicable methods for monitoring response regulator aspartate phosphorylation both *in vitro* and *in vivo* using Phos-tag-based reagents. *Anal. Biochem.*, **376**, 73–82.
43. Desnoyers,G., Morissette,A., Prevost,K. and Masse,E. (2009) Small RNA-induced differential degradation of the polycistronic mRNA iscRSUA. *EMBO J.*, **28**, 1551–1561.
44. Choi,J. and Groisman,E.A. (2013) The lipopolysaccharide modification regulator PmrA limits *Salmonella* virulence by repressing the type three-secretion system Spi/Ssa. *Proc. Natl. Acad. Sci. U.S.A.*, **110**, 9499–9504.
45. Bae,W., Jones,P.G. and Inouye,M. (1997) CspA, the major cold shock protein of *Escherichia coli*, negatively regulates its own gene expression. *J. Bacteriol.*, **179**, 7081–7088.
46. Chamnongpol,S. and Groisman,E.A. (2000) Acetyl phosphate-dependent activation of a mutant PhoP response regulator that functions independently of its cognate sensor kinase. *J. Mol. Biol.*, **300**, 291–305.
47. Sanowar,S., Martel,A. and Moual,H.L. (2003) Mutational analysis of the residue at position 48 in the *Salmonella enterica* Serovar Typhimurium PhoQ sensor kinase. *J. Bacteriol.*, **185**, 1935–1941.
48. Henkin,T.M. and Yanofsky,C. (2002) Regulation by transcription attenuation in bacteria: how RNA provides instructions for

transcription termination/antitermination decisions. *Bioessays*, **24**, 700–707.
49. Orr,M.W., Mao,Y., Storz,G. and Qian,S.B. (2020) Alternative ORFs and small ORFs: shedding light on the dark proteome. *Nucleic. Acids. Res.*, **48**, 1029–1042.
50. Liu,M., Tolstorukov,M., Zhurkin,V., Garges,S. and Adhya,S. (2004) A mutant spacer sequence between -35 and -10 elements makes the P*lac* promoter hyperactive and cAMP receptor protein-independent. *Proc. Natl. Acad. Sci. U.S.A.*, **101**, 6911–6916.
51. Cromie,M.J. and Groisman,E.A. (2010) Promoter and riboswitch control of the Mg^{2+} transporter MgtA from *Salmonella enterica*. *J. Bacteriol.*, **192**, 604–607.
52. Phadtare,S., Inouye,M. and Severinov,K. (2004) The mechanism of nucleic acid melting by a CspA family protein. *J. Mol. Biol.*, **337**, 147–155.
53. Choi,J. and Groisman,E.A. (2020) *Salmonella* expresses foreign genes during infection by degrading their silencer. *Proc. Natl. Acad. Sci. U.S.A.*, **117**, 8074–8082.
54. Westermann,A.J., Forstner,K.U., Amman,F., Barquist,L., Chao,Y., Schulte,L.N., Muller,L., Reinhardt,R., Stadler,P.F. and Vogel,J. (2016) Dual RNA-seq unveils noncoding RNA functions in host-pathogen interactions. *Nature*, **529**, 496–501.
55. Plant,J. and Glynn,A.A. (1974) Natural resistance to *Salmonella* infection, delayed hypersensitivity and *Ir* genes in different strains of mice. *Nature*, **248**, 345–347.
56. Cunrath,O. and Bumann,D. (2019) Host resistance factor SLC11A1 restricts *Salmonella* growth through magnesium deprivation. *Science*, **366**, 995–999.
57. Alix,E., Miki,T., Felix,C., Rang,C., Figueroa-Bossi,N., Demettre,E. and Blanc-Potard,A.B. (2008) Interplay between MgtC and PagC in *Salmonella enterica* serovar Typhimurium. *Microb. Pathog.*, **45**, 236–240.
58. Kafri,R., Bar-Even,A. and Pilpel,Y. (2005) Transcription control reprogramming in genetic backup circuits. *Nat. Genet.*, **37**, 295–299.
59. Ray,S., Da Costa,R., Das,M. and Nandi,D. (2019) Interplay of cold shock protein E with an uncharacterized protein, YciF, lowers porin expression and enhances bile resistance in *Salmonella* Typhimurium. *J. Biol. Chem.*, **294**, 9084–9099.
60. Ray,S., Da Costa,R., Thakur,S. and Nandi,D. (2020) *Salmonella* Typhimurium encoded cold shock protein E is essential for motility and biofilm formation. *Microbiology (Reading)*, **166**, 460–473.
61. Cao,A. and Moi,P. (2002) Regulation of the globin genes. *Pediatr. Res.*, **51**, 415–421.
62. Manning,L.R., Russell,J.E., Padovan,J.C., Chait,B.T., Popowicz,A., Manning,R.S. and Manning,J.M. (2007) Human embryonic, fetal, and adult hemoglobins have different subunit interface strengths. Correlation with lifespan in the red cell. *Protein Sci.*, **16**, 1641–1658.
63. Miller,S.I. and Mekalanos,J.J. (1990) Constitutive expression of the *phoP* regulon attenuates *Salmonella* virulence and survival within macrophages. *J. Bacteriol.*, **172**, 2485–2490.
64. Shin,D., Lee,E.J., Huang,H. and Groisman,E.A. (2006) A positive feedback loop promotes transcription surge that jump-starts *Salmonella* virulence circuit. *Science*, **314**, 1607–1609.
65. Xia,B., Ke,H. and Inouye,M. (2001) Acquirement of cold sensitivity by quadruple deletion of the *cspA* family and its suppression by PNPase S1 domain in *Escherichia coli*. *Mol. Microbiol.*, **40**, 179–188.
66. Johansson,J., Mandin,P., Renzoni,A., Chiaruttini,C., Springer,M. and Cossart,P. (2002) An RNA thermosensor controls expression of virulence genes in *Listeria monocytogenes*. *Cell*, **110**, 551–561.
67. Hoe,N.P. and Goguen,J.D. (1993) Temperature sensing in *Yersinia pestis*: translation of the LcrF activator protein is thermally regulated. *J. Bacteriol.*, **175**, 7901–7909.
68. Giuliodori,A.M., Brandi,A., Gualerzi,C.O. and Pon,C.L. (2004) Preferential translation of cold-shock mRNAs during cold adaptation. *RNA*, **10**, 265–276.
69. Weber,M.H., Beckering,C.L. and Marahiel,M.A. (2001) Complementation of cold shock proteins by translation initiation factor IF1 in vivo. *J. Bacteriol.*, **183**, 7381–7386.
70. Hofweber,R., Horn,G., Langmann,T., Balbach,J., Kremer,W., Schmitz,G. and Kalbitzer,H.R. (2005) The influence of cold shock proteins on transcription and translation studied in cell-free model systems. *FEBS J.*, **272**, 4691–4702.
71. Altuvia,S., Zhang,A., Argaman,L., Tiwari,A. and Storz,G. (1998) The *Escherichia coli* OxyS regulatory RNA represses *fhlA* translation by blocking ribosome binding. *EMBO J.*, **17**, 6069–6075.
72. Desnoyers,G. and Masse,E. (2012) Noncanonical repression of translation initiation through small RNA recruitment of the RNA chaperone Hfq. *Genes Dev.*, **26**, 726–739.