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Effective mucosal live attenuated *Salmonella* vaccine by deleting phosphotransferase system component genes *ptsI* and *crr*

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Salmonella enterica **is a major human pathogen that causes invasive non-typhoidal Salmonellosis (iNTS), resulting in significant morbidity and mortality. Although a number of pre-clinical and clinical studies have reported on the feasibility of developing a safe and effective vaccine against iNTS, there have been no licensed *Salmonella* vaccines available to protect against NTS strains. Vaccine formulations of highest priority for NTS are live attenuated vaccines, which can elicit effective induction of intestinal mucosal and intracellular bacteria-specific cell mediated immune responses. Since glucose is crucial for intracellular survival and replication in host cells, we constructed strains with mutations in components of the glucose uptake system, called the phosphotransferase system (PTS), and compared the relative virulence and immune responses in mice. In this study, we found that the strain with mutations in both *ptsI* and *crr* (KST0556) was the most attenuated strain among the tested strains, and proved to be highly effective in inducing a mucosal immune response that can protect against NTS infections in mice. Thus, we suggest here that KST0556 (Δ*ptsI*Δ*crr*) is a potential live vaccine candidate for NTS, and may also be a candidate for a live delivery vector for heterologous antigens. Moreover, since PTS is a well-conserved glucose transporter system in both Gram-negative and Gram-positive bacteria, the *ptsI* and *crr* genes may be potential targets for creating live bacterial vectors or vaccine strains.**

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Introduction

Salmonella enterica is a facultative Gram-negative intracellular bacterium and is also a common food-borne pathogen for both humans and animals that can result in high mortality and tremendous economic losses (Crump *et al.*, 2008; Batz *et al.*, 2012). *S. enterica* subsp. *enterica* serovars Typhi and Paratyphi are human-restricted pathogens that cause systemic enteric fever, whereas *S. enterica* subsp. *enterica* serovars Enteritidis and Typhimurium could infect a broad range of hosts and are responsible for invasive nontyphoidal *Salmonella* (iNTS) diseases (Thomas *et al.*, 2011; LaRock *et al.*, 2015). Nontyphoidal *Salmonella* (NTS) causes acute self-limiting gastroenteritis in humans with 93 million cases and 155,000 deaths annually, and an estimated case fatality of 20–25% in infants, the elderly, and immunocompromised people in Africa (Majowicz *et al.*, 2010; Wiedemann *et al.*, 2014). In fact, iNTS was the second most common invasive bacterial disease (29%), followed by *Streptococcus pneumoniae* infection in sub-Saharan Africa (Feasey *et al.*, 2012; Ao *et al.*, 2015). Antibiotic treatments are the most effective method for clearing iNTS infections, which tend to fail in patients with compromised host immunity and infected with antibiotic-resistant NTS. Therefore, vaccinations provide a rational route for solving this problem.

Currently, two types of licensed *Salmonella* vaccines, both for typhoid fever, are available as safe and efficacious for people aged 2 years or older (Cryz *et al.*, 1993; Khan *et al.*, 2012). However, there is currently no vaccine available for targeting iNTS directly. Vivotif®, a live oral vaccine containing a mutated *S.* Typhi Ty21a, could effectively elicit intestinal antibodies against O-antigens, which exhibit partial cross-protective efficacy against *S.* Typhimurium and *S.* Enteritidis (Engels *et al.*, 1998; DeRoeck *et al.*, 2005, 2008; Kantele *et al.*, 2012). Vi polysaccharide subunit vaccine confers similar levels of humoral immune responses and protection against typhoid fever as Vivotif®, but offers no cross-reactive immune responses against other serotypes (Fraser *et al.*, 2007; Tennant and Levine, 2015). However, it has been shown that live attenuated oral vaccines such as *S.* Typhi Ty21a generally provide better protection, compared to vaccines containing killed bacteria or bacterial subunits, against intracellular bacteria including *Salmonella*, because live attenuated oral vaccines induce cell-mediated and mucosal immune responses (Lundin *et al.*, 2002; Wahid *et al.*, 2014). Live attenuated *S.* Typhimurium (WTO5), which has mutations in both *aroC* and

ssaV, was tested in clinical trial I, but it was discontinued due to prolonged stool shedding of WTO5 for up to 23 days in healthy volunteers (Hoiseth and Stocker, 1981; Hindle *et al.*, 2002). In addition, several live attenuated vaccine strains have been developed by mutating virulence gene(s) and/or metabolic gene(s), but one obstacle in developing live attenuated vaccines is that it is difficult to attain satisfactory attenuation without compromising immunogenicity (Galen *et al.*, 2011).

The structurally conserved and ubiquitous pathways of central metabolism provide resources and energy for the biosynthesis of cellular macromolecules (Varki, 2017). Thus, genes involved in the central metabolism of aromatic amino acids and nucleotides have been widely used as important targets in the construction of auxotrophic attenuated strains (Hoiseth and Stocker, 1981; O'Callaghan *et al.*, 1988; Garcia-Del Portillo *et al.*, 1999). Carbohydrates are basic building blocks to many essential metabolic pathways in bacteria, and each carbon source is often consumed through a dedicated transportation and utilization pathway (Dills *et al.*, 1980). In addition, carbon utilization is likely to be closely connected with pathogenic mechanisms via direct or indirect regulation of the infection process (Steeb *et al.*, 2013). More than 20 carbohydrates may be transported into the bacterial cytoplasm by the phosphotransferase system (PTS), which is widely present among bacteria (Deutscher *et al.*, 2006). Among these sugars, glucose is the most preferred sugar for transport through the PTS. In the process of glucose metabolism, a phosphoryl group derived from phosphoenolpyruvate (PEP) is transferred sequentially to the general cytoplasmic PTS proteins, enzyme I (EI; PtsI) and HPr (PtsH), and in the case of glucose, the phosphoryl group is further transferred to the carbohydrate-specific cytoplasmic $EIIA^{Glc}$ (Crr), membrane-bound $EIICB^{Glc}$ (glucose permease), and glucose, followed by the transport of glucose-6 phosphate into bacteria (De Reuse and Danchin, 1988). Previously, we demonstrated the potential of PTS components as targets for developing live attenuated *Salmonella* vaccines. Deletion of the *ptsI* gene in *S.* Typhimurium impaired its glucose and glycerol uptake, which lead to attenuated virulence in a mouse colitis model. Furthermore, oral immunization by this strain was highly immunogenic, inducing both humoral and cell-mediated immnnnune activities in this mouse model (unpublished data).

Another PTS component, $EIIA^{Glc}$ (Crr), is a bi-functional protein involved in both the uptake of glucose and other sugars, and activation of adenylate cyclase (CyaA), resulting in increased cyclic AMP (cAMP) levels (Maze *et al.*, 2014). The cAMP activates the transcription factor cAMP receptor protein (CRP), which modulates essential cellular processes as well as the expression of several hundred genes, including virulence genes (Curtiss and Kelly, 1987). In this study, we explored whether Crr could be used as another target in PTS for developing a live attenuated vaccine strain. We found that the Crr mutant has the disadvantage of showing reactogenicity when a large amount is administered in mice, but mutations in both *ptsI* and *crr* showed synergistic reduction of its virulence, with high immunogenicity.

Materials and Methods

Reagents

All antibodies, purified proteins, antibiotics, and other reagents used in this study were purchased from Sigma-Aldrich.

Bacterial strains and growth conditions

The *S.* Typhimurium strains used in this study were derived from strain UK1 (ATCC68169) (Luo *et al.*, 2011). All mutants were constructed using lambda Red recombinase-mediated replacement as described previously (Datsenko and Wanner, 2000). Primers used in this study are listed in Table 2. The bacterial strains and plasmids used in this study are listed in Table 1. The *Salmonella* strains were grown at 37°C in Luria-Bertani (LB) broth (Difco), M9 minimum (Sigma-Aldrich) as described previously (Maze *et al.*, 2014). The media were supplemented with kanamycin (50 μg/ml), chloramphenicol (25 μg/ml), or ampicillin (100 μg/ml), if required.

Cell invasion and replication assays

The experimental procedures were performed essentially as described previously (Bowden *et al.*, 2009). Mouse macrophage like cell lines (RAW 264.7, ATCC® SC-6004™) were cultured in Dulbecco's modified Eagle medium (DMEM) (Gibco) supplemented with 10% fetal bovine serum (FBS) (Gibco), 100 U/ml penicillin, and 100 μg/ml streptomycin at 37°C with 5% carbon dioxide. The cells were plated in

Table 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant genotype	Source or reference
Escherichia coli		
DH5α	$F^-r^-m^+$Ø80d*lacZ*ΔM15	Gibco BRL
Salmonella Typhimurium		
KST0134	UK1, clinical isolate from horse	ATCC68169
KST0555	UK1, *ΔptsI*	Lim *et al.* (2015)
KST0556	UK1, *ΔptsIΔcrr*	This study
KST0584	UK1, *Δcrr*	This study
Plasmid		
pKD46	P_{BAD}-*gam-beta-exo oriR101 repA101*^{LS}; Amp^R	Datsenko and Wanner (2000)
pKD13	FRT $Kana^R$ FRT PS1 PS4 *oriR6K*γ; Amp^R	Datsenko and Wanner (2000)
pCP20	*cI*857 λP_R flp oripSC101^{ts}; Amp^R Cm^R	Datsenko and Wanner (2000)

Kana, kanamycin; Cm, chloramphenicol; Amp, ampicillin.

Table 2. Primers used for mutant strains construction in this study

Name	Sequence (5′→3′)
ptsI-F	AAG TTT TTT TTC CGG GTT CTT TTA AAA ATC AGT CAC AAG TAA GGT AGG GTT CAT ATG AAT ATC CTC CTT A
ptsI-R	GCA GTT CCT GTT TGT AGA TTT CAA TCT CTT TGC GCA GCG CGC GAA CTT CTT GTA GGC TGG AGC TGC TTC
crr-F	AAT CTG CTA ATC CAC GAG ATG CGG CCC AAT TTA CTG CTT AGG AGA AGA TCC ATA TGA ATA TCC TCC TTA
crr-R	AAT CAG TTC TTT GAT TTC ATC CAT GTT GGA GAT AAC AAC CGG AGT CAG GGT GTA GGC TGG AGC TGC TTC
ptsI-D-F	TTC AAA TCT TCC TTT CGC GGC
ptsI-D-R	CAG TAC GGA CGG TGA CAT TG
crr-D-F	TTA AGA AGA TTA TCC GTA ACA CGA A
crr-D-R	TGC GGA TAA CCG GAG TTT CAC

F, forward primer; R, reverse primer; D, diagnosis primer.

48-well cell culture plates (SPL) at 1×10^5 cells/well and incubated for 24 h. *Salmonella* strains were grown in LB broth with appropriate concentrations of antibiotic, overnight at 37°C with shaking. The overnight cultures of *Salmonella* were washed with phosphate buffered saline (PBS) (137 mM NaCl; 2.7 mM KCl; 10 mM Na_2HPO_4; 1.8 mM KH_2PO_4; Lonza), resuspended in DMEM, and then added to the prepared cell monolayers at a multiplicity of infection (MOI) of 1. After 1 h, the cells were washed three times with PBS, and then pre-warmed medium containing 100 μg/ml gentamycin was added 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 X-100 for 30 min. Finally, the cell lysate was serially diluted with PBS, and the diluents were spotted on LB agar plates to determine the number of colony forming units (CFU).

Fluorescence microscopic analysis

To visualize infected *Salmonella*, RAW 264.7 cells were plated on 15 μ-Chamber 12-well glass slides (Ibidi) and then infected with KST0134 (WT), KST0555 (Δ*ptsI*), KST0556 (Δ*ptsI*Δ*crr*), and KST0584 (Δ*crr*) strains 50 MOI at 37°C for 1 h. DMEM containing with gentamicin (100 μg/ml) was used to eliminate extracellular bacteria. After 18 hpi, the cells were fixed with 4% paraformaldehyde for 30 min and permeabilized with 0.5% Triton X-100 in PBS for 20 min. The cells were then washed three times with PBS and blocked with 3% BSA in PBS up to 2 h. Intracellular *Salmonella* was detected with FITC-conjugated anti-rabbit *Salmonella*-specific antibody (1:1,000 dilution; Abcam). The nucleus was stained with 150 ng/ml 4',6-Diamidine-2'-phenylindole dihydrochloride (DAPI) (Life Technologies). All images were captured on the Olympus CX41 imaging system.

Mice and animal experiments

All animal experiments were approved by the Committee on the Use and Care of Animals at Korea Atomic Energy Research Institute (KAERI), and were performed according to accepted veterinary standards. Six-week-old male BALB/c mice were purchased from Orient Bio. To measure the virulence of UK1 and its isogenic mutants, mice groups (n = 5) were infected orally with 10^8 CFU of *Salmonella* KST0134 (WT) and its isogenic mutants, KST0555 (Δ*ptsI*), KST0584 (Δ*crr*), or KST0556 (Δ*ptsI*Δ*crr*). Water and food were withdrawn 4 h before the infection, and resupplied at 2 hpi (Coombes *et al.*, 2005). After 6 days post-infection (dpi), the spleens, mesenteric lymph nodes, and caecum were aseptically isolated. The number of viable bacteria in each organ was determined by plating the serially diluted homogenates or blood on LB agar plates. To further verify the protection capacity of KST0556 (Δ*ptsI*Δ*crr*), five mice were immunized with 10^7 or 10^5 CFU KST0556 (Δ*ptsI*Δ*crr*) orally or intraperitoneally (*i.p.*) in 100 μl PBS, respectively. The same doses of bacteria were used to boost twice the immunization at every two-week interval after the primary immunization. On 42 days after the first immunization, 10^8 CFU UK1 was inoculated orally to the immunized mice. Mortality and body weight changes were observed and recorded.

Measurement of mice immunoglobulin

Blood samples from the mice were taken at day 14, 28, and 42 after the first immunization. The serum was isolated through centrifugation and stored at -80°C for long-term use. *S.* Typhimurium (UK1) were cultured in LB and harvested at mid-log phase. The absorbance of *Salmonella* pellet was adjusted to 0.1 at 600 nm by diluting in PBS. Next, 100 μl of *Salmonella* was used for coating wells of 96-wells immune plates (SPL). The plates were incubated overnight at 4°C to allow adherence of bacterial cells onto the wells. The plates were washed five times using PBS containing 0.05% Tween 20 (PBS-T), followed by blocking with PBS containing 1% bovine serum albumin for 1 h at room temperature. After blocking, the diluted sera were added to each well, incubated at room temperature for 1 h, followed by washing out the unbound samples with PBS-T. Appropriate dilutions of goat anti-mouse Ig (Sigma-Aldrich), goat anti-mouse IgG-UNLB (Southern-Biotech), goat anti-mouse IgM-UNLB, and goat anti-mouse IgA antibodies conjugated with horseradish peroxidase (HRP) were added to the wells and incubated for 30 min at RT. The plates were then washed 5 times with PBS-T, and then 100 μl of TMB substrate reagent (BD biosciences) was added. When the colors developed, 50 μl of 2 N H_2SO_4 was added, and the absorbance was measured at 450 nm using a Victor X3 light plate reader (Perkin-Elmer).

Statistical analysis

Data were presented as the means ± SD. For ELISA, the statistical significance was determined by the Student's *t*-test. For survival rates in the lethal challenge experiments, statistical significance was evaluated by Log rank (MantelCox) analysis. GraphPad Prism software was used to plot graphs. P value < 0.05 was considered statistically significant.

Results

Impaired carbohydrate uptake and intracellular replication by deleting *ptsI*

Previous studies have indicated that *Salmonella* strains lacking the *ptsI* gene, encoding an initial component of PTS, impaired growth in minimal media containing only glucose or glycerol (Lim *et al.*, 2015). We constructed several single or double mutant strains for PTS components, and compared their growth in nutrient-rich and minimal medium. In nutrient-rich LB broth, there was no obvious growth defect observed in the mutants KST0555 (Δ*ptsI*), KST0584 (Δ*crr*), and KST0556 (Δ*ptsI*Δ*crr*) compared to isogenic KST0134 (WT) (data not shown). To further investigate whether PTS mutants could use glucose or glycerol as a carbon source, the strains were cultured in M9 minimum medium supplemented with glucose or glycerol. No growth was observed for KST0134 (WT) and PTS mutants in minimal medium lacking carbohydrates (data not shown). In minimal media supplemented with glucose, KST0584 (Δ*crr*) showed comparably similar growth patterns as that of KST0134 (WT), whereas KST0555 (Δ*ptsI*) and KST0556 (Δ*ptsI*Δ*crr*) did not reach the stationary phase until 24 h in culture (Fig. 1A). However, when the *Salmonella* strains were grown in M9 supplemented with glycerol, only KST0555 (Δ*ptsI*) showed impaired growth, which did not reach an OD_{600} above 0.5 until after 15 h (Fig. 1B). The mutation on *ptsI* affects not only the utilization of glucose but also glycerol. Since KST0556 (Δ*ptsI*Δ*crr*) was not able to utilize glucose, but the growth of KST0556 (Δ*ptsI* Δ*crr*) was restored in the media supplemented with glycerol, KST0556 (Δ*ptsI*Δ*crr*) should be glycerol auxotroph.

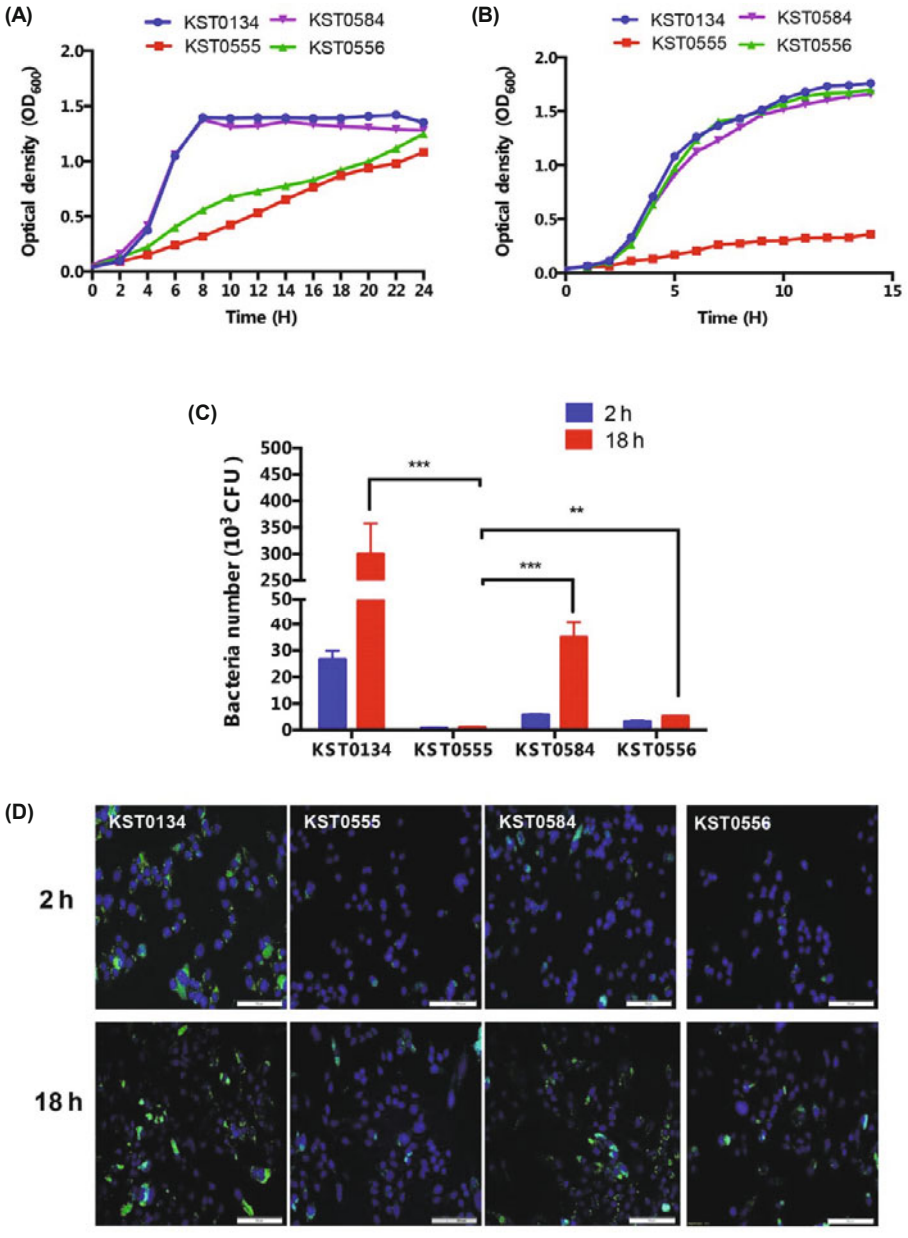

Fig. 1. Impaired growth and survival of *Salmonella* wild-type (WT) and the 3 phosphotransferase system (PTS) mutants in minimal medium or RAW 264.7 cells. (A, B) Growth curves of KST0134 (blue), KST0555 (red), KST0584 (purple), and KST0556 (green) in the minimal M9 broth supplemented with 0.5% glucose (A) or 0.5% glycerol (B). (C, D) Cell invasion and replication of WT and PTS mutants in mouse RAW 264.7 cells. RAW 264.7 cells were infected with WT or PTS mutants at a multiplicity of infection (MOI) of 1, and the bacterial viability were counted at 2 (blue) or 18 (red) hours post-infection (hpi). Data are representative of three independent experiments and are shown as the means ± SD. $^{**}P < 0.005$ and $^{***}P < 0.001$ compared with WT (C). *Salmonella* was visualized using an immunofluorescence assay (D). RAW 264.7 cells infected with WT or PTS mutants were stained with FITC-conjugated *Salmonella*-specific IgG (green) at 2 or 18 hpi. Nuclei were stained with DAPI (blue).

Host nutrients, such as lipids, carbohydrates, amino acids, and nucleotides, are essential for *Salmonella* infection, proliferation, and disease progression (Steeb *et al.*, 2013). Among them, glucose is the primary energy source required for intracellular replication of *S.* Typhimurium in mice and macrophages (Postma *et al.*, 1993; Bowden *et al.*, 2009). To evaluate whether these genes were involved in *Salmonella* virulence and surveillance in mouse macrophages, an invasion and replication assay was conducted using RAW 264.7 cells (Fig. 1C). The invasion ability (2 hpi) of KST0134 (WT) was approximately 35 ± 3.60, 4 ± 0.23, and 8 ± 1.34 fold higher than KST0555 (*ΔptsI*), KST0584 (*Δcrr*), and KST0556 (*ΔptsI Δcrr*), respectively. Intracellular replication (18 hpi) of KST0134 (WT) and KST0584 (Δ*crr*) increased more than 10.3 ± 0.6 and 7.6 ± 0.4 fold at 2 hpi, thereby indicating their successful replication in macrophages. In contrast, there were no obvious or small CFU changes at 18 hpi in macrophages infected with either KST0555 (*ΔptsI*) or KST0556 (*ΔptsIΔcrr*), indicating a lack of or lower replication ability in macrophages. These data suggested that glucose was a key carbon source for *Salmonella* to replicate in the macrophages.

To directly visualize the states of *Salmonella* invasion and replication capacity in macrophages, immunohistochemistry using an FITC-conjugated *Salmonella*-specific monoclonal antibody was performed (Fig. 1D). The fluorescence signals of KST0134 (WT) in macrophages were significantly stronger than those of the three *Salmonella* isogenic mutant strains at both 2 and 18 hpi. The signal intensity for KST0584 (*Δcrr*) was slightly lower than that of KST0134 (WT), but was obviously lower in KST0555 (*ΔptsI*) and KST0556 (*ΔptsIΔcrr*) compared to that of the WT at both 2 and 18 hpi. These data indicated that PtsI is a key component required for the uptake of glucose as a carbohydrate source.

Higher attenuation by double deletions of *ptsI* and *crr* genes in a mouse colitis model

To compare the virulence of the PTS isogenic mutants, mice were inoculated orally with 10^8 CFU of KST0134 (WT), KST0555 (*ΔptsI*), KST0584 (*Δcrr*), and KST0556 (*ΔptsIΔcrr*). After inoculation, mice were weighed and monitored regu-

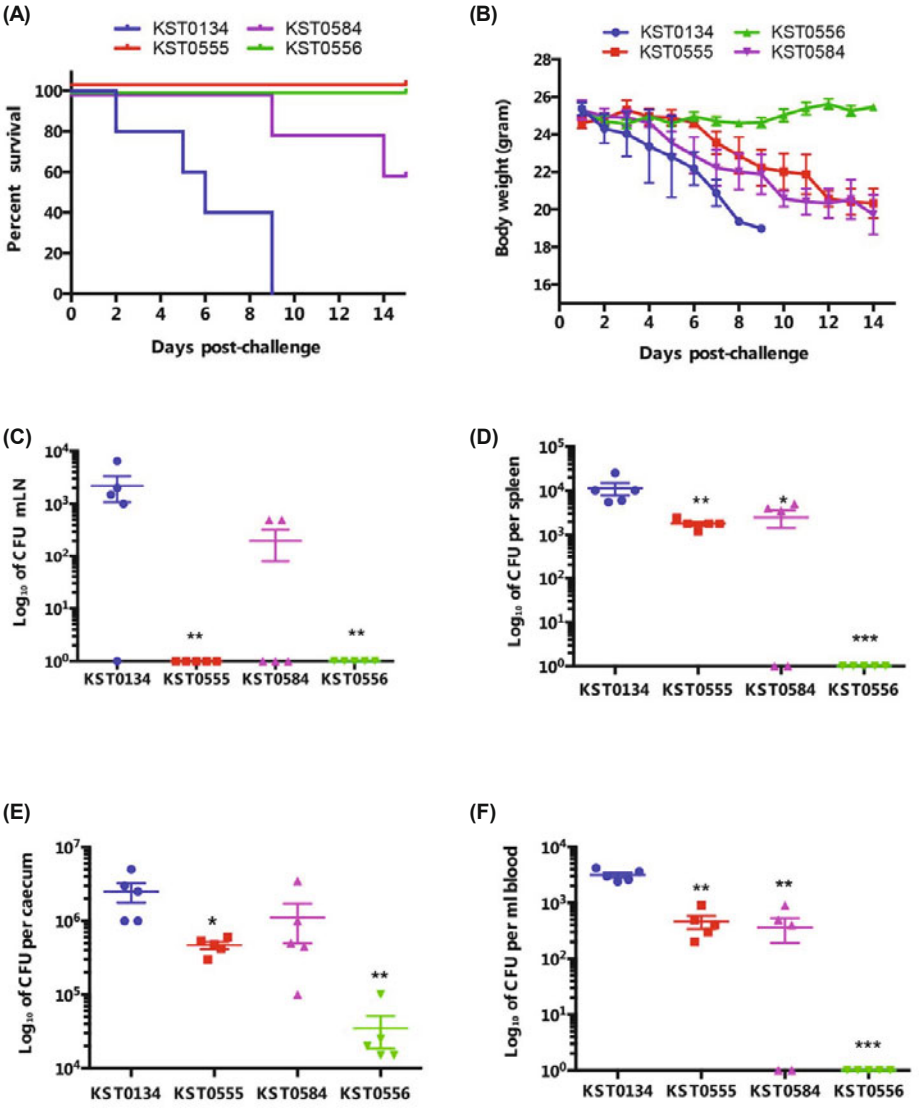

Fig. 2. Attenuation of PTS mutants in a mouse colitis model. (A) Survival curve and the change in body weight of BALB/c mice (n = 5) orally infected with KST0134 (blue), KST0555 (red), KST0584 (purple), and KST0556 (green). Mice were pretreated with 20 mg of streptomycin, followed by oral inoculation with 10^8 CFU of each *Salmonella* strain, and survival was monitored for 14 days. (B) The body weights of the infected mice were recorded and plotted for 14 days. (C–F) *Salmonella* colonization in mice organs. Mice were infected orally with 10^8 CFU of WT or PTS mutants, and the number of bacteria in the mesenteric lymph nodes (C), spleen (D), caecum (E), and blood (F) were counted. Data are mean ± SD. Asterisks indicate significant difference comparted with WT group. $^*P < 0.05$, $^{**}P < 0.005$, $^{***}P < 0.001$ compared with WT.

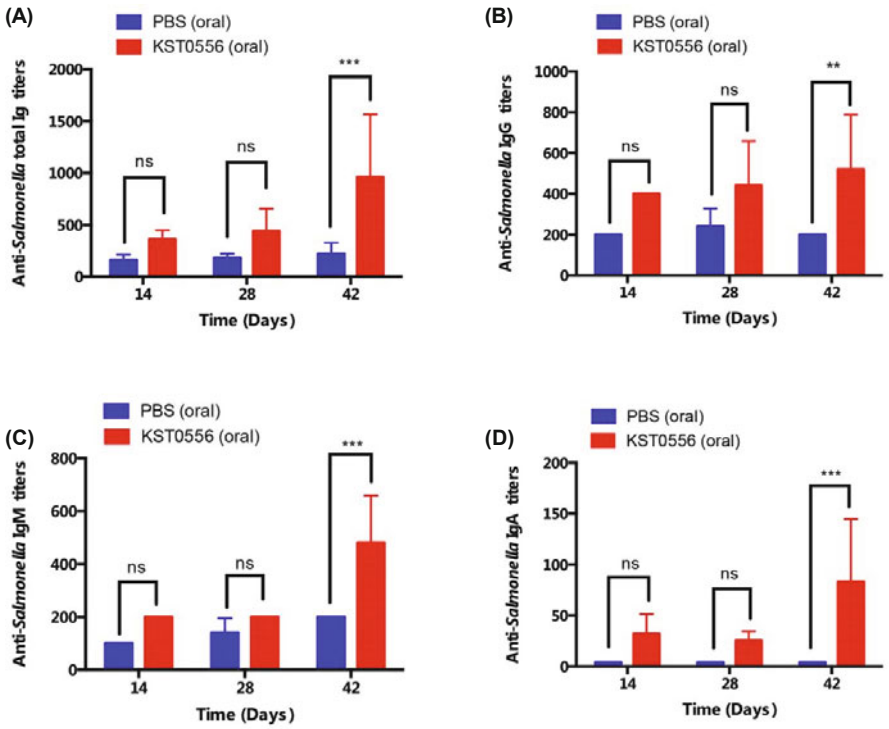

Fig. 3. Humoral and mucosal immune responses induced by KST0556 oral immunization. (A–D) Mice (n = 5) were vaccinated orally three times on days 0, 14, and 28 with 10^7 CFU of KST0556. The serum and feces were collected every two weeks. Anti-*Salmonella* specific total Ig (A), IgG (B), IgM (C), and IgA (D) titers were determined by ELISA. Blue and red bars indicate antibody titers of PBS and KST0556 vaccinations, respectively. Data are mean ± SD of three independent experiments. Asterisks indicate significant difference between PBS and KST0556 vaccination groups. $P < 0.05$, $^{**}P < 0.005$, $^{***}P < 0.001$.

larly for 14 days or until death occurred (Fig. 2A). All mice infected with KST0134 (WT) died at 9 dpi with a dramatic loss of their body weight. Although 60% or 100% of the mice survived for 2 weeks after infecting with KST0584 (*Δcrr*) or KST0555 (*ΔptsI*), respectively, their body weights were obviously reduced, and was more than 20% reduced at 14 dpi. In contrast, no significant mortality and relatively constant body weights were observed in mice infected with KST0556 (*ΔptsIΔcrr*) (Fig. 2B), indicating that deletion of both *ptsI* and *crr* would further reduced the virulence of *S.* Typhimurium *in vivo*.

To determine the colonization ability of *Salmonella* in or-

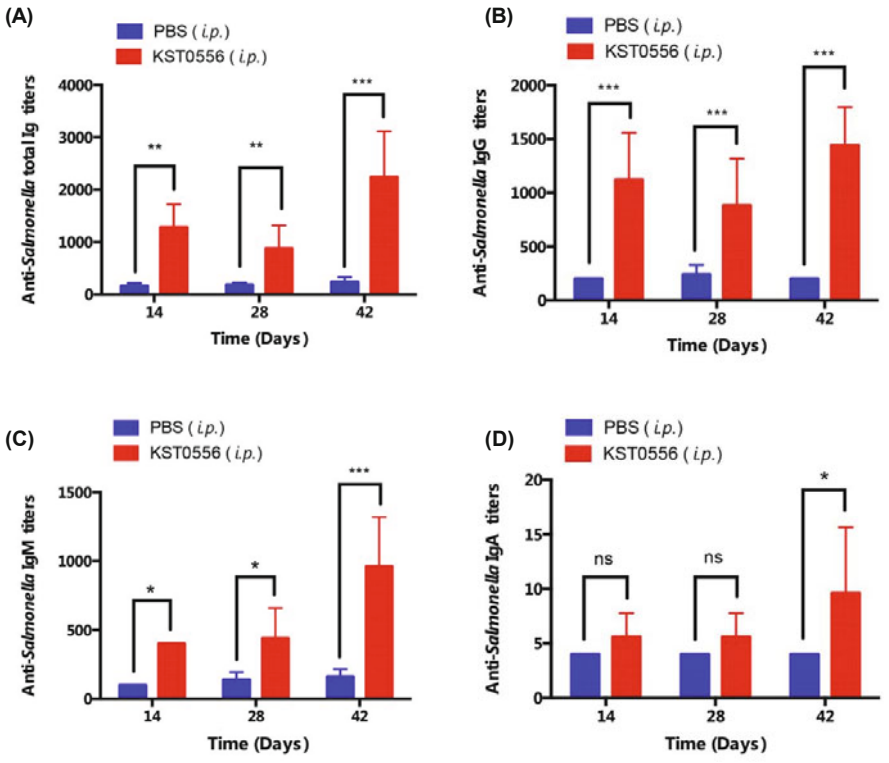

Fig. 4. Humoral and mucosal immune responses induced by intraperitoneal immunization with KST0556. Mice (n = 5) were vaccinated three times on day 0, 14, and 28 with 10^5 CFU of bacteria through *i.p.* injection. (A–D) The serum and feces were collected every two weeks. Anti-*Salmonella*-specific total Ig (A), IgG (B), IgM (C), and IgA (D) titers were determined by ELISA. Blue and red bars indicate antibody titers of PBS and KST-0556 vaccinations, respectively. Data are mean ± SD of three independent experiments. Asterisks indicate significant difference between PBS and KST0556 vaccination groups. $P < 0.05$, $^{**}P < 0.005$, $^{***}P < 0.001$.

gans and blood, mice were euthanatized at 6 dpi, and the number of bacteria in blood, spleens, mesenteric lymph nodes, and caecum were counted by plating on LB agar (Fig. 2C–F). Even though mice infected with KST0134 (WT) showed higher colonization or invasion of *Salmonella* cells in all organs and in the blood compared to those infected with the mutant strains, we could still detect high levels of *Salmonella* in the organs and blood of mice infected with KST0555 (*ΔptsI*) or KST0584 (*Δcrr*), suggesting that mutants were attenuated, but still showed partial invasiveness. In contrast, KST0556 (*ΔptsIΔcrr*) could not efficiently colonize the mouse cecum; the colonization was 71.42 ± 1.5, 13.37 ± 4.05, and 31.74 ± 0.80 fold lower than KST0134 (WT), KST0555 (*ΔptsI*), and KST0584 (*Δcrr*), respectively. Furthermore, no bacteria were detected in the blood, LN, and spleen of KST0556 (*ΔptsIΔcrr*)-infected mice, indicating that KST-0556 (*ΔptsIΔcrr*) had the lowest virulence among the PTS mutants, and was likely to be non- or extremely low-invasive.

Protective immune responses by live KST0556 vaccination

Next, we examined whether KST0556 (*ΔptsIΔcrr*) could be utilized as a live vaccine candidate. Each group of mice (n = 5) was immunized three times with 10^7 CFU orally or 10^5 CFU *i.p.* over a period of two weeks. Every two weeks before immunization, blood and feces were collected and evaluated for titers of *Salmonella*-specific antibodies. As shown in Fig. 3, mice immunized orally with KST0556 (*ΔptsIΔcrr*) showed higher total Ig, IgG, and IgM in the serum as well as IgA in feces compared to mice immunized with PBS at 42 days post-immunization. However, at day 14 and 28 (14 days after first and second immunization), there was no significant increase of all antibody titers in both serum and feces. Of note, increase in IgA was the most significant compared with other subtypes, indicating that oral immunization of KST0566 (*ΔptsI Δcrr*) induced more efficient mucosal immune responses. When immunized with KST0556 (*ΔptsIΔcrr*) via *i.p.* injection, the total Ig, IgG, and IgM titers were increased significantly even after the first immunization (14 days) (Fig. 4A–C). In contrast, the IgA level in feces was modestly increased only after 42 days, but it had a much lower titer compared to the feces of mice immunized orally (Figs. 3D and 4D). These results indicated that KST0556 (*ΔptsIΔcrr*) could stimulate humoral immune responses via both oral and *i.p.* immunization, and oral immunization could induce mucosal responses effectively.

To investigate whether vaccination with KST0556 (Δ*ptsI*Δ*crr*) could protect against a *S.* Typhimurium UK1 challenge, mice were immunized and boosted with 10^7 CFU orally or 10^5 CFU via *i.p.* as described above. At 14 days after the last immunization, mice were orally infected with 10^8 CFU of KST0134 (WT). As depicted in Fig. 5, all unvaccinated mice died at 5–9 dpi. Furthermore, the body weights of infected mice dramatically decreased to approximately 30% before death. On the contrary, the KST0556 (Δ*ptsI*Δ*crr*)-immunized mice showed effective protection against the lethal challenge. The above data suggested that the *S.* Typhimurium strain lacking both *ptsI* and *crr* was capable of inducing protective humoral and mucosal immune responses against an extremely high lethal dose of *S.* Typhimurium.

Discussion

Infections by iNTS are the leading cause of hospitalization and death in Africa and other developing countries (Ao *et al.*, 2015). Due to an increase in antibiotic-resistant *Salmonella* worldwide, new therapeutic methods for these infections are urgently required. Vaccinations are thought to be the most effective and economic strategy for managing enteric infectious diseases, including rotavirus, *Vibrio cholerae*, *Shigella*

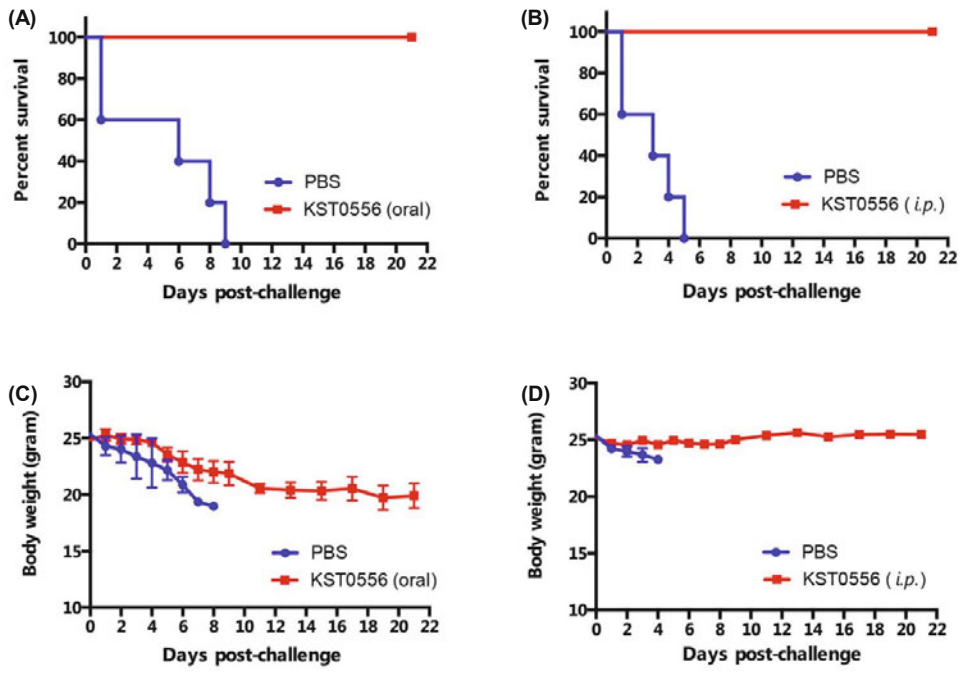

Fig. 5. Protective effect of KST0556 immunization against *Salmonella* challenge in mice. (A, C) Groups of mice (n = 5) were immunized orally with 10^7 CFU of KST0556 three times every two weeks, followed by an oral challenge with 10^8 CFU of *S.* Typhimurium UK1 7 days after the last vaccination. Mouse survival (A) and body weight changes (C) were monitored for 22 days. (B, D) Groups of mice (n = 5) were immunized *i.p.* with 10^5 CFU of KST0556 three times every two weeks, followed by an oral challenge with 10^8 CFU of *S.* Typhimurium UK1 7 days after the last vaccination. Mouse survival (B) and body weight changes (D) were monitored for 22 days.

spp. infections (Girard *et al.*, 2006; Meiring *et al.*, 2017; Church *et al.*, 2018), but no promising vaccines have been developed against iNTS so far. For enteric infectious diseases, live attenuated vaccines are the most attractive solution for preventing the spread and invasion of NTS, because they can effectively induce the host mucosal and cell-mediated immune responses (Tennant and Levine, 2015). In addition, oral administrations are easy to manage, do not require trained medical professionals, have reduced vaccination costs, and generate less waste (Tennant and Levine, 2015). In this study, we targeted the glucose metabolic pathway to construct an auxotrophic vaccine strain. Our previous study showed that deletion of the *ptsI* gene caused a dramatic reduction of its virulence and effective humoral and cell-mediated immune responses (Lim *et al.*, 2015). However, this single mutant strain KST0555 (Δ*ptsI*) was only slightly invasive and did not activate the mucosal immune response, which might be critical to combat enteric bacterial infections. Thus, we evaluated the invasiveness and immune responses of several strains with mutations in PTS components to identify a better vaccine strain.

Glucose is a major substrate required for the intracellular replication of *S.* Typhimurium in macrophages and epithelial cells (Bowden *et al.*, 2014). The key glycolytic genes, *pfkA* and *pfkB*, have central roles for the ability of *Salmonella* to replicate inside macrophages and mice, and the *ΔpfkAB* mutant was highly attenuated *in vivo* (Paterson *et al.*, 2009). In addition, the genes encoding components of PTS, which plays a critical role in transporting glycolytic substrates, have been shown to be connected with *Salmonella* pathogenesis (Postma *et al.*, 1993). PTS is the most well-known and important carbon uptake system consisting of enzyme I (EI encoded by *ptsI*), histidine phosphocarrier protein (HPr encoded by *ptsH*), and the membrane-associated enzyme II (EII). Among several EII components, $EIIA^{Glc}$ encoded by *crr* is involved in highly specific glucose transport (Le Bouguenec and Schouler, 2011). Our previous study found that deletion of *ptsI* alone caused downregulation of several virulence-related genes, which caused a reduction in virulence and replication in mice (Lim *et al.*, 2015). However, although KST0555 (Δ*ptsI*) was attenuated as much as other vaccine strains, it was slightly invasive, resulting in weight loss in mice when inoculated at a very high dose (10^8 CFU). We made additional mutations in *crr* because $EIIA^{Glc}$ is an activator of adenylate cyclase, which regulates many virulence genes directly or indirectly (Poncet *et al.*, 2009). Not surprisingly, this double mutant strain KST0556 (Δ*ptsI*Δ*crr*) was highly attenuated in a mouse oral infection model compared to *ptsI* or *crr* single mutant strains KST0555 (Δ*ptsI*) and KST0584 (Δ*crr*), respectively. Taken together, although all three mutant strains were highly attenuated, only KST0556 (Δ*ptsI*Δ*crr*) is much more suitable to use as a vaccine strain. However, the molecular mechanism of how the double mutations caused the synergistic reduction in virulence needs to be investigated further.

Live attenuated vaccines must balance attenuation with immunogenicity. For example, a *Mycobacterium tuberculosis* strain with mutations in *phoP* and *fadD26* virulence genes is hyper-attenuated with low-immunogenicity, which leads to reduced protective immune responses compared to the BCG vaccine (Solans *et al.*, 2014). Thus, a balance between attenuation and immunogenicity should be considered as the primary point of focus in the development of live attenuated vaccine strains. In fact, our previous study indicated that vaccination with KST0555 (*ΔptsI*) elicited effective humoral and cell-mediated immune responses, but no mucosal immune response (Lim *et al.*, 2015). The strain KST0556 (*ΔptsI Δcrr*) used in this study was hyper-attenuated, which led to a lack of or extremely low colonization in the spleen and mesenteric lymph nodes, while its vaccination would likely induce protective levels of mucosal and humoral immune responses. We hypothesized that the deletion of both *ptsI* and *crr* would increase the non-glucose carbohydrate (glycerol) utilization rate to help *Salmonella* survive and replicate under harsh environments, thereby providing persistent stimulation. We found that the survival of KST0556 (*ΔptsI Δcrr*) in macrophages was significantly higher compared to that of KST0555 (*ΔptsI*), indicating that the higher immune response elicited by KST0556 (*ΔptsIΔcrr*) may be due to its ability to survive and replicate in RAW 264.7 cells.

In the present study, the *S.* Typhimurium strain lacking *ptsI* and *crr,* encoding components of the PTS system, showed additional attenuation in mice, but provided protective levels of humoral and mucosal immune responses in mice. These findings support the notion that strains that have a deficiency in utilizing glucose could be considered potential attenuated vaccine candidates.

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Conflict of Interest

The authors have no conflicts of interest to declare.

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