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Understanding the multifaceted roles of the phosphoenolpyruvate: Phosphotransferase system in regulation of *Salmonella* virulence using a mutant defective in *ptsI* and *crr* expression

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ABSTRACT

The phosphoenolpyruvate (PEP):carbohydrate phosphotransferase system (PTS) catalyzes the translocation of sugar substrates with their concomitant phosphorylation in bacteria. In addition to its intrinsic role in sugar transport and metabolism, numerous recent studies report the versatility of the PTS to interconnect energy and signal transduction in response to sugar availability. In this study, the role of PTS in *Salmonella* virulence regulation was explored. To decipher the regulatory network coordinated by the PTS during *Salmonella* infection, a transcriptomic approach was applied to a transposon insertion mutant with defective expression of *ptsI* and *crr*, which encode enzyme I and enzyme IIA^{Glc} of the PTS, respectively. There were 114 differentially expressed genes (DEGs) exhibiting two-fold or higher expression changes in the transposon mutant strain, with 13 up-regulated genes versus 101 down-regulated genes. One-third of the DEGs were associated with energy production and carbohydrate/amino acid metabolism pathways, implicating the prominent role of the PTS in carbohydrate transport. With regard to regulation of virulence, the tested mutant decreased the expression of genes associated with quorum sensing, *Salmonella* pathogenicity islands, flagella, and the PhoPQ regulon. We investigated the possibility of PTS-mediated regulation of virulence determinants identified in the transcriptomic analysis and proposed a regulatory circuit orchestrated by the PTS in *Salmonella* infection of host cells. These results suggest that *Salmonella* divergently controls virulence attributes in accordance with the availability of carbohydrates in the environment.

1. Introduction

The phosphoenolpyruvate (PEP):carbohydrate phosphotransferase system (PTS), unique to prokaryotes, catalyzes the uptake of a large number of sugar substrates, particularly hexoses (such as glucose, fructose, and mannose) and hexitols (such as mannitol, sorbitol, and xylitol), generally called PTS sugars (Postma et al., 1993). The PTS is composed of two cytoplasmic proteins, enzyme I (EI) and the histidine phosphocarrier protein (HPr) common to most PTS sugars, and enzyme II (EII) complexes responding to specific sugars. The PTS components transfer phosphate from PEP to the incoming sugar: first, a phosphate group from PEP is transferred to EI, then relayed to EII via HPr; finally, EII transports the incoming sugar with concomitant phosphorylation (Deutscher et al., 2006). In addition to sugar transport activities, emerging experimental evidence suggests a pleiotropic role of the PTS in an extensive regulatory network integrating carbohydrate uptake with diverse signal transduction mechanisms. The PTS takes part in numerous regulatory functions, from those related to carbon, nitrogen, and phosphate metabolism, to chemotaxis, potassium transport, and the virulence of certain pathogens (Milohanic et al., 2014). PTS components exert their regulatory functions by phosphorylating the target proteins directly or by interacting with them in phosphorylation-dependent manners. For phosphorylation-mediated regulation, certain transcriptional regulators containing specific PTS regulation domains are phosphorylated by PTS components and their regulatory activities are modulated. Besides transcriptional regulators, the target proteins include non-PTS transporters, catabolic enzymes, and histidine kinases of two-component signal transduction systems. In the latter case, the components of the PTS phosphorylation cascade interact with target proteins in phosphorylated or unphosphorylated states, either promoting or compromising their functions (Galinier and Deutscher, 2017).

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The genus *Salmonella* consists of two species, *S. bongori* and *S. enterica*; the latter is a dangerous human and animal pathogen prevalent worldwide (Lamas et al., 2018). *S. enterica* subsp. *enterica*, one of six *S. enterica* subspecies, consists of more than 1500 serotypes with distinct host ranges (Timme et al., 2013). *S. enterica* serovars Typhi and Paratyphi are highly host-adapted pathogens that provoke systemic typhoid fever exclusively in humans and some other primates. *S. enterica* serovars Enteritidis and Typhimurium cause acute self-limiting gastroenteritis in humans and have a broad host range including cattle, swine, and poultry (Larock et al., 2015). Apart from gastroenteritis, these non-typhoidal serotypes are especially problematic in immunocompromised individuals due to bacteremia and endovascular infection leading to focal infection (Hohmann, 2001). Invasive non-typhoidal salmonellosis (iNTS) is associated with a case fatality of 20–25% in infants, the elderly, and immunocompromised people, particularly in Africa (Ao et al., 2015; Feasey et al., 2012; Haeusler and Curtis, 2013).

S. Typhimurium possesses the *pts* operon composed of *ptsH*, *ptsI*, and *crr* genes, which encode HPr, EI, and a component of the glucose-specific EII complex, $EIIA^{Glc}$, respectively (De Reuse and Danchin, 1988). *S.* Typhimurium with a defective PTS showed attenuation in invasion into host cells and proliferation inside host macrophages, implicating regulation of virulence by PTS (Bowden et al., 2014, 2009). Especially, *S.* Typhimurium lacking EI, which catalyzes the first reaction of PTS, exhibited significantly reduced fitness during infection in mice. Absence of the *ptsI* gene resulted in a nearly three-log increase of the 50% lethal dose (LD_{50}) in an intraperitoneally (IP) infected mouse model (Kok et al., 2003). Furthermore, mice administered intravenously (IV) with *ptsI* mutants exhibited no visible clinical signs of illness on day seven post-infection (Mastroeni et al., 2016). Taken together, these results indicate that EI exerts prominent roles in the regulation of *S.* Typhimurium virulence in parallel with sugar translocation. However, it remains unanswered how EI takes part in modulating *Salmonella* virulence during host infections. To understand the underlying mechanism exerted by EI, a transcriptomic approach was applied in a *S.* Typhimurium *ptsI* mutant wherein transposon Tn*10* was inserted at position 1,477 bp of the 1,739 bp *ptsI* gene, causing transcriptional interruption of *ptsI* and adjacent *crr*. The comprehensive transcriptomic analysis revealed that the transposon insertion mutant concurrently downregulated multiple virulence determinants, including the *lsr* operon, *Salmonella* pathogenicity island (SPI) genes, flagella genes, and the PhoPQ regulon. These results provide an insight into new roles of PTS in coordinating the expression of virulence determinants in *Salmonella.*

2. Material and methods

2.1. Bacterial strains, plasmids, and growth conditions

S. Typhimurium UK1 was used as a parent strain throughout this study. The *ptsI*::Tn*10* mutant strain was constructed in our previous study (Lim et al., 2015). All deletion, epitope tagging, and *lacZY* fusion strains were constructed using the phage λ-derived Red recombination system (Datsenko and Wanner, 2002). In brief, chloramphenicol (cam) and kanamycin (kan) resistance cassettes were PCR-amplified using pKD3 and pKD13 as templates, respectively, and two-primer sets designed to produce DNA fragments of cam and kan resistance cassettes with both 3′ and 5′ termini homologous to regions adjacent to the target sites in the chromosome. The PCR products were introduced into a *Salmonella* strain harboring pKD46 for homologous recombination. The antibiotic resistance cassettes inserted into the chromosome were removed using flippase (FLP) recombinase produced by pCP20. For construction of the transcriptional *lacZY* fusion, the resolved strain with a recombination scar in place of the kan resistance cassette was transformed with pCE70 to replace the scar sequence with *lacZY* (Ellermeier et al., 2002). In the case of horizontal transfer of genetic materials between *Salmonella* strains, including SL1344, 14028, and UK1 strains, the phage P22-mediated transduction was used as described previously (Chan et al., 1972). To construct a plasmid pPC, pts1/pts2 and pts3/pts4 primer sets were used to amplify the promoter region for the *ptsHIcrr* operon (447 bp) and *ptsI* and *crr* DNA fragments (2275 bp). The pts2 primer is complementary to pts3; thus, in another PCR reaction using pts1 and pts4 primers, two separate PCR products were combined. The final PCR product was digested with SphI and BamHI and cloned into pZC320 (Shi and Biek, 1995). Bacterial strains, plasmids, and primers used in this study are listed in Table S1 and S2, respectively. Bacterial strains were cultured in Luria-Bertani (LB) medium (Difco, Franklin Lakes, NJ, USA) at 37 °C with or without shaking at 200 rpm. Tetracycline, kanamycin, and ampicillin (Sigma-Aldrich, St. Louis, MO, USA) were used at 15, 50, and 100 μg/ml, respectively, when required.

2.2. Macrophage survival assay

Murine macrophage-like cell line RAW264.7 (ATCC®, Manassas, VA, USA) was cultured in Dulbecco's modified Eagle medium (DMEM; Life Technologies, Carlsberg, CA, USA) supplemented with 10% fetal bovine serum (FBS; Lonza, Basel, Switzerland) at 37 °C with 5% CO_2 and seeded into 24-well plates at 2×10^5 cells/well. In order to compare the survival ability between wild-type and the *ptsI*::Tn*10* mutant strains, *Salmonella* cells cultured overnight were washed with phosphate buffered saline (PBS), resuspended in DMEM, and added to monolayered macrophage cells at a multiplicity of infection (MOI) of ten. The plate was incubated at 37 °C with 5% CO_2 for 30 min. Extracellular bacteria were removed by washing the infected macrophage cells with PBS three times and replenishing the macrophages with DMEM containing 100 μg/ml gentamycin (Life Technologies) for 2 h. The medium was subsequently replaced with DMEM containing gentamycin at 20 μg/ml and the cells were further incubated for 8 h. Macrophage cells were washed with PBS and lysed in 1% Triton X-100 (Sigma-Aldrich) for 10 min. The cell lysate was serially diluted using PBS and plated on LB agar to enumerate intracellular bacteria.

2.3. RNA isolation and microarray

Salmonella cells at the stationary phase in LB broth were centrifuged at 10,000 × g for 5 min and subjected to total RNA isolation using an SV Total RNA Isolation Kit (Promega, Madison, WI, USA) according to the manufacturer's instructions. For microarray probe preparation, a mixture of 75 μg RNA and 3 μg hexamer was first denatured at 65 °C for 10 min, then further incubated at 42 °C in a solution containing SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA, USA), dNTP mix, Cy3- or Cy5-conjugated dUTP (Amersham Biosciences, Buckinghamshire, UK), RNasin, and DTT for 2 h. Reverse transcripts were denatured with 1 M HCl and purified using a PCR purification kit (Qiagen, Venlo, Netherlands). Probes labeled with Cy3 or Cy5 were resuspended in 20 μl distilled water. Equivalent amounts of probes prepared from the wild-type and the *ptsI*::Tn*10* mutant strains with different cyanine dyes were mixed in 2× hybridization solution containing 50% formamide, 10 × SSC, and 0.2% SDS and denatured by boiling for 5 min. The heat-treated probes were applied to a non-redundant pan-*Salmonella* ORF chip and incubated in a hybridization chamber (Corning, Corning, NY, USA) at 42 °C overnight. Detailed construction methods for DNA chips have been described previously (Porwollik et al., 2004).

2.4. Microarray data analysis

Microarray data analysis was conducted as described previously (Lim et al., 2015). Briefly, scans were performed with a Scan Array 5000 laser scanner using ScanArray 2.1 software (Packard BioChip Technologies, Billerica, MA, USA) and signal intensities were quantified using QuantArray 3.0 software (Packard BioChip Technologies). Printtip loess normalization within an array and scale normalization

between arrays using the R statistical programming environment (http://www.bioconductor.org) was applied for nine data points for each probe on the array, obtained from three biological replicates with swapped dyes. The significance threshold for change in gene expression was set at a *p*-value less than 0.001 and a B-value above zero.

2.5. β-galactosidase assay

β-galactosidase activity was assessed in triplicate according to the standard method by (Miller, 1972), with the enzymatic activity expressed in Miller units.

2.6. Quantitative real-time PCR (qRT-PCR) analysis

Total RNAs isolated as described above were treated with a TURBO DNA-free kit (Ambion, Austin, USA) to remove DNA remnants. cDNA was synthesized using an Omniscript Reverse Transcription kit (Qiagen, Hilden, Germany) and random hexamers (Invitrogen). cDNA was mixed with 2 × iQ™ SYBR® Green Supermix (Bio-Rad Laboratories, Hercules, CA, USA) and 0.2 μM specific primers in a 20 μl reaction volume and subjected to qRT-PCR using an iCycler iQ® real-time PCR detection system (Bio-Rad Laboratories). The mRNA expression level of the target gene was normalized to the *rpoD* expression level. The primers used in this study are presented in Table S2.

2.7. Primer extension analysis

Salmonella total RNAs were isolated using TRIzol reagent (Life Technologies, Invitrogen, Thermo Fisher Scientific) according to the manufacturer's instructions. Primer extension analysis was conducted to compare the activity of promoters under different conditions as described previously (Lim et al., 2007). To examine *phoP* promoter activities, a P_{phoP} primer complementary to + 52 to + 82 relative to the translation start site was used. A control primer extension assay was also conducted using primer P_{ompA} (Schechter et al., 2003). The primer sequences are presented in Table S2.

2.8. SDS-PAGE and western blotting

For SDS-PAGE analysis of secreted proteins, *Salmonella* cells were cultured in LB broth to the stationary phase and centrifuged at 10,000 × g for 5 min to collect the cell-free spent medium as described previously (Niemann et al., 2011). After filtering the spent medium using a 0.22-μm pore-sized filter, the secreted proteins in the filtrate were precipitated using 20% trichloroacetic acid (TCA; Sigma-Aldrich). Concentrated protein samples were dissolved in 1 × Laemmli sample buffer. Whole cell lysates were prepared by dissolving bacterial cell pellets directly in 1 × Laemmli sample buffer. Proteins were separated using 10% or 12% polyacrylamide gels and stained using colloidal blue. Protein bands on the gel were cut out and subjected to liquid chromatography coupled with LTQ-Orbitrap mass spectrometry (Thermo Fisher Scientific, San Jose, USA) for protein identification when required. Proteins on SDS-PAGE gels were transferred to PVDF membranes (Bio-Rad Laboratories) and the membrane was blocked with 5% skim milk to prevent nonspecific protein binding. FLAG epitope-tagged SsrB, hemagglutinin (HA) epitope-tagged PhoP, and DnaK were identified using anti-FLAG M2 (1:10,000 dilution; Sigma-Aldrich), anti-HA (1:10,000 dilution; Sigma-Aldrich), and anti-DnaK (1:10,000 dilution; Sigma-Aldrich) primary antibodies, respectively, in combination with a horseradish peroxidase-conjugated secondary antibody (1:700 dilution; Santa Cruz Biotechnology, Dallas, TX, USA). Western blotting was performed using the West-ZOL® plus western blot detection system (iNtRON Biotechnology, Seongnam, South Korea) and the blot images were visualized using the ChemiDoc™ MP System (Bio-Rad Laboratories).

2.9. Motility assay

Salmonella cells at the stationary phase in LB broth were diluted to 10^9 CFU/ml and an aliquot of 10 μl was spotted onto 0.35% LB agar plates. The plates were incubated at 37 °C for 5 h at least and the diameter of the bacterial growth halos was measured to compare motility between strains.

2.10. Statistical analysis

The data were presented as the mean ± standard deviation from a minimum of three independent tests using different biological replicates and analyzed using the Student's *t*-test. A *p*-value of 0.05 or less was regarded as statistically significant.

3. Results and discussion

3.1. Comprehensive transcriptomic analysis of a ptsI::Tn10 mutant strain

The fate of intracellular *Salmonella* is determined by pre-growth conditions prior to macrophage infection (Sridhar and Steele-Mortimer, 2016; Van Der Velden et al., 2000). Shin et al. demonstrated previously that PTS genes (*ptsG* and the *pts* operon of *ptsHI* and *crr*) were post-transcriptionally regulated by oxygen concentrations: positive regulation of mRNA transcription by oxygen and negative regulation of protein expression by oxygen (Shin et al., 2008). To determine the optimum conditions under which the PTS participates in *Salmonella* infections in host cells, wild-type *S.* Typhimurium and a *ptsI*::Tn*10* mutant strain were cultured to the stationary phase with or without agitation prior to infection in murine macrophages. Bacterial cell numbers internalized by macrophages were comparable between the wild-type and the mutant strain regardless of oxygen concentrations. However, wild-type *Salmonella* cultured with agitation proliferated about 1.8-fold more than that cultured statically at 10 h post-infection (Fig. 1A), suggesting that stirring cultivation stimulates *Salmonella* to adapt to intracellular niches more promptly than static cultivation. Although the transposon mutant strain exhibited a decrease in intracellular survival independent of oxygen concentrations (Fig. 1A), stirring cultivation conditions were chosen for transcriptomic analysis of the *ptsI*::Tn*10* mutant strain because *Salmonella* became highly competitive to survive inside macrophages under this growth condition.

To define the repertoire of *Salmonella* genes whose expressions are altered by a transposon insertion in the tested mutant strain, we performed DNA microarray analysis using total RNA extracted from the stirring cultivation condition. Statistical analysis and sorting of DEGs with expression changes more than two-fold showed that 13 and 101 genes were up- and down-regulated, respectively, in the *ptsI*:Tn*10* mutant as compared with wild-type *Salmonella* (Tables S3). It was apparent that a large number (~37%) of down-regulated genes were associated with energy production and carbohydrate/amino acid metabolism pathways (Fig. 1B). Glucose is the predominant carbon source available for intracellular *S.* Typhimurium inside murine macrophages (Bowden et al., 2009). Thus, the defective intracellular replication of the mutant was likely to be ascribed in part to a shortage of available glucose in the cells. As expected, the expression of *crr*, located downstream of *ptsI*, was significantly decreased in the *ptsI*::Tn*10* mutant, which has a transposon Tn*10* insertion downstream of the +1 site of the $P_{crr\text{-}1}$ promoter (Table S3 and Fig. S1). It has been reported that a five-fold reduction in $EIIA^{Glc}$ activity does not influence glucose metabolism (Van Der Vlag et al., 1995). However, it cannot be ruled out that the decreased *crr* expression might lead to the attenuation of *Salmonella* virulence as described below. A list of the top 15 genes most significantly down-regulated in the *ptsI*::Tn*10* mutant strain is presented in Table 1. Interestingly, DEGs down-regulated in the mutant strain included well-known virulence determinants associated with quorum sensing, motility, SPI, and PhoPQ. Henceforth, we validated the

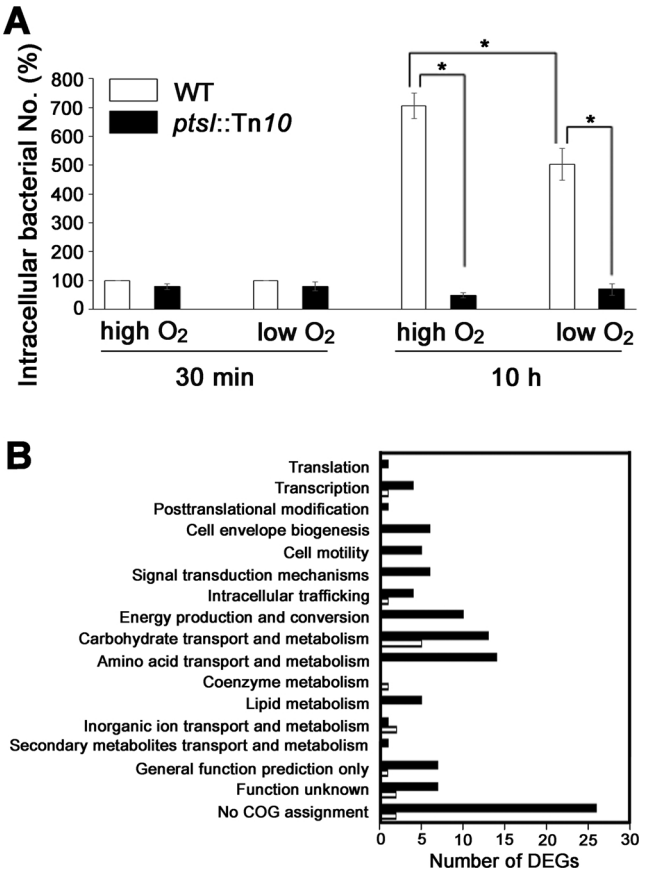

Fig. 1. Comprehensive transcriptomic analysis of *Salmonella ptsI*::Tn*10* strain. (A) Comparison of *Salmonella* intracellular survival depending on pre-culture conditions. *Salmonella* cells including wild-type and *ptsI*::Tn*10* strains were pre-cultured with or without agitation before addition to macrophages. Intracellular *Salmonella* was enumerated at 30 min and 10 h post-infection and normalized using the numbers of wild-type strain at 30 min for each condition. Bacterial numbers with differences in *p*-values < 0.05 are denoted with asterisks. (B) Functional categorization of differentially expressed genes (DEGs) in the *ptsI* mutant strain. Genes up (wild bar)- or down (black bar)-regulated 2-fold or more in the *ptsI* mutant strain as compared to those in the wild-type strain were classified according to clusters of orthologous groups (COG).

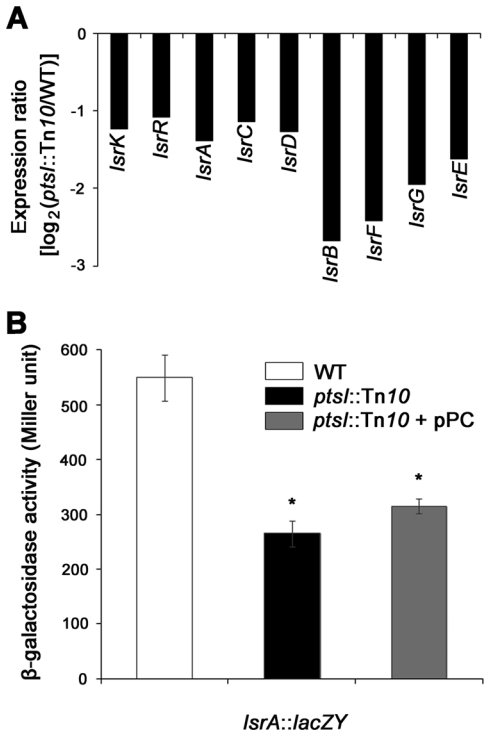

Fig. 2. Down-regulation of the *lsr* operon in the *ptsI* mutant strain. (A) Expression of the *lsr* operon in the microarray analysis. Relative expression of *lsr* operon genes between the *ptsI* mutant and wild-type strains was expressed as $\log_2$ (fold change). (B) A transcriptional *lacZY* fusion was introduced into the chromosomal *lsrA* gene of the wild-type and the *ptsI* mutant strains. The *ptsI* mutant strain was transformed with the pPC plasmid expressing *ptsI* and *crr* under the *ptsHIcrr* operon promoter. Asterisks indicate different β-gal activities of *p*-value < 0.05 in comparison with the wild-type strain.

transcriptomic analysis of the *ptsI* mutant and explored the underlying mechanism of the PTS in *Salmonella* virulence regulation

3.2. *Salmonella* PTS is associated with multiple virulence determinants

3.2.1. Positive regulation of the lsr operon by the PTS

S. Typhimurium has the LuxS/autoinducer-2 (AI-2) quorum sensing system for interspecies signaling. Extracellular AI-2 is imported into the cytoplasm via the ATP transporter LsrACDB encoded by the *lsrACDBFGE* operon and subsequently phosphorylated by a cytoplasmic kinase, LsrK. Phosphorylated AI-2 in turn inactivates the transcriptional regulator LsrR, which inhibits the transcription of the bidirectional *lsr*

Table 1
Top 15 genes exhibiting decreased expression in the ptsI mutant strain.

Gene	Locus tag	Description	$\log_2$ (*ptsI*::Tn*10*/WT)	*p*-value
	STM3132	putative xylanase/chitin deacetylase	−2.91	5.8E-14
lsrB	STM4077	autoinducer 2 (AI-2)-binding protein	−2.67	9.4E-12
	STM3133	putative amidohydrolase	−2.51	6.0E-13
fliC	STM1959	flagellin, filament structural protein	−2.50	5.3E-10
pipB2	STM2780	secreted effector protein	−2.50	4.4E-11
crr	STM2433	glucose-specific IIA component	−2.48	1.7E-09
	STM2779	hypothetical protein	−2.44	3.1E-12
	STM2585	Gifsy-1 prophage: similar to transpose	−2.44	8.8E-06
lsrF	STM4078	putative AI-2 aldolase	−2.42	1.1E-10
sipC	STM2884	cell invasion protein	−2.37	7.0E-11
pagC	STM1246	PhoP-regulated membrane protein	−2.35	7.6E-07
sopB	STM1091	Secreted effector protein	−2.25	1.3E-07
gltI	STM0665	glutamate/aspartate transporter subunit	−2.25	1.6E-07
ssrB	STM1391	*Salmonella* pathogenicity island-2 regulator	−2.23	5.6E-12
acs	STM4275	acetyl-CoA synthetase	−2.18	8.3E-10

operons of *lsrACDBFGE* and *lsrRK* by binding to the *lsr* operon promoter sites (Xue et al., 2009). We observed that *lsr* operon genes were down-regulated in the *ptsI* mutant strain (Fig. 2A and Table S3). Positive-regulation of the *lsr* operon by the PTS was validated using a transcriptional *lacZY* fusion to the *lsr* operon promoter (Fig. 2B). Several studies suggest the correlation between the PTS and quorum sensing in *Escherichia coli*. AI-2 internalization through the LsrACBD transporter required the PTS, in particular phosphorylated EI (Pereira et al., 2012). Besides, the transcription of the *lsr* operon was activated by cyclic AMP (cAMP), which was produced by the activity of adenylate cyclase with the aid of phosphorylated $EIIA^{Glc}$ (Krin et al., 2002; Milohanic et al., 2014). Recently, it has been revealed that unphosphorylated HPr binds to LsrK and inhibits phosphorylation of internalized AI-2 by LsrK, which in turn leads to LsrR-mediated *lsr* operon repression (Ha et al., 2018). The *ptsI*::Tn*10* mutant strain has defects not only in EI production but also in $EIIA^{Glc}$ expression due to the transposon insertion mutation (Fig. S1). Therefore, the likelihood was that unphosphorylated HPr and unphosphorylated $EIIA^{Glc}$, due to the lack of EI responsible for their sequential phosphorylation, might cause LsrR repression of the *lsr* operon and dampen cAMP-mediated activation of the *lsr* operon, respectively, in the *ptsI* mutant strain. However, a derivative of the pZC320 plasmid, pPC, expressing *ptsI* and *crr,* did not complement the decreased *lsr* operon expression in the *ptsI*::Tn*10* mutant strain, implying the complexity of the PTS regulatory network (Fig. 2B).

3.2.2. Positive regulation of SPI-1 and flagella-associated genes by the PTS

Salmonella has acquired discrete chromosomal loci termed SPIs during its evolution as a pathogen. Among multiple SPIs, SPI-1 genes encode structural components constituting a type III secretion system (T3SS1) and its secretion substrates called effectors. T3SS1 translocates diverse effector proteins into the cytoplasm of host cells and facilitates *Salmonella* invasion into host epithelial cells, inducing intestinal inflammation (Ilyas et al., 2017). Transcriptomic analysis of the *ptsI* mutant strain revealed overall down-regulation of SPI-1 genes (Fig. 3A and Table S3). The expression of a chromosomal *lacZY* fusion to *invF*, a primary transcriptional regulator gene of SPI-1, was also decreased in the *ptsI* mutant strain (Fig. 3B), suggesting the impact of a malfunctioning PTS on SPI-1 via its cognate regulator's response. Decreases in SPI-1 gene expression brought about decreased secretion of SipA and SipC effectors into the culture supernatant of the *ptsI* mutant strain (Fig. 3C). However, the introduction of the pPC plasmid did not fully complement the altered SPI-1 expression in the *ptsI* mutant strain (Fig. 3B).

The expression of genes associated with flagellar filament structure, including *fliC* and *fljB* encoding two different types of filament subunits and *fliD* encoding the filament cap, was significantly decreased in the *ptsI* mutant strain (Fig. 3A and Table S3). In accordance with the transcriptional changes, the secretion of phase 2 flagellin FljB into bacterial culture medium was significantly decreased in the *ptsI* mutant strain (Fig. 3C). The impaired motility of the mutant strain on semi-solid agar was likely attributable to the decreased expression of both types of flagellins and their cap protein (Fig. 3D). Introduction of the pPC plasmid restored bacterial motility in the mutant strain. Mutations in flagella genes leading to immotility impeded *Salmonella* adhesion and invasion ability during host infection (Eichelberg and Galán, 2000; Haiko and Westerlund-Wikström, 2013). However, considering that *Salmonella* cells were added to phagocytic macrophages and comparable internalization occurred regardless of bacterial strains in our study

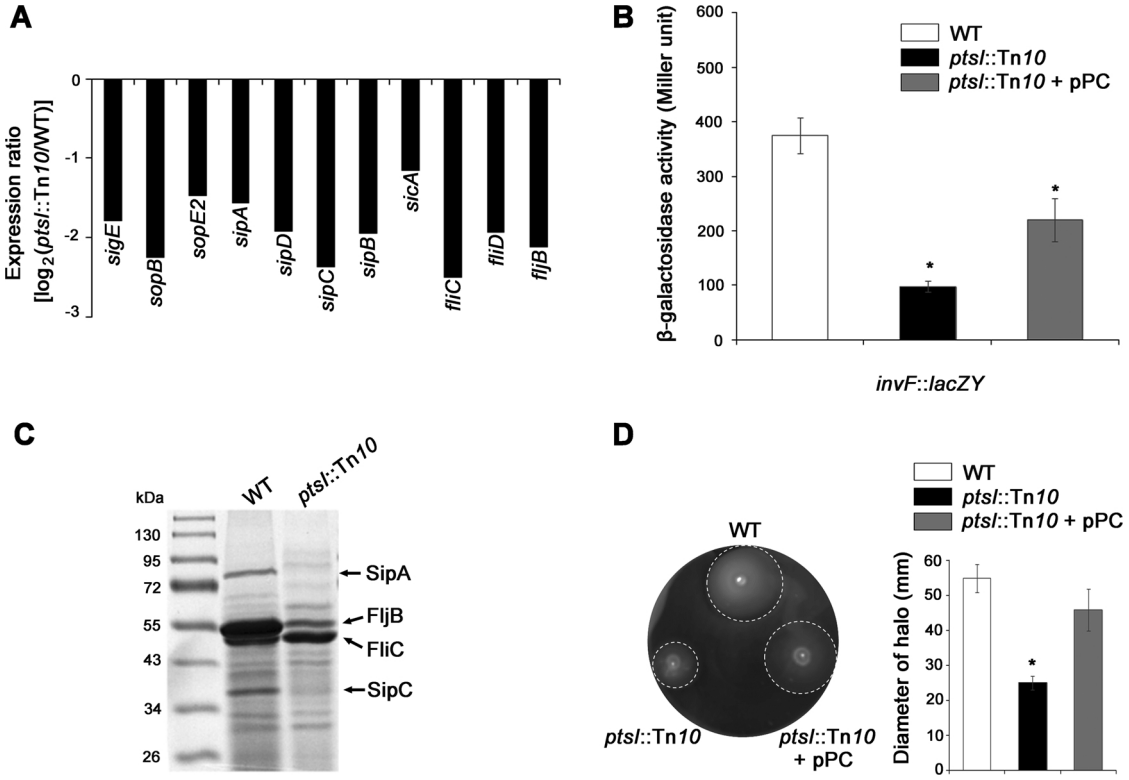

Fig. 3. Down-regulation of *Salmonella* pathogenicity island 1 (SPI-1) and flagella genes in the *ptsI* mutant strain. (A) Expression of SPI-1 and flagella genes in the microarray analysis. Expression fold changes between the *ptsI* mutant and wild-type strains are expressed in $\log_2$ scale. (B) Expression of *invF* was compared between wild-type and the *ptsI* mutant strains using a transcriptional *lacZY* fusion to P_{invF} promoter. Plasmid pPC was introduced into the *ptsI* mutant strain to complement the defective PTS expression. β-gal activities with *p*-value < 0.05 in comparison to the wild-type strain were denoted by asterisks. (C) Comparison of secreted proteins between wild-type and the *ptsI* mutant strains. Proteins secreted into the culture medium were analyzed using SDS-PAGE and mass spectrometry. (D) Bacterial motility was compared in the wild-type strain and the *ptsI* mutant strains either harboring pPC or not. Diameters of bacterial halos on semi-solid agar plates were plotted in triplicate tests and those with *p*-value < 0.05 in comparison to the wild-type strain were indicated with an asterisk. Images of (C) and (D) are representative photographic images.

(Fig. 1A), the attenuated replication of the *ptsI* mutant strain was hardly associated with its compromised motility before internalization into host cells. Nevertheless, flagella structure is surely implicated in *Salmonella* virulence; *Salmonella* cells unable to control *fliC* expression were remarkably cleared out during systemic infection in mice (Miao et al., 2010).

Considering the down-regulation of the *lsr* operon in the *ptsI* mutant (Fig. 3), the positive regulation of SPI-1 and flagella genes by the PTS might be attributed in part to LsrR, which directs carbohydrate availability status to virulence regulation. Choi et al. have reported that LsrR represses the expression of SPI-1 genes as well as flagella genes, although they failed to identify LsrR binding sites in their promoters (Choi et al., 2012). Unphosphorylated PTS components under excessive glucose concentrations or the lack of EI as the phosphoryl donor (the *ptsI* mutation *per se*) might liberate the LsrR repressor from inhibitory AI-2 binding, leading to inhibition of SPI-1 and flagella expression as described above.

3.2.3. Positive regulation of SPI-2 and its associated genes by the PTS

A different T3SS encoded by SPI-2 (T3SS2) is crucial for *Salmonella* to survive inside epithelial cells and macrophages and to spread throughout deeper tissues such as liver and spleen during systemic infection (Waterman and Holden, 2003). It is interesting to note that the majority of SPI-2 genes altered their expression negatively as a result of the transposon insertion in *ptsI* gene: of 32 genes (from *ssaU* to *ssrB*) associated with T3SS2, 17 genes were repressed in the mutant strain as compared with the wild-type strain (Fig. 4A and Table S3). In addition, four genes (*sopD2*, *pipB*, *pipB2*, and *sseJ*), located apart from SPI-2 but encoding T3SS2 effectors, showed significant decreases in their expression due to Tn*10* insertion in *ptsI* gene (Fig. 4A and Table S3). In accordance with the transcriptomic analysis results, a transcriptional *lacZY* fusion to the promoter of the *sseA* operon was expressed about 4-fold lower in the *ptsI* mutant strain than that in the wild-type strain (Fig. 4B), though the pPC plasmid failed to complement the *ptsI* mutant strain. The transcription of SPI-2 genes is dependent on the two-component system (TCS) SsrAB encoded within SPI-2 (Deiwick et al., 2003). To confirm the findings of the transcriptome analysis, the protein levels of the response regulator SsrB were compared between wild-type and *ptsI* mutant strains, which were modified to produce chromosomally FLAG-tagged SsrB, respectively. As predicted, SsrB was significantly decreased in the *ptsI* mutant (Fig. 4C). The down-regulation of SPI-2 in the *ptsI* mutant strain might be associated with the SPI-1 master regulator HilD, which mediates transcriptional cross-talk between SPI-1 and SPI-2 (Puente et al., 2008). Alternatively, a direct correlation of SPI-2 with the PTS has been suggested by (Mazé et al., 2014). $EIIA^{Glc}$ encoded by *crr* binds to T3SS2 components including SsaK, SsaN, and SsaQ directly or indirectly and stabilizes the cytoplasmic compartments of the injectisome. Therefore, the lack of $EIIA^{Glc}$ abolishes secretion and translocation of T3SS2 effectors. Considering the decreased *crr* expression in the *ptsI* mutant strain due to the location of the Tn*10* transposon insertion (Fig. S1), the integrity of T3SS2 might be impaired in the *ptsI*::Tn*10* mutant. Intriguingly, we observed the possibility of feedback control in T3SS2 machinery. The mRNA levels of T3SS2-associated genes were decreased in the absence of SsaK (Fig. 4D). SsaK, along with SsaN and SsaQ, forms the C-ring complex located at the peripheral cytoplasmic face of the T3SS2 apparatus and its absence causes incomplete T3SS2 assembly, abolishing the translocation of effectors through the secretion machinery (Niemann et al., 2011; Yoshida et al., 2014). Unstable T3SS2 assembly in the *ptsI* mutant strain might trigger a feedback control in SPI-2, suppressing excessive T3SS2 gene expression, as illustrated in a regulatory model (see Conclusions). The possibility of SPI-2 regulation in response to carbohydrate metabolism should be further evaluated.

3.2.4. Positive regulation of the PhoPQ regulon by the PTS

The PhoPQ (PhoP, response regulator; PhoQ, sensor kinase) TCS

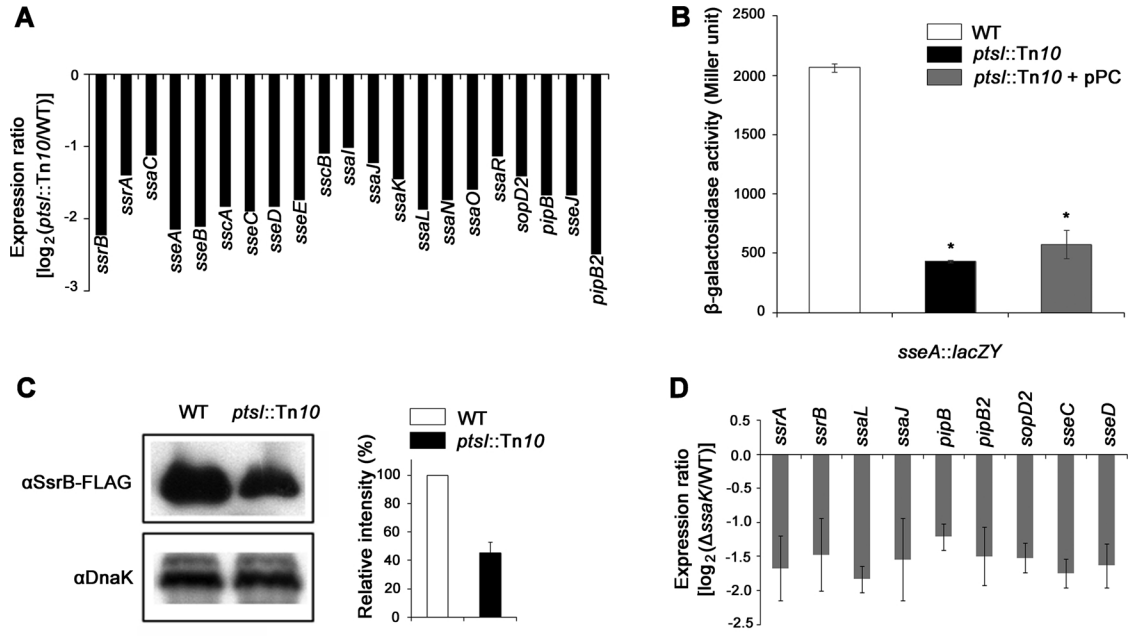

Fig. 4. Down-regulation of *Salmonella* pathogenicity island 2 type III secretion system (T3SS2) genes in the *ptsI* mutant strain. (A) Expression of T3SS2-associated genes in the microarray analysis. Gene expression was compared between the *ptsI* mutant and wild-type strains and the ratio is expressed in $\log_2$ scale. (B) Gene *sseA*, the first gene forming one of the major SPI-2 transcriptional units, was fused to the *lacZY* fragment in wild-type and the *ptsI* mutant strains and its expression was monitored using a β-gal assay. The *ptsI* mutant strain was transformed with a pPC plasmid, which expressed *ptsI* and *crr* using their own promoters. In comparison with the wild-type strain, differences with p-value < 0.05 were indicated with asterisks. (C) Expression of SsrB in wild-type and *ptsI* mutant strains. Both strains were genetically modified to produce SsrB tagged with FLAG at its C-terminus. DnaK, an abundant cytosolic protein, was used as a control to compare the protein levels between lanes. SsrB intensity was normalized with that of DnaK using ImageJ software (https://imagej.nih.gov/ij/) and its relative abundance in three independent tests was expressed as a percentage. A representative western blotting image is shown. (D) Expression comparison of T3SS2 genes between wild-type and Δ*ssaK* strains using qRT-PCR. mRNA levels were normalized using *rpoD* levels in each strain and their relative expression ratios (Δ*ssaK*/wild-type) were plotted.

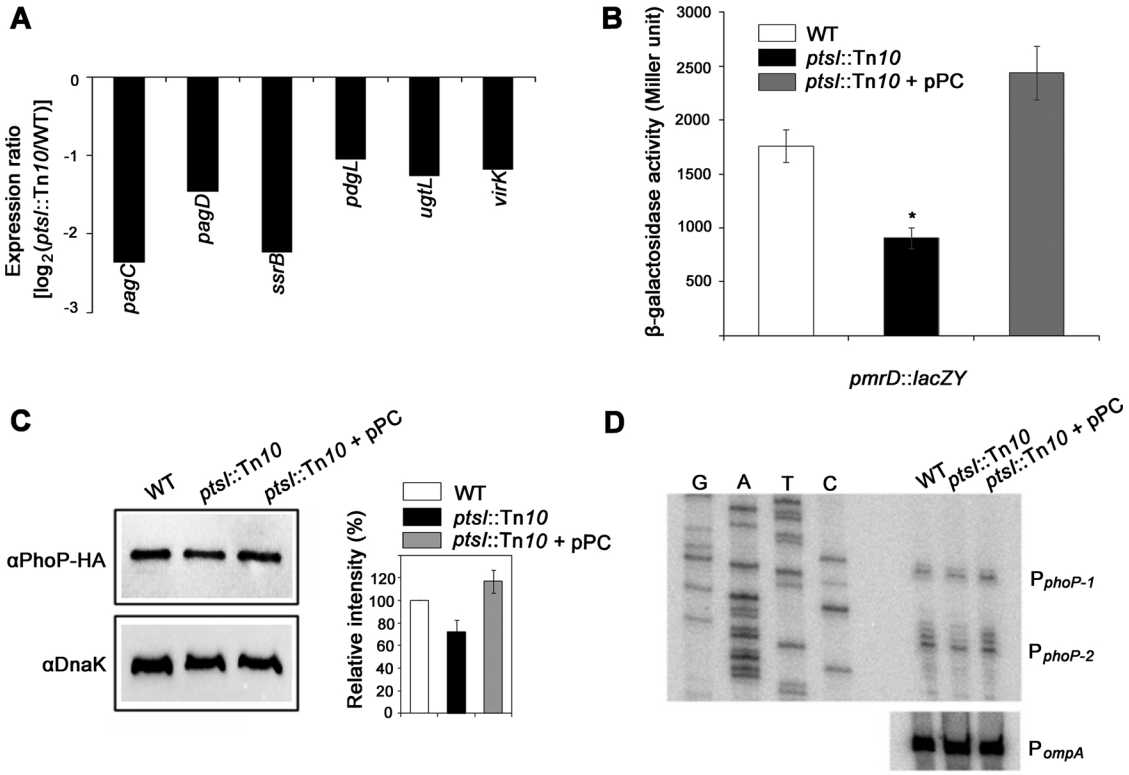

Fig. 5. Down-regulation of the PhoPQ regulon in the *ptsI* mutant strain. (A) Expression of six genes with PhoP-binding sites in their promoter regions in the microarray analysis. Relative expression ratio between the *ptsI* mutant and wild-type strains is shown in $\log_2$ (fold change). (B) Gene *pmrD* involved in the PhoPQ and PmrAB regulons was fused with *lacZY* in the chromosome of the wild-type and the *ptsI* mutant strains and a pPC plasmid was further introduced into the *ptsI* mutant strain. Different β-gal activities with p-value < 0.05 in comparison with the wild-type strain were indicated with an asterisk. (C) PhoP tagged with HA at its C-terminus was analyzed in wild-type and *ptsI* mutant strains using western blotting. The *ptsI*::Tn*10* mutant strain was complemented with pPC. ImageJ software was used to compare PhoP production between strains using DnaK as an internal control for normalization and the relative PhoP production from three independent tests is plotted in a graph. (D) Activities of *phoP* promoters were compared in wild-type strain and the *ptsI* mutant strains using primer extension analysis. pPC plasmid was introduced into the *ptsI* mutant strain to supplement the lack of *ptsI* and *crr* genes. Transcription from the *ompA* promoter was examined as a control to show equivalent RNA levels between lanes. Images of (C) and (D) are representative photographic images.

exerts a pivotal role, conveying diverse environmental cues to virulence regulation during the infection process (Groisman, 2001). The transposon mutation caused a significant decrease in expression of *pagC* (Table 1), which is one of the well-known PhoP-regulated genes (Gunn et al., 1995). Of the 23 PhoP-activated genes with PhoP-binding sites in their promoter regions (Zwir et al., 2012), six genes including *pagC* were down-regulated in the *ptsI* mutant strain (Fig. 5A and Table S3). Another PhoP-activated gene, *pmrD*, encodes a connector protein that links two TCSs, PhoPQ and PmrAB (Kox, 2002; Zwir et al., 2012). Transcriptional *lacZY* fusion analysis on *pmrD* confirmed the down-regulation of PhoP-activated genes by the Tn*10* mutation (Fig. 5B). To investigate if the defective PTS influenced PhoPQ regulon expression through PhoP, its protein level was compared between wild-type and *ptsI* mutant strains. The Tn*10* insertion mutation decreased PhoP levels to 70% of the wild-type levels (Fig. 5C). The *phoPQ* operon is transcribed from two promoters, $P_{phoP\text{-}1}$ and $P_{phoP\text{-}2}$: the former is induced by activating signals in the presence of PhoPQ, while the latter is independent of PhoPQ and maintains a basal activity for PhoPQ homeostasis (Soncini et al., 1995). When the activity of the two *phoP* promoters was examined using primer extension analysis, the activity of $P_{phoP\text{-}2}$ was decreased in the *ptsI* mutant strain in accordance with the decreased PhoP levels (Fig. 5D). Unlike the *lsr* operon and SPI genes, complementation of the *ptsI* mutant strain using plasmid-borne *ptsI* and *crr* in *trans* restored the PhoP production, $P_{phoP\text{-}2}$ activity and *pmrD* expression to those of the wild-type strain (Fig. 5B–D). Altogether, these observations suggest that the PTS can affect PhoPQ-regulated gene expression in a PhoP-dependent manner. Considering that *Salmonella* exploits the PhoPQ TCS to cope with acidification inside *Salmonella*-containing vacuoles (SCVs) (Prost et al., 2007), the vacuolar compartments for intracellular *Salmonella* replication, the significant attenuation in intracellular replication (Fig. 1A) was partially attributable to the down-regulation of the PhoPQ regulon in the *ptsI* mutant strain.

4. Conclusions

An increasing number of studies report the pivotal role of the PTS in fine-tuning *Salmonella* virulence in response to available carbohydrate sources in the environment. In the same context, our transcriptomic analysis of the *ptsI*::Tn*10* mutant strain demonstrated that incomplete PTS caused concurrent down-regulation of multiple virulence determinants, implying an extensive regulatory network integrating carbohydrate transport pathway and virulence regulation. Typical virulence determinants associated with the PTS include the quorum sensing *lsr* operon, SPIs, flagella genes, and the PhoPQ regulon. A plausible mediator between PTS activity and these discrete virulence factors may be the LsrR regulator of quorum sensing. LsrR relieved from AI-2 inactivation due to inactive LsrK kinase might branch off to multiple regulatory circuits of the *lsr* operon, SPI-1, and flagella cascade. Unphosphorylated HPr directly inhibits LsrK activity, while phosphorylated $EIIA^{Glc}$ indirectly activates *lsrK* expression via cAMP. Apart from quorum sensing, $EIIA^{Glc}$ is also required for the translocation of T3SS2 effectors. We propose a regulation model wherein multiple virulence determinants are concurrently down-regulated in the *ptsI*::Tn*10* mutant strain described in this study (Fig. 6). The pleiotropic PTS regulatory circuit including the PhoPQ regulon and both SPI-1 and SPI-2, two crucial virulence factors required for *Salmonella* systemic

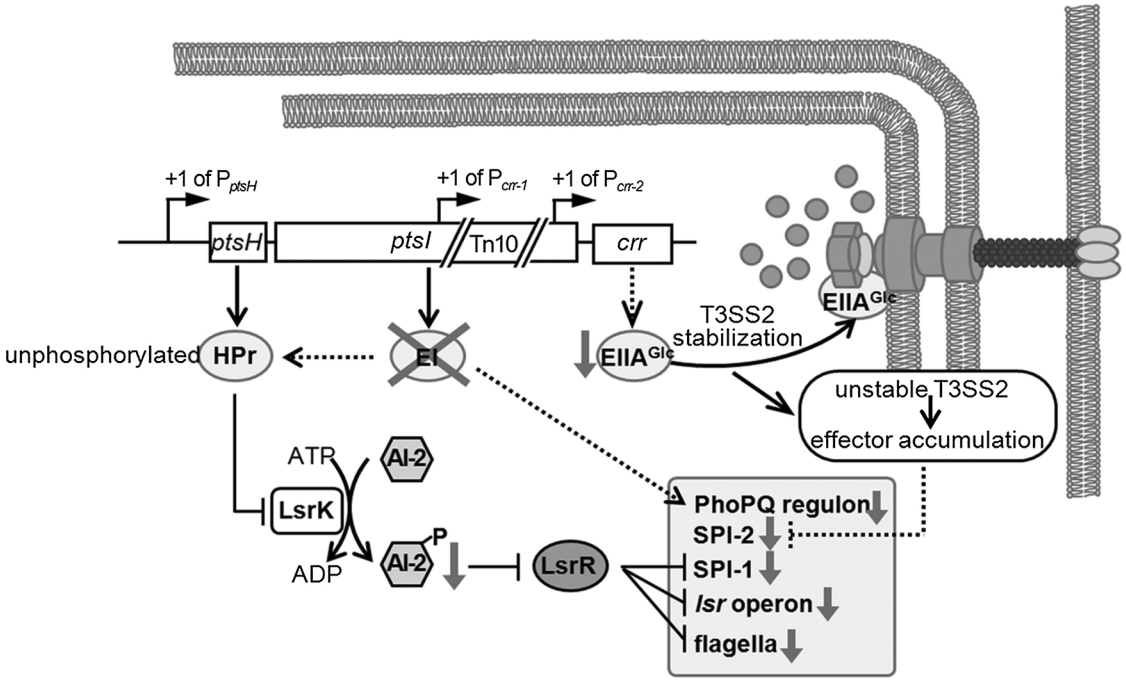

Fig. 6. Model for the roles of PTS components in *Salmonella* virulence regulation in the *ptsI*::Tn*10* mutant strain. Production of EI and $EIIA^{Glc}$ is supposed to be blocked (EI marked with X) or dampened ($EIIA^{Glc}$ with a downward grey arrow) due to a transposon Tn*10* insertion downstream of the transcription start site of *crr* (+1 site of P_{crr-1}) in the *ptsI*::Tn*10* mutant strain. Black lines with blunt ends show inhibition and grey downward arrows indicate decreased expression or production as the consequence. Regulation deduced from this study is shown in dotted lines. An outlined box contains primary virulence determinants down-regulated by the Tn*10* insertion mutation described in this study. Grey circles are T3SS2 effectors accumulated in the cytosol.

infection, accounted for the diminished intracellular survival of the *ptsI* mutant strain. Although SPI-1 is primarily required for *Salmonella* invasion into host cells, some T3SS1 effectors, such as SipA, SigD, and SopE2, play roles in intracellular proliferation in cooperation with T3SS2 effectors. Further investigation is required to unravel the extensive regulatory network orchestrated by the PTS.

Conflicts of interest

The authors declare that they have no conflicts of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.micres.2019.04.002.

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