

Tumor-targeting bacterial therapy with amino acid auxotrophs of GFP-expressing *Salmonella typhimurium*

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Here we report a genetically modified bacteria strain, *Salmonella typhimurium* A1, selected for anticancer activity *in vivo*. The strain grows in tumor xenografts. In sharp contrast, normal tissue is cleared of these bacteria even in immunodeficient athymic mice. *S. typhimurium* A1 is auxotrophic (Leu/Arg-dependent) but apparently receives sufficient support from the neoplastic tissue to grow locally. Whether additional genetic lesions are present is not known. In *in vitro* infection, the GFP-expressing bacteria grew in the cytoplasm of PC-3 human prostate cancer cells and caused nuclear destruction. These effects were visualized in cells labeled with GFP in the nucleus and red fluorescent protein in the cytoplasm. *In vivo*, the bacteria caused tumor inhibition and regression of xenografts visualized by whole-body imaging. The bacteria, introduced *i.v.* or intratumorally, invaded and replicated intracellularly in PC-3 prostate cancer cells labeled with red fluorescent protein grafted into nude mice. By day 15, *S. typhimurium* A1 was undetectable in the liver, lung, spleen, and kidney, but it continued to proliferate in the PC-3 tumor, which stopped growing. When the bacteria were injected intratumorally, the tumor completely regressed by day 20. There were no obvious adverse effects on the host when the bacteria were injected by either route. The *S. typhimurium* A1 strain grew throughout the tumor, including viable malignant tissue. This result is in marked contrast to bacteria previously tried for cancer therapy that were confined to necrotic areas of the tumor, which may account, in part, for the strain's unique antitumor efficacy.

red fluorescent protein | bacterial therapy | auxotrophy | leucine | arginine

Cancer research has long sought a magic bullet that, in contrast to most current therapeutic protocols, would selectively target and destroy malignant cells. This goal has long remained elusive in large part because of the extreme genetic and phenotypic variability of most tumors. There is, however, an intriguing pathway that recurrently attracts interest, *i.e.*, the restricted colonization of malignancies by facultative anaerobic bacteria. It has been known for almost 60 years that anaerobic bacteria can selectively grow in tumors (1–16). The conditions that permit anaerobic bacterial growth, *i.e.*, impaired circulation, and extensive necrosis, are found in many tumors, so that bacterial therapeutic conduits may offer entrée to a wide variety of malignancies.

Novel approaches to developing tumor-therapeutic bacteria have recently been described. Fujimori and coworkers (14, 15) demonstrated that the anaerobic bacterium *Bifidobacterium longum* could selectively grow in the hypoxic regions of solid tumors. Vogelstein and coworkers (17) created a strain of *Clostridium novyi* depleted of its lethal toxin 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). The main efficacy of these anaerobic bacteria was in combination with chemotherapy.

Salmonella typhimurium was first attenuated by purine and other auxotrophic mutations (12, 18, 19). The resulting nutritional requirements were apparently met within the tumor environment where the bacteria replicated to at least 1,000-fold their concentration in normal tissues (12). *Salmonella* lipid-A was also genetically modified by disrupting the *msbB* gene to reduce septic shock (12). Melanomas in mice treated with *Salmonella msbB* mutant were 6% the size of tumors in untreated controls (12). However, these *Salmonella* variants did not cause tumor regression or eradication. Recently, the *S. typhimurium* with attenuated lipid-A has been evaluated in a phase I clinical trial (20). Bacteria labeled by GFP or luciferase and injected *i.v.* into live animals were seen to replicate in solid tumors and in their metastases, including tumors of the breast, prostate, brain, and fibrosarcoma (21). Antitumor efficacy was not observed in these experiments.

In the experiments reported here, we first mutagenized *S. typhimurium* and selected auxotrophs. We then used these in an *in vivo* tumor-killing assay to select an appropriate strain. Such a screen requires a means of quickly evaluating the results of genetic manipulations of the bacteria.

We used whole-body fluorescence imaging techniques to follow the infecting GFP-labeled bacteria (22) and their effect on the red fluorescent protein (RFP)-labeled target tumors (23–28). The whole-animal results were obtained expeditiously and used as a secondary screen for tumor targeting by preselected, mutagenized bacterial strains. We report here the development and observation of a strain of *S. typhimurium* that selectively grows in and kills tumor cells with discrimination and efficacy.

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. Cells (2.0×10^8) in 40 μ l of 10% glycerol were mixed with 2 μ l of pGFP (Clontech) vector and placed on ice for 5 min before electroporation with a Gene Pulser apparatus (Bio-Rad) according to the manufacturer's instructions. Electroporation was done at 1.8 kV with the pulse controller at 1,000- Ω parallel resistance.

Induction of Mutations with Nitrosoguanidine (NTG) and Selection for Auxotrophs. Freshly prepared NTG (1 mg/ml in sterile water) was added to the washed culture to a final concentration of 100 μ g/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 to express any mutations that were

Abbreviations: NTG, nitrosoguanidine; RFP, red fluorescent protein; cfu, colony-forming units.

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Selection of *Salmonella typhimurium* A1-GFP Mutant

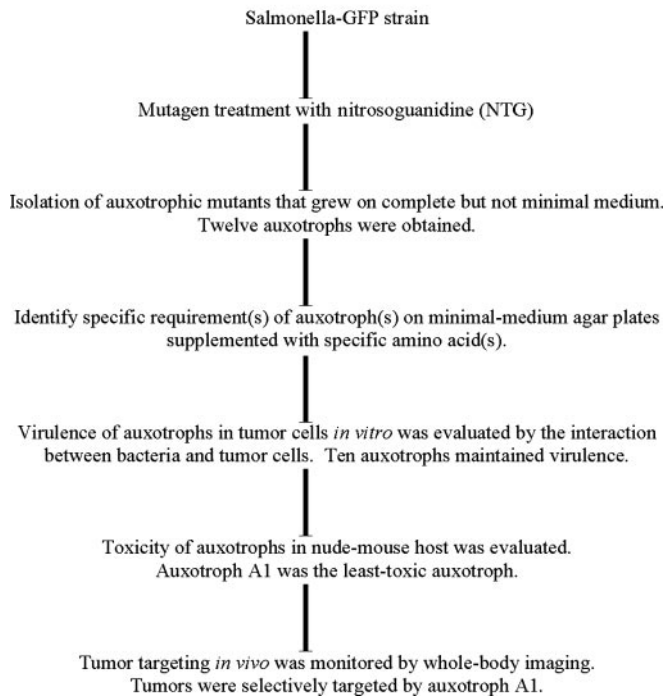


Fig. 1. Schematic diagram of the steps used to isolate the *S. typhimurium* A1 mutant with selective tumor-targeting efficacy.

induced. Bacterial colonies were replica-plated in supplemented minimal agar plates containing specific amino acids to identify the requirements of the auxotrophs. Auxotroph A1, which required Leu and Arg, was identified (Fig. 1).

RFP Gene Transduction of PC-3 Cell Line. For RFP gene transduction, $\approx 60\%$ confluent PC-3 human prostate cancer cells were incubated with a 1:1 precipitated mixture of retroviral supernatants of PT67 packaging cells and F12K medium containing 7% FBS (Gemini Bioproducts, Calabasas, CA) for 72 h. The packaging cells produced the pLNCX2 vector (Clontech) containing the DsRed2-expressing RFP and the neomycin resistance gene (29). RFP-expressing cells were selected in medium containing 200–1,000 $\mu\text{g}/\text{ml}$ neomycin increased in a stepwise manner. Clones expressing RFP were isolated with cloning cylinders (Bel-Art Products, Pequannock, NJ) by trypsin/EDTA and were amplified and transferred by conventional culture methods in the absence of selective agent (28).

RFP Gene and GFP–Histone H2B Gene Transduction of PC-3 Cell Lines (29). For RFP and GFP gene transduction, 70% confluent human PC-3 cells were used. To establish dual-color cells, clones of PC-3-expressing RFP in the cytoplasm (PC-3–RFP) were initially established as described above.

For establishing dual-color cells, PC-3–RFP cells were then incubated with a 1:1 precipitated mixture of retroviral supernatants of PT67 packaging cells and culture medium. The histone H2B–GFP fusion gene was inserted into the pLHCX vector (Clontech) that also contains the hygromycin resistance gene. To select the double transformants, cells were incubated with hygromycin 72 h after transfection. The level of hygromycin was increased stepwise up to 400 $\mu\text{g}/\text{ml}$. These sublines stably expressed GFP in the nucleus and RFP in cytoplasm.

Animal Tumor Model and Bacteria Infection. Nu/nu mice, 6 weeks old, male, were used for growth models and infection studies

(please see below). PC-3–RFP tumor cells (2×10^6) were injected s.c. on the flank of the nude mice and grown for 30 days. For intratumoral injection, auxotroph A1 was grown overnight on LB medium. Bacteria were harvested at late logarithmic phase, washed, and diluted with PBS and injected directly into the central areas of the RFP-labeled tumors under fluorescence guidance. Two injection sites of 50 μl each for a total of 100 μl and 10^9 colony-forming units (cfu) per tumor were used. For i.v. injection, auxotroph A1 was grown and diluted as above and injected into the tail vein of nude mice with RFP orthotopic tumors (10^7 cfu per 100 μl of PBS). 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.

Analysis of Infection *in Vivo*. After injection as described above, tissue samples were obtained from lung, liver, spleen, kidney, heart, and tumor. Normal tissues and tumors were excised and weighed and observed under fluorescence microscopy to determine the extent of bacterial infection. For biodistribution studies, cfu of the tumors and normal mouse tissues were determined at different time points after inoculation by harvesting these tissues and homogenizing and plating supernatants on nutrient media. Tissues were also prepared for standard frozen sectioning and hematoxylin and eosin staining for histopathological analysis.

Fluorescence Dual-Color Imaging of Bacterial–Host Interaction (28). Macroimaging was carried out in a fluorescence light box (Lighttools Research, Encinitas, CA). A C5810 three-chip cool color charge-coupled device camera (Hamamatsu Photonics, Bridgewater, NJ) was used. An Olympus BH 2-RFCA fluorescence microscope equipped with a mercury 100-W lamp power supply and GFP filter set (Chroma Technology, Brattleboro, VT) was used for microimaging. Images were processed for contrast and brightness and analyzed with the use of IMAGE PRO PLUS 4.0 software (Media Cybernetics, Silver Spring, MD).

Results and Discussion

Bacterial Culture Conditions Conferring Virulence in Tumor Cells. The intracellular virulence of *S. typhimurium* was determined by observing infected PC-3 cells by fluorescence microscopy (Fig. 2). The bacteria used in these experiments fluoresced green (GFP) and were clearly visible against the red fluorescence (RFP) of the cell cytoplasm. The cell nucleus, which bacteria rarely enter, was selectively labeled by fusing GFP with histone H2B (29). The resulting nuclear fluorescence facilitated observation of the apoptotic nuclear disintegration. Bacterial virulence proved to be strongly affected by the bacterial growth state, with late-logarithm culture the most effective. Late-logarithmic-phase *S. typhimurium* could infect $>90\%$ of the PC-3 tumor cells, whereas early-logarithmic-phase *S. typhimurium* was poorly invasive.

Isolation of Tumor-Selective Mutants of *S. typhimurium*. The selection of strain A1 is outlined in Fig. 1. Amino acid auxotrophs of *S. typhimurium* were selected after NTG mutagenesis. These strains were then selected for growth in tumor cells *in vitro*. The mutants were then selected for growth in tumors *in vivo*. Strain A1 is remarkable for its selectivity for growth in tumors compared with normal tissues. Strain A1 was the most discriminating of the 12 bacterial mutants tested. Strain A1 proved to be a Leu and Arg double auxotroph, although there may be other genetic alterations (Fig. 1).

Growth of *S. typhimurium* A1 in Cancer Cells *in Vitro*. The mutant A1, which expressed GFP, could invade and replicate intracellularly

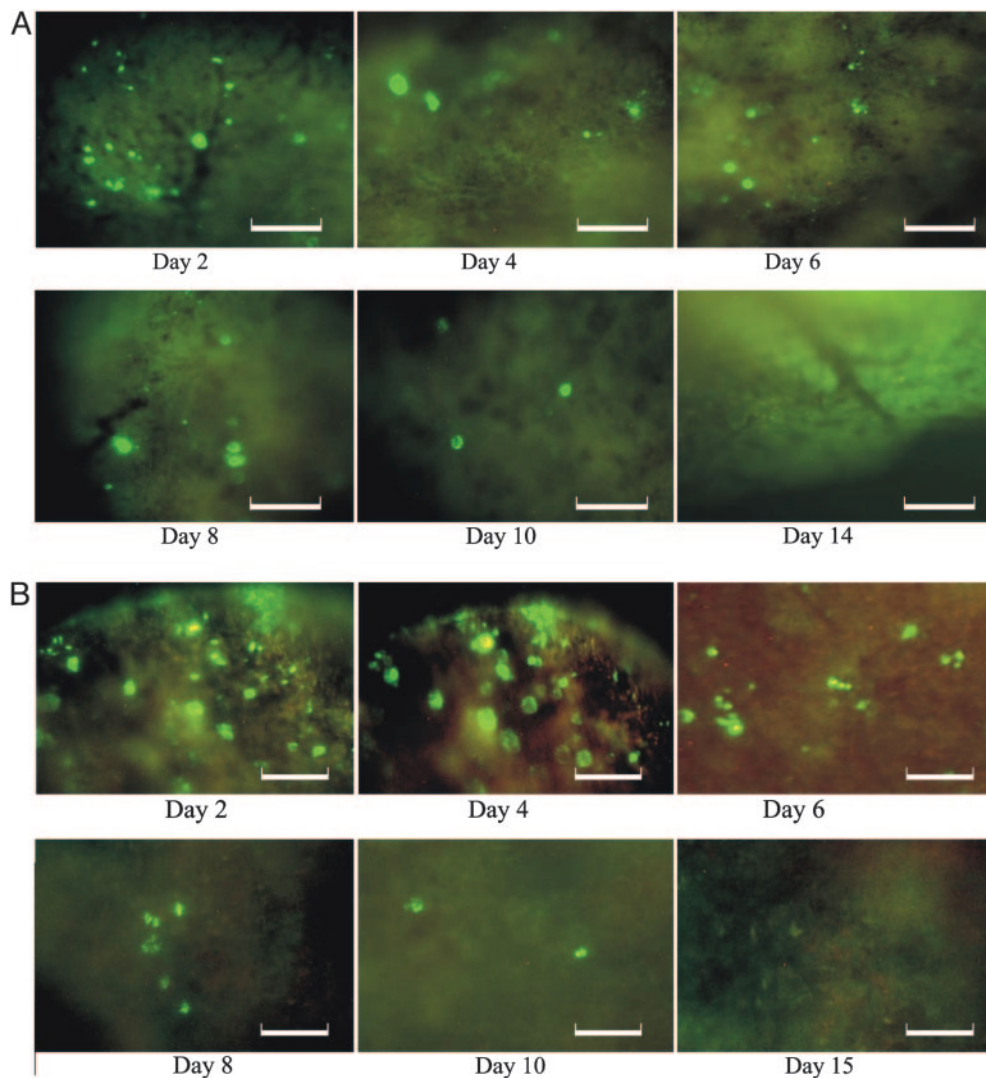


Fig. 4. Selective growth of *S. typhimurium* A1 in PC-3 human prostate tumor-bearing nude mice. After infection of *S. typhimurium*-GFP as described above, various organs were removed from the animal and imaged under fluorescence microscopy without any processing. Infection was determined by fluorescence visualization of bacterial colonies on the liver (A) or spleen (B). (Bars: 182 μm .)

infection (Fig. 4). However, by day 6, the number of bacteria had markedly regressed in the normal tissue. By day 15, GFP-labeled bacteria could no longer be seen in the spleen, liver, kidney, and lung (Fig. 4). These results agreed with those obtained by culturing bacterial isolated from the mouse organs. In contrast, the bacteria grew continuously in the PC-3 tumor (Fig. 4). The tumor/liver bacterial ratios were $\approx 2,000$ – $10,000$:1 by day 4 after injection, and, by days 10–15, bacteria had become undetectable external to the tumor. These results show that the A1 auxotroph could selectively target the PC-3 tumor *in vivo* with essentially complete clearance from normal tissue even in the T cell-deficient athymic nude mouse. It would be interesting to see future experiments that test the A1 auxotroph in syngeneic models by using immunocompetent mice. Similar results would be expected.

Frozen sections of RFP-expressing PC-3 tumor tissue show the extensive intracellular growth *in vivo* of GFP-labeled *S. typhimurium* A1 (Fig. 2B). Paraffin sections of the infected PC-3 tumor showed in even greater detail that strain A1 grew throughout the tumor (Fig. 2C). The growth of auxotroph A1 in the viable tumor tissue is in contrast to published observa-

tions of other tumor-targeting anaerobic organisms that appear to be restricted to the necrotic areas of the tumor (12, 17).

***S. typhimurium* A1 Suppresses PC-3 Tumor Growth.** GFP-labeled *S. typhimurium* A1 bacteria (10^7 cfu per 100 μl) were inoculated by means of tail-vein injection in PC-3-bearing nude mice. Tumor size was determined by fluorescence imaging at the indicated time points. *S. typhimurium* A1 selectively colonized the PC-3 tumor and suppressed its growth (Figs. 5A and B). An even more dramatic result was obtained by direct intratumoral injection of *S. typhimurium* A1 (Fig. 5C). The tumor completely disappeared within 15–26 days after the start of treatment (Fig. 5C). Subsequently, the tumor-bearing nude mice were cured and survived for as long as non-tumor-bearing mice.

We report here a new strategy for developing tumor-targeting bacteria for cancer therapy. We selected a *S. typhimurium* strain first for amino acid auxotrophy and subsequently for tumor targeting. This strain invaded and replicated intracellularly in human PC-3 prostate cancer cells *in vitro* and induced apoptosis. In these experiments, the double-labeling of tumor cells with RFP and GFP and bacteria with GFP enabled visualization of intracellular bacterial growth and resulting cellular disintegra-

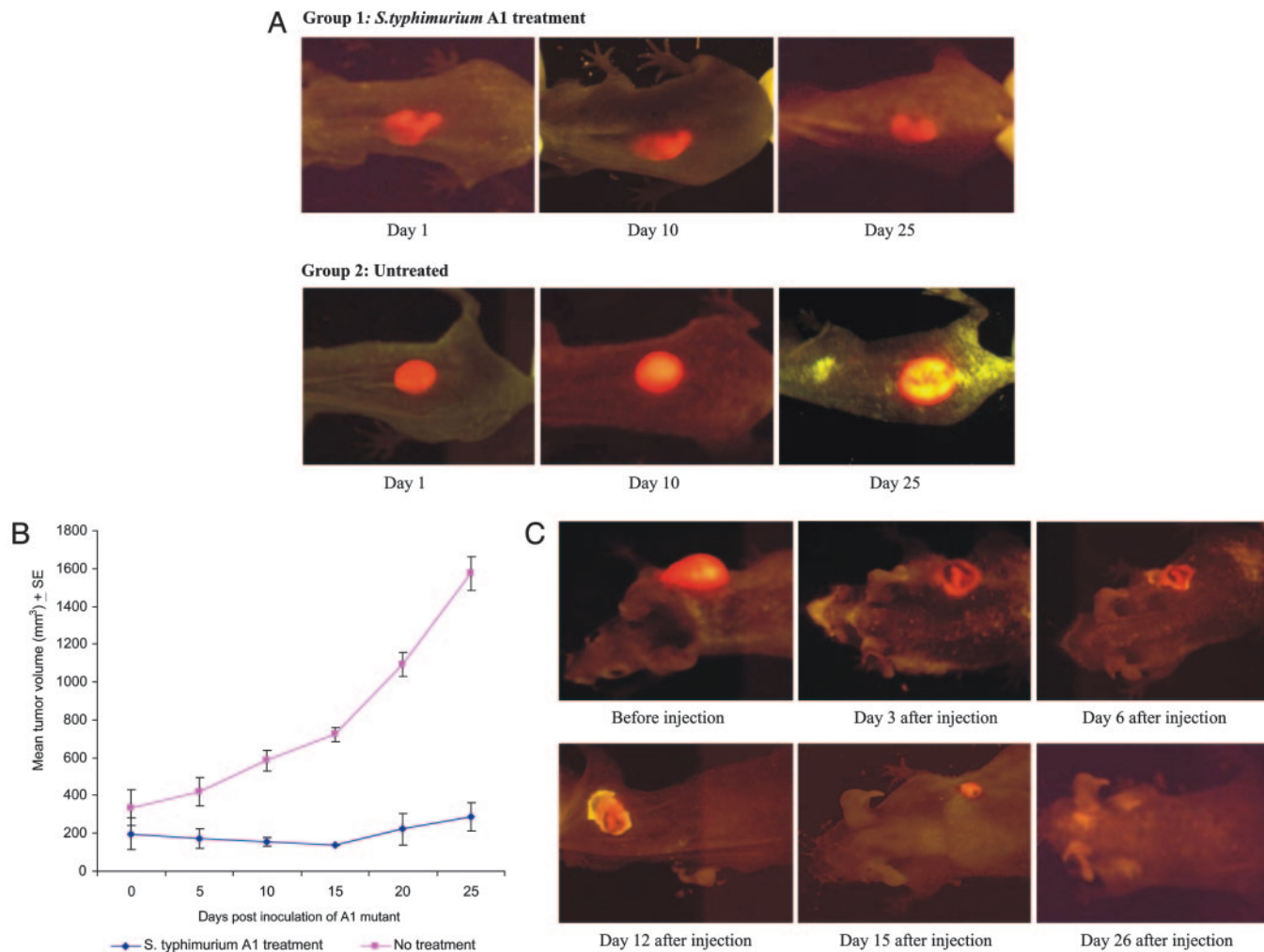


Fig. 5. Antitumor efficacy of *S. typhimurium* A1. (A) Whole-body imaging of the efficacy of *S. typhimurium* A1 on the growth of a PC-3 human prostate tumor after i.v. injection. NCR nude mice (aged 6–8 weeks) were implanted s.c. on the mid-right side with 2×10^6 RFP-labeled PC-3 human prostate tumor cells (2×10^6) with a 22-gauge needle. *S. typhimurium* were grown overnight on LB medium and then diluted 1:10 in LB medium. Bacteria were harvested at late-logarithmic phase and washed with PBS, then diluted in PBS and injected directly into the tail vein (10^7 cfu per 100 μ l of PBS). Tumors were visualized by fluorescence imaging at indicated time points after infection. (B) Quantitative efficacy of *S. typhimurium* A1 on the growth of a PC-3 human prostate cancer in nude mice after i.v. injection. Tumor size was measured at each time point after infection. Each point represents the mean for $n = 10$ animals. Tumor growth and bacterial infection were performed as described for A. (C) Whole-body imaging of efficacy of *S. typhimurium* A1 on the growth on a PC-3 human prostate tumor after intratumor injection. NCR nude mice (aged 6–8 weeks) were implanted s.c. on the mid-right side with RFP-labeled PC-3 human prostate tumor cells (2×10^6) with a 22-gauge needle. *S. typhimurium* were grown overnight on LB medium and then diluted 1:10 in LB medium. Bacteria were harvested at late-logarithmic phase and washed with PBS, then diluted in PBS and injected directly in the center area of the tumor by using two separate sites with injections of 50 μ l each, for a total of 100 μ l and 10^9 cfu per tumor.

tion in real time. For *in vivo* experiments, RFP labeling of the tumor cells allowed imaging in real time. Tumor regression and eventual eradication could be visualized. Wild-type *S. typhimurium* rapidly killed nude mice, whereas the A1 strain fails to progressively grow in normal tissue and is not fatal. The bacteria had disappeared from the liver, lung, spleen, and kidney by day 15. However, strain A1 selectively grew in the tumor and suppressed tumor growth after tail-vein injection. The most dramatic effect occurred after intratumor injection. In this case, the tumor completely disappeared, and the mice were completely cured with no obvious adverse effect on the host. These results distinguish *S. typhimurium* A1 from the previously described *S. typhimurium* variants (12, 18–20), which could only slow tumor growth. The present results also distinguish *S. typhimurium* A1 from the *Clostridia* and other anaerobic bacteria that also did not induce tumor eradication by themselves (17). The tumor-dependent growth of *S. typhimurium* A1 clearly

depends on the nutritional milieu within the malignant tissue. A similar milieu can probably be obtained in a wide variety of tumors. Recent unpublished results have demonstrated that *S. typhimurium* A1 can eradicate a human patient breast tumor in nude mice (M.Z. and R.M.H., unpublished observations). Future experiments will also investigate efficacy of auxotrophic tumors in syngeneic models with fully functional immune systems. Additional genetic changes in strain A1 may also account for its tumor selectivity. The A1 auxotroph grows throughout the tumor, including viable regions, which is in sharp contrast to previously described bacteria that are mostly confined to necrotic regions. This unique growth pattern may account, in part, for its strong antitumor activity. Future experiments should test auxotrophic *S. typhimurium* for different amino acids to determine whether there is tumor dependency for specific auxotrophic mutations or combinations. These results suggest a great potential for genetically modified bacteria as cancer therapeutics.

