Molecular Therapy
Oncolytics
Brief Report

Bacterial cancer therapy using the attenuated fowl-adapted *Salmonella enterica* serovar Gallinarum

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We report here a novel anti-cancer therapy based on an avian-host-specific serotype *Salmonella enterica* serovar Gallinarum (*S. Gallinarum*) deficient in ppGpp synthesis. To monitor the tumor targeting, a bioluminescent ΔppGpp *S. Gallinarum* was constructed and injected intravenously into mice bearing syngeneic and human xenograft tumors. Strong bioluminescent signals were detected specifically in all grafted tumors at 2 days post-injection (dpi). The bacterial counts in normal and tumor tissue at 1 dpi revealed that ΔppGpp *S. Gallinarum* reached $>10^8$ CFU/g in tumor tissue and 10^6–10^7 CFU/g in endothelial organs; counts were much lower in other organs. At 16 dpi, ΔppGpp *S. Gallinarum* counts in tumor tissue decreased to $\sim10^6$ CFU/g, while those in the other organs became undetectable. A strong anti-cancer effect was observed after the injection of ΔppGpp *S. Gallinarum* into BALB/c mice grafted with CT26 colon cancer cells. This could be attributed to reduced virulence, which allowed the administration of at least a 10-fold greater dose (10^8 CFU) of ΔppGpp *S. Gallinarum* than other attenuated strains of *S. enterica* serovar Typhimurium ($\leq10^7$ CFU). An advantage of the avian-specific *S. Gallinarum* as a cancer therapeutic should be a reduced capacity to cause infections or harm in humans.

INTRODUCTION

It has been shown that live bacteria selectively accumulate and replicate in tumors and destroy cancer cells in animal models. Thus, the use of live bacteria for cancer therapy has recently attracted much attention. Accumulated bacteria initiate a series of immune-mediated anti-tumor responses, although the underlying mechanism remains unclear.[1–4] Different variants of the genera *Bifidobacterium*, *Clostridium*, *Lactococcus*, *Shigella*, *Vibrio*, *Listeria*, *Escherichia*, and *Salmonella* have been analyzed in animal models of cancer.[5–9] Among these, the facultative anaerobe *Salmonella enterica* serovar Typhimurium has been used most widely in experimental rodent models because of its amenability to genetic manipulation.[10–15] However, rodents are highly susceptible to *S. Typhimurium* infection, and thus the bacteria need to be attenuated.

Among the many *S. Typhimurium* mutants that have been evaluated, VNP20009, A1-R, and ΔppGpp are the most widely studied.[3] VNP20009 is a genetically modified auxotrophic strain with 108 kb deletion, *purI*$^-$, *msbB*$^-$, and many SNPs.[16] The mutation in *msbB* gene resulted in a defective lipidA that crippled the associated virulence. Strain A1-R was chemically engineered to be auxotrophic for leucine and arginine,[17] whereas ΔppGpp lacks a signaling molecule (ppGpp) that positively or negatively regulates gene transcription.[18] In *S. Typhimurium*, ppGpp regulates transcription of all virulence genes encoded by *Salmonella* pathogenicity islands[19,20]; thus, the mutant defective in ppGpp synthesis, catalyzed by the *relA* and *spoT* gene products, was attenuated by over one million-fold compared with the wild-type strain.[21] ΔppGpp *S. Typhimurium* strains carrying various anti-cancer protein drugs have been constructed and deployed to treat xenografted cancers in rodent models.[22–24]

S. enterica serotypes are divided into two groups on the basis of the disease caused and host tropism.[25] Those with broad tropism include serovars Typhimurium and Enteritidis, which can cause enteritis in a wide range of host species; these two serotypes are responsible for the majority of food-borne *Salmonella*-induced enteritis cases in humans. Restricted host range serotypes rarely cause disease outside of their natural host, in which they cause systemic typhoid-like disease. Examples of these serotypes include *S. Typhi* in humans and *S. Pullorum* and *S. Gallinarum* in the chicken, which cause pullorum disease and fowl typhoid, respectively.[26,27]These diseases are attributed to the characteristics of Gallinarum/Pullorum serotype, which is incapable of entering the follicle-associated epithelium of mammalian Peyer's patches; therefore, Gallinarum/Pullorum is unable to survive and multiply in the cells of the mammalian reticuloendothelial system *in vivo* and *in vitro*.[28–31]

Here, we constructed a novel anti-cancer strain on the basis of *S. Gallinarum*. We reported previously that a ppGpp-defective

https://doi.org/10.1016/j.omto.2023.100745.

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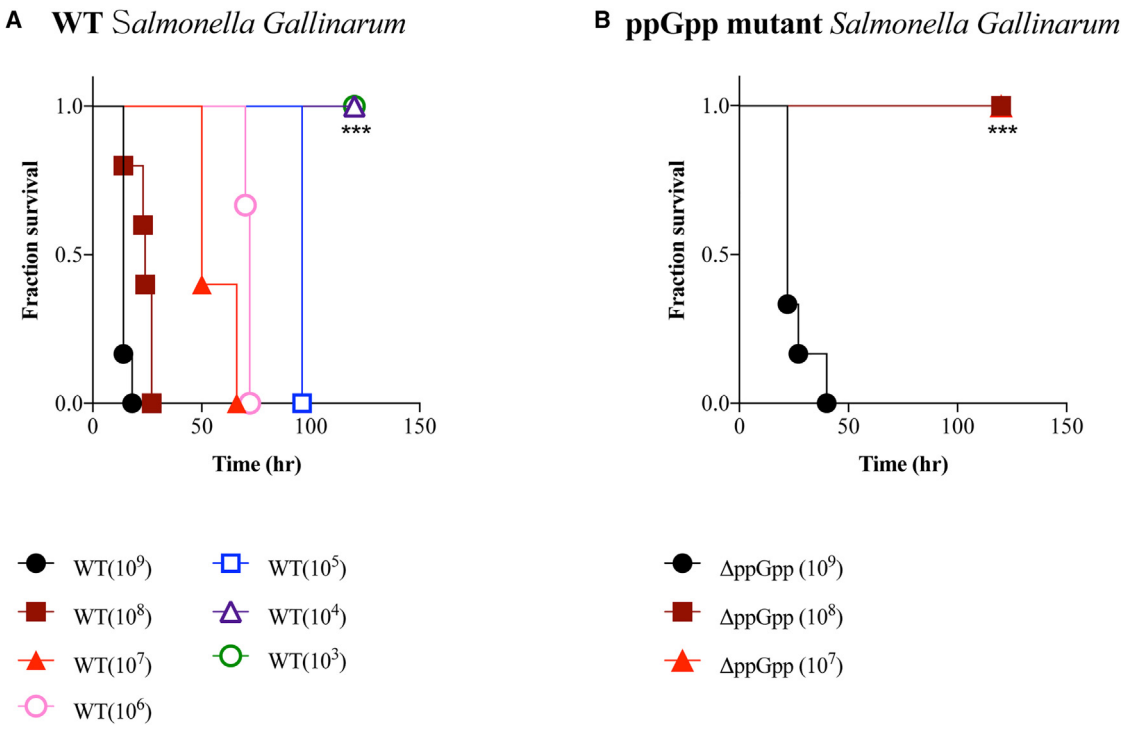

Figure 1. Mice survival following injections of wild-type and ΔppGpp *S. Gallinarum*

Survival of BALB/c mice (n = 5 per group) after intravenous injection of wild-type (A; SG3021) or ΔppGpp *S. Gallinarum* (B; SG2023) at the indicated dosages (*; < 0.025 and ****p < 0.0001).

mutant of *S. Gallinarum* was highly attenuated in a white leghorn chicken model[32] and that it was internalized by rodent-derived cells far less efficiently than wild-type bacteria; also, it failed to replicate intracellularly *in vitro*.[31] We tested the ppGpp-defective *S. Gallinarum* in rodent cancer models and demonstrated a strong anti-cancer effect with few side effects.

RESULTS

In an attempt to deploy *S. Gallinarum* as a cancer therapy agent, we first examined the survival of BALB/c mice injected intravenously (i.v.) with wild-type bacteria. We found that although *S. Gallinarum* causes typhoid only in fouls, all mice injected with $>10^5$ CFU died (Figure 1A). Therefore, we created a ppGpp-defective mutant by disrupting ppGpp synthetase I and II, encoded by *relA* and *spoT* genes, respectively.[18] It has been reported that a defect in ppGpp confers S. *Typhimurium* and *Escherichia coli* auxotrophic for several amino acids. The ppGpp-defective *S. Gallinarum* also required several amino acids, including branched chain amino acids, lysine, and serine, to grow (Table S1).

We then examined the tumor-targeting ability of the ppGpp-defective *S. Gallinarum* in tumor-bearing mice. To do this, we used bioluminescent bacteria and a noninvasive *in vivo* imaging system (IVIS). Bioluminescent bacteria were generated by transforming bacteria with an expression plasmid containing the *luxCDABE* operon from *Photobacterium leiognathi*.[33] One major obstacle is that bacteria tend to lose the plasmid carrying the reporter gene, which is not needed for bacterial survival. In addition, it is impractical to use an antibiotic resistance gene as a selective determinant in animal experiment. Therefore, we used a balanced-lethal host-vector system (the *glmS* gene system) in *S. Gallinarum*. This system takes an advantage of the phenotype of the GlmS$^-$ mutant, which undergoes lysis in animals lacking the nutrients required for proliferation of the mutant (i.e., D-glucosamine [GlcN] or N-acetyl-D-glucosamine [GlcNAc], both of which are components of peptidoglycan synthesis).[34–36] A novel balanced-lethal host-vector system was constructed in which the *glmS* gene of *S. Gallinarum* was incorporated into a plasmid that would complement the chromosomal *glmS* mutation. A 1.8 kb DNA fragment carrying the *glmS* of *S. Gallinarum* was obtained by PCR amplification and cloned into pLux containing the *lux* operon,[33,37] thereby generating *GlmS$^+$pLux* (Figure 2A). Luminescent signals from the bacteria carrying this plasmid that would be readily visualized by IVIS. We confirmed that the GlmS$^-$ mutant *S. Gallinarum* strain could not grow in ordinary bacterial growth medium unless supplemented with GlcNAc or GlcN and that the mutant was complemented with the *GlmS$^+$pLux*.

The ppGpp-defective strain carrying either a wild-type or mutated *glmS* was transformed with GlmS$^+$pLux carrying an ampicillin resistance marker, grown in Luria-Bertani (LB) broth, and sub-cultured (1:1,000) every 12 h. Bacteria carrying the plasmid were counted on GlcNAc-supplemented LB plates containing ampicillin. The results revealed that 99% of the plasmid carried by wild-type bacteria was cast out by day 4, while plasmids carried by the GlmS$^-$ mutant were fully

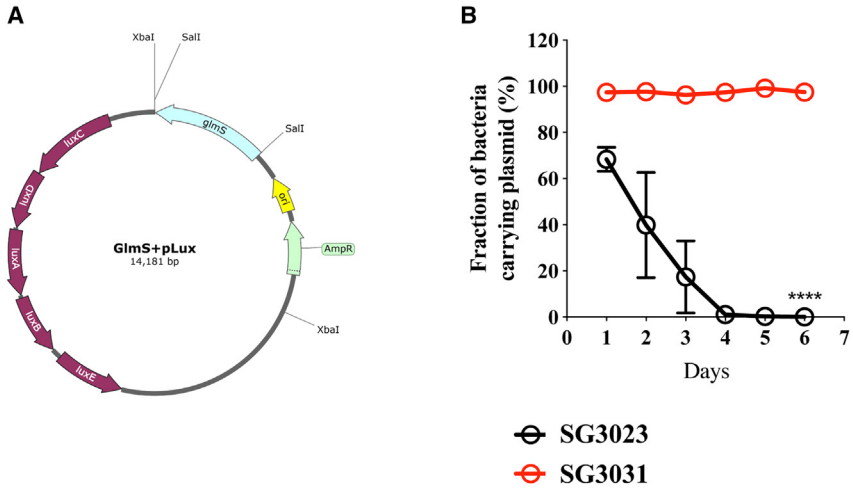

Figure 2. The balanced-lethal host-vector system (*glmS* gene system) in *S. Gallinarum*

(A) Plasmid map of *GlmS*$^{+}$*pLux*. (B) The GlmS^{-} mutant strain (SG3031) or the parental strain (SG3023) carrying GlmS^{+}pLux was sub-cultured (1:1,000) in LB broth every 12 h. The fraction of bacteria carrying the plasmid at the indicated time was determined by culture on GlcNAc-supplemented LB plates containing ampicillin (50 mg/mL). Each graph is representative of three independent experiments. Data represent mean ± SD, and asterisks indicate a significant difference between the wild-type and the GlmS^{-} mutant (****$p < 0.0001$).

maintained (Figure 2B). Therefore, the ppGpp-defective strain carrying a mutated *glmS* gene and transformed with *GlmS*$^{+}$*pLux* was used for bioluminescence imaging.

Tumor targeting by ΔppGpp *S. Gallinarum*

Various rodent tumor models were generated by grafting cultured tumor cells into the right thigh of different mouse strains. BALB/c mice received CT26 colon cancer cells or mouse 4T1 breast cancer cells, C57/BL6 mice received B16F10 melanoma cells, and BALB/c athymic nu^{-}/nu^{-} mice received human AsPC-1 pancreatic cancer cells. When the grafted tumors reached ~0.5 cm^{3} (after about 14 days), ΔppGpp *S. Gallinarum* harboring the mutation in *glmS* (1×10^{8} CFU) and carrying *GlmS*$^{+}$*pLux* were injected i.v. Bioluminescent signals generated by the bacteria were imaged at on the indicated days using an IVIS100 (Caliper, Hopkinton, MA) equipped with a cooled charge-coupled detector (CCD) camera (Figure 3). Following bacterial injection (~20 min, 0 days post-injection [dpi]), bioluminescent signals were detected mainly in the reticuloendothelial system, namely, the liver and spleen. The bioluminescent signals from 2 dpi were detected exclusively in grafted tumor tissues; these signals diminished gradually over time. The signal generated in AsPC-1 peaked at 4 dpi. It should be noted that *S. Gallinarum* targeted all four different tumor grafts in mice used in this study. We also injected ΔppGpp *S. Gallinarum* carrying the wild-type *glmS* gene and the *GlmS*$^{+}$*pLux* into BALB/c mice bearing CT26 tumors and tumor-associated bacteria were counted at 7 dpi using media plates containing ampicillin (Figure S1). The results showed that >90% of bacteria excreted the plasmid, demonstrating the necessity of using the balanced-lethal host-vector system. The presence of ΔppGpp *S. Gallinarum* in the grafted tumor tissue was demonstrated by staining bacteria in CT26 tumors at 3 dpi: bacteria were detected at the border between the proliferative and necrotic regions, as shown previously for *E. coli*[21] (Figure 4).

Anti-cancer efficacy of ΔppGpp *S. Gallinarum* in the CT26 tumor mouse model

The *in vivo* anti-tumor activity of ΔppGpp *S. Gallinarum* was accessed by injecting 1×10^{8} ΔppGpp *S. Gallinarum* into BALB/c mice bearing CT26 colon tumors. As a control, we also injected some mice with the ΔppGpp *S. Typhimurium* (1×10^{7}) used routinely in this laboratory.[22–24,38,39] Tumors in mice treated with 1×10^{8} ΔppGpp *S. Gallinarum* were significantly smaller than those in mice injected with 1×10^{7} *S. Typhimurium*, although the latter retarded growth better than PBS (negative control) (Figures 5A and 5B). Consequently, the group treated with ΔppGpp *S. Gallinarum* survived for longer than the groups treated with ΔppGpp *S. Typhimurium* or PBS (Figure 5C). The median overall survival of mice treated with 1×10^{7} *S. Typhimurium* was ~24 days, while that of the mice treated with 1×10^{8} *S.* Gallinarum was ~53 days. Compared with the PBS control (~15 days), treatment with 1×10^{8} *S. Gallinarum* extended median overall survival by ~3.5-fold. Under these conditions, we counted the actual number of bacteria in the internal organs and tumor tissue (Figure 6). At 1 dpi, the highest bacterial load was observed in the tumor (10^{8}–10^{9} CFU/g), although this decreased gradually to ~10^{6} CFU/g. This was consistent with the observation that bioluminescence generated by *S. Gallinarum* carrying *GlmS*$^{+}$*pLux* in the grafted tumor declined gradually over time (see Figure 3). The number of *S. Gallinarum* in the liver, spleen, and bone at 1 dpi was between 10^{6} and 10^{7} CFU/g; that in lung, kidney, and heart was ~10^{4} CFU/g; and that in the serum and eye was 10^{1}–10^{3} CFU/g. Most notably, *S. Gallinarum* was cleared completely from these organs by 16 dpi, presumably because of host immune responses. At this time, the ratio of *S. Gallinarum* between the tumor and the reticuloendothelial system, spleen, liver, and other organs by 16 dpi would be infinite. Roughly the same pattern of ΔppGpp *S. Typhimurium* distribution was observed in these organs at 1 dpi (Figure S2). The number of *S. Typhimurium* in these organs, however, did not decrease much over the 16 days.

DISCUSSION

In this study, we deployed 1×10^{8} ΔppGpp *S. Gallinarum* into the various tumor-bearing mice through the i.v. route (Figures 1B and 3). Mice tolerated injection of up to 10^{8} CFU easily. This is higher than the dose of ppGpp-defective *S. Typhimurium* used in our laboratory (~10^{7} CFU).[22–24,39] Similar experiments with *S. Typhimurium* A1-R, which is capable of selectively infecting and attacking viable tumor tissue,[40] as well as experiments with the *purI*$^{-}$, *msbB*$^{-}$ mutant (VN20009)[41,42] and the LPS-defective mutant Δ*rfaG*/Δ*rfaD*,[38] revealed that mice tolerated a lower dose (10^{6}–10^{7} CFU) of these bacteria.

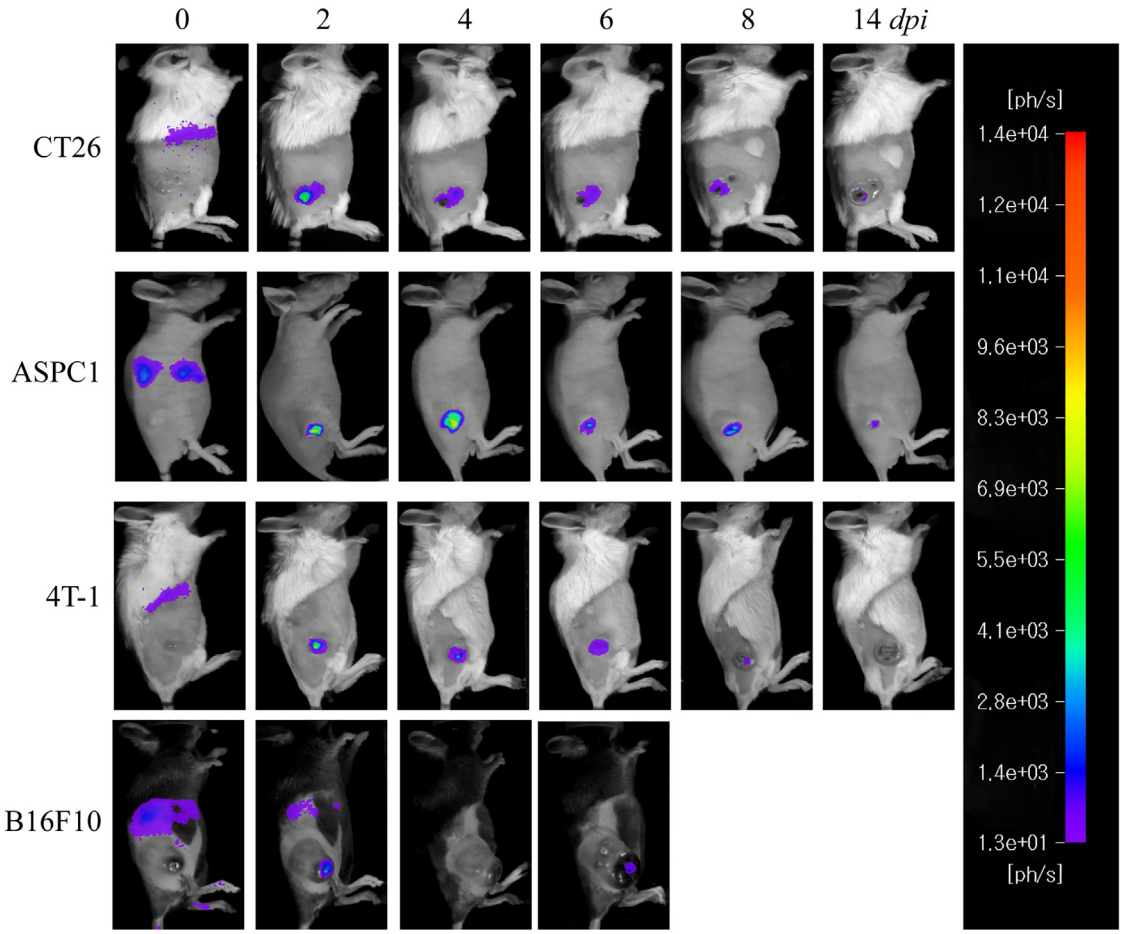

Figure 3. Tumor-targeting ability of *S. Gallinarum* (SG2031) transformed with *GlmS⁺pLux*

The bacteria were administered intravenously to mice (n = 3 per group) grafted with tumors. Bioluminescence signals were measured at the indicated days post-infection (dpi) using IVIS100 equipped with a cooled charge-coupled detector (CCD) camera. The y axis indicates photons × 10^5 · S^{-1} · cm^{-2} · sr^{-1}. Images labeled "0 dpi" were taken 20 min after bacterial injection.

Tumor-bearing mice tolerated a dose of 10^8 CFU *E. coli*.[41,43] Assuming that *E. coli* is almost completely avirulent, 10^8 CFU may be the most tolerable dose that does not trigger nonspecific catastrophe.

Endotoxemia induced by lipopolysaccharides (LPS) is a significant concern when using gram-negative bacteria for cancer therapy. In our case, especially because we used 1×10^8 *S. Gallinarum*, the endotoxemia is perceivable concern. A single *E. coli* cell is known to contain 2×10^6 molecules, which corresponds to an amount of about 20 fg.[44,45] Assuming LPS in *E. coli* and *S. Gallinarum* are about the same, the LPS content in the 1×10^8 *S. Gallinarum* should be ~2 μg. In general, the lethal dose of LPS typically falls within the range of 100 μg or more per mouse.[46,47] Therefore, 2 μg of LPS in the 1×10^8 *S. Gallinarum* would be negligible. However, the lethal dose of LPS required to induce endotoxemia in different animals vary enormously depending on various factors, including the species, age, and health status, as well as the specific LPS source and route of administration.[48]

Bacteria-mediated cancer therapy relies on its ability to induce a systemic anti-tumor immune response.[1–4,11,49] We observed an abrogated anti-cancer effect mediated by *S. Gallinarum* in the tumor-bearing mice depleted of either cytotoxic T lymphocytes ($CD8^+$ T cells) or natural killer (NK) cells (Figure S3).The mechanism underlying the anti-tumor effect of *S. Gallinarum* would be much the same as that mediated by *S. Typhimurium*, in which cytotoxic $CD8^+$ T cells or NK cells play pivotal roles.[50,51] It is not yet certain, however, whether the striking anti-tumor effects observed with ppGpp-defective *S. Gallinarum* can be ascribed to reduced virulence, which allows the administration of a 10-fold greater dose than for ppGpp-defective *S. Typhimurium*, or to the intrinsic anti-tumor characteristics of *S. Gallinarum*. One factor clearly attributable to the reduced virulence of ppGpp-defective *S. Gallinarum* is rapid clearance from internal organs. *S. Gallinarum* appears to have several advantages over other strains as a cancer therapeutic: (1) it is safer than *S. Typhimurium* in humans and rodents, (2) it poses no health threat to the persons handling it in laboratories and clinics, and (3) using the

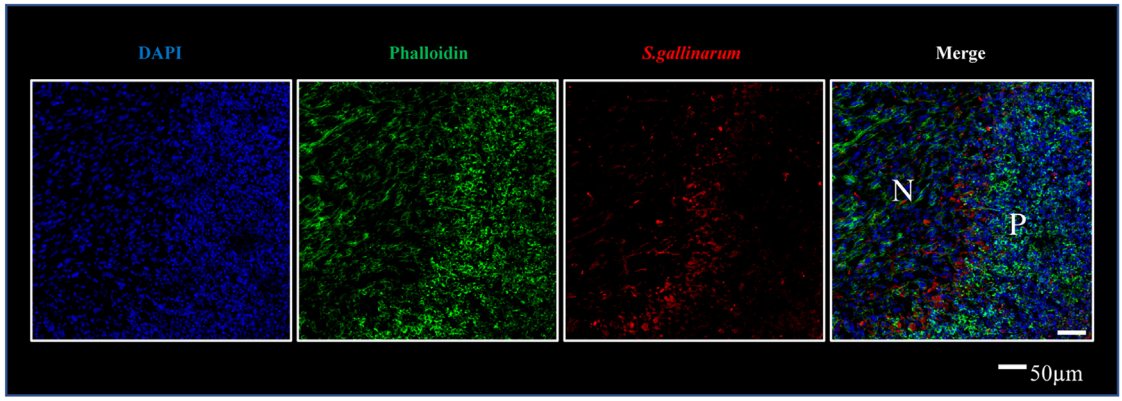

Figure 4. Bacterial colonization in tumor tissue

The subcutaneous CT26 tumor tissues shown in Figure 3 were excised at 3 dpi and processed prior to staining with DAPI (nuclei), Alexa Fluor 488-conjugated phalloidin (actin), and a *Salmonella*-specific antibody. The confocal images are shown at 200× magnification. N, necrotic region; P, proliferative region.

balanced-lethal host-vector system means that the plasmids encoding anti-tumor protein-encoding genes are stably maintained.

MATERIALS AND METHODS

Bacterial growth

Salmonella spp were grown in LB broth (Difco Laboratories) containing 1% NaCl, with vigorous aeration at 37°C. For solid support, 1.5% granulated agar (Difco Laboratories) was included. Antibiotics were obtained from Sigma-Aldrich. When necessary, antibiotics were added at the following concentrations: ampicillin, 50 μg/m; kanamycin, 50 μg/mL; and chloramphenicol, 15 μg/mL. GlcNAc was added (100 mg/mL) to the medium as required.

Bacterial strains

The *S. Gallinarum* mutants were derived from clinical isolate SG3021 obtained from the liver of a chicken with fowl typhoid on a South Korean broiler farm (Table 1). All bacterial strains were constructed using the method developed by Datsenko and Wanner.[52] The ppGpp-defective mutants were generated by sequential introduction of *relA::kan* and *spoT::cat* into the genome of SG3021. The open reading frame was replaced by a gene carrying either *kan* or *cat* by PCR amplification with a pair of 60 nt primers that include 40 nt homology extensions and 20 nt priming sequences; pKD13 was used as a template (Table 2). The PCR products were purified using DNA cleanup kit (#28104; Qiagen) and electroporated into bacteria carrying a lambda Red helper plasmid (pKD46). The mutants were confirmed by PCR using the original and test primers (Table 2). SG3023 was derived from SG3022 after eliminating antibiotic resistance genes using a helper plasmid expressing the FLP recombinase, pCP20. The GlmS^{-} deletion mutant was generated in the same way.

Measurement of plasmid stability

Overnight cultures were sub-cultured (1:1,000) every 12 h in fresh LB medium. Samples were taken every 24 h and serially diluted. Appropriate volumes were spread in triplicate on GlcNAc-supplemented LB plates with or without ampicillin. The number of colonies was used to calculate the total number of viable cells (CFU) and the fraction of bacteria containing the plasmid.

Plasmid GlmS^{+}*pLux*

The luminescence-expressing plasmid (pLux) was described previously.[33] Briefly, pLux containing the *lux* operon (*luxCDABE*, ~9.5 kb) of *P. leiognathi* was inserted into the pUC19 plasmid backbone via the *XbaI* restriction enzyme sites.[37] To construct a plasmid containing both the lux operon cassette and *glmS*, the *glmS* gene of *S. Gallinarum* was amplified using forward primer 5′-AA GTC GAC ATG TGT GGA ATT GTT GGC-3′ and reverse primer 5-GG GTC GAC TTA CTC TAC GGT AAC CGA TTT C-3′, with genomic DNA as a template. This 1.8 kb fragment was digested with *Sal I* and ligated into the same site in the pLux vector to generate *GlmS^{+}pLux* (Figure 2A).

Tumor cell lines

Murine CT26 colon carcinoma cells, 4T1 breast carcinoma cells, B16F10 melanoma cells, and human ASPC1 pancreas adenocarcinoma cells were obtained from the American Type Culture Collection. The CT26 and 4T1cells were grown in high-glucose DMEM containing 10% fetal bovine serum and 1% penicillin-streptomycin. The murine B16F10 melanoma cells and ASPC1 cells were grown in RPMI 1640 containing 10% fetal bovine serum and 1% penicillin-streptomycin.

Bacterial distribution in internal organs in rodents

For the viable counts of *Salmonella* spp, groups of BALB/C mice (n = 5) were injected with 1×10^7 ΔppGpp *S. typhimurium* and 1×10^8 ΔppGpp *S. Gallinarum* through the i.v. route and sacrificed on the days specified (day 1, 5, 10, or 16). The organs were collected and the tissues were homogenized in sterile PBS containing 0.05% Tween 20 with homogenizer. The *Salmonella* was recovered from the homogenates and quantified by plating on agar plates containing kanamycin (50 μg/mL) and chloramphenicol (15 μg/mL).

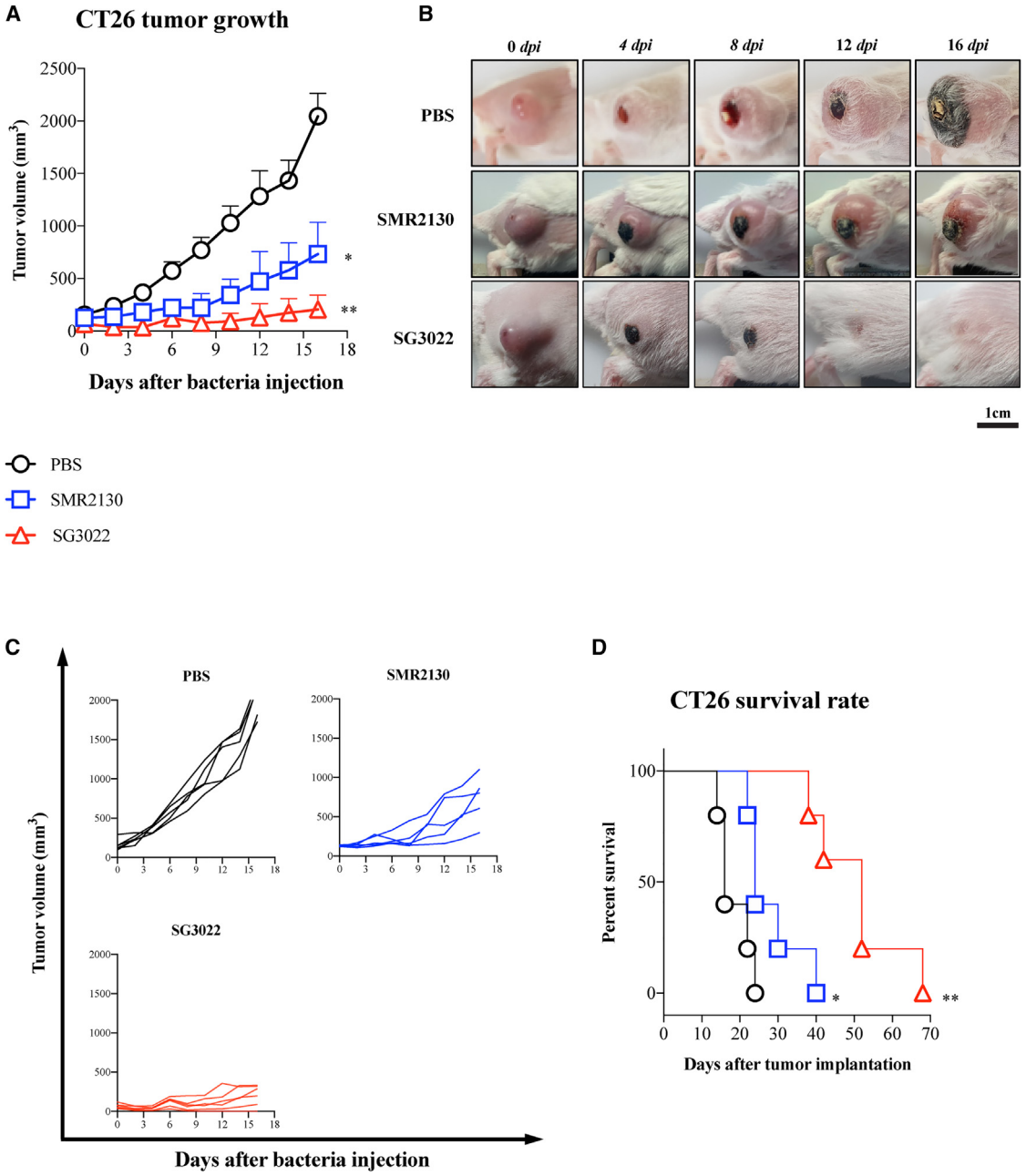

Figure 5. Anti-tumor effects of ΔppGpp *S. Gallinarum* (1×10^8 CFU, SG3023) compared with ΔppGpp *S. Typhimurium* (1×10^7 CFU, SMR2130) injected intravenously into BALB/c mice (n = 5/group) mice bearing CT26 colon tumors

(A) Average tumor volumes; error bars correspond to the 95% confidence interval (CI). Asterisks indicate significant differences from the PBS-treated control (*$p < 0.05$ and **$p < 0.005$). (B) Representative gross morphological changes. (C) Tumor volumes in the individual mouse. (D) Kaplan-Meier survival curves for tumor-bearing mice receiving the treatments described above. *$p < 0.03$ and **$p < 0.005$.

Experiments using rodents as cancer models

Male mice (5–6 weeks old, 20–30 g body weight) were purchased from Samtako Company. All animal care, experimental, and euthanasia procedures were performed in accordance with approved protocols. Mice carrying subcutaneous tumors were generated as follows: tumor cells cultured *in vitro* were harvested, suspended in 30 μL PBS, and injected subcutaneously into the right thigh at the indicated doses (1×10^6 cells for CT26, 4T-1 and B16F10 cells; and 1×10^7 cells for ASPC1). When tumors reached ~100 mm^3, ppGpp-defective *S. Gallinarum* or *S. Typhimurium* (1×10^8 and 1×10^7 CFU/mL in PBS, respectively)

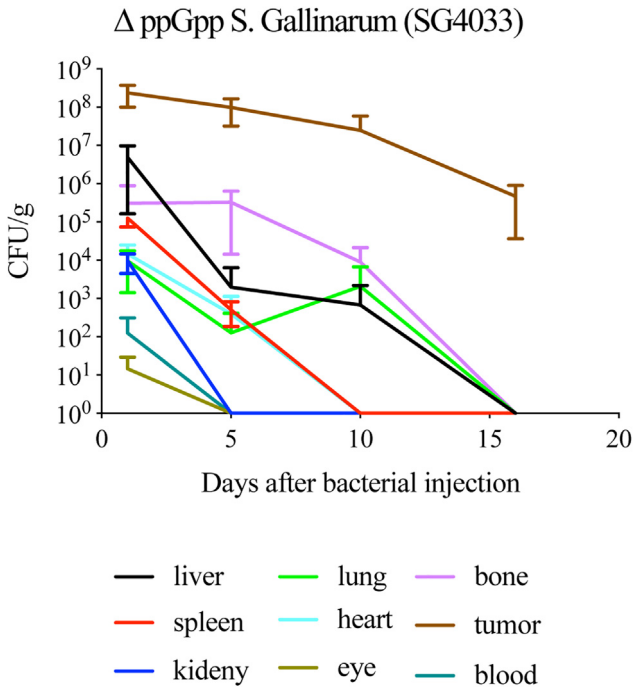

Figure 6. Distribution of bacteria in organs on the indicated days post-intravenous injection of 1 × 10^8 Δ ppGpp *S. Gallinarum* (SG3022) into BALB/c mice (n = 5/group) carrying CT26 tumors
Data represent mean ± SD.

were administrated through the tail vain (day 0). The tumor volume was calculated using the following formula: $(L \times H \times W)/2$, where L is the length, W is the width, and H is the height of the tumor in millimeters. Mice with tumor volumes $\geq$1,500 mm^3 were euthanized according to the guidelines of the Animal Research Committee. Survival rates were estimated using the Gehan-Breslow-Wilcoxon test. All animal experiments were approved and performed by the Institutional Animal Use and Care Committee of Chonnam National University (CNU IACUC-H-2020-7).

Optical bioluminescence imaging

To image bacterial bioluminescence, animals were anesthetized with isoflurane (2%)[24] and placed in the light-tight chamber of the IVIS100, which was equipped with a cooled CCD camera. Photons emitted from luciferase-expressing bacteria were collected and integrated over 1 min periods. Pseudocolor images indicating photon counts were overlaid on photographs of the mice using the Living Image software version 2.25 (Caliper).

Immunofluorescence staining and confocal microscopy

To stain *Salmonella* inside the tumor, isolated tissues were fixed overnight at room temperature in PBS containing 3.4% formalin and embedded in optimal cutting temperature (OCT) compound (Tissue-Tek). The tissues were then frozen and sliced into 7 mm sections using a microtome-cryostat. The sections were collected on aminopropyl triethoxysilane-coated slides, washed with PBS (pH 7.4) to remove the OCT, and incubated overnight at 4°C with an anti-*Salmonella* LPS antibody (1:100; #ab8274; Abcam), followed by an Alexa Fluor 568-conjugated goat anti-mouse (1:100; #A11031; Life Technologies) and Alexa Fluor 488-conjugated phalloidin (1:1,000; #W21404; Invitrogen) secondary antibodies and for the detection of F-actin (1:1,000, #MA1-80729; Thermo Fisher Scientific), respectively. After staining nuclei with DAPI/Antifade (1:200; Invitrogen), the samples were mounted and images were captured under a confocal microscope (LSM 510; Zeiss Laboratories). Representative images are shown in the figures.

Table 1. Bacterial strains

Strain	Description	Reference or source
S. Gallinarum		
SG3021	wild-type clinical isolate	Jeong et al.[31]
SG3022	Δ *relA::kan*, Δ *spoT::cat*	This work
SG3023	Δ *relA*,Δ*spoT*	This work
SG3030	Δ *relA*,Δ*spoT*, *glmS::kan*	This work
SG3031	Δ*relA*,Δ*spoT*, Δ*glmS*	This work
S. Typhimurium		
SHJ2037	*relA::kan*, *spoT::cat*	Song et al.[19]
SMR2130	Δ *relA*,Δ*spoT*	Song et al.[19]

Statistical analyses

Statistical analysis was performed using SPSS 18.0 (SPSS Inc., Chicago, IL). A two-tailed Student's t test was used to determine the significance of differences in tumor growth between the control and treatment groups. A p value of 0.05 was considered to indicate statistical significance. All data are expressed as mean ± SD.

DATA AND CODE AVAILABILITY

All data generated or analyzed in this article are available from the corresponding author upon request.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.omto.2023.100745.

ACKNOWLEDGMENTS

This work was supported by the Starting Growth Technological R&D Program (TIPS Program; grant S3130592) funded by the Ministry of SMEs and Startups (MSS; Korea) in 2021. J.-H.J. was supported by a National Research Foundation of Korea (NRF) grant (MSIT) (2020R1A5A2031185). D.L. was supported by a 2023 Research Grant from Kangwon National University and a National Research Foundation of Korea grant funded by the Korean government (MSIP) (NRF-RS-2023-00210053).

AUTHOR CONTRIBUTIONS

Conceptualization, H.E.C. and J.-H.J.; methodology, K.K., T.D., E.S., and D.L.; investigation, E.S., T.D., K.K., and D.L.; writing – original

Table 2. Primers used

Primer name	Sequence 5′-3′
relA::kan F	GTG GAT CGC AAG CCT GGG AAT TTC CAG CCA GCA GTC GTG TGA GCG CTT AGG TGT AGG CTG GAG CTG CTT C
relA::kan R	GTG CAG TCG CCG TGC ATC AAT CAC ATC CGG CAC CTG GTT CAG CTT ACC GAA TTC CGG GGA TCC GTC GAC C
spoT::cat F	TTA AGC GTC TTC GGC AGG CGT ATC TCG TTG CAC GTG ACG CTC ACG AGG GCT GTA GGC TGG AGC TGC TTC
spoT::cat R	GCC AGA TGT ACG CGA TCG CGT GCG GTA AGG CGA ATA AAG GTA CTA TAG ACC ATA TGA ATA TCC TTA G
glmS::kan F	TTA CTC AAC CGT AAC CGA TTT TGC CAG GTT ACG CGG CTG GTC AAC GTC GGT GCC TTG ATT GTG TAG GCT GGA GCT TCG AA
glmS::kan R	ATG TGT GGA ATT GTT GGC GCG ATC GCG CTT CGT GAT GTA GCT GAA TCC TTC TTG AAG GTC ATA TGA ATA TCC TTC GTT CC

draft, D.L.; writing – review & editing: H.E.C., J.-H.J., and D.L. All authors have read and agreed to the published version of the manuscript.

DECLARATION OF INTERESTS

The authors declare no conflicts of interest.

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