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Activation of master virulence regulator PhoP in acidic pH requires the *Salmonella*-specific protein UgtL

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Abstract

The ability to respond to changes in pH is critical for all organisms. In *Salmonella enterica*, the sensor PhoQ responds to a mildly acidic pH by phosphorylating, and thereby activating, the virulence regulator PhoP. This PhoP/PhoQ two-component system is conserved in a subset of Gram-negative bacteria. PhoQ has been thought to be sufficient to activate PhoP in mildly acidic pH. However, we found that the *Salmonella*-specific protein UgtL, which was horizontally acquired by *Salmonella* before the divergence of *S. enterica* and *S. bongori*, was also necessary for PhoQ to activate PhoP under mildly acidic pH conditions, but not for PhoQ to activate PhoP in response to low Mg²⁺ or the antimicrobial peptide C18G. UgtL increased the abundance of phosphorylated PhoP by stimulating autophosphorylation of PhoQ, thereby increasing the amount of the phosphodonor for PhoP. Deletion of *ugtL* attenuated *Salmonella* virulence and further reduced PhoP activation in a strain bearing a form of PhoQ that is not responsive to acidic pH. Thus, when *Salmonella* experiences mildly acidic pH, PhoP activation requires PhoQ to detect pH and UgtL to amplify the PhoQ response. Our findings reveal how acquisition of a foreign gene can strengthen signal responsiveness in an ancestral regulatory system.

INTRODUCTION

pH governs critical biological processes including gene expression, energy generation, and enzymatic functions (1). The failure to control or respond to changes in pH results in organelle malfunction, leading to disease (2–4) and cell death (5). Thus, organisms monitor changes in extracytoplasmic or cytoplasmic H⁺, or both, or chemical or physical modifications triggered by acidification (6, 7). For example, the *Escherichia coli* CadC, *Helicobacter pylori* ArsS, and *Salmonella enterica* PmrB proteins detect acidic pH in the periplasm (8–10), whereas the *E. coli* Tsr and Tar proteins and the *S. enterica* EnvZ and PhoQ proteins respond to a decrease in cytoplasmic pH (11–13). Here, we report that full

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activation of the *Salmonella* sensor PhoQ by a mildly acidic pH requires a protein to amplify the response to this signal.

PhoP/PhoQ is a two-component system that controls virulence, Mg²⁺ homeostasis, and resistance to antimicrobial peptides in several species of Gram-negative bacteria (14, 15). In the intracellular pathogen *S. enterica* serovar Typhimurium (16), mildly acidic pH (13, 17), low Mg²⁺ (18), or certain antimicrobial peptides (19) activate the sensor PhoQ, which promotes the phosphorylated state of its cognate response regulator PhoP (PhoP-P). PhoP-P is the active form of the PhoP protein: PhoP-P binding to promoter regions (20) stimulates or represses gene transcription (20, 21), depending on the particular target gene, in vivo. In the absence of inducing conditions, PhoQ operates as a phosphatase that dephosphorylates PhoP-P. PhoQ harbors two transmembrane regions that define a periplasmic domain (18) that is required for sensing Mg²⁺ (18, 22) and the antimicrobial peptide C18G (19), but not for sensing acidic pH (13, 23). The cytosolic domain of PhoQ mediates the response to a mildly acidic pH and is sufficient to do so, albeit not as efficiently as the full-length protein (13). The curtailed response to acidic pH by a mutant bearing only the cytoplasmic domain of PhoQ implies that another region of PhoQ or an additional factor(s), or both, is required for full PhoQ activation by acidic pH.

The PhoP protein controls the expression of ~9% of *Salmonella* genes (24). PhoP-activated genes include several that encode proteins that modify the lipid A portion of lipopolysaccharide (25, 26) or confer resistance to antimicrobial peptides (26–28), or both. XXXXXXXX. Curiously, a strain lacking the horizontally acquired PhoP-activated gene *ugtL* has a lipid A profile resembling that of a *phoP* null mutant (29) and exhibits hypersusceptibility to the antimicrobial peptide magainin 2, although neither phenotype is as strong as in the *phoP* null (29). Transcription of *ugtL* from a heterologous promoter restores magainin 2 resistance to a mutant defective in *slyA* (30), a PhoP-activated gene required for transcription of many PhoP-activated horizontally acquired genes (31–33). The deduced amino acid sequence of the *Salmonella*-specific *ugtL* gene bears no similarity to predicted proteins in sequence databases.

We report that activation of the ancestral PhoP/PhoQ system by acidic pH requires the horizontally acquired UgtL protein and that this activation is necessary for *Salmonella* virulence. UgtL promoted PhoQ autophosphorylation in vitro and enhanced PhoP phosphorylation in vivo, resulting in transcription of PhoP-activated genes. UgtL was specifically required for PhoP activation by a mildly acidic pH and was dispensable for activating PhoP under other PhoQ-stimulating conditions. Our findings indicate that PhoP activation under acidic pH requires a protein for signal sensing (PhoQ) and a separate protein (UgtL) for amplification of that response. Moreover, they argue that activation of the PhoP-dependent virulence program is a derived state resulting from the acquisition of *ugtL* and potentially additional genes.

RESULTS

UgtL is required for PhoP/PhoQ activation by mildly acidic pH

Because the transcriptional regulatory targets of a two-component system may be subject to additional levels of control, the abundance of transcripts controlled by the response regulator is not necessarily a reliable indicator of activation of the system. Instead, the direct output of a two-component system is the amount of phosphorylated response regulator (34). Therefore, to investigate activation of the PhoP/PhoQ system, we examined the amount of PhoP-P by using Phos-tag gels (35) and Western blotting with antibodies recognizing PhoP. Phos-tag gels allow the separation of the phosphorylated and unphosphorylated forms of the PhoP protein in crude extracts from bacteria grown under different conditions (36).

We examined the amounts of PhoP-P in isogenic wild-type and *ugtL* *Salmonella* strains grown under different conditions that induce the sensor PhoQ. These experiments were performed in a *hns-FLAG* derivative background in which the PhoP/PhoQ system was activated in mildly acidic pH similarly to wild-type *Salmonella* (fig. S1). When wild-type *Salmonella* was exposed to a mildly acidic pH, PhoP-P represented ~40% of the total PhoP protein and unphosphorylated PhoP the remaining ~60% (Fig. 1A). By contrast, the fraction of PhoP-P was very much reduced in the *ugtL* mutant (Fig. 1A) under mildly acidic conditions. The defect of the *ugtL* mutant is due to lack of UgtL protein, as opposed to the *ugtL* mutation compromising expression of a gene(s) located downstream, because a plasmid expressing the *ugtL* gene from a heterologous promoter restored the abundance of PhoP-P but the empty vector control did not (Fig. 1B). UgtL was required for normal PhoP-P abundance during growth specifically in mildly acidic pH because the *ugtL* mutant displayed normal PhoP activation when PhoQ was induced by the antimicrobial peptide C18G or low Mg²⁺ conditions (Fig. 1A). As expected, PhoP-P was not detected in wild-type or *ugtL* *Salmonella* grown under non-inducing conditions (neutral pH, high Mg²⁺ and no C18G) (Fig. 1A), and both strains contained similar amounts of unphosphorylated PhoP (Fig. 1A).

The *ugtL* mutant displayed reduced mRNA abundance of the PhoP-activated horizontally acquired genes *mgtC*, *pcgL* and *pagC* when cells were grown in mildly acidic pH (Fig. 1C), in agreement with the notion that PhoP-P is the form of PhoP that promotes gene transcription (20). By contrast, the *ugtL* mutant retained wild-type mRNA abundance of the *ompC* and *kdpE* genes, which are under transcriptional control of the response regulators OmpR (37) and KdpD (38), respectively (Fig. 1C). Altogether, these results establish that *Salmonella* requires UgtL to achieve wild-type PhoP-P amounts when the PhoQ inducing signal is a mildly acidic pH.

UgtL enhances PhoP-P abundance even in neutral pH

If UgtL activates PhoP by helping PhoQ sense a mildly acidic pH, then *ugtL* expression under non-inducing conditions would not be expected to increase the abundance of PhoP-P. However, transcription of the *ugtL* gene from a heterologous promoter increased PhoP-P amounts even when *Salmonella* was grown under non-inducing conditions for PhoQ (Fig.

1B). These results indicate that stimulation of PhoP-P by UgtL is not dependent on a mildly acidic pH.

UgtL increases PhoP-P abundance in a PhoQ-dependent manner

UgtL may increase PhoP-P amounts by stimulating PhoP phosphorylation or by decreasing PhoP-P dephosphorylation, or both. Thus, PhoQ is a likely UgtL target because PhoQ-P is both the only known phosphodonor for wild-type PhoP (39) and the only known PhoP-P phosphatase (40). Alternatively or in addition, UgtL may prevent the PhoP-activated protein MgrB from inhibiting PhoQ's autokinase activity (41), increase the basal amount of the PhoP protein, which could facilitate PhoP phosphorylation from a non-cognate phosphodonor, or both.

If UgtL targets PhoQ, then inactivation of the *ugtL* gene should not alter PhoP-P amounts in the absence of PhoQ. Because PhoQ-P is the only known phosphodonor for wild-type PhoP (39), it is not possible to evaluate the effects of *ugtL* mutation PhoP phosphorylation in a *phoQ* mutant background. Therefore, we investigated the role of *ugtL* in a *phoP*phoQ* strain, which lacks the *phoQ* gene but produces a PhoP variant that autophosphorylates from the small molecular weight phosphoryl donor acetyl phosphate (39). Isogenic *ugtL*⁺ and *ugtL*⁻ strains in the *phoP*phoQ* background displayed similar PhoP-P amounts (Fig. 1D), which is in contrast to the lower PhoP-P amounts displayed by the *ugtL* mutant in a *phoP*⁺*phoQ*⁺ (wild-type) genetic background (Fig. 1D). Thus, UgtL acts in a PhoQ-dependent manner.

The UgtL-dependent increase in PhoP-P is independent of MgrB because the *ugtL mgrB* double mutant displayed lower PhoP-P amounts than the *mgrB* single mutant (fig. S2). In agreement with previous reports that examined transcription of PhoP-activated genes in *E. coli* during growth in low Mg²⁺ (42), PhoP-P amounts were higher in the *mgrB* mutant than in wild-type *Salmonella* grown in mildly acidic pH (fig. S2). Moreover, amounts of PhoP-P were higher in the *mgrB ugtL* double mutant than the *ugtL* single mutant (fig. S2). These results indicate that MgrB activity is not dependent on the presence of UgtL or specific to a particular PhoQ-inducing condition, and that the UgtL-dependent enhancement of PhoP-P does not require the presence of MgrB.

The positive feedback loop that PhoP exerts on its own promoter (43) was also dispensable for UgtL activity because PhoP-P amounts were still lower in the *ugtL* mutant than in the isogenic *ugtL*⁺ strain when the chromosomal *phoPphoQ* operon was transcribed from a PhoP-independent promoter (21) (fig. S3). Cumulatively, these data indicate that UgtL enhances PhoP-P abundance by altering one or more PhoQ activities.

UgtL binds to the sensor PhoQ

Independent approaches demonstrated that UgtL interacted directly with PhoQ. First, we used the bacterial two-hybrid system wherein β -galactosidase activity was examined in an *E. coli* strain lacking its own adenylate cyclase but expressing two fragments (T25 and T18) of the *Bordetella pertussis* adenylate cyclase that, when brought in close proximity by interactions of chimeric proteins, restore activity (44). The β -galactosidase activity of a strain co-expressing the T25 adenylate fragment fused to the N terminus of UgtL (T25-

UgtL) and the T18 adenylate fragment fused to the C terminus of PhoQ (PhoQ-T18) was ~50 times higher than that produced by bacteria carrying the negative control plasmids (Fig. 2A). This activity was even higher than that produced by the strain with the positive control plasmids (Fig. 2A). By contrast, there was little β -galactosidase activity when the UgtL fusion protein (T25-UgtL) was co-expressed with T18 fused to the N terminus of PhoQ (T18-PhoQ) (Fig. 2A). Likewise, no β -galactosidase activity was produced by strains expressing T18 fusions to either the N or C terminus of PhoQ (T18-PhoQ or PhoQ-T18) along with a T25 fusion to CigR (T25-CigR) (Fig. 2A), an inner membrane protein used as a negative control.

As an independent test for the interaction between UgtL and PhoQ, we used immunoprecipitation of the UgtL-HA and PhoQ-FLAG proteins and corresponding controls synthesized with the PURExpress in vitro transcription-translation system (45). These tagged proteins were reconstituted into liposomes (46) because PhoQ and UgtL are integral membrane proteins (18, 29). Antibodies recognizing the FLAG epitope pulled down UgtL-HA, and antibodies recognizing the HA epitope pulled down PhoQ-FLAG (Fig. 2B, S4). In these experiments, there was a background signal that indicated a small amount of nonspecific binding of the UgtL-HA and PhoQ-FLAG proteins to antibodies recognizing the FLAG and HA epitopes, respectively. The UgtL-PhoQ interaction appears to be specific because the antibodies recognizing the HA epitope did not pull down EnvZ-FLAG, a sensor kinase used as a negative control, and the antibodies recognizing the FLAG epitope did not pull down UgtL-HA, from liposomes harboring UgtL-HA and EnvZ-FLAG (Fig. 2B, S4).

UgtL interacts with a region of PhoQ not involved in acidic pH sensing

The PhoQ protein harbors two transmembrane domains that define a periplasmic region necessary for sensing Mg^{2+} (22) and C18G (19). The large C-terminal region of PhoQ is required for the response to acidic pH and harbors the catalytic and dimerization domains of PhoQ. To determine whether UgtL interacts with regions of PhoQ implicated in signal sensing, we investigated the behavior of a strain expressing PhoZ in place of PhoQ. PhoZ is a chimeric protein consisting of the N-terminal portion of PhoQ including its periplasmic, transmembrane and HAMP (i.e., Histidine kinase, adenylyl cyclases, Methyl-accepting proteins and Phosphatases, mediating signaling output) domains, fused to the catalytic and dimerization domains from EnvZ (Fig. 2B, S4). The N-terminal 266 amino acid region from PhoQ present in PhoZ was sufficient for interaction with UgtL-HA (Fig. 2B, S4). Therefore, UgtL binds to a region of PhoQ that is distinct from the cytosolic region previously implicated in sensing a mildly acidic pH (13).

UgtL promotes PhoQ autophosphorylation from ATP

We wondered how UgtL binding to PhoQ (Fig. 2) increases PhoP-P abundance in vivo (Fig. 1A). UgtL could promote PhoP phosphorylation by stimulating PhoQ autophosphorylation, by enhancing phosphotransfer from PhoQ-P to PhoP, by inhibiting dephosphorylation of PhoP-P by PhoQ, or by modifying several of these activities. In phosphorylation assays using in vitro-synthesized proteins, UgtL accelerated autophosphorylation of the full length PhoQ protein (Fig. 3A). In contrast, neither phosphotransfer from PhoQ to PhoP (Fig. 3B) nor PhoP-P dephosphorylation by PhoQ (Fig. 3C) were affected by the presence of UgtL in

the reaction. These results assign a biochemical function to UgtL, a protein with no homologs in sequence databases.

The transmembrane and periplasmic regions of UgtL are required to enhance PhoP-P abundance

UgtL is an inner membrane protein (29). The deduced amino acid sequence of the *ugtL* gene predicts two transmembrane domains that define a 24 amino acid periplasmic region (47) (Fig. 4A). To define the portion of UgtL required for PhoQ activation, we examined PhoP phosphorylation in a *ugtL* deletion strain expressing various truncated variants of a C-terminally FLAG-tagged UgtL protein (Fig. 4A). The use of FLAG-tagged variants enabled us to probe the amounts of the investigated proteins. A strain expressing the UgtL-FLAG protein exhibited the same proportion of PhoP-P:PhoP proteins as a strain expressing the wild-type, untagged UgtL protein (fig. S5.)

Like full-length UgtL, one specifying the UgtL variant UgtL(1–87), which includes only the first 87 amino acids of UgtL, retained normal PhoP activation when cells were grown in mildly acidic pH conditions (Fig. 4B). Expression of UgtL(21–87) or UgtL(31–87) also increased PhoP-P amounts in mildly acidic pH (Fig. 4B), although these latter two UgtL variants displayed slightly lower activity than did full-length UgtL or UgtL(1–87) (Fig. 4B). By contrast, plasmids expressing UgtL(60–87), UgtL(31–69), or UgtL(1–69) failed to increase PhoP-P amounts in acidic pH (Fig. 4B). The FLAG-tagged UgtL(31–69) and UgtL(1–69) proteins were produced in similar abundance as the wild-type UgtL-FLAG protein (Fig. 4B). Therefore, the failure of these variants to enhance PhoP-P abundance is likely due to loss of activity as opposed to decreased expression or stability. Taken together, these results indicate that the first predicted transmembrane domain and periplasmic region of UgtL are necessary to increase PhoP-P abundance, but the 30 N-terminal and 45 C-terminal amino acids of UgtL are not. These results leave open the possibility that the periplasmic domain of UgtL alone, if artificially targeted to the periplasm, could be able to bind to PhoQ and enhance PhoP phosphorylation.

UgtL functions independently of the cytoplasmic pH-sensing domain of PhoQ

The UgtL protein appears to stimulate PhoQ's autokinase activity independently of PhoQ's ability to sense a mildly acidic pH, because expression of the *ugtL* gene from a heterologous promoter increased PhoP-P amounts even when *Salmonella* was grown under non-inducing conditions (neutral pH) for PhoQ (Fig. 1B). If this is the case, PhoP-P amounts should be lower in a double mutant lacking the *ugtL* gene and carrying a PhoQ mutation (*phoQ^{SB}*) that renders the protein insensitive to mildly acidic pH (13) compared to either single mutant. As hypothesized, the ratio of PhoP-P to PhoP was the highest in wild-type *Salmonella*, somewhat reduced in the *phoQ^{SB}* mutant, and greatly reduced in the *ugtL* single mutant and the acidic pH-blind *phoQ^{SB} ugtL* double mutant (Fig. 4C). Thus, PhoQ-promoted phosphorylation of PhoP triggered by an acidic pH entails PhoQ detecting a change in pH (or a downstream effect of a change in pH) and the UgtL protein enhancing PhoQ autophosphorylation.

PhoP promotes *ugtL* transcription in mildly acidic pH

Given that UgtL enhances PhoQ activation in mildly acidic pH (Fig. 1), we wondered how the *ugtL* gene is expressed under different PhoQ-inducing conditions. The abundance of *ugtL* transcripts was much higher in wild-type *Salmonella* experiencing low Mg²⁺ or a mildly acidic pH than when the organism was grown under non-inducing conditions (fig. S6). The transcriptional induction of the *ugtL* gene is PhoP-dependent because little *ugtL* mRNA accumulation was observed in the isogenic *phoP* mutant (fig. S6). The transcriptional induction of the *ugtL* gene in mildly acidic pH implies that the UgtL protein is present and therefore available to activate the sensor PhoQ under acidic conditions.

UgtL-dependent resistance to magainin 2 requires PhoP

Our laboratory reported growth in low Mg²⁺ renders both *ugtL* and *phoP* null mutants hypersensitive to the antimicrobial peptide magainin 2 (29). A plasmid in which the *ugtL* gene is transcribed from a heterologous promoter complemented the *ugtL* mutant but not a *phoP* mutant in this context (fig. S7). These results suggest that UgtL-mediated magainin 2 resistance requires PhoP itself, a PhoP-dependent product(s), or both.

ugtL is required for full virulence

It has been proposed that activation of the PhoP/PhoQ system by mildly acidic pH is critical for *Salmonella* virulence because inhibition of acidification of the *Salmonella*-containing vacuole prevents the expression of PhoP-activated genes in phagocytic (48, 49) and non-phagocytic cells (50), limits replication inside macrophages (51, 52), and attenuates virulence in mice (53). Given that the *ugtL* gene is necessary for full PhoP activation under a mildly acidic pH, we reasoned that inactivation of the *ugtL* gene would attenuate *Salmonella* virulence.

Intraperitoneal inoculation of BALB/c and C3H/HeN mice with *ugtL* mutant *Salmonella* demonstrated that virulence of the *ugtL* strain was indeed attenuated compared to wild-type *Salmonella* (Fig. 5A–B). Natural resistance-associated macrophage protein 1 (NRAMP1) is a divalent metal ion transporter that is present in macrophage phagosomal membranes and has been implicated in mammalian resistance to several intracellular pathogens that remain within acidic phagosomes, including *Salmonella* (54). Whereas C3H/HeN mice are wild-type with respect to NRAMP1, BALB/c mice harbor mutant alleles of SCL11A1 (54, ref). Both BALB/c and C3H/HeN mice were susceptible to infection by wild-type *Salmonella* and resistant to infection by *phoQ* mutants. Whereas no mice survived more than seven days after inoculation with wild-type *Salmonella*, mice infected with *ugtL* *Salmonella* survived longer, with some surviving more than 14 days post inoculation (Fig. 5A–B). These results reinforce the notion that the ability of the PhoP/PhoQ system to respond to a mildly acidic pH is necessary for *Salmonella* pathogenesis.

DISCUSSION

We have established that activation of the response regulator PhoP during growth under mildly acidic pH requires two proteins providing two different functions: the sensor PhoQ is responsible for detecting changes in pH (or a downstream effector of such changes), and the

protein UgtL boosts the response of PhoQ to mildly acidic pH (Fig. 1 and 6). In addition, UgtL confers resistance to the antimicrobial peptide magainin 2 (29). This activity of UgtL is independent of the promotion of PhoP-P abundance (Fig. 1) because wild-type and *ugtL* *Salmonella* display similar PhoP-P amounts during growth in low Mg^{2+} (Fig. 1A), a condition in which the *ugtL* mutant exhibits hypersusceptibility to magainin 2 (fig. S7). That a given protein may have more than one target is also illustrated by another PhoP-activated protein, MgtC, which binds to and inhibits the F_1F_0 ATP synthase (55) and binds to and stabilizes PhoP (56).

Horizontal acquisition of foreign gene broadens the responsiveness of an ancestral regulatory system

The UgtL protein is highly conserved in the pathogenic species *S. enterica* (98% amino acid sequence identity), which includes the human pathogen serovar Typhi. Because the *phoP* gene is required for virulence of *S. enterica* serovar Typhi (57) and the PhoQ proteins from the Typhi and Typhimurium serovars are 99% identical, *ugtL* may also contribute to typhoid pathogenesis.

The UgtL protein from the non-pathogenic species *S. bongori* exhibits 55% amino acid identity to *S. enterica* UgtL. This unusually low amino acid identity is intriguing given that *S. bongori* PhoQ is insensitive to mildly acidic pH but can be activated by other PhoQ-inducing signals (13). The presence of *ugtL* in both *S. enterica* and *S. bongori* suggests that this gene was acquired before the two *Salmonella* species diverged from a common ancestor. This also suggests that the ability of *Salmonella* to respond to acid through PhoQ activation and UgtL-mediated enhancement of acid-induced PhoQ activation may be an important determinant of a species' virulence.

Mildly acidic pH also stimulates the transcription of PhoP-activated genes in *E. coli* (42), yet *E. coli* lacks *ugtL*. Instead, the *E. coli*-specific protein SafA mediates this activation (58). Like UgtL, the SafA protein enhances the abundance of PhoP-P under non-inducing conditions for PhoQ when expressed from a heterologous promoter (58). The absence of sequence similarity between SafA and UgtL makes these proteins functional paralogues rather than homologues.

The *safA* gene is under transcriptional control of the *E. coli*-specific EvgA/EvgS two-component system, which is also activated by acidic pH (58). In contrast, the *Salmonella* *ugtL* gene is under transcriptional control of the PhoP/PhoQ system (fig. S6) (30, 59). Therefore, SafA enables *E. coli* to activate PhoP-dependent genes in response to the signals acting on the sensor EvgS, whereas UgtL enables *Salmonella* to heighten the response to a particular PhoQ-inducing signal (acidic pH). Our findings identify an instance of convergent evolution whereby a gene horizontally acquired through foreign DNA enhances the responsiveness of an ancestral regulatory system.

UgtL promotes sensor phosphorylation without participating in signal sensing

The process of signal transduction typically involves sensors that modify the enzymatic activity of proteins directly responsible for transmission of a signal. For example, upon binding to (2*S*,4*S*)-2-methyl-2,3,3,4-tetrahydroxytetrahydrofuryl borate, the sensor LuxP

stimulates the phosphatase activity of the LuxQ protein in *Vibrio harveyi* (60, 61). Likewise, G protein-coupled receptors respond to receptor-specific stimuli by activating a downstream G protein that, in turn, initiates an intracellular signaling cascade (62). In contrast, other proteins limit signal transduction processes. For instance, binding of the *E. coli* LysP protein to the pH sensor CadC renders it unresponsive to acidic pH (63). The mode of action of UgtL differs from those described above. That is, the output of the sensor PhoQ reflects both its direct response to a mildly acidic pH and tuning of that response by UgtL.

Activation of PhoQ by acidic pH is required for *Salmonella* virulence

The PhoQ protein responds to a mildly acidic pH (13, 17), low Mg²⁺ (18), certain antimicrobial peptides (19), unsaturated long chain fatty acids (64), acetate concentrations (65), and the redox state in the periplasmic space (66). Which of these signals activates PhoQ during *Salmonella* infection of its mammalian hosts? It has been proposed that acidic pH and low Mg²⁺ are not relevant signals because a *Salmonella* strain with amino acid substitutions in PhoQ that render PhoQ less responsive to these two signals retained wild-type virulence in mice (67).

The data presented here strongly suggest that PhoQ activation by mildly acidic pH is critical for *Salmonella* virulence because a *ugtL* null mutant is defective for both PhoP/PhoQ activation by mildly acidic pH (Fig. 1) and virulence in mice (Fig. 5). This notion is supported by the virulence attenuation exhibited by a *Salmonella* strain with a PhoQ variant impaired in its ability to respond to a mildly acidic pH (13), which, like the *ugtL* mutant (Fig. 1), responds normally to both low Mg²⁺ and the antimicrobial peptide C18G (13). Furthermore, *Salmonella* replication within macrophages and virulence in mice are compromised upon inhibition of phagosome acidification (51–53). An additional signal(s), protein(s), or both must activate PhoP/PhoQ during infection because the *Salmonella* strains lacking *ugtL* or bearing amino acid substitutions in PhoQ that hinder the response to mildly acidic pH are not as attenuated for virulence as the *phoQ* null mutant (13, 28) (Fig. 5).

MATERIALS AND METHODS

Bacterial strains, plasmids, oligodeoxynucleotides, and growth conditions

Bacterial strains and plasmids used in this study are listed in Table S1. All *S. enterica* serovar Typhimurium strains were derived from the wild-type strain 14028s (28) and constructed by phage P22-mediated transductions as described (68). Bacteria were grown at 37°C in Luria-Bertani (LB) broth or N-minimal media (69) supplemented with 0.1% casamino acids, 38 mM glycerol, and the indicated pH (pH 7.6 or pH 4.9) and concentrations of MgCl₂. *E. coli* DH5α was used as the host for preparation of plasmid DNA (70). *E. coli* BTH101 *cya* was used for the bacterial two-hybrid assay (44). To induce plasmid expression, isopropyl β-D-1-thiogalactopyranoside (IPTG) was added at 20 μM for experiments with the UgtL truncation variants and at 200 μM for the magainin 2 assays. When necessary to select for plasmid maintenance, appropriate antibiotics were added at the following final concentrations: ampicillin at 50 μg/ml, chloramphenicol at 20 μg/ml, kanamycin at 50 μg/ml, and tetracycline at 10 μg/ml. The antimicrobial peptide C18G was

used at a final concentrations of 5 µg/ml, and bacteria were treated for 90 min. DNA oligonucleotides used in this study are listed in Table S2.

In vivo detection of phosphorylated PhoP

PhoP and PhoP-P were separated on 12.5% polyacrylamide gels containing acrylamide-Phos-tagTM ligand (Phos-tagTM Consortium) as described by the manufacturer. Gels were copolymerized with 50 µM Phos-tagTM acrylamide and 100 µM MnCl₂. Whole-cell extracts were prepared as described and normalized by OD₆₀₀ (35). The samples were electrophoresed on Phos-tagTM gels with standard running buffer [0.4% (w/v) SDS, 25 mM Tris, 192 mM glycine] at 4°C under 20 mAmp for 5 h, transferred to nitrocellulose membranes, and analyzed by immunoblotting using polyclonal rabbit antibodies recognizing PhoP (1:2,000) and polyclonal mouse antibodies recognizing AtpB (1:5,000). Secondary horseradish peroxidase-conjugated antisera recognizing rabbit and mouse antibodies (GE healthcare) were used at 1:5,000 dilution. The blots were developed with the Amersham ECL Western Blotting Detection Reagents (GE Healthcare) or SuperSignal West Femto Chemiluminescent system (Pierce). The data are representative of three independent experiments, which produced similar results.

Construction of mutant strain

To generate an *hns-FLAG* strain, a *cat* cassette was introduced in the 3' end of the *hns* gene as follows: the *cat* fragment was amplified from plasmid pKD3 using primers 15748/15749, then introduced into wild-type *Salmonella* (14028s) harboring plasmid pKD46 as described (71). The *cat* cassette was removed using plasmid pCP20 (71).

Construction of plasmids

Plasmid pUgtL(1–87) was constructed as follows: the *ugtL* gene was amplified from wild-type *Salmonella* (14028s) using primers 2240/16060, then introduced between the BamHI and HindIII sites of pUHE21–2*lacI*^H (43). Plasmid pUgtL-FLAG was constructed as follows: the *ugtL* coding region was amplified from wild-type *Salmonella* (14028s) using primers 2240/3143, then introduced between the BamHI and HindIII sites of pUHE21–2*lacI*^H (43). Plasmids for expressing truncated UgtL-FLAG were constructed as follows: various truncated FLAG-tagged *ugtL* genes were amplified using primers 2240/16096 (for 1–87), 16096/16098 (for 21–87), 16096/16099 (for 31–87), 16096/16104 (for 61–87), 16098/16105 (for 21–69), 16099/16105 (for 31–69), or 2240/16105 (for 1–69) from strain 14028s, then introduced between the BamHI and HindIII sites of pUHE21–2*lacI*^H (43). Plasmid pPhoZ, constructed by a former lab member, harbors the DNA sequence specifying the N-terminal 266 amino acids of PhoQ fused in frame to the C-terminal 218 amino acids of EnvZ between the BamHI and HindIII sites of pUHE21–2*lacI*^H (43). Plasmid pUT18-PhoQ and pUT18C-PhoQ were constructed as follows: the *phoQ* gene was amplified from wild-type *Salmonella* (14028s) using primers 11824/11825, then introduced between BamHI and KpnI sites of pUT18 or pUT18C (72). Plasmid pKT25-UgtL was constructed as follows: the *ugtL* gene was amplified from wild-type *Salmonella* (14028s) using primers 16041/16042, then introduced between XbaI and KpnI sites of pKT25 (72). Plasmid pKT25-CigR was constructed as follows: the *cigR* gene was amplified from wild-type *Salmonella* (14028s) using primers 16108/16109, then introduced between XbaI and KpnI sites of pKT25 (72).

Quantitative RT-PCR

Total RNA was isolated using the 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 abundance of the *mgtC*, *phoP*, *ugtL*, *pmrD* and *rrs* genes were measured by quantification of cDNA using Fast SYBR Green PCR Master Mix (Applied Biosystems) and following primers (*mgtC*: 6962/6963; *pagC*: 6964/6965; *pcgL*: 6627/6628; *ompC*: W2033/W2034; *kdpE*: 7923/7924) and monitored using a QuantStudio6 machine (Applied Biosystems). Data were normalized to the abundance of 16S ribosomal RNA amplified with primers 3023 and 3024.

Bacterial two-hybrid assay

E. coli BTH101 harboring derivatives of plasmids pUT18 and pKT25 were grown overnight in LB broth containing ampicillin (100 µg/ml) and kanamycin (50 µg/ml), added at 1:100 dilution to 1 ml of the same fresh media containing isopropyl β-D-1-thiogalactopyranoside (IPTG; 0.5 mM) and grown with shaking at 30°C overnight as described (44, 72). β-galactosidase activities were determined using a kinetic assay as described (73).

Pull-down assay with in vitro transcription-translation system

Pull-down assay was performed as described with some modifications (55). All proteins for in vitro synthesis were produced using the cell-free PURExpress in vitro protein synthesis system (New England Biolabs) in the presence of 0.12 mg/ml proteoliposomes at 37°C for 3 h. Proteoliposomes were prepared using soybean L-α-phosphatidylcholine (Sigma) in buffer (20 mM Tricine, 20 mM succinic acid, 80 mM NaCl and 0.6 mM KOH, adjusted to pH 8.0) to a concentration of 32 mg/ml as described (46). DNA templates were prepared according to the manufacturer's instructions. To synthesize UgtL-HA, PhoQ-FLAG, PhoZ-FLAG, and EnvZ-FLAG, we used primers 16061/16062 for UgtL-HA, 16063/16064 for PhoQ-FLAG, 16063/16065 for PhoZ-FLAG, 16065/16066 for EnvZ-FLAG. At the end of the reaction, samples were diluted in TBS buffer (Tris-buffered saline) with 20 times the reaction volumes. Diluted reactions were mixed in 500 µL TBS containing 0.12 mg/ml proteoliposomes and incubated at room temperature for 2 h. Then samples were pulled down with magnetic beads conjugated to antibodies recognizing the HA epitope at 4°C for 1 h or with magnetic beads conjugated to antibodies recognizing the FLAG epitope at room temperature for 2 h. Pulled down samples were analyzed by Western blotting using antibodies recognizing the HA or FLAG epitopes.

Autophosphorylation, phosphotransfer and dephosphorylation assays

For autophosphorylation assays, 0.68 µM PhoQ-Strep was incubated with 4.71 µM of UgtL-HA in TKM buffer (50 mM Tris-HCl [pH 8.0], 50 mM KCl, 1 mM MgCl₂) at 37°C for 15 min. The reaction was started by addition of 30 µM of ATP containing 30 µCi of [γ -³²P] ATP in a 50 µl reaction mixture at room temperature. An 8 µl aliquot was mixed with 5 × SDS loading buffer at different times to stop the reaction. Samples were kept on ice until they were loaded onto a 10% Bis-Tris SDS-PAGE gel to separate the protein from free nucleotides.

For phosphotransfer assays, phosphorylated PhoQ-Strep was generated by incubating 0.68 μM of PhoQ-Strep with or without 4.71 μM of UgtL-HA in TKM buffer with 30 μM of ATP containing 30 μCi of [γ - ^{32}P] ATP at room temperature for 1 h. The PhoQ-P was recovered using a Micro Bio-Spin 6 chromatography column (Bio-Rad) by removing excess ATP and ADP generated from ATP. The phosphotransfer reaction was started by adding 0.68 μM of PhoP proteins in a 50 μl reaction mixture. An 8 μl aliquot was mixed with 5 \times SDS loading buffer at different time points to stop the reaction. The samples were kept on ice until they were loaded onto 10% Bis-Tris SDS-PAGE gel.

For dephosphorylation assays, phosphorylated PhoP-His6 was prepared as described (74). Briefly, 300 pmol of GST-YgiYc beads were incubated with 20 μCi [γ - ^{32}P] ATP in 50 μl of TBS-MD buffer at room temperature for 12 h, and then the beads were washed with TBS buffer five times to remove free ATP. 3 nmol of purified PhoP-His6 was incubated with phosphorylated GST-YgiYc beads in 60 μl of TBS-MD buffer at room temperature for 4 h. After incubation, the PhoP-P was recovered using a Micro Bio-Spin 6 chromatography column that had been pre-equilibrated with TBS. The dephosphorylation reaction was started by adding 0.68 μM of PhoP-P to 0.68 μM of PhoQ-Strep with or without 4.71 μM of UgtL-HA pre-incubated with 100 μM of ADP in 50 μl reaction mixture. An 8 μl aliquot was mixed with 5 \times SDS loading buffer at different times to stop the reaction. Samples were kept on ice until they were loaded onto a 10% Bis-Tris SDS-PAGE gel.

Amounts of PhoP-P and PhoQ-P were quantified using a BAS-5000 imaging system (FujiFilm) and a phosphorimaging plate (FujiFilm).

Mouse virulence assay

Six-week-old female BALB/c or C3H/HeN mice were purchased from Charles River Laboratories. Five mice in each group were infected intraperitoneally with 0.1 ml of PBS containing $\sim 10^2$ (for BALB/c) or $\sim 2 \times 10^4$ (for C3H/HeN) *Salmonella* that had been grown overnight in LB broth and resuspended and diluted in PBS. Mouse survival was monitored every 12 hours for three weeks. Virulence assays were conducted twice with similar outcomes, and data for each experimental group correspond to groups of five mice. All 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.

Antimicrobial peptide killing assay

Antimicrobial peptide susceptibility assays were conducted as described (29). Bacteria were grown in N-minimal media with 10 μM Mg^{2+} and pH 7.6 with IPTG (200 μM) to mid-log phase. Bacterial cells were then diluted to $1\sim 2 \times 10^5$ colony forming units (CFU)/ml in the same media. Magainin 2 was dissolved and diluted with autoclaved distilled water. Samples of 5 μl of peptide solution were placed in wells of 96-well plate and 45 μl of the bacteria samples were added. After 1 h incubation at 37°C with aeration, samples were serially diluted in LB and plated on LB agar plates for enumeration. The percentage survival was calculated as following: survival (%) = CFU of peptide-treated culture/CFU of no peptide culture \times 100.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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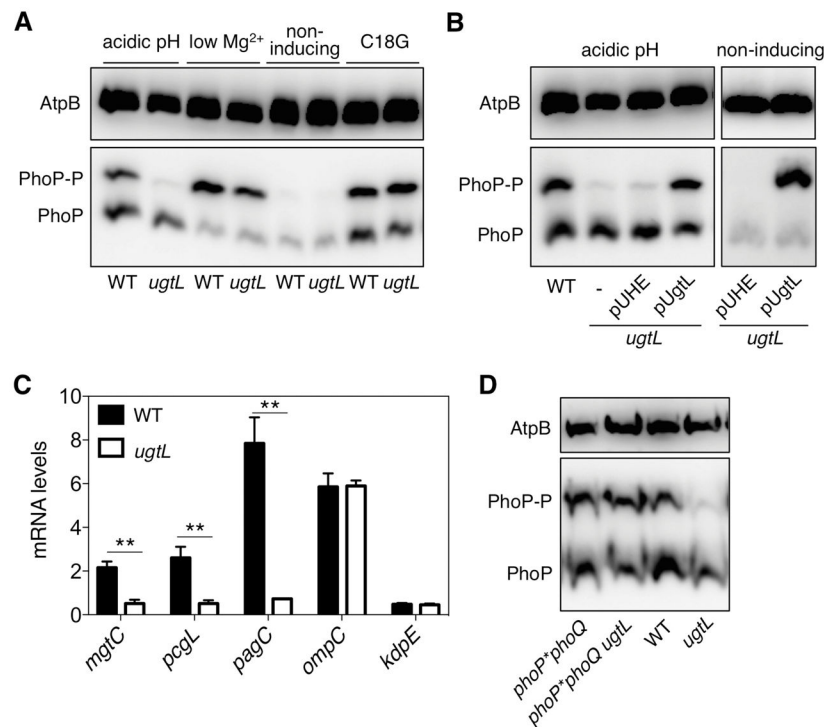


Fig. 1. The *ugtL* gene is required for promoting the phosphorylated state of PhoP in acidic conditions in a PhoQ-dependent manner

(A) Phos-tag Western blot analysis of crude extracts prepared from wild-type (WT) *Salmonella enterica* (JC805) and the isogenic *ugtL* mutant (JC925) strains grown in N-minimal media with 1 mM of Mg²⁺ at pH 4.9 (acidic pH), 10 μM Mg²⁺ at pH 7.6 (low Mg²⁺), 1mM Mg²⁺ at pH 7.6 (non-inducing), or the antimicrobial peptide C18G to mid-log phase using antibodies recognizing PhoP or the loading control AtpB. (B) Phos-tag Western blot analysis of crude extracts prepared from wild-type *Salmonella* (JC805) and *ugtL* mutants (JC925) harboring either the empty vector (pUHE) or a plasmid expressing UgtL (pUgtL) grown in inducing conditions (acidic pH) or non-inducing conditions to mid-log phase using antibodies recognizing PhoP or the loading control AtpB. (C) Abundance of *mgtC*, *pcgL*, *pagC*, *ompC*, and *kdpE* transcripts in wild-type (JC805) and *ugtL* (JC925) *Salmonella* grown in acidic pH to mid-log phase. The mean and SD from three independent experiments are shown. Unpaired students T-test were performed between wild-type and isogenic *ugtL* mutant strains; ** $p < 0.01$. (D) Phos-tag Western blot analysis of crude extracts prepared from wild-type (JC805), *ugtL* (JC925), *phoP*phoQ* (JC1014), and *phoP*phoQ ugtL* (JC1056) *Salmonella* grown in acidic pH to mid-log phase using antibodies directed against PhoP or the loading control AtpB. Data are representative of three independent experiments.

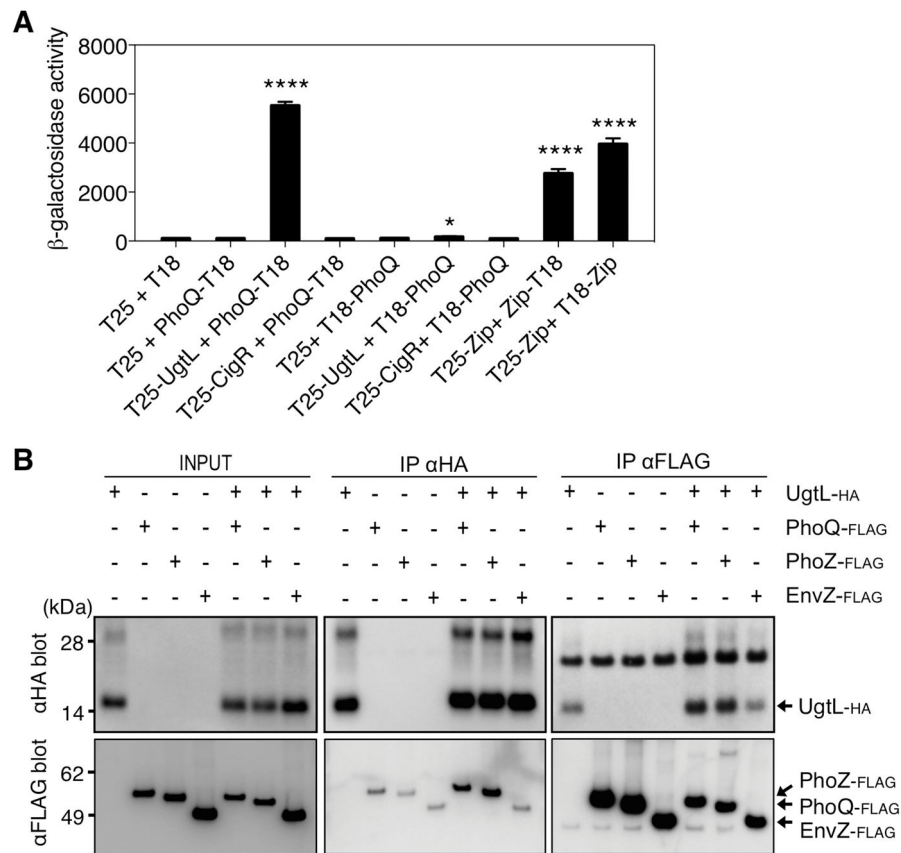


Fig. 2. The UgtL and PhoQ proteins interact

(A). β -galactosidase activity in bacterial two-hybrid system assays in *E. coli* BTH101 expressing the indicated fusion proteins. In each experimental condition, the bacteria carried the two *Bordetella pertussis* adenylate cyclase fragments T25 and T18 either alone or fused to UgtL, CigR, or PhoQ in the indicated combinations. The adenylate cyclase fragment was fused to the N terminus in fusion proteins T25-UgtL, T25-CigR, and T18-PhoQ and to the C terminus in fusion protein PhoQ-T18. T25-Zip, Zip-T18, and T18-Zip were used as positive controls. The mean and SD from three independent experiments are shown. Unpaired students T-test were performed between strains harboring empty vectors with the other combinations; * $p < 0.05$, **** $p < 0.0001$. (B) Pulldown assays showing interactions between in vitro-synthesized UgtL-HA, PhoQ-FLAG, PhoZ-FLAG, and EnvZ-FLAG proteins. Samples were analyzed by Western blotting using antibodies recognizing the HA epitope and the FLAG epitope. Densitometry of each blot with ImageJ software is shown below the blots in the same order using arbitrary units (AU). Dashed lines in the densitometry graphs indicate signals from nonspecific binding of the UgtL-HA and FLAG-tagged proteins to antibodies recognizing the FLAG and HA epitopes, respectively. The data are representative of two independent experiments, which produced similar results.

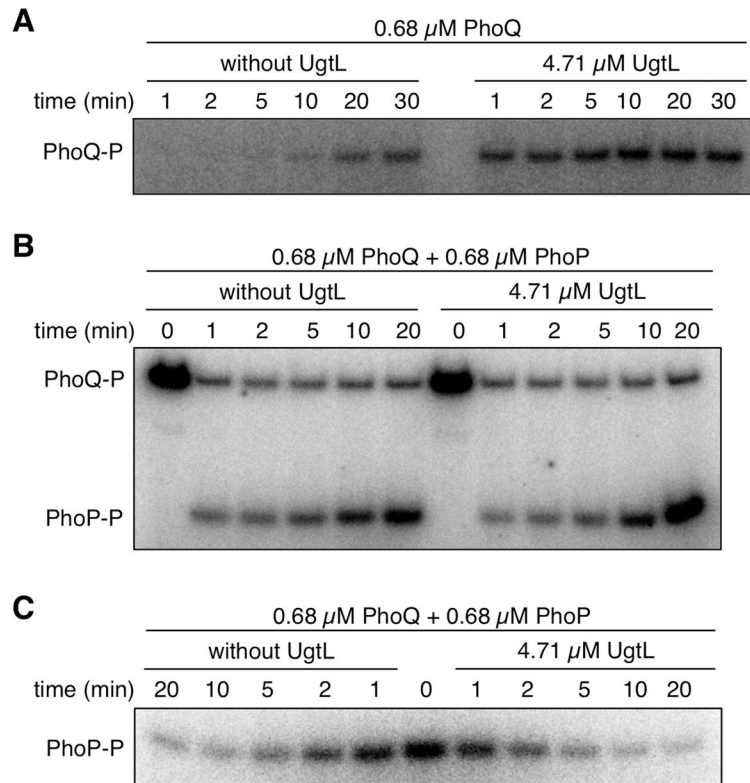


Fig. 3. UgtL promotes autophosphorylation of PhoQ in vitro

(A) Amounts of PhoQ-P at the indicated time points in the presence or absence of UgtL and 32 P-labeled ATP. (B) Amounts of PhoQ-P and PhoP-P at the indicated times after addition of PhoP to reaction mixtures containing PhoQ-P or PhoQ-P + UgtL. (C) Amounts of PhoP-P at the indicated times after addition of PhoP-P to reaction mixtures containing PhoQ or PhoQ + UgtL. The data are representative of two independent experiments, which produced similar results.

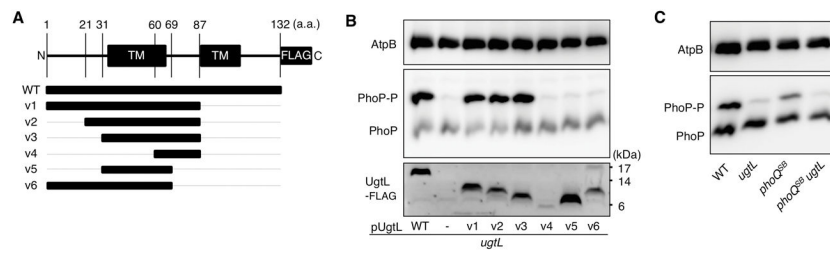


Fig. 4. UgtL and the cytoplasmic domain of PhoQ respond independently to mildly acidic pH
(A) Schematic of the full-length (FL) UgtL-FLAG protein and the truncated variants v1–v6.
(B) Phos-tag Western blot (top and middle) or Western blot (bottom) analysis of crude extracts prepared from *Salmonella ugtL* mutant (EG13682) harboring plasmids expressing FL FLAG-tagged UgtL or the truncated variants as indicated. Phos-tag Western blots were probed with antibodies recognizing PhoP and AtpB (loading control), and the Western blot was probed with antibodies recognizing the FLAG epitope. Data are representative of three independent experiments, which produced similar results. **(C)** Phos-tag Western blot analysis of crude extracts prepared from wild-type (WT) *Salmonella* (JC805), *ugtL* mutant *Salmonella* (JC925), *Salmonella* expressing a PhoQ variant (PhoQ^{SB}) that is insensitive to acidic pH (JC1102; *phoQ^{SB}*), and *phoQ^{SB} ugtL* *Salmonella* (JC1123) grown in acidic pH to mid-log phase. Blots were probed with antibodies recognizing PhoP and AtpB. Data are representative of three independent experiments, which produced similar results.

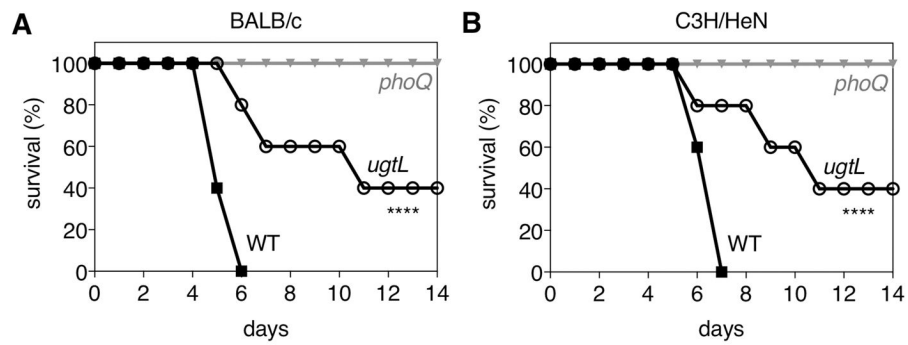


Fig. 5. UgtL is required for maximal *Salmonella* virulence in mice

Survival of BALB/c (A) or C3H/HeN (B) mice inoculated intraperitoneally with wild-type (14028s), *ugtL* (EG13682) or *phoQ* (MS5996s) *Salmonella*. Data are representative of N = 2 independent experiments, which produced similar results, n=5 mice per each experimental group. Mantel-Cox test was performed between wild-type and *ugtL* *Salmonella* infected mice; **** $p < 0.0001$.

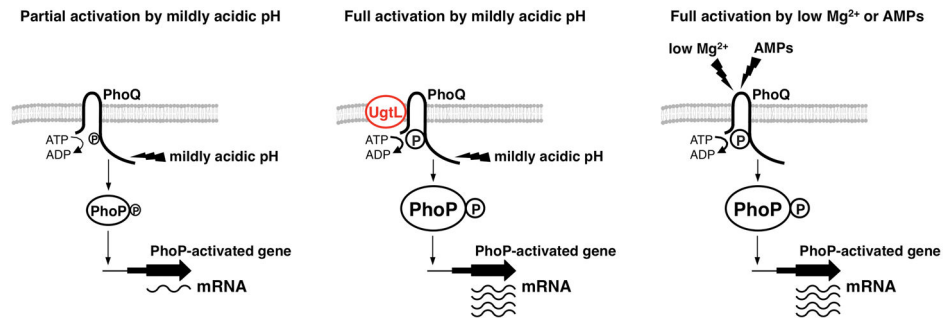


Fig. 6. Activation of the sensor PhoQ in mildly acidic pH requires the *Salmonella*-specific UgtL protein to intensify the response of PhoQ to a mildly acidic pH

(A) The sensor PhoQ responds to acidification of the cytoplasm through its cytoplasmic domain. Mildly acidic pH causes PhoQ to autophosphorylate, after which it functions as a phosphodonor to the response regulator PhoP. Phosphorylated PhoP (PhoP-P) binds to promoters and promotes transcription of PhoP-activated genes. (B) UgtL enhances PhoQ autophosphorylation in response to acidic conditions, which results in increased abundance of PhoP-P and of PhoP-activated mRNAs. UgtL is required for *Salmonella* to achieve full PhoP-dependent gene transcription when the PhoQ-inducing signal is mildly acidic pH. (C) The periplasmic domain of PhoQ mediates PhoQ activation in response to conditions of low Mg²⁺ or the antimicrobial peptide C18G. In response to either of these stimuli, PhoQ undergoes autophosphorylation and then functions as a phosphodonor to PhoP. In this context, PhoQ-mediated activation of PhoP is sufficient for full transcription of PhoP-activated genes and is not dependent on UgtL.