Salmonella pathogenicity island 2 expression negatively controlled by EIIA^{Ntr}–SsrB interaction is required for *Salmonella* virulence

Jeongjoon Choi[a,1], Dongwoo Shin[b,1], Hyunjin Yoon[a], Jiae Kim[a], Chang-Ro Lee[c], Minjeong Kim[a,2], Yeong-Jae Seok[c], and Sangryeol Ryu[a,3]

[a]Department of Food and Animal Biotechnology, Department of Agricultural Biotechnology, Center for Agricultural Biomaterials, and Research Institute for Agriculture and Life Sciences, Seoul National University, Seoul 151-921, Korea; [b]Department of Molecular Cell Biology, Samsung Biomedical Research Institute, Sungkyunkwan University School of Medicine, Suwon 440-746, Korea; and [c]Department of Biophysics and Chemical Biology and Institute of Microbiology, College of Natural Sciences, Seoul National University, Seoul 151-742, Korea

Edited* by Sankar Adhya, National Cancer Institute, National Institutes of Health, Bethesda, MD, and approved October 20, 2010 (received for review January 22, 2010)

SsrA/SsrB is a primary two-component system that mediates the survival and replication of *Salmonella* within host cells. When activated, the SsrB response regulator directly promotes the transcription of multiple genes within *Salmonella* pathogenicity island 2 (SPI-2). As expression of the SsrB protein is promoted by several transcription factors, including SsrB itself, the expression of SPI-2 genes can increase to undesirable levels under activating conditions. Here, we report that *Salmonella* can avoid the hyperactivation of SPI-2 genes by using *ptsN*-encoded EIIA^{Ntr}, a component of the nitrogen-metabolic phosphotransferase system. Under SPI-2–inducing conditions, the levels of SsrB-regulated gene transcription increased abnormally in a *ptsN* deletion mutant, whereas they decreased in a strain overexpressing EIIA^{Ntr}. We found that EIIA^{Ntr} controls SPI-2 genes by acting on the SsrB protein at the posttranscriptional level. EIIA^{Ntr} interacted directly with SsrB, which prevented the SsrB protein from binding to its target promoter. Finally, the *Salmonella* strain, either lacking the *ptsN* gene or overexpressing EIIA^{Ntr}, was unable to replicate within macrophages, and the *ptsN* deletion mutant was attenuated for virulence in mice. These results indicated that normal SPI-2 gene expression maintained by an EIIA^{Ntr}–SsrB interaction is another determinant of *Salmonella* virulence.

virulence gene regulation | nitrogen-metabolic phosphotransferase system (PTS) | protein–protein interaction

During systemic infection of mammalian hosts, *Salmonella* employs two distinct type III secretion systems (TTSSs) to modify the host cell response. The first system is encoded by genes clustered in *Salmonella* pathogenicity island 1 (SPI-1) and translocates SPI-1–encoded effector proteins to mediate invasion into host cells (1). When engulfed by macrophages, *Salmonella* cells express the second TTSS and its substrate effectors from SPI-2; this step inhibits the bacteria-killing processes that occur inside macrophages, thus facilitating the survival of *Salmonella* (2).

The expression of SPI-2 genes is under the control of several transcription factors. Among them, the SsrA/SsrB two-component system, which is encoded by the *ssrA* and *ssrB* genes located within SPI-2, seems to have the most direct effect on SPI-2 expression. The purified C terminus of SsrB binds to multiple promoters of SPI-2 genes encoding the TTSS and secreted effectors (3). As the SsrB protein is a response regulator, the DNA-binding activity, which is controlled by the phosphorylation state (4), the cognate sensor kinase, SsrA, should be activated to phosphorylate the SsrB protein within macrophages. Although such phagosomal signals promoting the SsrA activity have not yet been identified, the SsrB-regulated genes are expressed in *Salmonella* cells grown in minimal medium at acidic pH and/or with low Mg^{2+} concentration (5–8).

Similar to many other two-component regulatory systems, the expression of SsrA/SsrB is positively autoregulated; the SsrB protein binds to *ssrA* and *ssrB* promoters and activates their transcription (4). The OmpR and PhoP proteins (response regulators of the OmpR/EnvZ and PhoP/PhoQ two-component systems, respectively) also control the expression levels of SsrA and SsrB. The OmpR regulator directly activates the transcription of *ssrA* and *ssrB* genes (9), whereas the PhoP protein does so for *ssrB* transcription and controls *ssrA* gene expression at the posttranscriptional level via an as yet undetermined mechanism (10).

Recently, small DNA-binding proteins that negatively control the transcription of SPI-2 genes have been discovered. One is the histone-like protein, H-NS, which selectively represses the transcription of horizontally acquired genes in *Salmonella* resulting from its association with AT-rich regions of these loci (11). Consistent with these findings, the H-NS protein represses expression of the SsrB-target genes within SPI-2, which is relieved by activation of the SsrB protein (3). Another small protein, YdgT, also functions as a negative regulator of SPI-2 expression; deletion of the *ydgT* gene increases transcription levels of the SsrB-regulated SPI-2 genes (12). The *ydgT* mutant strain is attenuated for virulence in mice, suggesting that the maintenance of appropriate SPI-2 expression level may be another determinant of *Salmonella* pathogenesis (12, 13).

Together with enzyme I^{Ntr} (EI^{Ntr}) and Npr, enzyme IIA^{Ntr} (EIIA^{Ntr}) is assumed to constitute the nitrogen-metabolic phosphotransferase system (PTS). Unlike enzymes II of the carbohydrate PTS, which have been implicated in various physiological events (14, 15), substantially less is known about the functions of EIIA^{Ntr}. In *Escherichia coli*, the *ptsN* gene encoding EIIA^{Ntr} was identified as a suppressor of some lethal phenotypes; inactivation of the *ptsN* gene recovered viability of an *era* mutant lacking essential GTPase activity (16), whereas overexpression of EIIA^{Ntr} compensated for the loss of σ^E activity (17). In addition, *E. coli* EIIA^{Ntr} was reported to interact directly with the TrkA potassium (K^+) transporter and the KdpD sensor kinase to control cytoplasmic K^+ levels (18, 19).

Author contributions: J.C., D.S., C.-R.L., and S.R. designed research; J.C., D.S., H.Y., J.K., C.-R.L., and M.K. performed research; J.C., D.S., H.Y., Y.-J.S., and S.R. analyzed data; and J.C., D.S., and S.R. wrote the paper.

The authors declare no conflict of interest.

*This Direct Submission article had a prearranged editor.

[1]J.C. and D.S. contributed equally to this work.

[2]Present address: Radiation Research Center for Biotechnology, Korea Atomic Energy Research Institute, Jeongeup 580-185, Korea.

[3]To whom correspondence should be addressed. E-mail: sangryu@snu.ac.kr.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1000759107/-/DCSupplemental.

In the present study, we showed that EIIA^{Ntr} acts on the SsrB response regulator and allows SPI-2 genes to be expressed at normal levels under inducing conditions. Here, we present evidence that EIIA^{Ntr} interacts with the SsrB protein to relieve its binding to the SPI-2 promoter. We demonstrated that in the absence of EIIA^{Ntr}, *Salmonella* overexpresses SPI-2 genes and is attenuated for virulence in a mammalian host.

Results

EIIA^{Ntr} Negatively Controls the Expression of SPI-2 Genes. The *ptsN* gene encodes EIIA^{Ntr}, a component of the nitrogen-metabolic PTS (20). Because it has been reported that EIIA^{Ntr} performs gene regulatory function in *E. coli* (18, 19) and *Pseudomonas putida* (21), we wanted to search for *Salmonella* genes whose expression levels are affected by EIIA^{Ntr}. We conducted DNA microarray analysis using RNA isolated from wild-type and *ptsN* deletion strains grown in Luria–Bertani broth (LB) medium to stationary phase. In the absence of EIIA^{Ntr}, the transcription levels of various SPI-2 genes encoding TTSS and its substrate effector proteins increased by two- to threefold (Table S1).

The expression of SPI-2 genes is induced in *Salmonella* grown in minimal media at acidic pH but repressed at neutral pH (6, 22). We grew the wild-type and *ptsN* deletion strains harboring a *lacZ* fusion to either the *ssaG* or *sseA* promoter in M9 minimal medium adjusted to pH 7.0 or 5.8. We found that the growth of the strains was similar at respective pH values (Fig. S1). In the wild-type strain grown in acidified minimal medium, the transcription levels of *ssaG* and *sseA* genes were induced after 4 h, reaching the maximum by 6 h (Fig. 1*A*). In contrast, in the *ptsN* deletion strain, the expression of these two genes was promoted after only 2 h and increased continuously to levels about fivefold higher than those of the wild-type strain by 10 h (Fig. 1*A*). Note that the regulatory effect of EIIA^{Ntr} required a low pH because deregulation of the *ssaG* and *sseA* genes resulting from *ptsN* deletion did not occur at neutral pH (Fig. 1*A*). By qRT-PCR, we determined that overexpression of SPI-2 genes in the *ptsN* mutant was indeed due to the lack of EIIA^{Ntr}. Under acidic growth conditions, the *ssaR*, *sseB*, *sseE*, and *ssrB* mRNA levels were 9- to 13-fold higher in the absence of *ptsN* than in its presence; these levels were restored by expression of EIIA^{Ntr} from a low-copy number plasmid (Fig. 1*B*). Consistent with the transcription data, the SPI-2 protein expression levels were also altered by deletion of the *ptsN* gene. The *ptsN* deletion strain showed much higher levels of SsrB protein expression than the wild-type strain, but introduction of the EIIA^{Ntr}-expressing plasmid restored the wild-type levels (Fig. 1*C*).

To examine whether EIIA^{Ntr} overexpression could repress SPI-2 transcription, we constructed pJJ14 in which EIIA^{Ntr} was expressed from the *lac* promoter of a multicopy plasmid. When the wild-type strains carrying a *lacZ* fusion with either the *ssaG* or *sseA* gene and harboring the plasmid pJJ14 were grown in acidified minimal medium, the transcription levels of these two genes decreased by 2.4-fold on addition of isopropyl-β-D-thiogalactopyranoside (IPTG) (Fig. S2). Taken together, these results indicated that under SPI-2–inducing conditions, EIIA^{Ntr} negatively regulates SPI-2 genes, resulting in their expression at the appropriate levels.

EIIA^{Ntr}-Mediated Regulation of SPI-2 Genes Requires SsrB Protein. As the DNA-binding activity of EIIA^{Ntr} has not been reported, the regulatory function of EIIA^{Ntr} would be mediated by a transcription factor(s) controlling the expression of SPI-2 genes. We hypothesized that the EIIA^{Ntr}-mediated control of SPI-2 expression could be dependent on SsrB, a response regulator of the SsrA/SsrB two-component system, because this protein is known to be a key regulator that directly activates the transcription of multiple SPI-2 genes (3). Moreover, *ssrB* is one of the SPI-2 genes showing increased expression level in the *ptsN* deletion

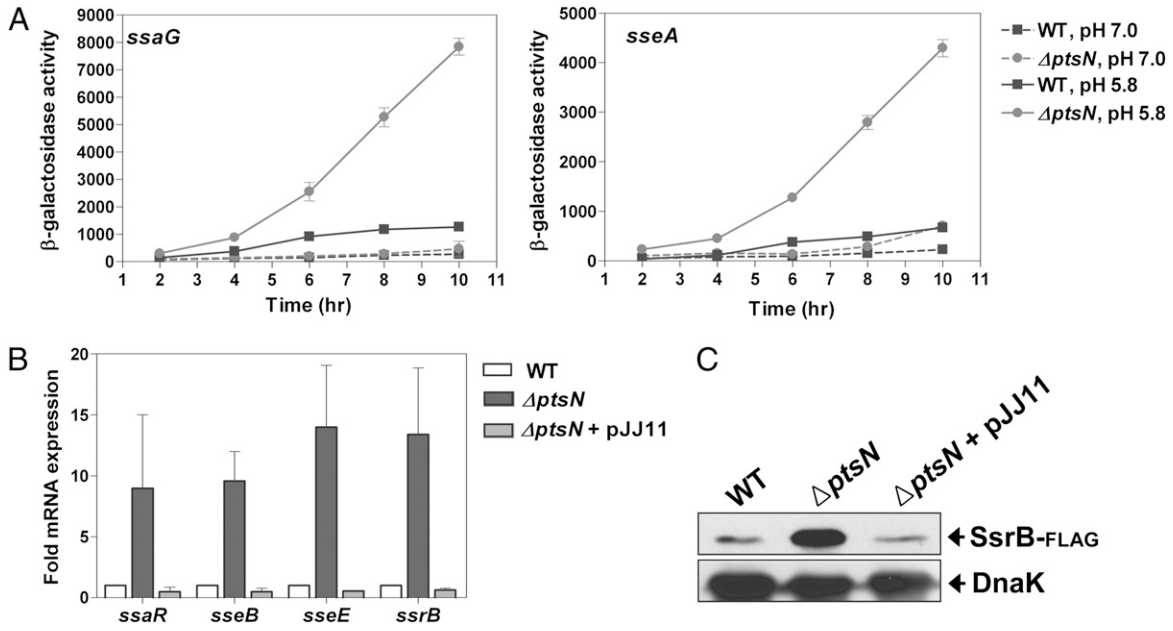

Fig. 1. EIIA^{Ntr} is necessary for normal expression of SPI-2 genes under their inducing conditions. (*A*) Transcription levels of the *ssaG* and *sseA* genes were determined by β-galactosidase assay. Overnight cultures of the wild-type (WT) and *ptsN* deletion (*ΔptsN*) strains carrying a transcriptional *ssaG–lacZ* (P4P70, SR3207) or *sseA–lacZ* (SR3266, SR3267) fusion construct on the chromosome were diluted in M9 minimal medium adjusted to pH 7.0 or 5.8, and β-galactosidase activity (Miller units) was determined at the designated time points. (*B*) mRNA levels of SPI-2 genes, *ssaR*, *sseB*, *sseE*, and *ssrB*, were determined by qRT-PCR. The wild-type (SL1344) and *ptsN* deletion (SR3203) strains and the SR3203 strain harboring the pJJ11 plasmid (i.e., a low-copy number plasmid expressing EIIA^{Ntr}) were grown in M9 minimal medium at pH 5.8 to stationary phase. (*A* and *B*) Values shown are the means and SD of three independent experiments. (*C*) Western blotting analysis was performed on cell extracts prepared from the wild-type (SR3002), *ΔptsN* (SR3251), and pJJ11-harboring SR3251 strains, which were grown in M9 minimal medium at pH 5.8.

strain (Fig. 1 *B* and *C*). Consistent with previous reports (3, 6, 23), deletion of the *ssrB* gene markedly impaired transcriptional activation of the *ssaG* gene at acidic pH (i.e., 11-fold reduction; Fig. 2*A*). We showed that the absence of SsrB abolished the regulatory effect of EIIA^{Ntr} on SPI-2 gene expression. Under acidic pH conditions, the strain harboring the functional *ssrB* gene but lacking the *ptsN* gene overexpressed *ssaG*, whereas the *ptsN* mutation failed to increase the levels of *ssaG* gene transcription in the absence of SsrB (Fig. 2*A*).

EIIA^{Ntr} Controls the SsrB Protein at the Posttranscriptional Level. These observations raise the question of how *ssrB* expression was increased in the *ptsN* deletion strain. EIIA^{Ntr} may repress *ssrB* transcription. Alternatively, as the *ssrB* gene is positively autoregulated (4), the lack of EIIA^{Ntr} negatively controlling the stability and/or activity of the SsrB protein may result in overexpression of *ssrB*. If the latter case were true, the *ptsN* deletion would still activate further transcription of SsrB-regulated targets, even with expression of the *ssrB* gene from a heterologous promoter. In the *ssrB* deletion strain harboring the pJJ16 plasmid in which the SsrB protein was induced from the *lac* promoter, transcription of the *ssaG* gene recovered in acidified medium containing IPTG (Fig. 2*B*). Under the same experimental conditions, however, the lack of EIIA^{Ntr} again increased the transcription level of the *ssaG* gene by sevenfold (Fig. 2*B*). Therefore, our findings suggest that EIIA^{Ntr} negatively controls *ssrB* gene expression at the posttranscriptional level, which in turn down-regulates the transcription of SsrB-regulated genes including *ssrB* itself.

EIIA^{Ntr} Interacts with the SsrB Protein. EIIA^{Ntr} has been reported to bind directly to the TrkA and KdpD proteins and to control K^+ homeostasis in *E. coli* (18, 19). Therefore, we investigated whether *Salmonella* EIIA^{Ntr} interacts with the SsrB protein using a bacterial two-hybrid system (24). We constructed two plasmids in which EIIA^{Ntr} and SsrB were expressed as forms fused to the T25 and T18 catalytic domains, respectively, of *Bordetella pertussis* adenylate cyclase. Indeed, the coexpression of these two fusion proteins in the *E. coli* strain lacking endogenous adenylate cyclase activity resulted in about 95-fold higher levels of β-galactosidase activity compared with the control expressing the T25–EIIA^{Ntr} fusion protein and T18 fragment (Fig. 3*A*). This result indicated that EIIA^{Ntr} interacts with SsrB to complement adenylate cyclase activity. The EIIA^{Ntr}–SsrB interaction was likely to be specific because coexpression of the T25–EIIA^{Ntr} and T18–SsrA sensor fusion failed to promote *lacZ* expression over the control (Fig. 3*A*).

Next, we investigated EIIA^{Ntr}–SsrB interaction in vitro using purified proteins. We used the SsrB protein carrying thioredoxin fusion at its N terminus because this fusion facilitated overexpression of SsrB and increased SsrB solubility for protein purification. Note that expression of the thioredoxin-fused SsrB protein was able to restore *ssaG* transcription in *ssrB* mutant, indicating that the thioredoxin fusion did not interfere with regulatory function of SsrB (Fig. S3). We examined real-time interaction between EIIA^{Ntr} and SsrB using the surface plasmon resonance technique. When the SsrB protein was allowed to flow over a CM5 sensor chip with immobilized EIIA^{Ntr}, we could observe SsrB-binding to EIIA^{Ntr} (Fig. 3*B*). In contrast, when SsrB was exposed to immobilized EIIA^{Glc}, an enzyme II of the carbohydrate PTS, no interaction was detected between these two proteins (Fig. 3*B*). Taken together, the data demonstrate that EIIA^{Ntr} specifically binds to the SsrB response regulator.

EIIA^{Ntr} Prevents the SsrB Protein from Binding to Its Target Promoter. Binding of transcription factors to target promoters is an essential step for controlling gene expression. Thus, we explored

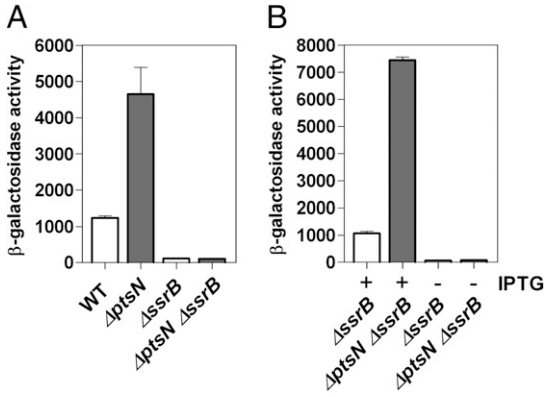

Fig. 2. EIIA^{Ntr} controls SPI-2 genes by acting on the SsrB protein at the posttranscriptional level. Transcription levels of the *ssaG* gene were determined by β-galactosidase activities (Miller units) expressed from strains harboring a *lacZ* transcriptional fusion to the *ssaG* gene. (*A*) The wild-type (P4P70) strain and strains with deletion of the *ptsN* (SR3207), *ssrB* (SR4001), and both *ptsN* and *ssrB* (SR4002) genes carrying an *ssaG–lacZ* fusion were grown in M9 minimal medium at pH 5.8. (*B*) The *ΔssrB* (SR4001) and *ΔptsNΔssrB* (SR4002) strains harboring a *ssaG–lacZ* fusion and the plasmid pJJ16 in which the SsrB protein is expressed from the *lac* promoter were grown in M9 minimal medium at pH 5.8 with or without 2 μM IPTG. Values shown are the means and SD of three independent experiments.

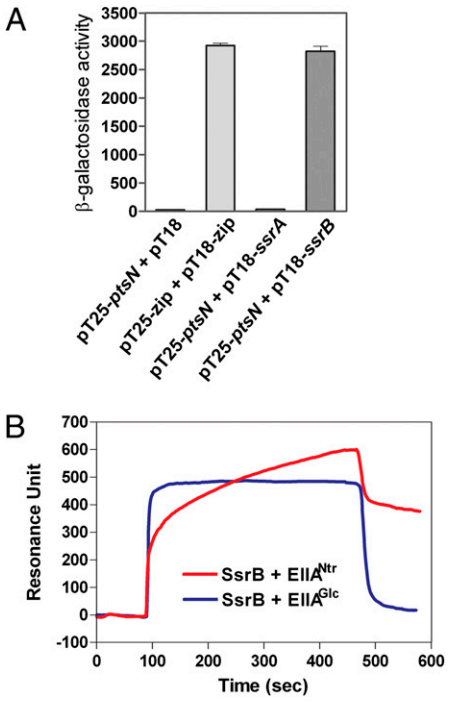

Fig. 3. EIIA^{Ntr} interacts with the SsrB protein both in vivo and in vitro. (*A*) EIIA^{Ntr}–SsrB interaction was assessed using a bacterial two-hybrid system (24). β-Galactosidase activity (Miller units) was determined in *E. coli* BTH101 strains harboring plasmids coexpressing T25-EIIA^{Ntr} and T18 (pT25-*ptsN* + pT18), T25-Zip and T18-Zip (pT25-zip + pT18-zip), T25-EIIA^{Ntr} and T18-SsrB (pT25-*ptsN* + pT18-*ssrB*), and T25-EIIA^{Ntr} and T18-SsrA (pT25-*ptsN* + pT18-*ssrA*). Values shown are the means ± SD of three independent experiments. (*B*) Interaction between SsrB and EIIA^{Ntr} was examined by conducting surface plasmon resonance spectroscopy. Purified EIIA^{Ntr} and EIIA^{Glc} (a control protein) were immobilized on the carboxymethylated dextran surface of a CM5 chip at 1.5 ng/mm^2 concentration. SsrB (4.5 ng/μL) was allowed to flow over the EIIA^{Ntr} and EIIA^{Glc} surfaces for 10 min in each sensorgram. The red-lined sensorgram indicated strong SsrB-binding to EIIA^{Ntr}, whereas no interaction was detected between SsrB and EIIA^{Glc} (blue-lined sensorgram).

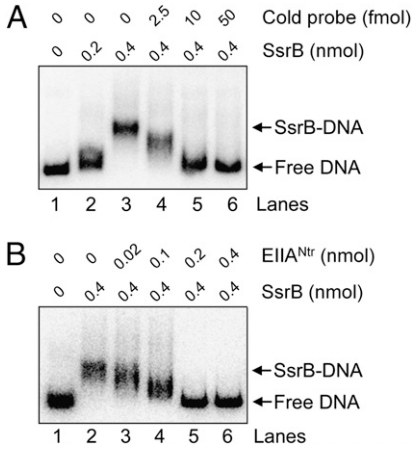

Fig. 4. EIIA^{Ntr} inhibits SsrB-binding to target promoter. EMSA experiments were conducted to examine interaction between the SsrB protein and the *ssaG* promoter. (*A*) The SsrB protein was incubated with the 5′ end-labeled *ssaG* promoter DNA (2.5 fmol) in the presence or absence of the unlabeled DNA probe. Concentrations of SsrB and cold probe in the reactions were indicated on top of the figure. (*B*) The SsrB protein was incubated with the radio-labeled DNA fragments containing *ssaG* promoter in the presence or absence of EIIA^{Ntr}. Amounts of SSrB and EIIA^{Ntr} in the reactions are described at the *Top*.

whether the EIIA^{Ntr}–SsrB interaction could interfere with SsrB binding to the target promoter by conducting electrophoretic mobility shift assay (EMSA) experiments. When the SsrB protein was incubated with 5′ end-labeled DNA probes, it bound to the *ssaG* promoter DNA to form the SsrB–DNA complex (Fig. 4*A*, lane 3). This SsrB binding was specific because the unlabeled *ssaG* promoter DNA in the reaction competed with the labeled probe for SsrB-binding (Fig. 4*A*, lanes 4–6). Interestingly, EIIA^{Ntr} did prevent SsrB from binding to the target DNA: as the concentrations of EIIA^{Ntr} increased in the reactions, the levels of the SsrB–DNA complex decreased to generate the free *ssaG* promoter DNA (Fig. 4*B*, lanes 3–6). This result was not due to competition between SsrB and EIIA^{Ntr} for DNA binding because EIIA^{Ntr} alone was unable to bind to the *ssaG* promoter (Fig. S4). Therefore, these results suggest that interaction between the SsrB protein and EIIA^{Ntr} prevents SsrB binding to its target promoters, and thus reduces SPI-2 gene transcription levels.

***Salmonella* Lacking EIIA^{Ntr}-Controlled SPI-2 Expression Shows Attenuated Virulence.** Expression of SPI-2 is required for the survival and replication of *Salmonella* within macrophages (25). When infected with murine macrophages, the wild-type strain replicated, but the *ptsN* deletion mutant failed to do so 18 h after phagocytosis (Fig. 5*A*). This was not due to phagocytic differences between these two strains because the numbers for both strains within macrophages were similar at 30 min postinfection (Fig. 5*A*). This phenotypic defect of the *ptsN* mutant was due to the lack of EIIA^{Ntr} because expression of the *ptsN* gene from a low-copy number plasmid enabled the mutant strain to replicate within macrophages (Fig. 5*A*). Next, we overexpressed EIIA^{Ntr} in the wild-type strain and found that this also prevented intraphagosomal growth of *Salmonella* (Fig. 5*B*). Therefore, our data suggest that both the loss of normal SPI-2 expression by *ptsN* deletion and the reduced SPI-2 activity by EIIA^{Ntr} overexpression impair *Salmonella* replication within macrophages.

As *Salmonella* should survive within macrophages during systemic infection of mammalian hosts, the *ptsN* mutant strain could be attenuated for virulence in mice. We inoculated the wild-type or *ptsN* deletion strain into a group of 10 mice via the i.p. route. All of the mice infected with wild-type *Salmonella* died within 9 d, whereas deletion of the *ptsN* gene prolonged the mean survival time by 6 d (Fig. 5*C*). We further verified the virulence phenotype of the *ptsN* deletion mutant by counting bacterial cells in the liver and spleen. Five days after infection,

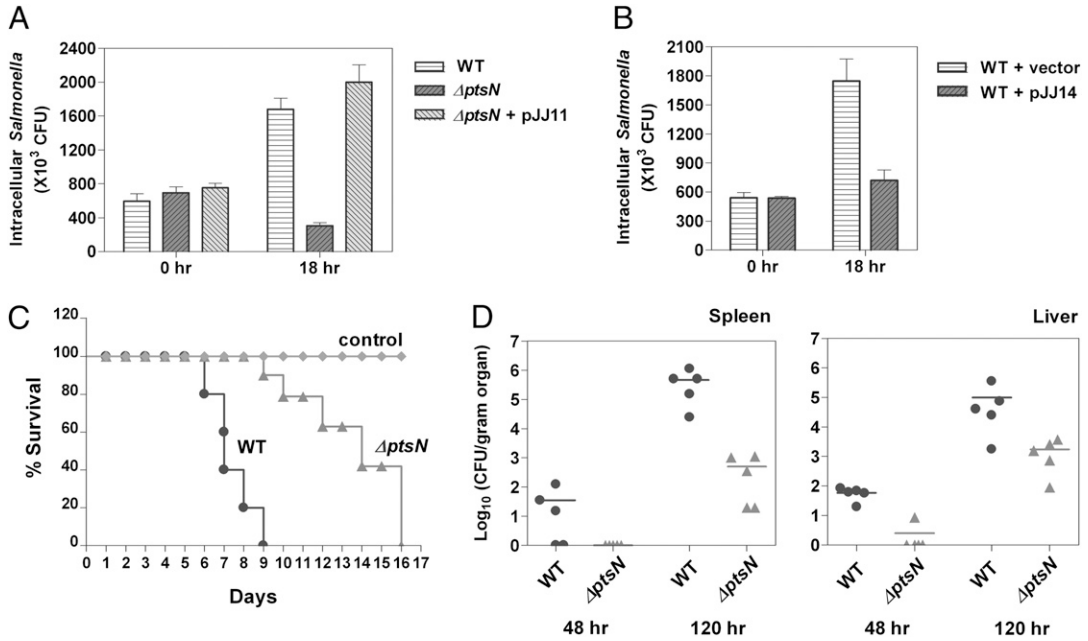

Fig. 5. Virulence phenotypes displayed by the *Salmonella* strains lacking or overexpressing the *ptsN* gene. (*A*) Macrophages J774A.1 were infected with the wild-type (SL1344) and Δ*ptsN* (SR3203) strains and SR3203 strain harboring the plasmid pJJ11. The numbers of gentamicin-resistant bacteria were determined at 30 min (indicated as 0 h) and 18 h after phagocytosis. (*B*) Gentamicin protection assay was conducted using the wild-type strain (SL1344) carrying the empty vector or the plasmid pJJ14. (*C* and *D*) BALB/c mice were intraperitoneally infected with the wild-type (SL1344) and *ptsN* deletion (SR3203) strains. Survival of mice was monitored daily (*C*), and the numbers of bacteria in the spleen and liver were determined (*D*).

the numbers of *ptsN*-deleted *Salmonella* were 1,000- and 100-fold lower than those of the wild-type bacteria in the spleen and liver, respectively (Fig. 5*D*). Thus, our results indicate that $EIIA^{Ntr}$ is necessary for virulence of *Salmonella* in mice.

Discussion

Once engulfed by macrophages, intracellular pathogens face harsh environments within the phagosome. By using the SPI-2–encoded TTSS, *Salmonella* translocates various effector proteins into the cytoplasm of a host cell to interfere with the bacteria-killing events (26). In this study, we demonstrated that $EIIA^{Ntr}$, a component of the nitrogen-metabolic PTS encoded by the *ptsN* gene, is necessary for SPI-2 genes to be expressed at the appropriate levels under their inducing conditions, and that lack of this regulation attenuates *Salmonella* virulence during systemic infection of mice.

In addition to genes encoding a TTSS, as well as effector proteins, SPI-2 possesses the *ssrA* and *ssrB* genes that express the SsrA/SsrB two-component regulatory system. The response regulator, SsrB, binds directly to the promoters and activates transcription of gene clusters within SPI-2 (3). In a strain lacking $EIIA^{Ntr}$, we found that the levels of SsrB-regulated gene expression were abnormally elevated under SPI-2–inducing conditions (Fig. 1). These observations raised questions regarding how $EIIA^{Ntr}$ maintains the expression of SPI-2 genes at the appropriate levels. On the basis of several observations, we propose that $EIIA^{Ntr}$ binds directly to the SsrB protein to relieve SsrB-binding to its target promoters. First, $EIIA^{Ntr}$-mediated regulation of SPI-2 genes was dependent on the SsrB protein because *ptsN* mutation failed to increase SPI-2 expression in the absence of SsrB (Fig. 2*A*). Second, $EIIA^{Ntr}$ negatively controlled SsrB at the posttranscriptional level because the *ptsN* deletion strain could still overexpress SPI-2 genes even when the SsrB protein was expressed from a heterologous promoter (Fig. 2*B*). Third, $EIIA^{Ntr}$ interacted with SsrB in vivo and in vitro (Fig. 3). Fourth, $EIIA^{Ntr}$ prevented binding of SsrB to its target promoters (Fig. 4*B*). However, as the SsrB protein should be phosphorylated to function as a transcription factor (4), we cannot exclude the possibility that the $EIIA^{Ntr}$–SsrB interaction may inhibit SsrA-mediated phosphorylation of SsrB or promote dephosphorylation of phosphorylated SsrB. Indeed, in *Bacillus subtilis*, the RapH protein promotes dephosphorylation of the Spo0A response regulator in vitro (27).

Although a sensor kinase and its cognate response regulator represent a basic two-component system mediating bacterial signal transduction, many proteins called "connectors" have been identified in two-component systems. Through an interaction with a sensor kinase or response regulator, connector proteins can modulate many steps affecting their activities (28). ComA, a response regulator of the ComA/ComP two-component system, activates the development of competence in *B. subtilis*. Similar to the role of *Salmonella* $EIIA^{Ntr}$ for SsrB, the Rap protein family members RapC, RapF, and RapH bind to the ComA protein and inhibit its binding to target promoter DNA (27, 29, 30). The *kdpFABC* operon encodes the high-affinity K^+ transporter KdpFABC, and its expression is regulated by the KdpD/KdpE two-component system. The *E. coli* $EIIA^{Ntr}$ controls *kdpFABC* transcription via direct interaction with the KdpD/KdpE two-component system (19). However, unlike the *Salmonella* $EIIA^{Ntr}$ interaction with the SsrB response regulator, the *E. coli* homolog binds to the KdpD sensor protein and stimulates its kinase activity, thereby increasing the levels of phosphorylated KdpE (19). In this sense, $EIIA^{Ntr}$ seems to be a connector protein acting on a sensor kinase and response regulator of different two-component systems.

In the nitrogen-metabolic PTS, a phosphoryl group on EI^{Ntr} is finally transferred to $EIIA^{Ntr}$ through NPr. The interactions between $EIIA^{Ntr}$ and its partner proteins are determined by the phosphorylation state of $EIIA^{Ntr}$. For interaction with the TrkA or KdpD protein, $EIIA^{Ntr}$ should be dephosphorylated (18, 19). We found that *Salmonella* $EIIA^{Ntr}$ carrying a mutation in the putative phosphorylation site (i.e., H73A substitution) can still interact with SsrB in vivo and maintain the normal levels of SPI-2 gene expression (Fig. S5 *A* and *B*). Moreover, phosphorylated $EIIA^{Ntr}$ could also bind to the SsrB protein in vitro (Fig. S5*C*). Thus, these results suggest that $EIIA^{Ntr}$ performs SPI-2 regulatory function regardless of its phosphorylation state.

Over the course of infection, *Salmonella* often uses two-component regulatory systems to sense signals within host cells and to promote the expression of genes that are necessary for intracellular survival. Thus, the inactivation of regulatory systems playing such roles would result in impairment of bacterial virulence. The PhoP protein is a response regulator of the PhoP/PhoQ two-component system and plays a key role in *Salmonella* pathogenesis (31). The *Salmonella pho-24* mutant, in which the PhoP protein is constitutively activated due to a mutation in PhoQ, and a strain that lacks the surge of PhoP activity by elimination of a positive feedback loop from the *phoPQ* operons, are attenuated for virulence in mice (32, 33). These results suggest that the controlled activity of regulatory systems is another determinant of bacterial virulence. Recently, transcription of SPI-2 genes was reported to be negatively controlled by small regulatory proteins, such as YdgT and H-NS (7). Under non-inducing conditions, these proteins are likely to be associated with regulatory regions of SPI-2 to silence gene transcription (12, 13). When activated within macrophages, the SsrB protein promotes transcription of SPI-2, possibly by counteracting the H-NS– and YdgT-mediated repression (7). Indeed, deletion of the *ydgT* gene attenuated *Salmonella* virulence in a mouse infection model, which may have resulted from the expression of SPI-2 genes with inappropriate timing (i.e., under repressing conditions) and/or at inappropriate levels under inducing conditions (12, 34).

The expression levels of SsrB are regulated by at least three response regulators. The SsrB protein increases its own levels via positive autoregulation (4) and the OmpR and PhoP proteins also do so by directly activating *ssrB* transcription (9, 10). Due to these multiple positive feedback loops converging on SsrB expression, without a means to control the levels of activated SsrB protein, SPI-2 genes would be expressed ectopically within phagosomes where the signals activating these response regulators exist. The results of the present study indicated that $EIIA^{Ntr}$ prevents SPI-2 genes from being expressed at undesirable levels under their activating conditions, the loss of which consequently attenuates *Salmonella* virulence during systemic infection of an animal host.

Finally, we notice the possibility that the alternative sigma factor σ^E might enhance $EIIA^{Ntr}$-mediated SPI-2 gene regulation inside macrophages. σ^E is required for *Salmonella*'s resistance to phagosomal defense molecules such as oxidative stress and antimicrobial peptides (35, 36), and thus a *Salmonella* mutant lacking σ^E is attenuated for survival within macrophages and virulence in mice (35, 37). Moreover, it was shown that antimicrobial peptides can activate σ^E (36). Interestingly, in *E. coli*, overexpression of σ^E promotes *ptsN* transcription, indicating that the *ptsN* gene is a member of σ^E regulon (38). Taken together, during *Salmonella*'s growth inside macrophages, σ^E activated by the phagosomal stresses could enhance $EIIA^{Ntr}$ expression, which tightly controls timing and/or levels of SPI-2 expression.

Materials and Methods

Bacterial Strains, Plasmids, and Growth Conditions. *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 S2. Phage P22-mediated transduction was performed as described previously (39). All *Salmonella* strains were grown aerobically at 37 °C in LB or M9 minimal medium at the adjusted pH. Antibiotics were used at the following con-

centrations: ampicillin, 50 μg/mL; chloramphenicol, 25 μg/mL; kanamycin, 50 μg/mL; and streptomycin, 50 μg/mL. A detailed description of construction of strains and plasmids is provided in *SI Materials and Methods*.

EMSA. EMSA experiments were performed as described previously (23, 40). DNA fragments corresponding to the *ssaG* promoter were amplified by PCR using ^{32}P-labeled primer EMSA–ssaG–F1 (5′-GGTAGTTTGGGACTACAGCCT-CATTTA-3′) and primer EMSA–ssaG–R1 (5′-AATCATCGATTCTGGGTTGAG-CAAATC-3′) with wild-type *Salmonella* chromosomal DNA as a template. The promoter DNA was purified from agarose gels using a gel extraction kit (Qiagen). The labeled DNA probe (2.5 fmol) was incubated with the thioredoxin-fused SsrB protein in the presence or absence of EIIA^{Ntr}–His$_6$ at 37 °C for 30 min in 20 μL of binding buffer (10 mM Tris, pH 7.5, 50 mM KCl, 5 mM $MgCl_2$, and 2.5% glycerol) containing 50 ng/μL poly(dI-dC). The reaction mixtures were resolved by 6% PAGE, and the radiolabeled DNA fragments were visualized using a BAS2500 system (Fuji Film).

Surface Plasmon Resonance Spectroscopy. Real-time interaction of SsrB with EIIA^{Ntr} or EIIA^{Glc} was monitored via surface plasmon resonance detection by using a BIAcore 3000 system (BIAcore) with some modifications as described previously (18). EIIA^{Ntr} and EIIA^{Glc} were immobilized onto the carboxymethylated dextran surface of a CM5 sensor chip, respectively. EIIA^{Ntr} and EIIA^{Glc} (100 μL, 10 μg/mL) in coupling buffer (10 mM sodium acetate, pH 5.0) were allowed to flow over the sensor chip at 5 μL/min to couple the proteins to the matrix by a *N*-hydroxysuccinimide/1-ethyl-3′-(3-dimethylaminopropyl) carbodiimide reaction (80 μL of mixture). On the basis of the assumption that 1,000 resonance units (RUs) correspond to a surface concentration of 1 ng/mm^2, EIIA^{Glc} and EIIA^{Ntr} were immobilized to surface concentration of 1.5 ng/mm^2, respectively. The standard running buffer was 10 mM Hepes (pH 7.2), 150 mM NaCl, 10 mM KCl, 1 mM $MgCl_2$ and 0.5 mM EDTA, and all reagents were introduced at a flow rate of 10 μL/min. The sensor surface was regenerated between assays by using the standard running buffer at a flow rate of 100 μL/min for 10 min to remove bound analytes. See *SI Materials and Methods* for details on the construction of bacterial strains and plasmids, RNA isolation and quantitative real-time RT-PCR, DNA microarray analysis, Western blotting analysis, bacterial two-hybrid assay, purification of EIIANtr and SsrB, and gentamicin protection assay. Primers used for construction of bacterial strains and plasmids are listed in Table S3 and those for qRT-PCR in Table S4.

ACKNOWLEDGMENTS. We thank Linda Kenney (Department of Microbiology and Immunology, University of Illinois, Chicago, IL) for kindly providing the *ssrBc* clone. This work was supported by grants from the 21C Frontier Microbial Genomics and Applications Center Program and by the World Class University program through the National Research Foundation of Korea, funded by the Ministry of Education, Science, and Technology (R32-2008-000-10183-0).

1. Galán JE, Collmer A (1999) Type III secretion machines: Bacterial devices for protein delivery into host cells. *Science* 284:1322–1328.
2. Cirillo DM, Valdivia RH, Monack DM, Falkow S (1998) Macrophage-dependent induction of the *Salmonella* pathogenicity island 2 type III secretion system and its role in intracellular survival. *Mol Microbiol* 30:175–188.
3. Walthers D, et al. (2007) The response regulator SsrB activates expression of diverse *Salmonella* pathogenicity island 2 promoters and counters silencing by the nucleoid-associated protein H-NS. *Mol Microbiol* 65:477–493.
4. Feng X, Walthers D, Oropeza R, Kenney LJ (2004) The response regulator SsrB activates transcription and binds to a region overlapping OmpR binding sites at *Salmonella* pathogenicity island 2. *Mol Microbiol* 54:823–835.
5. Hensel M (2000) *Salmonella* pathogenicity island 2. *Mol Microbiol* 36:1015–1023.
6. Xu X, Hensel M (2010) Systematic analysis of the SsrAB virulon of *Salmonella enterica*. *Infect Immun* 78:49–58.
7. Fass E, Groisman EA (2009) Control of *Salmonella* pathogenicity island-2 gene expression. *Curr Opin Microbiol* 12:199–204.
8. Coombes BK, Brown NF, Valdez Y, Brumell JH, Finlay BB (2004) Expression and secretion of *Salmonella* pathogenicity island-2 virulence genes in response to acidification exhibit differential requirements of a functional type III secretion apparatus and SsaL. *J Biol Chem* 279:49804–49815.
9. Feng X, Oropeza R, Kenney LJ (2003) Dual regulation by phospho-OmpR of *ssrA/B* gene expression in *Salmonella* pathogenicity island 2. *Mol Microbiol* 48:1131–1143.
10. Bijlsma JJ, Groisman EA (2005) The PhoP/PhoQ system controls the intramacrophage type three secretion system of *Salmonella enterica*. *Mol Microbiol* 57:85–96.
11. Navarre WW, et al. (2006) Selective silencing of foreign DNA with low GC content by the H-NS protein in *Salmonella*. *Science* 313:236–238.
12. Coombes BK, Wickham ME, Lowden MJ, Brown NF, Finlay BB (2005) Negative regulation of *Salmonella* pathogenicity island 2 is required for contextual control of virulence during typhoid. *Proc Natl Acad Sci USA* 102:17460–17465.
13. Silphaduang U, Mascarenhas M, Karmali M, Coombes BK (2007) Repression of intracellular virulence factors in *Salmonella* by the Hha and YdgT nucleoid-associated proteins. *J Bacteriol* 189:3669–3673.
14. Siebold C, Flükiger K, Beutler R, Erni B (2001) Carbohydrate transporters of the bacterial phosphoenolpyruvate: Sugar phosphotransferase system (PTS). *FEBS Lett* 504:104–111.
15. Deutscher J, Francke C, Postma PW (2006) How phosphotransferase system-related protein phosphorylation regulates carbohydrate metabolism in bacteria. *Microbiol Mol Biol Rev* 70:939–1031.
16. Powell BS, et al. (1995) Novel proteins of the phosphotransferase system encoded within the *rpoN* operon of *Escherichia coli*. Enzyme IIA^{Ntr} affects growth on organic nitrogen and the conditional lethality of an erats mutant. *J Biol Chem* 270:4822–4839.
17. Hayden JD, Ades SE (2008) The extracytoplasmic stress factor, sigmaE, is required to maintain cell envelope integrity in *Escherichia coli*. *PLoS ONE* 3(2):e1573.
18. Lee CR, Cho SH, Yoon MJ, Peterkofsky A, Seok YJ (2007) *Escherichia coli* enzyme IIA^{Ntr} regulates the K$^+$ transporter TrkA. *Proc Natl Acad Sci USA* 104:4124–4129.
19. Lüttmann D, et al. (2009) Stimulation of the potassium sensor KdpD kinase activity by interaction with the phosphotransferase protein IIA(Ntr) in *Escherichia coli*. *Mol Microbiol* 72:978–994.
20. Rabus R, Reizer J, Paulsen I, Saier MH, Jr (1999) Enzyme I(Ntr) from *Escherichia coli*. A novel enzyme of the phosphoenolpyruvate-dependent phosphotransferase system exhibiting strict specificity for its phosphoryl acceptor, NPr. *J Biol Chem* 274:26185–26191.
21. Cases I, Lopez JA, Albar JP, De Lorenzo V (2001) Evidence of multiple regulatory functions for the PtsN (IIA(Ntr)) protein of *Pseudomonas putida*. *J Bacteriol* 183:1032–1037.
22. Rappl C, Deiwick J, Hensel M (2003) Acidic pH is required for the functional assembly of the type III secretion system encoded by *Salmonella* pathogenicity island 2. *FEMS Microbiol Lett* 226:363–372.
23. Carroll RK, et al. (2009) Structural and functional analysis of the C-terminal DNA binding domain of the *Salmonella* typhimurium SPI-2 response regulator SsrB. *J Biol Chem* 284:12008–12019.
24. Karimova G, Pidoux J, Ullmann A, Ladant D (1998) A bacterial two-hybrid system based on a reconstituted signal transduction pathway. *Proc Natl Acad Sci USA* 95:5752–5756.
25. Waterman SR, Holden DW (2003) Functions and effectors of the *Salmonella* pathogenicity island 2 type III secretion system. *Cell Microbiol* 5:501–511.
26. Abrahams GL, Hensel M (2006) Manipulating cellular transport and immune responses: Dynamic interactions between intracellular *Salmonella enterica* and its host cells. *Cell Microbiol* 8:728–737.
27. Smits WK, et al. (2007) Temporal separation of distinct differentiation pathways by a dual specificity Rap-Phr system in *Bacillus subtilis*. *Mol Microbiol* 65:103–120.
28. Mitrophanov AY, Groisman EA (2008) Signal integration in bacterial two-component regulatory systems. *Genes Dev* 22:2601–2611.
29. Bongiorni C, Ishikawa S, Stephenson S, Ogasawara N, Perego M (2005) Synergistic regulation of competence development in *Bacillus subtilis* by two Rap-Phr systems. *J Bacteriol* 187:4353–4361.
30. Core L, Perego M (2003) TPR-mediated interaction of RapC with ComA inhibits response regulator-DNA binding for competence development in *Bacillus subtilis*. *Mol Microbiol* 49:1509–1522.
31. Groisman EA (2001) The pleiotropic two-component regulatory system PhoP-PhoQ. *J Bacteriol* 183:1835–1842.
32. Shin D, Lee EJ, Huang H, Groisman EA (2006) A positive feedback loop promotes transcription surge that jump-starts *Salmonella* virulence circuit. *Science* 314:1607–1609.
33. Miller SI, Mekalanos JJ (1990) Constitutive expression of the *phoP* regulon attenuates *Salmonella* virulence and survival within macrophages. *J Bacteriol* 172:2485–2490.
34. Vivero A, et al. (2008) Modulation of horizontally acquired genes by the Hha-YdgT proteins in *Salmonella enterica* serovar Typhimurium. *J Bacteriol* 190:1152–1156.
35. Testerman TL, et al. (2002) The alternative sigma factor sigmaE controls antioxidant defences required for *Salmonella* virulence and stationary-phase survival. *Mol Microbiol* 43:771–782.
36. Crouch ML, et al. (2005) The alternative sigma factor sigma is required for resistance of *Salmonella enterica* serovar Typhimurium to anti-microbial peptides. *Mol Microbiol* 56:789–799.
37. Humphreys S, Stevenson A, Bacon A, Weinhardt AB, Roberts M (1999) The alternative sigma factor, sigmaE, is critically important for the virulence of *Salmonella* typhimurium. *Infect Immun* 67:1560–1568.
38. Rhodius VA, Suh WC, Nonaka G, West J, Gross CA (2006) Conserved and variable functions of the sigmaE stress response in related genomes. *PLoS Biol* 4:e2.
39. Watanabe T, Ogata Y, Chan RK, Botstein D (1972) Specialized transduction of tetracycline resistance by phage P22 in *Salmonella* typhimurium. I. Transduction of R factor 222 by phage P22. *Virology* 50:874–882.
40. Lim S, et al. (2007) Mlc regulation of Salmonella pathogenicity island I gene expression via *hilE* repression. *Nucleic Acids Res* 35:1822–1832.