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ptsI gene in the phosphotransfer system is a potential target for developing a live attenuated *Salmonella* vaccine

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Abstract. *Salmonella enterica* serovar Typhimurium causes invasive non-typhoidal *Salmonella* diseases in animals and humans, resulting in a high mortality rate and huge economic losses globally. As the prevalence of antibiotic-resistant *Salmonella* has been increasing, vaccination is thought to be the most effective and economical strategy to manage salmonellosis. The present study aimed to investigate whether dysfunction in the phosphoenolpyruvate:carbohydrate phosphotransferase system (PTS), which is critical for carbon uptake and survival in macrophages, may be adequate to generate *Salmonella*-attenuated vaccine strains. A *Salmonella* strain (KST0555) was generated by deleting the *ptsI* gene from the PTS and it was revealed that this auxotrophic mutant was unable to efficiently utilize predominant carbon sources during infection (glucose and glycerol), reduced its invasion and replication capacity in macrophages, and significantly (P=0.0065) lowered its virulence in the setting of a mouse colitis model, along with a substantially decreased intestinal colonization and invasiveness compared with its parent strain. The reverse transcription-quantitative PCR results demonstrated that the virulence genes in *Salmonella* pathogenicity island-1 (SPI-1) and -2 (SPI-2) and the motility of KST0555 were all downregulated compared with its parent strain. Finally, it was revealed that when mice were immunized orally with live KST0555, *Salmonella*-specific humoral and cellular immune responses were effectively elicited, providing protection against *Salmonella* infection. Thus, the present promising data provides a strong rationale for the advancement of KST0555 as a live *Salmonella* vaccine candidate and *ptsI* as a potential target for developing a live attenuated bacterial vaccine strain.

Introduction

Non-typhoidal *Salmonella* (NTS) is a leading cause of bacterial bloodstream infections in infants, the elderly and immunocompromised people, particularly in sub-Saharan Africa, where the case fatality rate is 20-25% (1,2). In sub-Saharan Africa, invasive NTS (iNTS) has been reported to be the second most common invasive bacterial disease type (29%), followed by *Streptococcus pneumoniae* infection (3,4). *S. enterica* serovar Typhimurium is the most common iNTS isolate, followed by *S. enterica* serovar Enteritidis (3,4). Although *Salmonella* may be controlled using antibiotics, an increasing prevalence of multidrug-resistant strains has been reported over previous decades (5,6). Thus, vaccine development is a potential prospect for controlling the epidemic prevalence of iNTS in humans in addition to animals (7). At present, two types of licensed *Salmonella* vaccines for typhoid fever are considered safe and efficacious for people aged over 2 years (8); however, there is no vaccine directly targeting iNTS. Vivotif®, a live oral vaccine containing a mutated *Salmonella* (Ty21a), may effectively elicit intestinal antibodies against O-antigens, which likely exhibits cross-protective efficacy against NTS, including *S*. Typhimurium and *S*. Enteritidis (9-11).

With the limitations of these existing vaccines, numerous subunit and live-attenuated vaccines for iNTS diseases are being developed, and a number of them are undergoing clinical studies (12,13). Approaches to subunit vaccines mainly focus on the application of the O-antigens of lipopolysaccharides and surface virulence factors, including flagellin and outer membrane proteins (14-16). These subunit vaccines potentially have the advantage of cross-protection, safety and low production cost (17). However, numerous subunit vaccines against

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bacterial pathogens have not been successful as the conformational changes in conserved epitopes during preparation result in poor humoral and long-lasting immunity, and a single structurally conserved protein, even if it is a critical virulence factor, would not be sufficient to provide effective immunity (18,19). Previously, numerous novel strategies for developing live NTS vaccines have been introduced. Most of the live attenuated *Salmonella* vaccine strains have been constructed by deleting functional metabolic gene(s) and/or virulence gene(s) (20-23). Among the candidate vaccine strains for iNTS, only a few were tested in clinical studies. LH1160, a *phoPQ* mutant strain that controls the transcription of multiple genes necessary for intracellular survival, had been tested in phase 1 clinical trials, but an unacceptable fever was reported in two of six volunteers immunized with LH1160 (21,24). WT05 is another attenuated *S*. Typhimurium vaccine in which the *aroC* gene, involved in aromatic amino acid biosynthesis, and the *ssaV* gene, a component of a Type 3 secretion system (T3SS) apparatus of *Salmonella* pathogenicity island 2 (SPI-2), are deleted. However, this vaccine also failed in phase 1 clinical trial due to the prolonged stool shedding of the vaccine strain in healthy volunteers immunized with WT05 (25,26). More recently, CVD1921, which is mutated in the *guaBA* genes that are involved in the biosynthesis of guanine nucleotides and the *clpP* gene affecting flagella expression, was revealed to be notably attenuated with decreased shedding, systemic spread and clinical disease manifestations in the digestive tract of the non-human primate model (rhesus macaque) used (27).

When *Salmonella* are internalized into the intracellular compartment of host cells, they need to adapt to the metabolism of available nutrients, mainly the carbon source in host cells (28,29). Multiple carbon transport systems are known to be involved in the intracellular life cycle and linked to the virulence of *Salmonella* (30-32). For example, the carbohydrate phosphotransferase system (PTS) is composed of two cytoplasmic proteins, enzyme I (*ptsI*) and histidine protein (*ptsH*), which are common to all PTS sugars, and membrane-bound enzyme II (EII) complex (*crr*), which is critical for sugar uptake (33). In a previous study, the removal of two components genes, *ptsI* and *crr*, in PTS was reported, and was revealed to be adequate to construct *Salmonella*-attenuated vaccine strains to provide protective humoral and mucosal immune responses, but a less effective cell-mediated immune response (34).

In the present study, a new attenuated *Salmonella* strain vaccine was developed by deleting the *ptsI* (EI) gene in the carbohydrate phosphotransferase (*ptsH*, *ptsI* and *crr*) operon. Furthermore, the humoral, cellular and protective effects of the vaccine were evaluated in a mouse model.

Methods and materials

Ethics statement. The present study was performed in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. All animal experiments were ethically approved by the Committee on The Use and Care of Animals at the Korea Atomic Energy Research Institute (KAERI; approval nos. IACUC-2016-028, IACUC-2017-010 and IACUC-2018-007) and performed according to accepted veterinary standards set by the KAERI animal care center. An

overall weight-loss of 25% was considered to be an indication for euthanasia. All efforts were made to minimize the suffering of the animals employed in the present study. To euthanize the mice, a CO_2 inhalation method as specified by KAERI Institutional Animal Care and Use Committee guidelines was used. Less than five mice were placed in a visible cage chamber (L27xW21xH17) and injected with compressed grade B CO_2 gas in cylinders. A fill rate of 10-30% of the cage chamber volume per minute with CO_2, which was 1-3 liter(s) per min, was appropriate to achieve a balanced gas mixture to fulfill the objective of rapid unconsciousness with minimal distress to the animals. Expected time to unconsciousness was usually reached at within 1-3 min and the flow was maintained for additional 1 min once respiration had ceased. If both signs were observed, then the mice were removed from the cage.

Reagents. All chemical reagents used in the present study were purchased from Sigma-Aldrich (Merck KGaA).

Bacterial strains and growth conditions. All bacteria strains used in the present study are listed in Table I. *Salmonella* strains were grown at 37°C in Luria-Bertani (LB) broth (Difco; BD Biosciences), M9 minimum media (Sigma-Aldrich; Merck KGaA) or phagosome-mimicking MES-buffered medium [5 mM KCl, 7.5 mM $(NH4)_2SO_4$, 0.5 mM K_2SO_4, 1 mM KH_2PO_4, 8 mM $MgCl_2$, 0.1% casamino acids, 0.4% carbon source and 170 mM 2-(*N*-morpholino) ethanesulphonic acid, pH=5.6] (35). The media were supplemented with kanamycin (50 μg/ml), chloramphenicol (15 μg/ml) or ampicillin (100 μg/ml), if required.

Salmonella mutants construction. *S*. Typhimurium strains used in the present study were derived from wild type (WT) strain UK1 (KST0134) (36). The *ptsI*-deficient strain (KST0555) was constructed using λ red-recombinase-mediated replacement as described previously (37). In brief, a kanamycin resistance (Km^R) cassette was amplified from the pKD13 plasmid that was annealed to the flanking regions of the deletion-target sites by using the primers listed in Table II. The resulting PCR product was transferred to UK1 harboring the plasmid pKD46 by electrophoresis, and the mutant was isolated on a kanamycin plate. The generated mutant was transformed with pCP20 to excise the Km^R cassette. *Salmonella* strains containing HA or 3xFLAG epitope tag in HilA and HilD and transcriptional *lac* fusion with *sseA* promoter (*sseA::lacZY*) were constructed using P22 transduction as described previously (38,39).

β-galactosidase assay. A β-galactosidase assay was performed as described previously (40). Overnight cultures of *Salmonella* KST0134 (WT), KST0555 (Δ*ptsI*) and KST0650 (Δ*hilD*) were sub-cultured into fresh LB broth or MES-buffered minimum media. *Salmonella* bacteria were harvested at 2, 4, 6, 8, 10 and 12 h post-inoculation, followed by resuspension with 1 ml Z-buffer (0.06 M Na_2HPO_4, $7H_2O$, 0.04 M NaH_2PO_4, H_2O, 0.01 M KCl, 0.01 M $MgSO_4$, $7H_2O$ and 0.05 M β-mercaptoethanol; pH=7.0), and permeabilized with 20 μl 0.1% sodium dodecylsulfate and 40 μl chloroform. Following 10 min of incubation at room temperature, 200 μl O-nitrophenyl β-glactopyranoside (ONPG; 4 mg/ml) was

Table I. Strains and plasmids used in the present study.

A, *Escherichia coli*

Author, year	Strain	Relevant gene	Source	(Refs.)
na	DH5α	F⁻r⁻m⁺Ø80d*lac*ZΔM15	Gibco; Thermo Fisher Scientific, Inc.	na

B, *Salmonella* Typhimurium

Author, year	Strain	Relevant gene	Source	(Refs.)
na	UK1	Clinical isolate from horse ATCC68169	American Type Culture Collection	na
Abed *et al*, 2014	BH129	UK1, Δ*ptsI*(Tn:10)	na	(1)
	KST0555	UK1, Δ*ptsI*	The present study	
Abed *et al*, 2014	KST0650	UK1, Δ*hilD*	na	(1)
	KST0600	UK1 hilA::HA-Kana^R	The present study	
	KST0601	KST0555 hilA::HA-Kana^R	The present study	
	KST0597	UK1 hilD::HA-Kana^R	The present study	
	KST0598	KST0555 hilD::HA-Kana^R	The present study	
Febriani *et al*, 2010	KST0134	SL1344, *sseA*::*lacZY*-Kana^R	na	(2)
	KST0589	UK1, *sseA*::*LacZY*-Kana^R	The present study	
	KST0590	KST0555, *sseA*::*LacZY*-Kana^R	The present study	
	KST0583	UK1, Δ*hilD*, *sseA*::LacZY-Kana^R	The present study	

C, Plasmid

Author, year	Strain	Relevant gene	Source	(Refs.)
Ao *et al*, 2015	pKD46	P_{BAD}-*gam-beta-exo oriR101 repA101^{LS}*; Amp^R	na	(3)
Ao *et al*, 2015	pKD13	FRT Kana^R FRT PS1 PS4 *oriR6K*; Amp^R	na	(3)
Ao *et al*, 2015	pCP20	*c*I857 P_R flp oripSC101^{ts}; Amp^R Cm^R	na	(3)

na, not applicable; Kana, kanamycin; Cm, chloramphenicol; Amp, ampicillin.

added as the substrate, and the mixture was incubated at 30˚C for 30 min in a water bath until the sample turned bright yellow. The reaction was stopped by adding 1 M Na_2CO_3 (500 μl), and the incubation period, i.e., the time between the addition of the ONPG substrate and the addition of stop solution, was recorded. The optical density$_{420}$ was recorded, and the specific activity and protein concentration were determined. The β-galactosidase activity was expressed in Miller units as described previously (40). Miller units were calculated as follows:

$$1{,}000 \times \frac{(Abs420)-1.75*Abs550}{(Abs600 \text{ of culture sample})*(\text{volume of culture})*(\text{reaction time})}$$

Cell invasion and replication assays. Experimental procedures were performed as described previously (30). The mouse macrophage cell lines (RAW264.7; cat. no. ATCC® TIB-71) and rat small intestinal epithelial cells (IEC-6; cat. no. ATCC® CRL-1592™) were purchased from the Korean Cell Line Bank. Cells were cultured in DMEM (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.), 100 U/ml penicillin and 100 μg/ml streptomycin at 37˚C and 5% CO_2. The cells were plated in 48-well cell culture plates (SPL Life Sciences) at IEC-6 (1x10^5 cells) and RAW264.7 (5x10^4 cells) per well and incubated for 2 h at 37˚C. Overnight-cultured *Salmonella* were washed with phosphate buffered saline (PBS) and suspended with DMEM, followed by addition to the prepared cell monolayers at multiplicity of infection (MOI) of 10. Following 1 h, the cells were washed three times with PBS, followed by an addition of pre-warmed medium containing 100 μg/ml gentamicin to remove extracellular bacteria. At 2 and 18 h post-infection (hpi), the wells were washed with PBS three times and lysed with 0.5% Triton-X100. Finally, the cell lysate was serially diluted with PBS, and 10 μl of the diluents was spotted on LB/agar plates to determine the number of colony forming units (CFUs). CFUs in the range of 20 to 50 colonies per spot from each dilution were counted. All data were calculated from at least three independent experiments performed in triplicate.

Fluorescence microscopic analysis. Infected *Salmonella* cells were visualized by plating RAW264.7 cells on 15 μm Chamber

Table II. Primers used in the present study.

Gene	Forward primer sequence (5'-3')	Reverse primer sequence (5'-3')
hilA	ATTAAGGCGACAGAGCTGGA	ATAGCAAACTCCCGACGATG
invF	TGTCGCACCAGTATCAGGAG	AAATAGCGCGAAACTCAGGA
ssaG	GATATGCTCTCCCACATGGC	AGGCAAATTGCGCTTTAATC
ssaB	GGATTCATGCTGGCAGTTTT	GCAGGATGCCCATCAATAGT
sseA	AGGAGGCCGAGAAGGATTTA	TTCCTGACGGTATCTCCACC
ssrA	CATGTTTGTGGCACTATCCG	GCGAGCTAAGGTAGCCAATG
sipA	AAACGTTGATACCCCTGCTG	GACATGGCTTCGGCTATTGT
sipB	TGTTTCGCTGTCTCAACTGG	TCGCAGCGTCATAAACACTC
sipC	AAGTCAGTGACCTGGGGTTG	AACGGCACTGGAAGACATTC
sipD	ACTGCCGTGGTACCGTCTAC	ATAATGACACGCCGGACTTC
fthD	CAACGAAGAGATGGCAAACA	GACGCGTTGAAAGCATGATA
fliA	CCGCTGAAGGTGTAATGGAT	CCGCATTTAATAACCCGATG
fliZ	AAACATTTCCCACGATCTGC	CGGTAAAGGGGGATTTCTGT
flgM	CCTTTGAAACCCGTTAGCAC	GCCGTTTTTAATGCTTCGAC
fliC	AACGACGGTATCTCCATTGC	TACACGGTCGATTTCGTTCA
pts Imutant	AAGTTTTTTTTCCGGGTTCTTTTAAAAATCA	GCAGTTCCTGTTTGTAGATTTCTTTGCGCAG
Construction	GTCACAAGTAAGGTAGGGTTCATATGAATAT CCTCCTTA	CGCGCGAACTTCTTGTCAATCTAGGCTGGA GCTGCTTC

12-well glass slides (Ibidi GmbH), followed by infection with WT and KST0555 strains at a MOI of 10 at 37˚C for 1 h. DMEM containing gentamicin (100 μg/ml) was used to eliminate extracellular bacteria. Following 2 or 18 h of infection, the cells were fixed with 4% paraformaldehyde for 30 min at 4˚C and permeabilized with 0.1% Triton-X100 in PBS for 20 min. The cells were then washed three times with PBS and blocked with 3% bovine serum albumin (BSA; Sigma-Aldrich; Merck KGaA) in PBS for up to 2 h at room temperature. Intracellular *Salmonella* were detected by incubating the cells with fluorescein isothiocyanate (FITC)-conjugated anti-rabbit *Salmonella*-specific antibodies (cat. no. ab20320; 1:1,000; Abcam) at room temperature for 30 min. The nuclei were stained with 150 ng/ml 4',6-diamino-2-phenylindole for 5 min at 37˚C (Thermo Fisher Scientific, Inc.). All images were captured using an Olympus CX41 fluorescence microscope (Olympus Corporation).

Mice experiments. Animal housing conditions (specific pathogen-free) and the animal experimental design were ethically approved by the Committee on The Use and Care of Animals at the KAERI and performed according to accepted National Health Institute standards. A total of 90 six-week-old male BALB/c mice (weight, 19-21 g) were purchased from OrientBio Inc. Five mice were randomly assigned per individually ventilated housing cage (OrientBio Inc.) maintained in an animal biological safety level 2 facility at 22-23˚C on a 12 h:12 h light:dark cycle. Cages were covered with high efficiency particulate air-filtered microisolation lids (OrientBio Inc.) in a static airflow environment. Bedding (Aspen Shaving; OrientBio Inc.) at an approximate depth of 1.0 cm was changed weekly. Irradiated rodent diet food and sterile water were provided *ad libitum*, supported by the wire cage top. Conscious mice were weighed (PB602-S balance; Mettler Electronics Corp.) at regular intervals using the 10 sec dynamic weighing

method. For measuring the virulence of *Salmonella* in the settings of a mice colitis model, mice (n=5) were administered 20 mg streptomycin (Sigma-Aldrich; Merck KGaA) in sterile water. Water and food were withdrawn for 4 h prior to streptomycin administration. Subsequently, the animals were supplied with water and food. At 20 h following streptomycin treatment, water and food were withdrawn again for 4 h prior to the administration of *Salmonella* orally (41). At the time of mortality or 72 h post-infection (hpi), mouse spleens, mesenteric lymph nodes and ceca were aseptically isolated, and the number of viable bacteria in each organ were determined by plating serially diluted homogenates or blood on LB agar plates. For further verifying the protection capacity of the KST0555 vaccination, five mice were immunized orally with 10^7 CFU KST0555 in 100 μl PBS three times at two-week intervals. At 7 days following the last immunization, 10^7 CFU WT were inoculated orally, and mortality and body weight changes were observed and recorded for 2 weeks. In all experiments, mice were considered to have succumbed to mortality and euthanized when they reached 75% of their original bodyweight.

Reverse transcription-quantitative PCR (RT-qPCR) analysis. Log-phase cultures of WT (KST0134) and KST0555 were diluted 100-fold into fresh LB broth or MES-buffered minimum media (pH=5.6), followed by incubation for 4 h at 37˚C overnight with shaking (42). Total RNA was isolated from bacterial cultures by using an RNeasy® mini kit (Qiagen GmbH) according to manufacturer's protocol. For RT-qPCR analysis, cDNA was synthesized from 1 μg total purified RNA using a Primescript 1st strand cDNA synthesis kit (Takara Bio, Inc.) according to manufacturer's protocol. Primers for different genes were designed based on Primer Express software (version 2.0; Applied Biosystems, Thermo Fisher Scientific, Inc.) and are listed in Table II. RT-qPCR amplification was

performed using SYBR Premix EX Taq (Takara Bio, Inc.) on a Bio-Rad CFX Real-Time System (Bio-Rad Laboratories, Inc.). Thermocycling conditions were as follows: One cycle at 95˚C for 10 min, followed by 40 cycles at 95˚C for 15 sec, 60˚C for 30 sec, 72˚C for 30 sec, and a final extension at 72˚C for 10 min. The relative gene expression was quantified using the $2^{-\Delta\Delta Cq}$ method (43). The 16S rRNA (*rrsH*) was selected as a control to normalize the expression levels.

Agar plate motility assay. A motility assay was performed as described previously (44). Briefly, 5 μl overnight cultures grown in LB broth were spotted onto the surface of a swarm plate (LB; 0.5% Bacto agar and 0.5% glucose) and incubated at 37˚C for 24 h.

Western blot analysis. Whole *Salmonella* lysates (10^8 CFU) were lysed in buffer (1% v/v Triton X-100 and 0.1% w/v SDS in PBS) and loaded and separated on 12% Bis-Tris BOLT gels (Invitrogen; Thermo Fisher Scientific, Inc.), followed by transfer onto nitrocellulose membranes. The membranes were blocked with 5% dry skimmed milk (Bio-Rad Laboratories, Inc.) in PBS with 0.05% Tween-20 (PBS-T) for 30 min at room temperature and then incubated with monoclonal anti-FLAG antibody (1:3,000; cat. no. F2555; Sigma-Aldrich; Merck KGaA), monoclonal anti-HA antibody (1:4,000; cat. no. ab137838; Abcam) or rabbit polyclonal anti-DnaK (1:3,000; cat. no. ab80161; Abcam). Following primary antibody incubation, the membranes were incubated with an anti-mouse total immunoglobulin (Ig) or anti-rabbit IgG conjugated with horseradish peroxidase-conjugated secondary antibody (1:5,000; cat. no. A9044; Sigma-Aldrich; Merck KGaA; Merck KGaA) for 1 h at room temperature. The protein bands were visualized using Enhanced Chemiluminescent Western Blotting Substrate (Thermo Fisher Scientific, Inc.). Bio-Rad ChemiDoc™ Touch imaging system and Bio-Rad CFX Manager software (version 3.1; Bio-Rad Laboratories, Inc.) were used for data acquisition and analysis.

Measurement of mice immunoglobulin. Mice blood samples were obtained 7 days subsequent to the last immunization. The serum was isolated by centrifugation at 500 x g for 10 min at 4˚C and stored at -80˚C for long-term use. *Salmonella* were cultured in LB and harvested at mid-log phase. The absorbance of the *Salmonella* pellet was adjusted to 0.1 at 600 nm by dilution with PBS. Next, 96-well immunoplates (SPL Life Sciences) were coated with 100 μl *Salmonella* or 1 μg *Salmonella* lipopolysaccharide (LPS; Sigma-Aldrich; Merck KGaA) and incubated overnight at 4˚C to allow the immobilization of bacterial cells onto the wells. The plates were washed five times with PBS-T, followed by blocking with 1% BSA in PBS for 1 h at room temperature. Subsequent to blocking, diluted sera were added to each well and incubated at room temperature for 1 h, followed by the washing of unbound antibodies with PBS-T. Secondary antibodies (all 1:4,000) were added to the wells by and incubated for 30 min at room temperature. The secondary antibodies were as follows: Goat anti-mouse Ig-HRP (cat. no. A0412; Sigma-Aldrich, Merck KGaA), goat anti-mouse IgG-HRP (cat. no. 1030-05; SouthernBiotech), goat anti-mouse IgM-HRP (cat. no. 1020-05; SouthernBiotech), goat anti-mouse IgG1-HRP (cat.

no. 1071-05; SouthernBiotech), goat anti-mouse IgG2a-HRP (cat. no. 1080-05; SouthernBiotech), goat anti-mouse IgG2b-HRP (cat. no. 1090-05; SouthernBiotech) and goat anti-mouse IgG3-HRP (cat. no. 1100-05; SouthernBiotech). The plates were then washed 5 times with PBS-T, and 100 μl of 3,3',5,5'-tetramethylbenzidine substrate reagent (BD Biosciences) was added. When the color developed, 50 μl 2 N H_2SO_4 was added, and the absorbance was measured at 450 nm by using a Victor X3 light plate reader (PerkinElmer, Inc.). The assigned titer value was indicative of the last dilution in which A_{450} is <0.1.

Cytokine measurement. Splenocytes (1×10^5 cells/well) were plated onto a round bottom 96-well plates (SPL Life Sciences) and incubated with PBS or *S.* Typhimurium WT lysate prepared by sonication for 72 h at 37˚C. Interleukin-4 (IL-4) and interferon-γ (IFN-γ) levels in the supernatants were measured using mouse IL-4 (cat. no. 555232; BD Biosciences) and mouse IFN-γ (cat. no. 555138; BD Biosciences) ELISA kits, respectively.

Adoptive transfer of sera, CD4⁺ and CD8⁺ cell protection. A total of 5 mice per group were immunized with 10^7 CFU of KST0555 orally three times in two-weeks intervals. Mice spleens were isolated at 7 days following the last vaccination, and lymphocytes were prepared from a cell suspension by pressing organ segments through a cell strainer (BD Biosciences). The cell suspension was depleted of red blood cells by using the hypertonic pressure of distilled water and then washed three times with PBS, as previously described (45). Splenic CD4⁺ and CD8⁺ T cells were isolated using a CD4⁺ T cell isolation kit (cat. no. 130-104-454; Miltenyi Biotec GmbH) and a CD8⁺ T cell isolation kit (cat. no. 130-104-075; Miltenyi Biotec GmbH) according to the manufacturer's protocol. Mice serum was isolated as described above and heated at 55˚C for 30 min to inactivate the complements. Cell purity was verified to be >90% using flow cytometry with staining with phyco-erythrin-conjugated anti-mouse CD4 (1:200; cat. no. 100511, BioLegend, Inc.) or FITC-conjugated anti-mouse CD8a (1:200; cat. no. 100705; BioLegend, Inc.) for 15 min at room temperature. Mouse serum (300 μl per mice), enriched CD4⁺ T cells (10^6 cells per mice) or CD8⁺ T cells (10^5 cells per mice) were administered intraperitoneally to each recipient mouse. Subsequent to 24 h, the mice were administered a lethal dose of WT (10^7 CFU), and their mortality and body weight changes were recorded.

Statistical analysis. Data are expressed as the mean ± standard deviation. Data in the bar graphs and bacterial numbers between groups were compared using an unpaired Student's t-test. The survival of mice was determined using Kaplan-Meier survival analysis, and significance of the difference was analyzed using a Log rank test by using GraphPad Prism software (version 6.0; GraphPad Software, Inc.). P<0.05 was considered to indicate a statistically significant difference.

Results

Characterization of a ptsI mutant strain (KST0555). Since a previous study indicated that glucose and glycerol are important
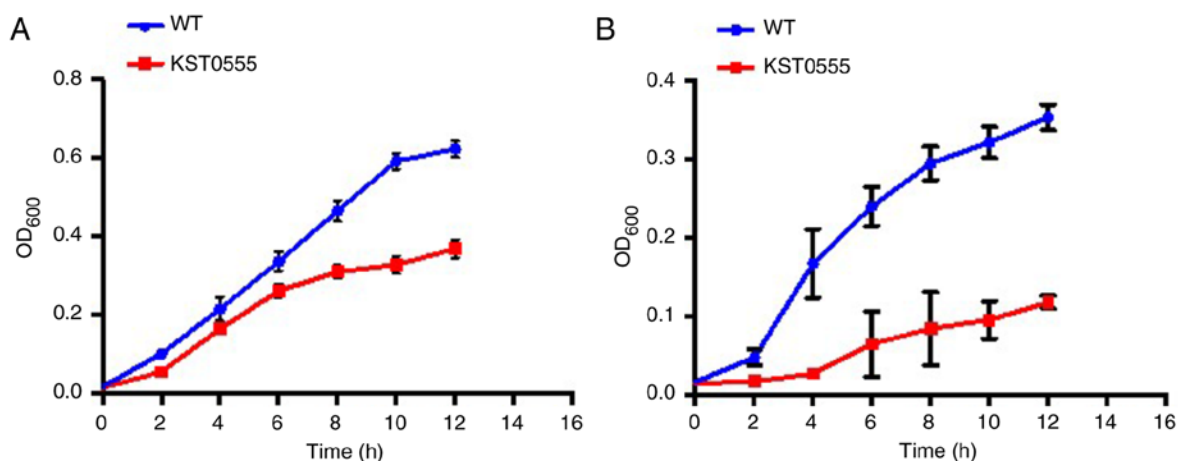
Figure 1. Impaired growth of KST0555 in the minimal M9 broth supplemented with glucose or glycerol. Growth curves of WT (blue) and KST0555 (red) in the minimal M9 broth with (A) 1% glucose and (B) 1% glycerol. WT, wild type; OD, optical density.

carbon sources in the systemic infection of *Salmonella* (30), the growth of KST0555 in minimal medium (M9) supplemented with these carbon sources was examined compared with that of its parent strain. Comparable growth was noted between KST0555 and WT (KST0134) in LB, and each of the strains were unable to grow in M9 medium with no carbon source (data not shown). When KST0555 was cultured in M9 supplemented with glucose, it exhibited substantially lower growth compared with the WT and did not reach the stationary phase in 12 h (Fig. 1A). Furthermore, its growth in M9 with glycerol was almost abolished (Fig. 1B) indicating that KST0555 was the auxotrophic mutant in the uptake of the carbon source.

Reduction of KST0555 virulence in vitro and in vivo. The present study then investigated the abilities of KST0555 to invade into and replicate within RAW264.7 and IEC-6 cells. Cell monolayers were infected with *Salmonella* at a MOI=10, and their relative invasion and replication ratio were calculated at 2 and 18 hpi (Fig. 2A and B). The invasion abilities (at 2 hpi) of KST0555 to RAW264.7 and IEC-6 cells were reduced significantly compared with that of the WT (P<0.005). In addition, the replication fold increase of WT were 12.6±2.9 times and 11.7±3.2 times, whereas those of KST0555 were only 1.3±0.2 and 1.5±0.3 times inside the RAW264.7 and IEC-6 cells at 18 hpi, respectively (P<0.005).

To directly visualize the invasion and replication of *Salmonella* within RAW264.7 cells, immunofluorescence was performed using a FITC-conjugated *Salmonella*-specific monoclonal antibody (Fig. 2C). A higher density of FITC signals was present in the cell monolayers infected with WT, compared with cells infected with KST0555 at 2 hpi, and the FITC signal was notably increased at 18 hpi, indicating efficient invasion and replication in RAW264.7 cells. However, KST0555 was barely detected at 2 and 18 hpi. The results were similar with the pattern of intracellular invasion and replication assays presented in Fig. 2A. All results suggest that the expression of *ptsI* is necessary for invasion and replication in host cells; in other words, its expression is likely required for the intracellular growth of *S*. Typhimurium.

To assess the toxicity of KST0555 *in vivo*, streptomycin-treated mice (n=5) were infected orally with the indicated doses of WT or KST0555, followed by monitoring of the mortality of mice for 21 days (Fig. 3A and B). When the mice (n=5) were inoculated with 1×10^7 CFU/mouse *Salmonella* WT strain, all mice succumbed to mortality at 10 days subsequent the challenge and their body weight were revealed to substantially decrease day by day. When the mice were inoculated orally with 1×10^8 CFU/mouse KST0555, the mice exhibited a noticeable drop in their body weight and 40% of the mice succumbed to mortality. The mice inoculated orally with either 5×10^7 or 1×10^7 CFU/mouse doses did not succumb to mortality, but the mice inoculated orally with 5×10^7 CFU/mouse exhibited a noticeable body weight loss by day 3 and exhibited a slow recovery in weight from day 11, indicating that the lethal dose 50 (LD_{50}) of KST0555 was $\sim10^8$ CFU/mouse, but the optimal oral vaccination dose in mice was lower compared with 1×10^7 CFU/mouse.

Salmonella infections typically are initiated by the ingestion of contaminated food and water, followed by the successful colonization in the distal ileum (46,47). The present study initially examined the colonized *Salmonella* in the mice organs at the time of mortality or 72 h subsequent to the oral inoculation of 1×10^7 CFU bacteria (Fig. 3C). Mice infected with the WT strain revealed substantially greater numbers of colonized *Salmonella*, estimated between 10^8 and 10^9 CFU/g, which was higher compared with the number of infection, indicating infected *Salmonella* colonized and replicated in the mice intestine. In contrast, it was revealed that there was <10^6 CFU/g *Salmonella* in the cecum of the mice infected with KST0555 at the time of mortality or 72 hpi.

Colonized *Salmonella* are occasionally translocated to systemic sites or to the peritoneal cavity to cause iNTS (48). The present study further examined the levels of *Salmonella* colonization in mice mesenteric lymphnodes, spleen, and blood (Fig. 3D-F) at the time of mortality or 72 hpi. A total of >10^5-10^9 CFU/tissue or CFU/ml *Salmonella* were detected in the mice organs and blood infected with WT, but no or <10^4 CFU/tissue or CFU/ml *Salmonella* were detected in mice organs and blood infected with KST0555, indicating that the KST0555 strain is attenuated and causes less pathological symptoms *in vivo*.
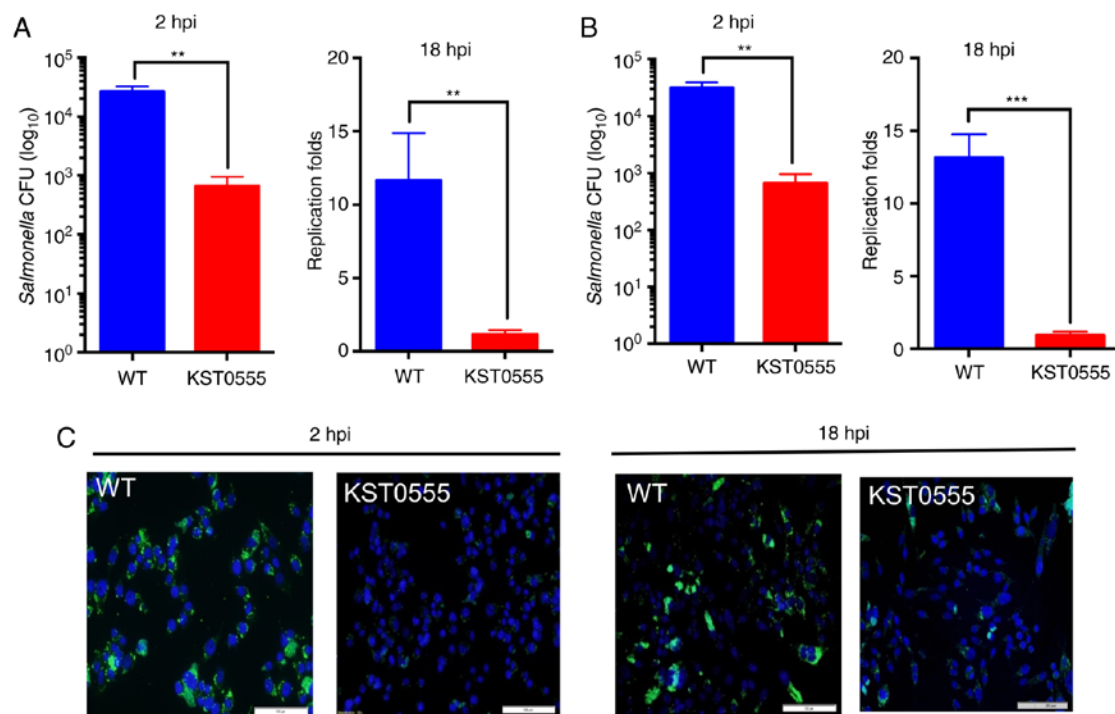
Figure 2. Importance of *ptsI* expression for *Salmonella* invasion and replication in mouse macrophages and epithelial cells. (A) Macrophages (RAW264.7 cells) or (B) IEC-6 cells were infected with WT or KST0555 at a multiplicity of infection of 10 and bacterial viability were counted at 2 or 18 hpi. Relative invasion and replication fold of KST0555 were calculated by comparing with WT. Data are representative of three independent experiments and are presented as the mean ± standard deviation. **P<0.005 and ***P<0.001 with comparisons shown by lines. (C) Adherence and invasion of *Salmonlla* were visualized using an immunofluorescence assay. WT or KST0555 infected RAW264.7 cells were stained with fluorescein isothiocyanate-conjugated *Salmonella*-specific immunoglobulin G (green) at 2 or 18 hpi. Scale bar, 100 μm. Nuclei were stained with DAPI (blue). IEC, intestinal epithelial cells; WT, wild type; hpi, hours post-infection; CFU, colony forming unit.

Downregulation of virulence-associated genes in KST0555. Next, the present study investigated the effect of the *ptsI* mutation on flagella expression and *Salmonella* motility. Surprisingly, only the expression of the *fliC* gene encoding a main flagellin subunit was significantly reduced by 95±3.7% in KST0555 compared with that in WT (P<0.005); however, *flhD, fliA, fliZ* and *flgM* were not significantly reduced (Fig. 4A). To verify whether the downregulation of *fliC* effectively influenced the motility of *Salmonella*, stationary phase cultures of WT or KST0555 were inoculated on motility agar medium followed by monitoring *Salmonella* migration diameters on agar plates following 18 h of incubation. Even though only *fliC* gene expression was impaired in KST0555, it exhibited significantly smaller halos compared with those of WT (P<0.001; Fig. 4B).

To determine whether the substantial reduction of a virulence phenotype in KST0555 was due to the downregulation of major virulent genes in *Salmonella*, the expression of the genes encoded in SPI-1 and -2 were examined, which have been attributed to numerous virulence properties of *Salmonella* (49,50). The majority of genes encoded within SPI-1 are well known to serve an active function in penetration into the intestinal epithelial barrier, and the expression of SPI-1 genes are regulated by the fundamental transcriptional regulators HilA, HilD and InvF (51). Thus, the present study firstly compared the expression of HilA and HilD proteins, central regulators of SPI-1 genes. As revealed in Fig. 4C, the expression of each of these proteins was reduced in KST0555 compared with in WT. In addition, *invF* mRNA levels, regulated by HilA and HilD,

were significantly downregulated in KST0555 compared with in WT, which explains why *invF*-dependent *sipBCDA* mRNA expression levels were reduced by ~80% in KST0555 compared with in WT (P<0.001; Fig. 4D).

HilD is also known to activate SPI-2 genes, the key virulence determinants for the survival/replication of *Salmonella* inside the host cells, through inducing the expression of the SsrA/B two-component system, the central positive regulator of SPI-2 (52). Thus, the expression of the key virulence factor *sseA* was compared in WT, KST0555 and KST0583 (*ΔhilD*) grown in LB or LPM using the *lacZ* gene fused to the promoter of *sseA*. As expected, the *sseA* expression was almost abolished in *ΔhilD* grown in only LB, whereas its expression was similar with WT when cultured in LPM, SPI-2 gene expression media (Fig. 4E and F). However, no *sseA* expression was revealed in KST0555 culture in LB and LPM. The expression levels of the downstream genes of the SseA promoter (SsaB, SsaG, SsrA and SseA) were examined using RT-qPCR. As shown in Fig. 4G, these genes were significantly downregulated in KST0555 culture in LB compared with the WT [*ssaB* (P=0.001), *ssaG* (P=0.0003), *ssrA* (P=0.0047) and *sseA* (P=0.0095)]. These results suggest that the reduced SPI-2 expression in KST0555 was in part due to the lower expression of HilD by a *ptsI* mutation, and additional mechanism(s) may be responsible for the downregulation of SPI-2 expression other than HilD in KST0555.

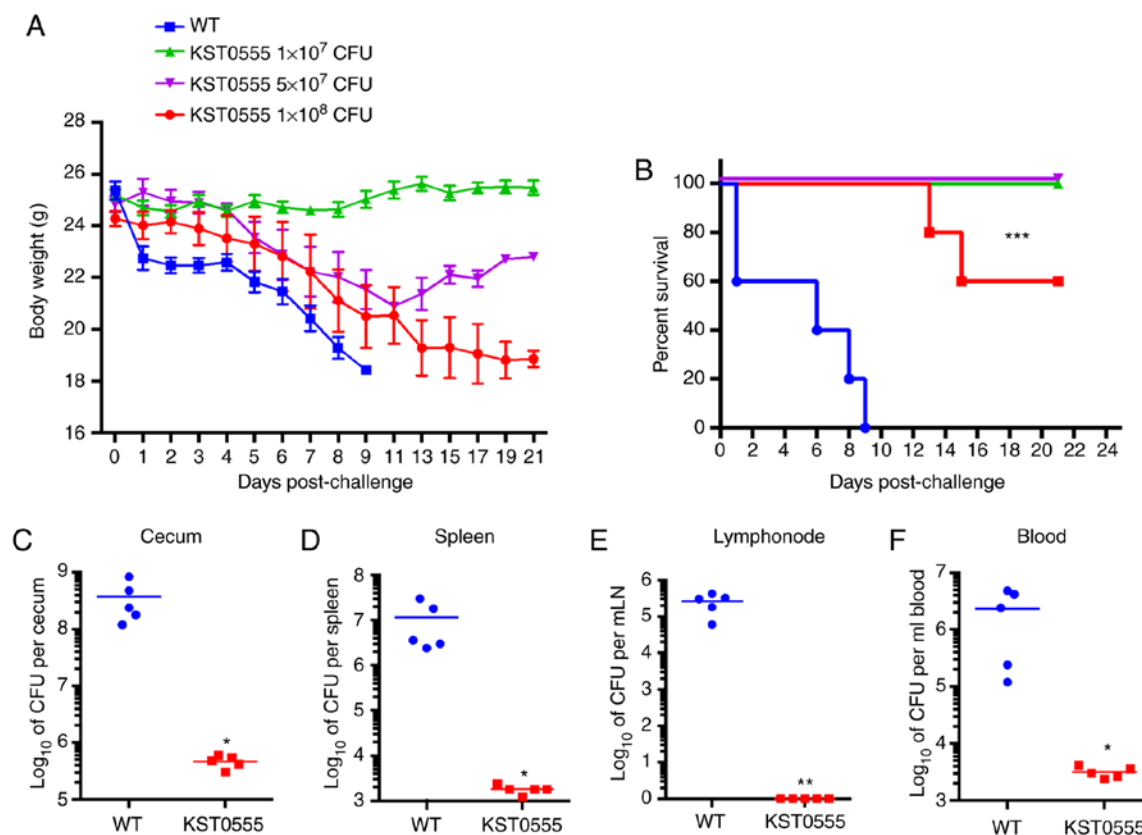*Vaccination of KST0555 elicits protective immune response against Salmonella.* To determine whether KST0555 may be

Figure 3. Virulence of KST0555 in a mouse colitis model. Survival curve of BALB/c mice (n=5) were pretreated with 20 mg streptomycin, followed by inoculation orally with $1x10^7$, $5x10^7$ and $1x10^8$ CFU KST0555 and $1x10^7$ CFU WT. (A) Mice survival and (B) body weight change was monitored for 21 days. Mice organs were dissected at the time of mortality or 72 hpi. The number of bacteria in the (C) cecum, (D) mLN, (E) spleen and (F) blood were counted. Data were presented as the mean ± standard deviation. *P<0.05, **P<0.05 and ***P<0.001 vs. WT. CFU, colony forming unit; WT, wild type; hpi, hours post-infection; mLN, mesenteric lymph nodes.

used as a live oral vaccine, the mice were immunized orally (n=5) three times in two-week intervals (0, 2 and 4 weeks) with 10^7 CFU of KST0555 and the levels of *Salmonella* and *Salmonella* LPS-specific antibodies in the serum were determined at 7 days following the last immunization (5 weeks). As presented in Fig. 5A, KST0555 vaccination significantly enhanced the serum levels of *Salmonella*-specific IgG (mean titer=1024) and IgM (mean titer=288) compared with those of PBS-immunized mice (mean titer=36-72; P<0.05). LPS-specific IgG and IgM were also elevated significantly by KST0555 vaccination compared with PBS-treated mice (P<0.05; Fig. 5B). When the subclasses of IgG specific for *Salmonella* (Fig. 5C) and LPS (Fig. 5D) were analyzed, *Salmonella* and LPS specific-IgG1, IgG2a, IgG2b and IgG3 were significantly increased compared with those of PBS groups (P<0.05). In addition, significant increases in Th1 and Th2-spcific cytokines (IFN-γ and IL-4) were detected in splenocytes from mice immunized with KST0555 when compared with PBS-immunized control mice (P<0.05) indicating that live KST0555 vaccine induces high antigen-specific antibody responses and T-cell mediated immune responses (Fig. 5E and F).

To directly determine the protective effect of a live KST0555 vaccine, the immunized mice were challenged orally with 10^7 CFU of WT at 7 days following the last vaccination (week 5). All PBS-immunized mice succumbed to mortality in 12 days post-injection along with a substantial reduction of

body weight daily, but 100% of KST0555-immunized mice were alive for >14 days post-injection without an obvious change in body weight (Fig. 5G and H). This suggests that a live KST0555 vaccine may provide a protective immune response.

Protection by adoptive transfer of serum, CD4+ or CD8+ T cells. The present study then investigated whether a live KST0555 vaccine may elicit functional humoral and cell-mediated immunities by an adoptive transfer experiment. Five mice in each group were administered intraperitoneally with 300 µl serum, $1x10^6$ CD4+ T cells or $1x10^5$ CD8+ T cells isolated from unvaccinated or live KST0555 ($1x10^7$ CFU) vaccinated mice as described above, followed by challenging with 10^7 CFU of WT *Salmonella*. Mice receiving the serum from live KST0555 immunized mice survived significantly longer compared with mice receiving unimmunized serum (P=0.0042; Fig. 6A), indicating that the antibodies raised by KST0555 vaccination were functional. The mice receiving splenic CD4+ T cells from KST0555-immunized mice survived 2 or 3 days longer compared with the naïve group, which was not significant, but modestly protected (P=0.0547; Fig. 6B). The mice receiving splenic CD8+ T cells from KST0555-immunized mice exhibited significant and sufficient protection compared with the transfer of those from the naïve group (P=0.0015; Fig. 6C), suggesting that a live oral KST0555 vaccine might induce an efficient cell-mediated immune response.
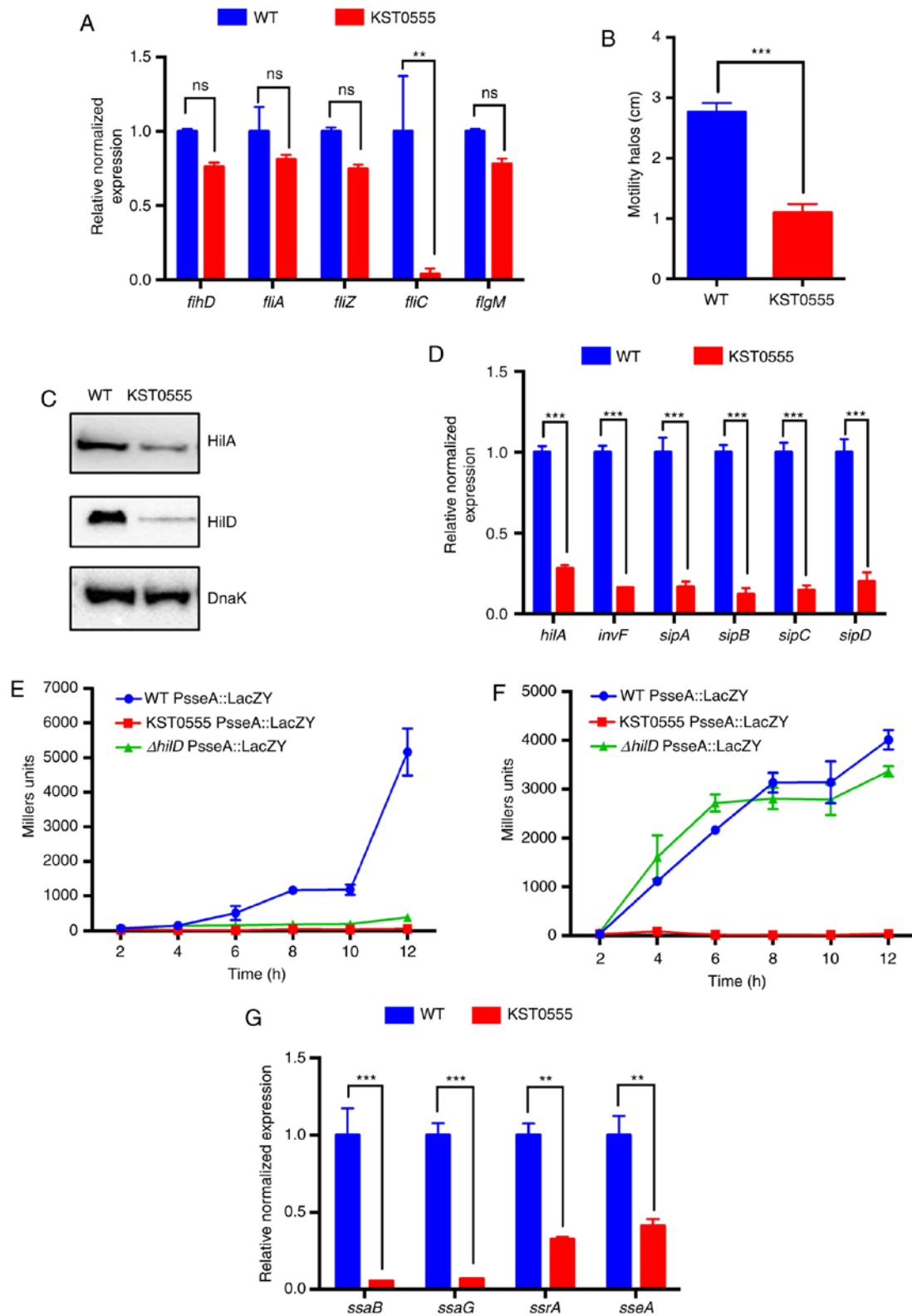
Figure 4. Downregulation of virulent genes and key regulator proteins of *Salmonella* in T3SS. Lower mobility caused by the deletion of the *ptsI* gene. (A) mRNA expression levels of flagella biosynthesis genes, *flhD*, *fliA*, *fliZ*, *fliC* and *flgM* in WT and KST0555 at early-log phase in LB broth were measured using RT-qPCR. (B) *Salmonella* motility was measured on an agar plate by inoculating 10 μl WT or KST0555 culture broth. The zone size of motility was measured at 18 h following inoculation. (C) Protein expression of *Salmonella* T3SS key regulators (HilA and HiD) in WT and KST0555 was examined using a HilA::FLAG and HilD::HA chromosomal fusion system. (D) Reduced expression of *Salmonella* SPI-1 genes and their regulators in KST0555. The expression of SPI-1 encoded genes (*hilA*, *invF*, *sopA*, *sipB*, *sipC* and *sipD*) in WT and KST0555 at the early-log phase in LB broth was quantified using RT-qPCR compared with that in WT. Reduced key virulence factor sseA expression in KST0555 and Δ*hilD* was abolished, compared with that in WT grown in (E) LB using the lacZ gene fused to the promoter of *sseA*, but only KST0555 still exhibited no expression of sseA, compared with Δ*hilD* and WT grown in (F) LPM. (G) Downregulation of SPI-2 encoded genes in KST00555. Transcription levels of SPI-2 encoded genes of KST0555 in LPM were quantified using RT-qPCR compared with that in WT. Data are representative of three independent experiments and presented as the mean ± standard deviation. Asterisks indicate significant differences between WT and KST0555. **P<0.05 and ***P<0.001 with comparisons shown by lines. WT, wild type; RT-qPCR, reverse transcription-quantitative PCR; LB, Luria-Bertani broth; LPM, MES-buffered minimum media; ns, non-significant.
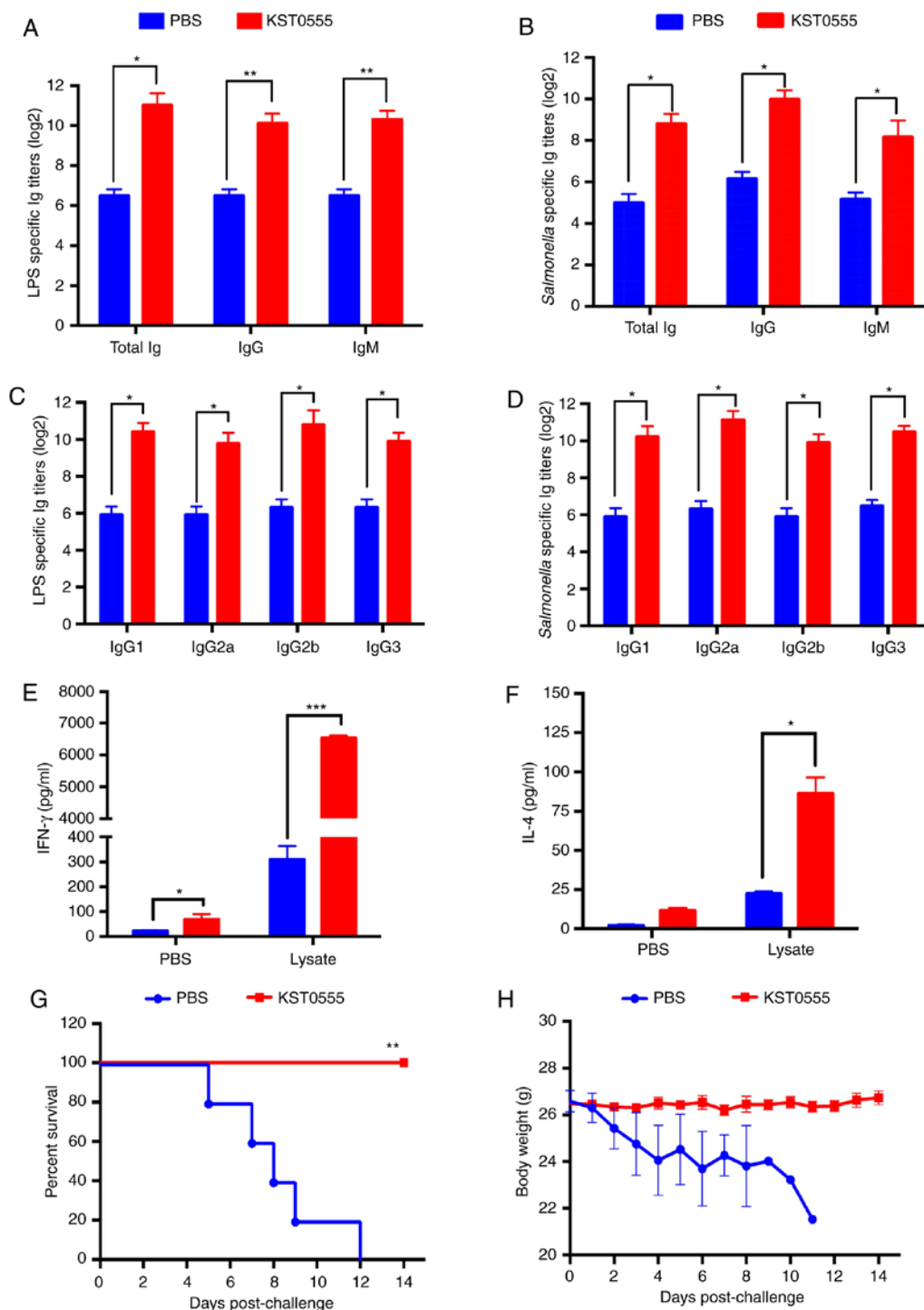
Figure 5. Protective effect of KST0555 immunization against *Salmonella* infections in mice. BALB/c mice (n=5) were orally immunized with KST0555 or PBS three times at two-week intervals. Serum levels of (A) *Salmonella*- or (B) LPS-specific total Ig, IgG and IgM were analyzed at 7 days following the last immunization. Data are representative of three independent experiments and are presented as the mean ± standard deviation. Subclass levels of (C) *Salmonella*- and (D) LPS-specific IgG1, IgG2a, IgG2b and IgG3 were analyzed at 7 days subsequent to the last immunization. Data are representative of three independent experiments and are presented as the mean ± standard deviation. Splenocytes isolated from mice were incubated with 10^8 CFU of *S*. Typhimurium WT lysate for 72 h, and concentrations of (E) IFN-γ and (F) IL-4 were measured from culture supernatants using an enzyme-linked immunosorbent assay. Bars indicate the mean of results of triplicate wells of a representative experiment. Error bars indicate standard deviation. Immunized mice were challenged orally with 10^7 CFU of WT at 7 days following the last immunization and (G) mouse survival and (H) body weight were monitored for 14 days. *P<0.05, **P<0.005 and ***P<0.001 vs. PBS group. PBS, phosphate buffered saline; Ig, immunoglobulin; LPS, lipopolysaccharide; CFU, colony-forming units; WT, wild-type; IFN-γ, interferon-γ; IL-4, interleukin-4.

Discussion

NTS infection is the leading cause of mortality in Africa and other developing countries (3). Previous comprehensive

epidemiological studies of iNTS indicate the urgent need of novel therapeutic methods for these infections (3,4,53). Live attenuated vaccines against NTS are a promising solution for the spreading of NTS, because they have numerous advantages
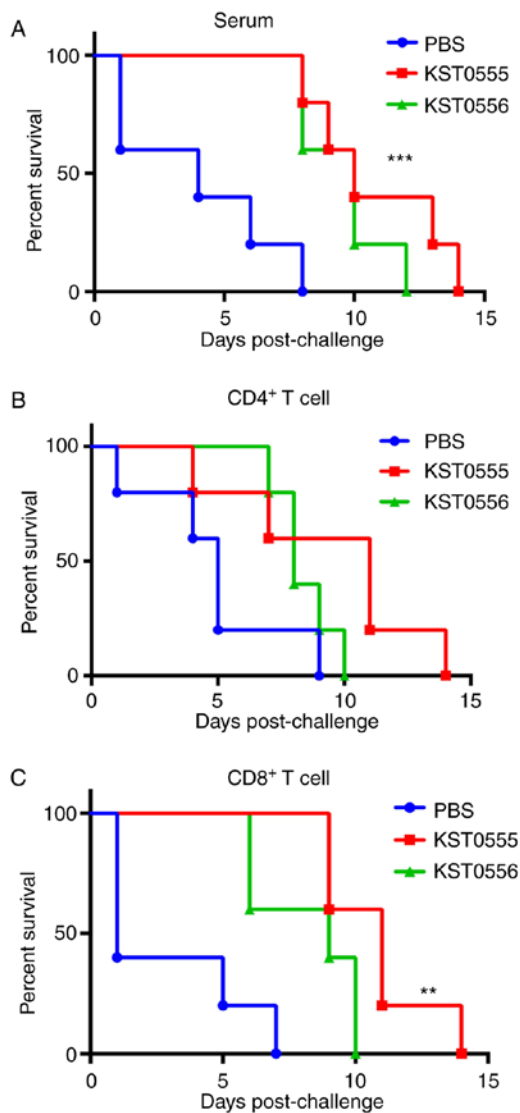
Figure 6. Protection by the adopted transfer of KST0555-vaccinated mice serum and CD8+ T cells against *Salmonella* infection. (A) Serum (300 μl) from PBS or KST0555-immunized mice was inoculated intraperitoneally into naïve BALB/c mice (n=5). At 12 h following inoculation, mice were challenged orally with 10^7 CFU of WT. Mouse survival was monitored for 14 days. Mice were immunized with KST0555 three times at 14-day intervals orally, and the spleen was dissected from the mice 7 days following final immunization. (B) Splenic CD4+ T cells or (C) CD8+ T cells from PBS- or KST0555-immunized mice were transferred intraperitoneally to naïve BALB/c mice (n=5). At 12 h following inoculation, mice were challenged orally with 107 CFU of WT, and mouse survival was monitored for 14 days. **P<0.05 and ***P<0.001 vs. the PBS group. PBS, phosphate buffered saline; CFU, colony-forming units; WT, wild-type.

over other vaccine types, including cost-effective production, induction of mucosal and cell-mediated immunities and no hazardous waste (21). In the present study, it was hypothesized that the disruption of *ptsI* in *S*. Typhimurium may create an attenuated strain, which is efficacious for vaccine application, by interfering with the uptake of PTS sugars, particularly glucose, the predominant carbohydrate source during infection. This strain, deficient in the uptake of PTS (glucose) and non-PTS (glycerol) sugars, exhibited a significantly (P=0.0172) reduced virulence *in vitro* and *in vivo* and elicited effective humoral and cell-mediated immune responses to protect against *Salmonella* infection.

The vaccine strain attenuated in metabolic gene(s) requires being metabolically active to reach immune inductive sites and elicit a biologically relevant protective immunity in the absence of overt disease. Furthermore, the hyperattenuation of vaccine strains may require the administration of multiple oral doses to achieve an effectively protective immune response (54,55). The deletion of *aroA*, a gene of the shikimate pathway associated with the synthesis of aromatic amino acids, is most commonly used as a metabolic mutation to attenuate *Salmonella* (56). The *guaBA* gene-deleted vaccine candidates, which impair guanine biosynthesis, are based on *S*. Typhimurium and *S*. Enteritidis isolates from sub-Saharan Africa; preclinical studies were conducted using a series of Center for Vaccine Development (University of Maryland School of Medicine) by using relevant animal models for human gastroenteritis, including streptomycin-pretreated mouse and rhesus macaque models (20,21). Another approach for developing live attenuated vaccines is the deletion of genes involved in the uptake of essential elements or nutrients that are indispensable for the growth of infectious microorganisms. In one example, the mutant in the *znuABC* operon encoding the high-affinity zinc transporter was revealed to be a safe and efficacious vaccine candidate in the setting of mouse and pig vaccine models (57-60).

In a previous study, the extreme attenuation was achieved by deleting two components genes, *ptsI* and *crr* genes, in PTS, despite the fact that a high number (>1x10^9 CFU) of $\Delta ptsIcrr$ was administrated without a noticeable body weight loss and its humoral immune responses may protect against a lethal challenge of its parent strain (34). However, the hyperattenuation of the $\Delta ptsIcrr$ strain also result in a high dose of CFU being needed, along with the non-activation of cell-mediated immune responses (34). In the present study, the KST0555 strain was defective in only one sugar transport gene (*ptsI*), but also highly attenuated in the mouse model. Although its targeting to immune sites, including spleen and mesenteric lymph nodes, was substantially reduced compared with that of the parent strain, its immune responses were sufficient to provide the protection against *Salmonella* infection via both humoral and cellular immunity.

In fact, numerous live attenuated *Salmonella* vaccine strains under pre-clinical and clinical studies have been revealed to have associations between the transcriptional and translational expression for two notable groups of virulence genes, SPI-1 and SPI-2 (21,24,61-63). Each are essential for colonization, invasion and intracellular proliferation and thus contribute to the successful infection of *S*. Typhimurium into the host cells by assembling T3SS (64). PTS is the most well-known and important carbon uptake system consisting of two general components: Enzyme I (EI) encoded by *ptsI* and histidine phosphocarrier protein (HPr) encoded by *ptsH* and the membrane-associated EII complex. EII complexes, consisting of the EIIA, EIIB and EIIC proteins, are usually carbon-specific; for example, EIIA^Glc encoded by *crr* stands for the glucose-specific EIIA component (65). The first PTS component, PtsI, autophosphorylates by using phosphoenolpyruvate as a phosphoryl donor and transfers the phosphate group through HPr to the EII complex to trigger the uptake of PTS sugars, including glucose, mannose, mannitol, fructose and cellobiose (33). Further evidence suggests that virulence functions and metabolism of pathogens are intertwined

by global regulatory networks, which serve an important function in the regulation of virulence traits (66). The SirA/BarA two-component system involved in various cellular responses, including carbohydrate metabolism, motility, biofilm formation and stress survival, may regulate the expression of SPI-1 and SPI-2 genes alone with the carbon storage regulatory (*csr*) systems (67,68). Since the expression of *sirA* and *csrA* is reduced by the mutation of the adenylate cyclase gene, which synthesizes cyclic AMP (cAMP) from ATP, or the cAMP receptor protein gene (*crp*) in *S*. Typhimurium (67) and deletion of the *ptsI* gene, prevents the formation of phosphorylated EIIA^{Glc}, the activator of adenylate cyclase, the reduction of SPI-1 and SPI-2 gene expression in KST0555 (Δ*ptsI*) is likely to be partially mediated via the cAMP/*crp* regulation mechanism (69). In addition, it was revealed that the reduction of virulence in KST0555 was not entirely due to the downregulation of SPI genes. One novel result was that only the *fliC* gene of the five flagella-associated genes tested was significantly downregulated in KST0555, which impaired *Salmonella* motility. Flagella-mediated motility is known to be required for the efficient colonization and induction of colitis in mice (70,71). Thus, further investigation on the mechanisms by which *ptsI* mutation affects the flagella gene expression of *S*. Typhimurium is warranted. The notable downregulation of SPI-1 and SPI-2 genes in *ptsI* mutants reveals the advantage of this mutant strain as an attenuated vaccine candidate. However, the comparison of the immunological efficacy of this strain with previously well-characterized vaccine strains and the regulation axis between carbon uptake and virulence in *Salmonella* should be investigated further at the molecular level.

In summary, the present study provides further strong evidence that the carbon transport system is an excellent target to develop a live *Salmonella* vaccine. Since the reduced virulence of KST0555 amy be attributed to not only the impairment of the sugar uptake but also the downregulation of virulence genes including SPI and flagellin, the molecular mechanism of the association between virulence and PTS should be studied further. However, these promising preclinical data provide a strong rationale for the advancement of KST0555 as a vaccine candidate. Furthermore, since PTS is widely conserved in gram-negative and gram-positive bacteria, the present study offers a novel insight for developing novel vaccine strains against various pathogenic bacteria.

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Availability of data and materials

The data used and/or analyzed in this study are available from the corresponding author upon reasonable request.

Authors' contributions

YZ and HSS conceived and designed the experiments. YZ, HSS, SML, KBA and HJJ performed the experiments. YZ, HSS, KBA, HCG, SR and SL analyzed the data. YZ, HSS, KBA and SL wrote the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The animal experiments were ethically approved by the Committee on The Use and Care of Animals at the Korea Atomic Energy Research Institute and performed according to accepted National Health Institute standards.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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