Programmed Delay of a Virulence Circuit Promotes Salmonella Pathogenicity

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ABSTRACT Signal transduction systems dictate various cellular behaviors in response to environmental changes. To operate cellular programs appropriately, organisms have sophisticated regulatory factors to optimize the signal response. The PhoP/PhoQ master virulence regulatory system of the intracellular pathogen Salmonella enterica is activated inside acidic macrophage phagosomes. Here we report that Salmonella delays the activation of this system inside macrophages using an inhibitory protein, EIICNtr (a component of the nitrogen-metabolism phosphotransferase system). We establish that EIICNtr directly restrains PhoP binding to its target promoter, thereby negatively controlling the expression of PhoP-activated genes. PhoP furthers its activation by promoting Lon-mediated degradation of EIICNtr at acidic pH. These results suggest that Salmonella ensures robust activation of its virulence system by suspending the activation of PhoP until a sufficient level of active PhoP is present to overcome the inhibitory effect of EIICNtr. Our findings reveal how a pathogen precisely and efficiently operates its virulence program during infection.

IMPORTANCE To accomplish successful infection, pathogens must operate their virulence programs in a precise, time-sensitive, and coordinated manner. A major question is how pathogens control the timing of virulence gene expression during infection. Here we report that the intracellular pathogen Salmonella controls the timing and level of virulence gene expression by using an inhibitory protein, EIICNtr. A DNA binding master virulence regulator, PhoP, controls various virulence genes inside acidic phagosomes. Salmonella decreases EIICNtr amounts at acidic pH in a Lon- and PhoP-dependent manner. This, in turn, promotes expression of the PhoP-activated virulence program because EIICNtr hampers activation of PhoP-regulated genes by interfering with PhoP binding to DNA. EIICNtr enables Salmonella to impede the activation of PhoP-regulated gene expression inside macrophages. Our findings suggest that Salmonella achieves programmed delay of virulence gene activation by adjusting levels of an inhibitory factor.

KEYWORDS PhoP, Salmonella, ptsN, virulence regulation

Living cells control gene expression in response to changes in their surroundings through signal transduction systems that detect environmental signals and convert them into cellular processes, including the control of gene expression, thereby altering cellular behavior. To behave appropriately, cells must precisely decide where and when to operate a certain process. Identification of the responding signals of such systems provides understanding about where an organism turns such systems on or off. However, little is known about how an organism decides the timing of system activation (i.e., kinetics of the system). In bacteria, signal responses are primarily mediated by two-component regulatory systems that comprise a signal sensor and a cognate response regulator, which is typically a transcriptional regulator (1). Here we show that
an inhibitory factor shapes the activation of the master virulence regulatory two-component system in the intracellular pathogen *Salmonella enterica*.

Given that intracellular pathogens experience an acidic pH inside the host phagosome (2–4), it is important for them to have a system that can respond to pH changes in order to survive and cause disease inside the host (5–9). The *Salmonella* PhoP/PhoQ two-component system is a master virulence regulatory complex (10, 11) that is activated by acidic pH (12, 13), low Mg$^{2+}$ (14), and certain antimicrobial peptides (15). This system is crucial for *Salmonella* virulence because the lack of either PhoP or PhoQ impairs *Salmonella* pathogenicity (10, 11). Activation of this system by acidic pH is critical for *Salmonella* virulence because the inhibition of acidification of the *Salmonella*-containing vacuole prevents expression of PhoP-activated genes in phagocytic (9, 16) and nonphagocytic (17) cells, limits replication inside macrophages (6, 18), and attenuates virulence in mice (19). Although “turn on” of the PhoP/PhoQ system is necessary for virulence, it is also important to precisely control this system because constant activation of this system renders *Salmonella* avirulent in mice (20).

The *ptsN* gene encodes EIIA$^{Ntr}$, a component of the nitrogen-metabolic phosphotransferase system (PTS) (21, 22). This nitrogen-metabolic PTS lacks a membrane-bound complex that controls the activities of sugar PTSs in response to particular sugar availabilities (21, 22). Recent studies have reported that EIIA$^{Ntr}$ is involved in various cellular functions, including potassium uptake (23, 24), the stringent response (25, 26), and amino sugar homeostasis (27). Moreover, we recently reported that EIIA$^{Ntr}$ promotes virulence by hampering SsrB, a transcriptional regulator of *Salmonella* pathogenicity island 2 (SPI-2) (28). Despite the fact that various regulatory functions of EIIA$^{Ntr}$ have been identified, the regulation of its own expression remains largely unknown.

Here we establish that *Salmonella* alters EIIA$^{Ntr}$ abundance, thereby controlling activation of the PhoP/PhoQ system during infection. Under acidic conditions, *Salmonella* reduces EIIA$^{Ntr}$ amounts by Lon-mediated degradation in a PhoP-dependent manner. EIIA$^{Ntr}$ hampers PhoP binding to its target DNA, thereby decreasing expression of PhoP-activated genes under acidic pH conditions. This double-negative regulation results in an overall positive feedback that furthers activation of the system. Our findings suggest that *Salmonella* ensures the timing and extent of its PhoP/PhoQ-mediated virulence program via regulation of an inhibitory factor during infection.

**RESULTS**

EIIA$^{Ntr}$ amounts decrease upon environmental acidification. To investigate the expression of EIIA$^{Ntr}$, we first investigated its transcription levels by measuring β-galactosidase activity produced by a $p_{rpoN}$-*lacZ* fusion given that the *ptsN* gene is located downstream of the *rpoN* gene, forming an operon (21). Although EIIA$^{Ntr}$ is a component of a nitrogen-metabolic PTS (21, 22), transcription levels of *rpoN* remained unaltered by 100-fold changes in the concentration of a nitrogen source (Fig. 1A). We next examined *rpoN* expression at different pH values or concentrations of Mg$^{2+}$, representing environmental conditions that *Salmonella* might encounter during infection (6, 14). However, none of those changes modified the expression of *rpoN* (Fig. 1B and C).

Despite the absence of notable changes in $p_{rpoN}$-*lacZ* expression, we examined EIIA$^{Ntr}$ protein amounts under these conditions. Similar to *rpoN* expression, EIIA$^{Ntr}$ amounts were not responsive to changes in nitrogen source (Fig. 1D). Surprisingly, however, EIIA$^{Ntr}$ abundance was significantly reduced when *Salmonella* was exposed to low-Mg$^{2+}$ or acidic-pH conditions (Fig. 1E and F).

This *rpoN*-independent alteration of EIIA$^{Ntr}$ levels (Fig. 1A to F) raised the possibility that transcription of the *ptsN* gene might not just be from the *rpoN* promoter. Indeed, primer extension analysis indicated the presence of a transcriptional start site 67 nucleotides upstream of the EIIA$^{Ntr}$ start codon (Fig. 1G). Therefore, we investigated the expression of a $p_{ptsN}$-*lacZ* transcriptional fusion in bacteria grown under the above-described conditions. However, none of those conditions altered the expression of
Taken together, these results suggest that *Salmonella* probably controls EIIA^Ntr^ levels through posttranscriptional regulatory mechanisms.

**PhoP decreases EIIA^Ntr^ abundance posttranscriptionally.** Given that an acidic pH and low Mg^{2+} are signals activating the sensor PhoQ (12–14), we hypothesized that the PhoP/PhoQ system might be involved in altering EIIA^Ntr^ amounts. To test this hypothesis, we examined the transcription and translation levels of EIIA^Ntr^ in isogenic wild-type and the *phoP* mutant *Salmonella* strains. Consistent with the expression of the *ptsN-lacZ* fusion gene from a plasmid (Fig. 1H to J), the chromosomal *ptsN-lacZ* fusion also showed similar β-galactosidase activities under acidic and neutral pH conditions (see Fig. S1A in the supplemental material). Moreover, mutation of the *phoP* gene did not alter *ptsN* expression (Fig. S1A). In contrast, EIIA^Ntr^ amounts were significantly higher in the *phoP* null mutant than in the wild type when grown under acidic conditions (Fig. 2A), indicating that PhoP reduces EIIA^Ntr^ amounts independent of its transcription.

The increased EIIA^Ntr^ abundance in the *phoP* mutant was restored by a plasmid expressing PhoP from a heterologous promoter (Fig. 2A). The absence of the cognate sensor kinase PhoQ also coordinated with a higher abundance of EIIA^Ntr^ (Fig. S1B) as in the *phoP* mutant (Fig. 2A), indicating that PhoP's action in altering EIIA^Ntr^ abundance is dependent on PhoP's phosphorylation. Furthermore, the lack of PhoP increased EIIA^Ntr^...
FIG 2 PhoP reduces EIIANtr abundance by destabilizing it via Lon protease. (A and B) Western blot analysis of crude extracts prepared from Salmonella expressing EIIANtr-FLAG from the normal chromosomal location (ptsN-FLAG), an isogenic phoP mutant, and the phoP mutant harboring a plasmid expressing PhoP under the control of an IPTG-inducible promoter (A) and Salmonella strains with deletions of the ptsN or ptsN and phoP genes harboring a plasmid expressing EIIANtr-FLAG from an IPTG-inducible promoter (B). Bacteria were grown in acidified M9 medium (pH 5.8) with or without IPTG (A, 0, 10, and 100 μM IPTG [from left to right]). Representative results from at least three independent experiments are shown. (C) Stabilities of ptsN mRNA were determined from wild-type (WT) and phoP mutant Salmonella. Bacteria were grown in M9 medium, pH 5.8, then the cultures were split in two and one was treated with 100 μg/ml of rifampin (Rif). Samples were collected at the indicated time points upon treatment. The means and SDs from three independent experiments are shown. (D to F) Western blot analysis of crude extracts prepared from ptsN-FLAG wild-type and isogenic phoP mutant strains (D), ptsN-FLAG wild-type and isogenic mutants with phoP, clpX, or lon gene deletions (E), and ptsN-FLAG wild-type and lon mutant strains (F). For ptsN-FLAG wild-type and isogenic phoP mutant strains, bacteria were grown in acidified M9 medium, and translation was stopped by addition of 200 μg/ml of chloramphenicol. Samples were collected at the indicated time points upon treatment. For ptsN-FLAG wild-type and isogenic mutants with phoP, clpX, or lon gene deletions, bacteria were grown in acidified M9 medium. For ptsN-FLAG wild-type and lon mutant strains, bacteria were grown in acidified M9 medium, and translation was stopped by the addition of 200 μg/ml of chloramphenicol (Cm). Samples were collected at the indicated time points upon treatment. $t_{1/2}$, half-life of EIIANtr. Representative results from at least three independent experiments are shown.

EIIANtr protein is degraded under acidic conditions in a PhoP-dependent manner. We next investigated how PhoP controls EIIANtr levels posttranscriptionally. PhoP may decrease EIIANtr levels by reducing the stabilities of ptsN mRNA and/or EIIANtr protein. ptsN mRNA showed similar levels of decay in both the wild type and the isogenic phoP mutant upon addition of rifampin to stop transcription (Fig. 2C). In contrast, the amount of EIIANtr decreased in the wild type after inhibition of protein synthesis with chloramphenicol treatment (half-life $t_{1/2} < 60$ min), whereas it remained constant in the strain lacking PhoP ($t_{1/2} > 120$ min) (Fig. 2D). Furthermore, this EIIANtr degradation was detected when Salmonella was grown at an acidic pH but not at a neutral pH (Fig. S2). These results suggest that PhoP boosts the degradation of EIIANtr protein in an acidic environment.

Lon protease mediates PhoP-dependent degradation of EIIANtr. Cytoplasmic proteases, including ClpXP and Lon, are involved in the proteolysis of cytosolic proteins in Gram-negative bacteria (29), and EIIANtr is a cytoplasmic protein. As PhoP counteracts ClpXP-mediated proteolysis of RpoS via IraP (30), we first investigated the potential role of ClpXP in controlling EIIANtr abundance. A Salmonella strain lacking ClpXP produced amounts of EIIANtr comparable to those of the wild type, unlike the phoP mutant (Fig. 2E). However, a lack of Lon increased the abundance of EIIANtr protein compared with that of the wild type, similar to the case with the phoP mutant strain (Fig. 2E). If Lon is responsible for the degradation of EIIANtr, the lon mutant should make EIIANtr stable. Like the phoP mutant (Fig. 2D), the lon mutant displayed sustained abundance of EIIANtr after chloramphenicol treatment ($t_{1/2} > 120$ min), whereas EIIANtr levels dwindle in the wild type ($t_{1/2} < 60$ min) (Fig. 2F). Furthermore, double deletion of the phoP and lon genes resulted in amounts of EIIANtr comparable to those in the phoP or

abundance (Fig. 2B) even when transcription of ptsN was induced by isopropyl-β-D-thiogalactopyranoside (IPTG), further supporting the notion that PhoP modulates the abundance of EIIANtr posttranscriptionally.
single-deletion mutants (Fig. S3). These results suggest that PhoP favors Lon protease-mediated degradation of EIIANtr.

We next wondered why Salmonella curtails EIIANtr amounts when it encounters an acidic pH, a PhoP-inducing condition inside macrophage phagosomes (31). To understand the role of EIIANtr, we investigated genes that are regulated by EIIANtr using a DNA microarray experiments with wild-type and isogenic ptsN mutant strains grown in acidified mini- mal medium. We found 768 differentially expressed genes in the ptsN mutant compared with the wild type (H11022x2-fold): 371 upregulated genes and 397 downregulated genes (Fig. S4). Consistent with a previous report (28), SPI-2 genes were more highly expressed in the ptsN mutant than in the wild type (Table S1). Interestingly, we found that transcript levels of PhoP-regulated genes were higher in the strain lacking EIIANtr than in the wild type (Table S1). We further verified the EIIANtr’s regulatory effects on PhoP-activated genes using quantitative reverse transcription-PCR (qRT-PCR): the ptsN mutant displayed 3- to 7-fold-higher transcript levels of PhoP-regulated genes than the wild type (Fig. 3A). Moreover, the elevated expression of those genes in the ptsN mutant was restored to wild-type levels by a plasmid expressing the ptsN gene from a heterologous promoter but not by the plasmid vector (Fig. 3A). Interestingly, plasmid-driven heterologous expression of an unphosphorylatable variant of EIIANtr (H73A) or a variant of EIIANtr mimicking the phosphorylated form (H73E) (25) was also able to rescue the expression of PhoP-regulated genes similarly to wild-type EIIANtr (Fig. 3A). These results suggest that EIIANtr modulates the expression of PhoP target genes independent of EIIANtr’s phosphorylation status and PhoP transcription.

Control of PhoP-regulated genes by EIIANtr requires PhoP. We next wondered whether EIIANtr controls expression of PhoP to regulate PhoP regulon. If EIIANtr directly controls PhoP-activated genes, EIIANtr should be able to regulate those genes in the absence of PhoP. However, the absence of PhoP abrogated the regulatory effects of EIIANtr on the expression of PhoP-activated genes (Fig. 3B), indicating that control of the PhoP regulon by EIIANtr requires PhoP. If the regulation of PhoP-regulated genes by EIIANtr is due to altered phoP transcription, heterologous expression of phoP from a

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EIIANtr negatively controls expression of PhoP-regulated genes. We next wondered why Salmonella curtails EIIANtr amounts when it encounters an acidic pH, a PhoP-inducing condition inside macrophage phagosomes (31). To understand the role of EIIANtr, we investigated genes that are regulated by EIIANtr using a DNA microarray experiments with wild-type and isogenic ptsN mutant strains grown in acidified mini- mal medium. We found 768 differentially expressed genes in the ptsN mutant compared with the wild type (>2-fold): 371 upregulated genes and 397 downregulated genes (Fig. S4). Consistent with a previous report (28), SPI-2 genes were more highly expressed in the ptsN mutant than in the wild type (Table S1). Interestingly, we found that transcript levels of PhoP-regulated genes were higher in the strain lacking EIIANtr than in the wild type (Table S1). We further verified the EIIANtr’s regulatory effects on PhoP-activated genes using quantitative reverse transcription-PCR (qRT-PCR): the ptsN mutant displayed 3- to 7-fold-higher transcript levels of PhoP-regulated genes than the wild type (Fig. 3A). Moreover, the elevated expression of those genes in the ptsN mutant was restored to wild-type levels by a plasmid expressing the ptsN gene from a heterologous promoter but not by the plasmid vector (Fig. 3A). Interestingly, plasmid-driven heterologous expression of an unphosphorylatable variant of EIIANtr (H73A) or a variant of EIIANtr mimicking the phosphorylated form (H73E) (25) was also able to rescue the expression of PhoP-regulated genes similarly to wild-type EIIANtr (Fig. 3A). These results suggest that EIIANtr modulates the expression of PhoP target genes independent of EIIANtr’s phosphorylation status and PhoP transcription.

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**FIG 3** EIIANtr reduces PhoP-regulated gene expression in a PhoP-dependent fashion. (A and B) mRNA levels of PhoP-activated pmrD, pagD, mig-14, and mgtA were determined in Salmonella wild-type, ptsN mutant, and ptsN mutant strains harboring a plasmid expressing EIIANtr, EIIANtr (H73A), or EIIANtr (H73E) (pPtsN, pPtsN(H73A), or pPtsN(H73E) or an empty vector (pVec)) (A) and Salmonella wild-type and isogenic mutants with deletions of the ptsN, phoP, or ptsN and phoP genes (B). Bacteria were grown in M9 (pH 5.8). (C) β-Galactosidase activities of Salmonella with a pPagD-lacZ fusion in the normal chromosomal location and isogenic mutants with deletions of the ptsN, phoP, or ptsN and phoP genes with the indicated plasmids (empty vector [pVec] or plasmid expressing PhoP [pPhoP]) were determined. Bacteria were grown in acidified M9 medium with 100 μM IPTG. The means and SDs from three independent experiments are shown.
plasmid should abolish the effect of EI\textit{A}\textsubscript{Ntr} on the expression of those genes. A lack of EI\textit{A}\textsubscript{Ntr} increased expression levels of PhoP-regulated genes, even when PhoP was produced from a heterologous promoter (Fig. 3C). These results indicate that EI\textit{A}\textsubscript{Ntr} regulates PhoP regulon in a PhoP-dependent manner.

EI\textit{A}\textsubscript{Ntr} hampers PhoP binding to its target promoter DNA. Given that EI\textit{A}\textsubscript{Ntr} regulates other regulatory systems via protein-protein interaction (23, 24, 28, 32), we next investigated whether EI\textit{A}\textsubscript{Ntr} interacts with the PhoP protein. We used the bacterial two-hybrid system, in which \(\beta\text{-galactosidase} \) levels are dependent on the proximity of fused proteins to fragments (i.e., T25 and T18) of the \textit{Bordetella pertussis} adenylate cyclase in an \textit{Escherichia coli} strain lacking its own adenylate cyclase (33). Coexpression of T25-EI\textit{A}\textsubscript{Ntr} and T18-PhoP resulted in approximately 141-fold-higher levels of \(\beta\text{-galactosidase} \) activity than in strains expressing T25–EI\textit{A}\textsubscript{Ntr} and T18 fragment or empty vectors (Fig. 4A), indicating that EI\textit{A}\textsubscript{Ntr} interacts with PhoP. This activity was comparable to that from the positive-control strain harboring T25 and T18 fragments fused to the leucine zipper of the transcription factor GCN4 (Fig. 4A). Consistent with the observation that unphosphorylatable EI\textit{A}\textsubscript{Ntr} (H73A) functions like the wild type in controlling the PhoP regulon (Fig. 3A), EI\textit{A}\textsubscript{Ntr} (H73A) displayed an interaction with PhoP similar to that of the wild-type protein (Fig. 4A).

PhoP promotes transcription of the PhoP regulon by binding to DNA (34) when PhoP is activated by PhoQ-mediated phosphorylation under inducing conditions (35) or by reducing acetylation of PhoP (36). Thus, the interaction of EI\textit{A}\textsubscript{Ntr} with PhoP could decrease PhoP activity by inhibiting the interaction of PhoP with the cognate kinase PhoQ (i.e., reducing phosphorylation), by promoting acetylation of PhoP, or by reducing deacetylation of PhoP. Alternatively, EI\textit{A}\textsubscript{Ntr} could interfere with PhoP binding to DNA.

If EI\textit{A}\textsubscript{Ntr} hampers PhoP phosphorylation by PhoQ, the lack of PhoQ should abolish the regulatory effects of EI\textit{A}\textsubscript{Ntr} on the PhoP regulon. Because PhoP is not active in the absence of PhoQ, we investigated the function of EI\textit{A}\textsubscript{Ntr} in a \textit{phoP}\textsuperscript{−} phoQ strain lacking PhoQ and expressing a PhoP variant that autophosphorylates from acetyl phosphate (35). EI\textit{A}\textsubscript{Ntr} reduced \textit{pagD} expression even in the absence of PhoQ (Fig. S5), indicating that the regulatory action of EI\textit{A}\textsubscript{Ntr} is independent of PhoQ. We next examined
whether acetylation of PhoP is responsible for ElIA
mediated regulation of PhoP target genes by mutating known acetylase (Pat) or deacetylase (CobB) (36). However, ElIA
displayed similar regulatory effects on pagD expression in the absence of Pat or CobB (Fig. S5).

To test whether EIIANtr inhibits PhoP’s DNA binding ability, a gel shift assay was conducted using purified PhoP and EIIANtr proteins with the phoP-activated pagD promoter. Purified PhoP bound to the pagD promoter DNA and formed a complex with the probe DNA in vitro (Fig. 4B). ElIA
prevented PhoP from binding to the target DNA: the PhoP-DNA complex decreased to generate the unbound pagD promoter DNA when amounts of ElIA
increased (although an excess of ElIA
could not fully dissociate PhoP from DNA), and ElIA
alone did not form a complex with the DNA (Fig. 4B). However, addition of an EIIA
paralogue, EIIAGlc, did not alter binding of PhoP to DNA, indicating that it is specific to EIIANtr (Fig. S6A). This inhibitory function of EIIANtr is specific, as another regulatory protein, PmrA, bound to the pbp promoter DNA regardless of EIIANtr (Fig. S6B). Taken together, these data suggest that EIIANtr reduces expression of the PhoP regulon by inhibiting PhoP binding to its target promoter DNA.

EIIANtr delays activation of PhoP target genes inside macrophages. We next examined if EIIANtr could control the expression of PhoP-activated genes during infection. To evaluate this, macrophages were infected with wild-type and mutant Salmonella strains harboring a gfp fusion with the promoter of the PhoP-activated gene pagD, and fluorescence was measured. Activation of pagD expression inside macrophages was completely dependent on PhoP, because the phoP mutant was unable to produce any fluorescence, in contrast to the wild type (Fig. 5A). The ptsN mutant showed higher and earlier activation of pagD expression than the wild type (Fig. 5A), indicating that EIIANtr inhibits PhoP activation inside macrophages. These results suggest that Salmonella delays expression of PhoP-activated genes inside macrophages via EIIANtr. The ptsN mutant also displayed accelerated activation of the pagD gene compared to that of the wild type in acidic pH (Fig. 5A). Consistent with a previous report (28), the lack of EIIANtr rendered Salmonella virulence attenuated in mice inoculated via the intraperitoneal route (Fig. 5B, left). Defective virulence of the ptsN mutant Salmonella was also observed when mice were inoculated via the oral route (Fig. 5B, right). These results are in agreement that EIIANtr controls the expression of
various virulence genes, including the PhoP and SsrB regulons (Fig. 3) (28). And it is possible that the delayed virulence gene expression by EIIA^{Ntr} might be critical for *Salmonella* pathogenicity.

**DISCUSSION**

In this study, we established that *Salmonella* employs EIIA^{Ntr} to delay the activation of its virulence program inside acidic phagosomes (Fig. 6). The master virulence regulator PhoP promotes the Lon-mediated degradation of EIIA^{Ntr} under acidic conditions (Fig. 1 and 2); this, in turn, favors the expression of PhoP-activated genes under acidic conditions (Fig. 3) and inside macrophages (Fig. 5A). Thus, the reduction of EIIA^{Ntr} amounts in the acidic phagosome allows delayed but robust activation of the *Salmonella* virulence program, including the PhoP/PhoQ system as well as SPI-2 genes (28) (Fig. 6), thereby enhancing its fitness inside the host (11, 28) (Fig. 5B).

Although nitrogen availability controls the phosphorylation status of EIIA^{Ntr} (37), total amounts of EIIA^{Ntr} remain unaltered in vivo (37) (Fig. 1D), suggesting that EIIA^{Ntr} phosphorylation status is important for its regulatory function. However, the role of EIIA^{Ntr} phosphorylation in its regulatory function is controversial: some EIIA^{Ntr} activities are dependent on its phosphorylation status (23, 24, 26, 27), whereas others are not (24, 25, 27, 28, 32, 38). EIIA^{Ntr} interacts with PhoP and decreases expression of PhoP-activated genes regardless of its phosphorylation status (Fig. 3A and Fig. 4A). Moreover, EIIA^{Ntr} accumulated in the *phoP* mutant of even in the absence of EII^{Ntr} (encoded by the *ptsP* gene), the phosphor donor for EIIA^{Ntr} (Fig. 5B). Together with previous reports (24, 25, 27, 28, 32, 38), these findings indicate that EIIA^{Ntr} operates some functions regardless of its phosphorylation status, suggesting that it is important to understand how bacteria control cellular amounts of EIIA^{Ntr} protein.

In this study, we established that *Salmonella* modulates EIIA^{Ntr} abundance via Lon-mediated degradation in a PhoP-dependent manner (Fig. 2). Moreover, we demonstrated that the *ptsN* gene has its own transcriptional start site, although the *ptsN* gene is considered a component of the *rpoN* operon (21). This raises the possibility that *Salmonella* may control expression of *ptsN* independent of *rpoN*. Indeed, a recent transcriptome sequencing (RNA-seq) study showed that nitrogen oxide...
shock reduces ptsN transcript levels but does not alter rpoN mRNA levels (39). Moreover, a recent study has shown that EliaNtr is degraded by Lon in the absence of GlnS and N-acetylglucosamine, although this degradation was not observed in the wild type (27). Furthermore, EliaNtr accumulates in the presence of acetylglucosamine in a degradation-independent manner (27). As a transcriptional regulator, PhoP probably induces an adapter-like protein that can alter degradability of EliaNtr by Lon in a PhoP-dependent manner; rather, PhoP brings EliaNtr to Lon. PhoP controls 9% of Salmonella genes (40) despite the fact that limited numbers of its direct targets are known. Given that EliaNtr regulates 768 genes (Fig. S4), PhoP perhaps controls a subset of genes via EliaNtr.

Our findings now provide mechanisms for how EliaNtr contributes to Salmonella virulence. EliaNtr tunes the timing and extent of virulence regulatory systems’ activation inside host cells (Fig. 3A and Fig. 5A) (28), thereby enabling Salmonella to properly manage its virulence program. In addition, there are other biological processes regulated by EliaNtr, and they are potentially involved in bacterial virulence, which includes ppGpp accumulation, metabolism to produce amino sugars, and potassium uptake (23, 24, 26, 27). Thus, Salmonella probably changes various processes by altering EliaNtr abundance during infection.

The function of EliaNtr described here may explain the different behaviors of PhoP-regulated genes in in vitro cultures and inside macrophages; the expression of PhoP-activated genes reaches maximal levels 5 to ~25 min after exposure to an environment that activates PhoQ (41), whereas it takes hours inside macrophages (16, 42) (Fig. 5A). Moreover, full activation of PhoP in acidic pH requires a PhoP-activated UgtL protein which amplifies the response of PhoQ to an acidic environment (43). Because EliaNtr binds to and hampers SsrB (28), induction of SsrB would favor PhoP activation by reducing the number of EliaNtr proteins interacting with PhoP. Moreover, inhibition of PhoP enables EliaNtr to efficiently hamper the SsrB regulon given that PhoP transcriptionally activates SsrB (44) and that EliaNtr hinders SsrB’s regulatory function (28).

Although the activation of the pagD gene inside macrophages was delayed in the wild type, maximal levels of activation were similar in the wild type and the ptsN mutant (Fig. 5A). In acidified defined media, however, the wild-type and the ptsN mutant strains did not show similar maximal levels of pagD expression (Fig. 3 and Fig. S7A). This might be due to difference in conditions that Salmonella experiences: acidic pH in defined media and complicated conditions inside acidic phagosomes. Moreover, PhoQ responds to multiple signals, including acidic pH, low Mg2+, antimicrobial peptides, and high osmolarity (45). EliaNtr reduces pagD gene expression not only at acidic pH (Fig. 3 and Fig. 5) but also under conditions stimulating PhoQ, low Mg2+ (Fig. S7B), and antimicrobial peptide C18G (Fig. S7C). Thus, we want to note that other PhoQ-inducing signals and/or other components inside phagosomes potentially contributing to the activation of the PhoP/PhoQ system inside phagosomes probably play a role in modulating EliaNtr-mediated function during infection.

Pathogens possess virulence genes that enable them to cause disease in the host. The EliaNtr gene can be defined as a virulence gene because it promotes Salmonella virulence in mice (28) (Fig. 5B). Paradoxically, EliaNtr antagonizes the functions of other virulence regulatory systems, such as PhoP/PhoQ and SsrB/SpiR, although the deletion of them highly attenuates Salmonella pathogenicity (11, 46). This inhibition of virulence regulators by EliaNtr delays the timing of their activation (Fig. 5A) and probably allows robust activation once the amount of active regulator(s) supersedes the inhibitory threshold created by EliaNtr (Fig. 6).

Why does Salmonella limit activation of virulence regulatory systems via EliaNtr during infection, although this may potentially decrease its pathogenicity? One possible explanation is that overactivation of those virulence systems might be harmful to Salmonella survival inside the host. Hyperconstitutive activation of the PhoP/PhoQ system actually attenuates Salmonella virulence in mice (20). Moreover, PhoP activates not only virulence factors but also antivirulence factors (47–49). Balancing those
virulence and antivirulence factors is probably important to achieving optimal fitness inside the host. In addition, it is possible that retarding induction of the PhoP-activated virulence program may allow Salmonella to efficiently replicate and spread to other cells. Because PhoP-activated SPI-2 genes result in macrophage death (50, 51) and early activation of SPI-2 genes accelerates cell death (48), delayed activation of SPI-2 genes probably allows Salmonella sufficient time to replicate inside the host cell.

Furthermore, Elia<sup>Ntr</sup> may also help Salmonella efficiently turn off those systems when unnecessary (e.g., when Salmonella escapes from phagocytes). Efficient transition between the “on” and “off” states of virulence regulatory systems allows the bacterial virulence program to operate efficiently and saves energy by reducing unnecessary usage.

**MATERIALS AND METHODS**

**Bacterial strains, plasmids, and growth conditions.** The *Salmonella enterica* serovar Typhimurium strains used in this study were derived from strain SL1344. The strains and plasmids used in this study are listed in Table S2A. Phage P22-mediated transduction was performed as described previously (52). All *Salmonella* strains were grown aerobically at 30 or 37°C in Luria-Bertani (LB) or M9 minimal medium at the desired pH and Mg<sup>2+</sup> concentrations to mid- to late log phase unless specified. Antimicrobial peptide C18G was treated at 5 μg/ml for an hour. Antibiotics were used at the following concentrations: ampicillin, 50 μg/ml; chloramphenicol, 25 μg/ml; and kanamycin, 50 μg/ml. Primers used for the construction of bacterial strains and plasmids are listed in Table S2B.

**Construction of mutant *Salmonella* strains.** To generate a *ptsN-FLAG* strain, a cat cassette was introduced in the 3’ end of the *ptsN* gene as follows: the cat fragment was amplified from pKD3 using primers *ptsN*-FLAG-F/*ptsN*-FLAG-R and then introduced into wild-type *Salmonella* (SL1344) harboring plasmid pKD46 as previously described (53). The cat cassette was removed with plasmid pCP20 (53).

To generate a *ptsP* mutant, a cat fragment was amplified from pKD3 using primers *ptsP*-Red-F/*ptsP*-Red-R and then introduced into wild-type *Salmonella* harboring plasmid pKD46 (53).

To generate a *phoP* mutant strain, a kan fragment was amplified from pKD13 using primers *phoP*-Red-F/*phoP*-Red-R and then introduced into wild-type *Salmonella* harboring plasmid pKD46 (53). Next, the *kan* cassette was removed with plasmid pCP20 (53).

To generate a *phoQ* mutant strain, a kan fragment was amplified from pKD13 using primers *phoQ*-Red-F/*phoQ*-Red-R and then introduced into wild-type *Salmonella* harboring plasmid pKD46 (53). Next, the *kan* cassette was removed with plasmid pCP20 (53).

To generate a *cobB* mutant strain, a *kan* fragment was amplified from pKD13 using primers *cobB*-P1-F-kan/*cobB*-P4-R-kan and then introduced into wild-type *Salmonella* harboring plasmid pKD46 (53).

To generate a *pat* mutant strain, a *kan* fragment was amplified from pKD13 using primers *pat*-P1-F-kan/*pat*-P4-R-kan and then introduced into wild-type *Salmonella* harboring plasmid pKD46 (53).

To generate a *clpP* mutant strain, a cat fragment was amplified from pKD3 using primers *clpP*-Red-F/*clpP*-Red-R and then introduced into wild-type *Salmonella* harboring plasmid pKD46 (53). Next, the *kan* cassette was removed with plasmid pCP20 (53).

To generate a *lon* mutant, a cat fragment was amplified from pKD3 using primers *lon*-Red-F/*lon*-Red-R and then introduced into wild-type *Salmonella* harboring plasmid pKD46 (53). Next, the *kan* cassette was removed with plasmid pCP20 (53).

To generate a strain with the p<sub>pHofP</sub>-lacZ fusion in the normal chromosomal location, pCP20 was introduced into SR3203 (the *ptsN* mutant). Next, the lacZ fusion was generated with plasmid pCE70 (54).

To generate a strain with the p<sub>pHofP</sub>-lacZ fusion in the normal chromosomal location, a cat fragment was amplified from pKD3 using primers *pagD*-1/*pagD*-2 and then introduced into wild-type *Salmonella* (SL1344) harboring plasmid pKD46 as previously described (53). The cat cassette was removed with plasmid pCP20 (53). Next, the lacZ fusion was generated with plasmid pCE70 (54).

**Construction of plasmids.** A plasmid expressing *ptsN*-FLAG was constructed as follows: the *ptsN*-FLAG cassette was removed with plasmid pCP20 (53). Next, the *lacZ* fragment was generated with plasmid pCE70 (54).

A plasmid expressing *phoP* gene was constructed as follows: the *phoP* coding region was amplified from wild-type *Salmonella* (SL1344) using primers *phoP*-com-F/*phoP*-com-R and then introduced between the EcoRI and BamHI sites of pUHE21-2-lacZ<sup>55</sup> (55).

A plasmid expressing *phoQ* gene was constructed as follows: the *phoQ* coding region was amplified from wild-type *Salmonella* (SL1344) using primers *phoQ*-com-F/*phoQ*-com-R and then introduced between the EcoRI and BamHI sites of pUHE21-2-lacZ<sup>55</sup> (55).

Plasmids expressing Elia<sup>Ntr</sup> variants (Elia<sup>Aw</sup> [H73A] and Elia<sup>Ae</sup> [H73E]) were constructed as follows: the pU14 plasmid was mutated using a QuickChange Lightning site-directed mutagenesis kit (Agilent Technologies) with primers *ptsN*-H73A-F/*ptsN*-H73A-R for H73A substitution and *ptsN*-H73A-F/*ptsN*-H73A-R for H73E substitution.

A plasmid expressing His<sub>6</sub>-tagged PhoP was constructed as follows: the *phoP* coding region was amplified from wild-type *Salmonella* (SL1344) using primers *pPhoP*-F/*pPhoP*-His6-R and then introduced between the BamHI and HindIII sites of pUHE21-2-lacZ<sup>55</sup> (55).
A plasmid expressing His6-tagged PmrA was constructed as follows: the pmrA coding region was amplified from wild-type Salmonella (SL1344) using primers pmrA-pF/pmR/pmR and pmrA-pF/pmR, respectively. Next, they were introduced between the EcoRI and BamHI sites of pET21a. A plasmid expressing T7-PPhoP fusion protein was constructed as follows: the phoP gene was amplified from wild-type Salmonella (SL1344) using primers phoP-F/phoP-R and then introduced between the BamHI and EcoRI sites of pET21a.

**Western blotting.** Salmonella strains expressing the EIIA^Amr^−FLAG protein from its normal chromosomal location or under the control of a heterologous promoter were grown as described in “Bacterial strains, plasmids, and growth conditions,” above. Bacteria were collected by centrifugation, and cell lysates were prepared using B-PER solution (Pierce). Cell lysates were separated by 2% SDS-PAGE, and selected fractions were dialyzed against buffer C (20 mM Tris (pH 8.0), 150 mM NaCl, and 250 mM imidazole). The fractions were then collected and analyzed by the ECL detection system (Amersham Biosciences).

**β-Galactosidase assay.** β-Galactosidase assays were carried out in triplicate, and the activity was determined as described previously (59).

**RNA isolation and quantitative RT (qRT)-PCR.** Salmonella strains were grown as described above, and total RNA was isolated using an RNeasy minikit (Qiagen). After DNase treatment of the isolated RNA, cdNA was synthesized using Omniscript reverse transcription reagents (Qiagen) and random hexamers (Invitrogen). Quantitative RT-PCR was carried out using 2× IQ SYBR Green Supermix (Bio-Rad), and real-time amplification of the PCR products was performed using the iCycler IQ real-time detection system (Bio-Rad). The primers used for detection of the gene transcripts are listed in Table S2B. Data were normalized to the abundance of 16S rRNA expression levels.

**RNA or protein stability analyses.** To test RNA stability, bacterial cultures were treated with 0.1 mg/ml of rifampin to stop transcription, and samples were collected at the desired time points. Total RNA was isolated, and mRNA levels were determined by qRT-PCR as described above. To test the protein stability, bacterial cultures were treated with 0.2 mg/ml of chloramphenicol to stop protein synthesis, and samples were collected at the desired time points. Protein levels were analyzed using Western blot analysis.

**Bacterial two-hybrid assay.** E. coli BTH101 organisms harboring derivatives of plasmids pUT18 and pK725 were grown overnight in LB broth containing ampicillin (100 μg/ml) and kanamycin (50 μg/ml), adding a 1:100 dilution to 1 ml of the same fresh medium containing 0.5 mM IPTG and employing shaking at 30°C overnight as previously described (33, 58).

**Purification of proteins.** His- tagged PhoP, His6-tagged PmrA and His6-tagged EIIA^Amr^− were expressed in E. coli BL21(DE3). Bacterial cells were grown in LB medium at 37°C until the optical density at 600 nm (OD600) reached 0.5, and the expression of those proteins was induced by addition of IPTG (0.5 M) followed by growth at 30°C for 8 h. Cells were harvested, washed, and suspended in buffer A (20 mM Tris [pH 8.0], 150 mM NaCl, and 20 mM imidazole). Then the cells were disrupted by sonication, and cell debris was removed by centrifugation at 20,000 × g for 4°C for 30 min. The supernatant was applied to a 1.5-ml nickel- nitrilotriacetic acid (Ni-NTA) agarose column equilibrated in buffer A, washed with a 1.5-column volume of the same buffer, and eluted using a gradient of buffer A and buffer B (20 mM Tris [pH 8.0], 50 mM NaCl, and 250 mM imidazole). The fractions were then collected and analyzed by SDS-PAGE, and selected fractions were dialyzed against buffer C (20 mM Tris [pH 8.0], 150 mM NaCl, and 10% glycerol).

**EMSA.** DNA fragments containing the promoter region of the pagD or pbgP gene were amplified by PCR using the primers EMSA-pagD-F/EMSA-pagD-R and EMSA-pbgP-F/EMSA-pbgP-R, respectively. Purified promoter DNA (80 fmol) was incubated with the desired concentrations of purified PhoP-His6, or PmrA-His6, with EIIA-Amr-His6, or EIIA-C6-His6, at room temperature for 20 min in 15 μl of binding buffer (10 mM Tris [pH 7.5], 0.5 mM EDTA, 1 mM MgCl2, 0.5 mM dithiothreitol [DTT], and 50 mM NaCl) containing 5 ng/μl of poly(dI-dC). Samples were prepared by addition of 3 μl of 6× electrophoretic mobility shift assay (EMSA) gel loading solution and separated by electrophoresis using a 6% nondenaturing polyacrylamide gel. DNA staining was performed according to the manufacturer’s instructions (EMSA kit; E33075; Thermo Fisher Scientific).

**Macrophenage infection assay.** The murine-derived macrophage line RAW264.7 was cultured in Dulbecco’s modified Eagle’s medium (DMEM; Life Technologies) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Life Technologies) at 37°C with 5% CO2. Macrophages were seeded in 24-well tissue culture plates at 5 × 10⁵ per well 1 day before infection with Salmonella. Confluent monolayers were inoculated with bacterial cells that had been grown overnight in LB broth, washed with phosphate-buffered saline (PBS), and resuspended in 0.1 ml of prewarmed DMEM at a multiplicity of infection of 20. Following a 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 μg/ml of gentamicin for 1 h to kill extracellular bacteria. Next, the wells were washed three times with prewarmed PBS and incubated with prewarmed medium supplemented with 10 μg/ml of gentamcin. At the desired time points, the cells were washed three times with prewarmed PBS and subjected to the following procedures. For green fluorescent protein (GFP) assessment, washed cells were scraped with 200 μl of PBS and subjected to fluorescence measurements at 510 nm. For CFU measurements,
washed cells were lysed with PBS containing 1% Triton X-100 and plated on LB agar plate at the proper dilutions.

**Mouse virulence assay.** Six-week-old female BALB/c mice were purchased from the Institute of Laboratory Animal Resources at Seoul National University. Five mice in each group were infected intraperitoneally or orally with 0.1 ml of PBS containing approximately 10^2 or 10^6 *Salmonella* cells grown in LB broth overnight, respectively. 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 the regulations of the Institutional Animal Care and Use Committee of Seoul National University.

**Transcriptomic analysis.** RNA labeling, hybridization to the microarrays, scanning, and data analysis were performed at Macrogen. Triplicates of total RNAs from wild-type and ptsN mutant strains grown in acidified M9 medium (pH 5.8) were purified as described above and subjected to microarray using a CombiMatrix chip for the *Salmonella Typhimurium* SL1344 genome (12,396 probes covering 4,441 genes). Arrays were scanned using the Axon GenePix 4000B scanner (Molecular Devices LLC). Image analysis and feature extraction were performed using Axon GenePix Pro software (Molecular Devices). The data were analyzed using Avadis Prophetic software version 3.3 (Strand Genomics). Fold changes were calculated by comparing averaged normalized signal intensities in wild-type versus ptsN mutant *Salmonella*. The t test was performed in parallel with the use of a false-discovery rate correction for multiple testing (*P* < 0.05) was used to pinpoint significantly different expression levels of genes. A cutoff of a 2-fold change for up- or downregulated expression was chosen to define genes that were differentially expressed.

**Mapping of the transcription start site (primer extension assay).** Reverse transcription was conducted using ptsN-P1 and Superscript II (Invitrogen). The ladder was generated with a template DNA that was amplified using primers ptsN-PE-F/ptsN-PE-R and the genomic DNA of SL1344.

**SUPPLEMENTAL MATERIAL**

Supplemental material for this article may be found at [https://doi.org/10.1128/mBio.00291-19](https://doi.org/10.1128/mBio.00291-19).

**FIG S1**, PDF file, 0.2 MB.

**FIG S2**, PDF file, 0.2 MB.

**FIG S3**, PDF file, 0.2 MB.

**FIG S4**, PDF file, 0.2 MB.

**FIG S5**, PDF file, 0.2 MB.

**FIG S6**, PDF file, 0.2 MB.

**FIG S7**, PDF file, 0.2 MB.

**FIG S8**, PDF file, 0.2 MB.

**TABLE S1**, PDF file, 0.8 MB.

**TABLE S2**, PDF file, 0.1 MB.

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J.C. and S.R. designed the research; J.C., H.K., Y.C., W.Y., and D.K. performed the experiments; J.C., H.K., and S.R. analyzed the data; and J.C. and S.R. wrote the paper.

We declare no conflict of interest.

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