Efficacy of a Genetically-modified *Salmonella typhimurium* in an Orthotopic Human Pancreatic Cancer in Nude Mice

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Abstract. *We report here a tumor-targeting strategy for pancreatic cancer using a modified auxotrophic strain of Salmonella typhimurium. The genetically-modified strain of S. typhimurium requires the amino acids arginine and leucine. These mutations preclude growth in normal tissue but do not reduce bacterial virulence in tumor cells. The tumor-targeting strain of S. typhimurium, termed A1-R and expressing green fluorescent protein (GFP), was administered to an orthotopic human pancreatic tumor expressing red fluorescent protein (RFP) in nude mice. After 7 days of treatment, the pancreatic cancer had regressed without the need of chemotherapy or any other treatment. This new strategy demonstrates the clinical potential of bacterial targeting for pancreatic cancer.*

It has been known for approximately 60 years that anaerobic bacteria can selectively grow in tumors (1-16). The conditions that permit anaerobic bacterial growth, such as impaired circulation and extensive necrosis, are found in many tumors. Several approaches to tumor-therapeutic anaerobic bacteria have been described. Dang *et al*. (17) created a strain of *Clostridium novyi* depleted of its lethal toxin (*C. novyi-NT*) and showed that *i.v.* administered C. novyi spores germinated within the avascular regions of tumors in mice and destroyed surrounding viable tumor cells (17). Also, cures can often be observed in mice by combining *C. novyi-NT* with radiation (18). It was recently shown that treatment of mice bearing large, established tumors with *C. novyi-NT,* in which a single dose of liposomal doxorubicin

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was added, could lead to eradication of the tumors. The bacterial factor responsible for the enhanced drug release was recognized as a protein termed liposomase (19, 20).

A genetically modified bacterial strain of *S. typhimurium*, termed *S. typhimurium* A1, has been previously developed by our laboratory (21). It is auxotrophic for leucine and arginine and expresses green fluorescent protein (GFP). When administered *i.v.* or intratumorally, A1 invaded and replicated intracellularly in PC-3 prostate cancer cells *in vivo* as well as *in vitro*. When A1 was injected intratumorally, the tumors regressed. There were no obvious adverse effects on the host when the bacteria were injected *i.v.* or intratumorally. The *S. typhimurium* A1 strain grew throughout the tumor, including viable malignant tissue. This result is in marked contrast to the anaerobic bacteria described above that were confined to necrotic areas of the tumor as discussed above. The ability to grow in viable tumor tissue may account, in part, for the unique antitumor efficacy of the A1 strain (21).

The A1 strain was re-isolated from A1-targeted tumor tissue *in vivo*. The idea was to increase the tumor targeting capability of the bacteria. As a result of this selective step, the tumor-cell targeting of the re-isolated A1 increased *in vivo* as well as *in vitro*. The re-isolated A1 bacteria, termed A1-R, administered *i.v.*, caused human breast cancer (22) and prostate cancer (23, 24) regression and cures in orthotopic nude-mouse models. A1-R was also found to be effective in nude mouse models of metastatic cancer (25, 26).

In the present study, it is demonstrated that *S. typhimurium* A1-R directly injected into a primary orthotopic pancreatic tumor growing in nude mice caused significant tumor regression.

Materials and Methods

GFP gene transfection of S. typhimurium. S. typhimurium (ATCC 14028) was grown at 37°C to midlogarithmic phase in liquid LB and harvested at 4°C. Bacteria (2.0×10^8) in 40 μL 10% glycerol were

mixed with 2 μL pGFP (Clontech Laboratories, Mountain View, CA, USA) vector and placed on ice for 5 min before electroporation with a Gene Pulser apparatus (Bio-Rad, Hercules, CA, USA) according to the manufacturer's instructions. Electroporation was done at 1.8 kV with the pulse controller at 1,000-Ω parallel resistance (21).

Induction of mutations with nitrosoguanidine (NTG) and selection for tumor targeting. Freshly prepared NTG (1 mg/ml in sterile water) was added to the washed bacterial culture to a final concentration of 100 μg/ml in Tris·maleic acid buffer at pH 6.0. The bacteria were incubated with NTG for 30 min. The NTG-treated cells were grown in nutrient broth. Bacterial colonies were replica-plated in supplemented minimal agar plates. Virulence of bacteria *in vitro* and toxicity in nude mice was evaluated. *S. typhimurium* A1, which is still virulent in tumor cells *in vitro* but non-toxic for host nude mice, was identified. The virulence for tumors *in vivo* was also confirmed. Strain A1 is remarkable for its selectivity for growth in tumors compared with normal tissues (21).

Re-isolation of S. typhimurium A1. *S. typhimurium* A1 auxotrophs expressing GFP were re-isolated as follows: the A1 bacteria were injected into the tail vein of a HT-29 human colon tumor-bearing nude mouse. Three days after infection, the tumor tissue was removed from the infected mouse. The tumor tissue was then homogenized and diluted with PBS. The resulting supernatant of the tumor tissue was cultured in LB agar plates at 37°C overnight. The bacteria colony with the brightest green fluorescence was picked up and cultured in 5 mL LB medium. This strain was termed A1-R (22).

RFP vector production. The RFP (DsRed-2) gene (BD Biosciences Clontech, Palo Alto, CA, USA) was inserted in the retroviral-based mammalian expression vector pLNCX (BD Biosciences Clontech) to form the pLNCX DsRed-2 vector. Production of retrovirus resulted from transfection of pLNCX DsRed-2 into PT67 packaging cells, which produce retroviral supernatants containing the DsRed-2 gene. Briefly, PT67 cells were grown as monolayers in DMEM supplemented with 10% FCS (Gemini Biological Products, Calabasas, CA, USA). Exponentially growing cells (in 10-cm dishes) were transfected with 10 μg expression vector using a LipofectAMINE Plus (Life Technologies, Grand Island, NY, USA) protocol. Transfected cells were re-plated 48 hours after transfection and 100 μg/mL G418 was added 7 hours after transfection. Two days later, the medium was changed to contain 200 μg/mL G418. After 25 days of drug selection, surviving colonies were visualized under fluorescence microscopy and RFP-positive colonies were isolated. Several clones were selected and expanded into cell lines after virus titering on the 3T3 cell line.

Production of histone H2B-GFP vector. The histone H2B gene has no stop codon, thereby enabling the ligation of the H2B gene to the 5'-coding region of the GFP gene (Clontech Laboratories). The histone H2B-GFP fusion gene was then inserted at the HindIII/CalI site of the pLHCX (Clontech Laboratories), which has the hygromycin resistance gene. To establish a packaging cell clone producing high amounts of histone H2B-GFP retroviral vector, the pLHCX histone H2B-GFP plasmid was transfected in PT67 cells using the same methods described above for PT67-DsRed2. The transfected cells were cultured in the presence of 200 to 400 μg/mL hygromycin (Life Technologies) for 15 days to establish stable PT67 H2B-GFP packaging cells.

RFP and histone H2B-GFP gene transduction of cancer cells. XPA1 human pancreas cancer cells (a gift from Dr. Anirban Maitra at Johns Hopkins University) were labeled with RFP and histone H2B-GFP. Clones expressing RFP in the cytoplasm were initially established. In brief, cancer cells were incubated with a 1:1 precipitated mixture of retroviral supernatants of PT67-RFP cells and RPMI 1640 (Mediatech, Inc., Herndon, VA) containing 10% fetal bovine serum for 72 hours. Fresh medium was replenished at this time. Cells were harvested with trypsin/EDTA 72 hours post-transduction and sub-cultured at a ratio of 1:15 into selective medium, which contained 200 μg/mL G418. The level of G418 was increased stepwise up to 800 μg/mL. RFP-expressing cancer cells were isolated with cloning cylinders (Bel-Art Products, Pequannock, NJ, USA) using trypsin/EDTA and amplified by conventional culture methods. For establishing dual-color cells, the cells were then incubated with a 1:1 precipitated mixture of retroviral supernatants of PT67 H2B-GFP cells and culture medium. To select the double transformants, cells were incubated with hygromycin 72 hours after transfection. The level of hygromycin was increased stepwise up to 400 μg/mL.

Intracellular growth of S. typhimurium A1-R. XPA1 cells labeled with RFP in the cytoplasm and GFP in the nucleus were grown in 24-well tissue culture plates to a density of 10^4 cells per well. *S. typhimurium* A1-R-expressing GFP were grown in LB and harvested at late-logarithmic phase, diluted in cell culture medium and added to the tumor cells ($1×10^5$ colony-forming units (CFU) per well). After 1 h incubation at 37°C, the cells were rinsed and cultured in medium containing gentamycin sulfate (20 μg/mL) to kill external but not internal bacteria. Interaction between bacteria and tumor cells was observed at the indicated time points. An Olympus BH 2-RFCA fluorescence microscope equipped with a mercury 100-W lamp power supply and GFP filter set (Chroma Technology, Brattleboro, VT, USA) was used for microimaging.

Orthotopic pancreatic cancer model in nude mice. Fifty microliters containing $2×10^6$ RFP-expressing XPA1-RFP cells were injected in the subcutaneous tissue in 4-week-old nude mice with a 1-mL 27G½ latex-free syringe (Becton Dickinson, Franklin Lakes, NJ). Subcutaneous tumors, at the exponential growth phase, were resected aseptically and cut with scissors and minced into approximately 3×3×3 mm pieces. Four-week-old mice were anesthetized by a ketamine mixture (10 μL ketamine HCL, 7.6 μL xylazine, 2.4 μL acepromazine maleate, and 10 μL H_2O) *via s.c.* injection. The abdomen was sterilized with alcohol. A subcostal incision was then made through the left upper abdominal pararectal line and peritoneum. The pancreas was carefully exposed and a tumor fragment was transplanted on the middle of the pancreas with an 8-0 suture. The pancreas was then returned into the peritoneal cavity, and the abdominal wall and the skin were closed with a 6-0 suture. Animals were kept in a sterile environment. All procedures of the operation described above were performed with a dissection microscope. All animal studies were conducted in accordance with the principles and procedures outlined in the National Institutes of Health Guide for the Care and Use of Laboratory Animals under assurance no. A3873-1.

Intra-tumoral bacterial therapy for pancreatic cancer. On day 7 after orthotopic implantation, the tumor was surgically exposed and imaged with the Olympus OV100 Small-Animal Imaging System
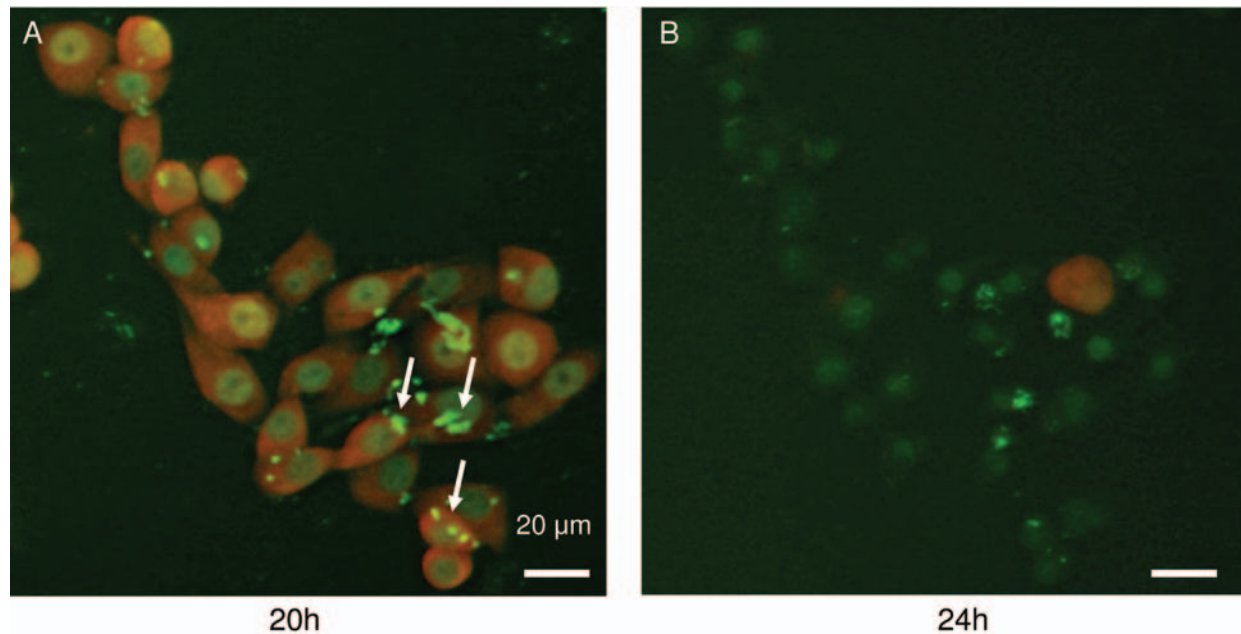
Figure 1. *Intracellular growth of S. typhimurium A1-R in vitro. XPA1 cells labeled with RFP in the cytoplasm and GFP in the nucleus. Interaction between bacteria and tumor cells was observed at the indicated time points under fluorescence microscopy. (A and B) GFP expressing S. typhimurium A1-R (arrows) was able to invade and replicate intracellularly in the dual-color XPA1 cell line in vitro. The cytopathic effects of A1-R on XPA1 cells after infection were visible using dual-color fluorescence. Intracellular bacterial infection leading to eventual cell fragmentation and cell death was observed (B). Bars, 20 μm.*

(Olympus Corp., Tokyo, Japan). The size of the tumor (fluorescent area [mm^2]) was measured. Three mice were treated with a low concentration of A1-R (10^7 CFU/mL), 3 were treated with a high concentration (10^8 CFU/mL), and 3 were used as untreated controls. Tumor volume (mm^3) was calculated with the formula V=1/2× (length × width^2). The bacteria were injected into the tumor. On day 14, the tumor was exposed again and the size was measured to determine the efficacy of treatment.

Statistical analysis. The experimental data are expressed as the mean±S.E. Statistical analysis was performed using the two-tailed Student's *t*-test.

Results

Intracellular growth of S. typhimurium A1-R. A1-R-GFP invaded and replicated intracellularly in XPA1 human pancreatic cancer cells expressing GFP in the nucleus and RFP in the cytoplasm. Intracellular bacterial infection led to cell fragmentation and cell death (Figure 1).

Intratumoral bacterial therapy for pancreatic cancer. The tumors were treated as illustrated in Figure 2. The average tumor size (fluorescent area) on day 7 was 3.2±1.9 mm^2 in the untreated group; 3.1±1.4 in the high-dose-bacteria treatment group; and 3.5±0.75 in the low-dose-bacteria treatment group. There was no significant difference among
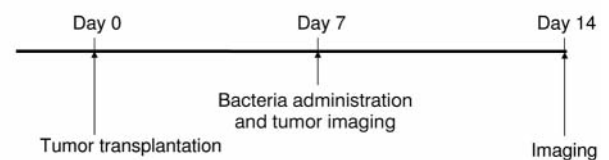
Figure 2. *Treatment schedule. On day 0, an XPA-1-RFP tumor fragment (1 mm^3), previously grown subcutaneously in nude mice, was transplanted on the pancreas of nude mice. On day 7, the tumor was surgically exposed. The size of the tumor (fluorescent area [mm^2]) was measured. Three mice were treated with a low-dose of A1-R (10^7 CFU/ml); 3 were treated with a high-dose of A1-R (10^8 CFU/ml); and 3 mice were used as untreated controls. On day 14, the tumors were exposed again and imaged quantitatively to determine the efficacy of treatment.*

those groups before treatment. On day 14, seven days after treatment, the tumor size was 19.9±4.3 mm^2 in the untreated group; 2.2±0.89 in the high-dose-bacteria treatment group; and 12.7±6.5 in the low-dose-bacteria treatment group. There was a significant difference between the untreated group and the high-dose-bacteria treatment group ($p<0.05$) (Figures 3 and 4).
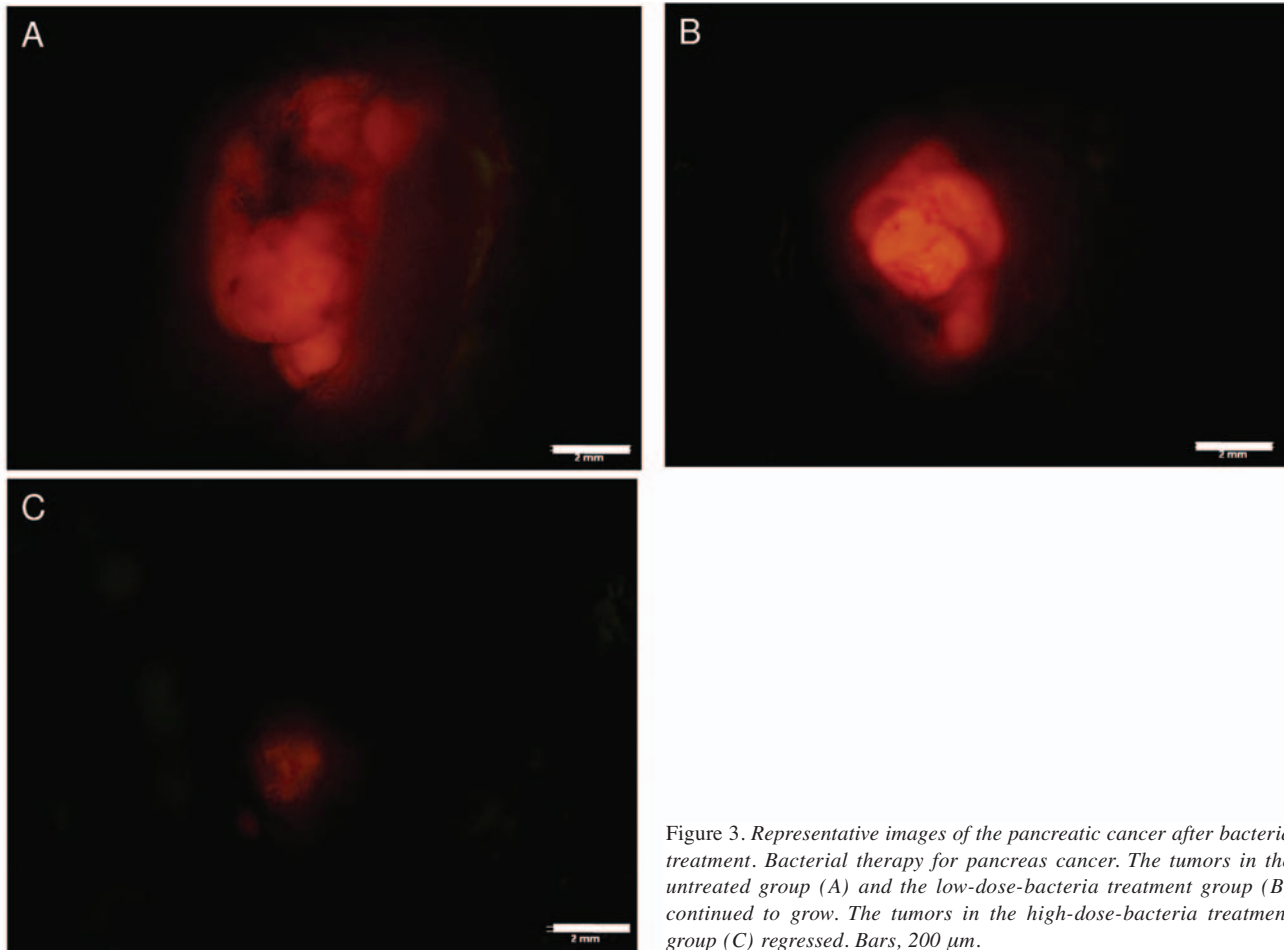
Figure 3. *Representative images of the pancreatic cancer after bacteria treatment. Bacterial therapy for pancreas cancer. The tumors in the untreated group (A) and the low-dose-bacteria treatment group (B) continued to grow. The tumors in the high-dose-bacteria treatment group (C) regressed. Bars, 200 μm.*
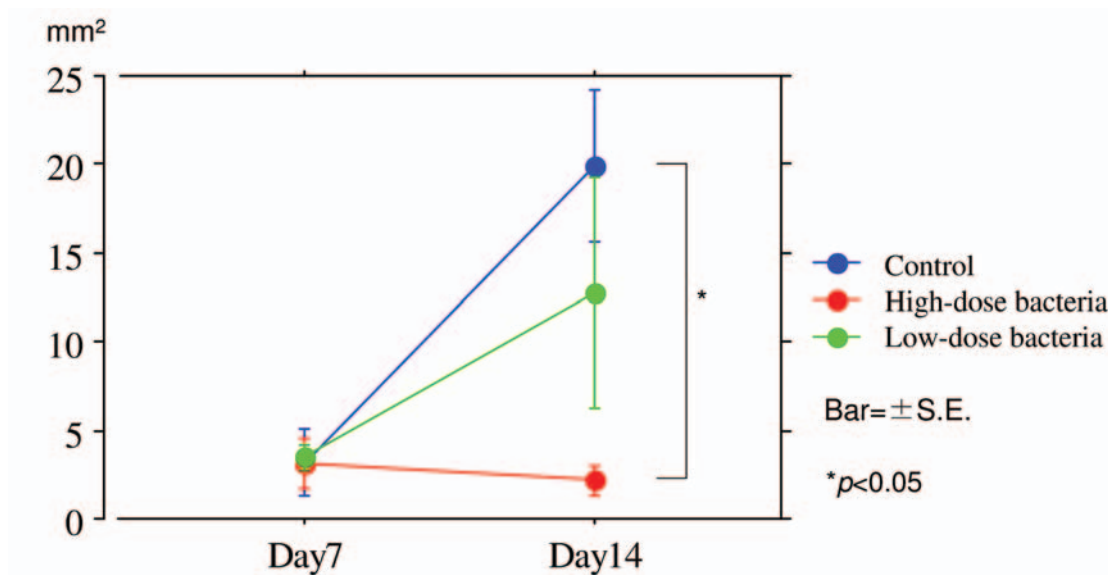

Figure 4. *Tumor size on day 7 and day 14. The average tumor size (fluorescent area) on day 7 was 3.2±1.9 mm^2 in the untreated group; 3.1±1.4 in the high-dose-bacteria treatment group; and 3.5±0.75 in the low-dose-bacteria treatment group. There was no significant difference among those groups before treatment. On day 14, seven days after treatment, tumor size was 19.9±4.3 mm^2 in the untreated group; 2.2±0.89 in the high-dose-bacteria treatment group; and 12.7±6.5 in the low-dose-bacteria treatment group. There was a significant difference between the untreated group and the high-dose bacteria treatment group (p<0.05).*

Discussion

A doubly auxotrophic leucine-arginine facultative anaerobic *S. typhimurium* strain has shown efficacy in a metastatic orthotopic nude-mouse model of pancreatic cancer, a notoriously treatment-resistant disease. The ultimate goal of this research is to bring tumor-targeting bacterial therapy to the clinic. To achieve this goal, several issues need to be addressed. First and foremost is safety. Strains containing deletion mutations leading to auxotrophy would have a lesser chance to revert to prototrophy than point mutants and therefore would be safer. It is essential that reversion to prototrophy does not occur, as this would lead to systemic infection of the patient. Therefore, future experiments will focus on developing deletion mutation–based auxotrophic tumor-targeting bacteria. *S. typhimurium* A1-R also needs to be evaluated in immunocompetent mice bearing syngeneic tumors (27). Tumor-targeting bacteria to be used in the clinic also need to have a broad range of efficacy against a particular type of tumor. The factors leading to a broad targeting range are still not well understood, and further work must be done to achieve this goal. Our results suggest that increased tumor cell adherence and invasion seem to be critical for increased tumor targeting by bacteria *in vivo*. The use of fluorescently-tagged bacteria greatly facilitates tumor-targeting studies both *in vitro* and *in vivo*. Thus, fluorescently-tagged bacteria may also be useful clinically.

Acknowledgements

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