A Bioluminescent Transposon Reporter-Trap Identifies Tumor-Specific Microenvironment-Induced Promoters in *Salmonella* for Conditional Bacterial-Based Tumor Therapy

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Abstract

Salmonella specifically localize to malignant tumors *in vivo*, a trait potentially exploitable as a delivery system for cancer therapeutics. To characterize mechanisms and genetic responses of *Salmonella* during interaction with living neoplastic cells, we custom designed a promoterless transposon reporter containing bacterial luciferase. Analysis of a library containing 7,400 independent *Salmonella* transposon insertion mutants in co-culture with melanoma or colon carcinoma cells identified five bacterial genes specifically activated by cancer cells: *adiY*, *yohJ*, *STM1787*, *STM1791*, and *STM1793*. Experiments linked acidic pH, a common characteristic of the tumor microenvironment, to a strong, specific and reversible stimulus for activation of these *Salmonella* genes *in vitro* and *in vivo*. Indeed, a *Salmonella* reporter strain encoding a luciferase transgene regulated by the *STM1787* promoter, which contains a *tusp* motif, showed tumor-induced bioluminescence *in vivo*. Furthermore, *Salmonella* expressing Shiga toxin from the *STM1787* promoter provided potent and selective anti-tumor activity *in vitro* and *in vivo*, demonstrating the potential for a conditional bacterial-based tumor-specific therapeutic.

Keywords

Salmonella; cancer; transposon; screen; reporter; bioluminescence

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Introduction

The evolving and highly heterogeneous landscape of tumor genetics and the tumor microenvironment pose a significant challenge for treating advanced solid tumors (1). Many characteristics of the tumor microenvironment, such as hypoxia, acidic pH, and a disorganized vascular architecture, limit delivery and efficacy of therapeutics and radiation treatments (2). Additionally, tumors undergoing targeted molecular therapy often relapse due to the utilization of autonomous parallel-redundant signaling pathways (3). Beyond the primary tumor, identifying disseminated disease that has metastasized to various organ sites is challenging, and systemically treating cancer often produces off-target toxicities. The ultimate anti-tumor therapy is one that overcomes these physiologic obstacles while simultaneously targeting tumors and avoiding normal tissue toxicity.

The remarkable ability of commensal and pathogenic bacterial strains to localize and preferentially grow within tumors has been well documented (4). The immune-privileged, hypoxic and nutrient-rich 'tumor soil' facilitates colonization by facultative anaerobic bacteria (5). These observations have spurred research into the diagnostic and therapeutic potential of genetically engineered and attenuated therapeutic strains of bacteria such as *Salmonella, Listeria* and *Clostridium* (5). *Salmonella* is one of the most studied of therapeutic bacteria, and upon systemic administration, is able to colonize xenograft tumors at rates 1,000 times greater than that of other organs, thereby abrogating tumor growth (6, 7). A firm understanding of the genetic programs involved in normal pathogenesis, characterization of spatiotemporal kinetics and dynamics during intra-tumoral colonization *in vivo*, genetic tractability, as well as the oncolytic capacity of *Salmonella* typhimurium have made *Salmonella* strains ideal candidates for anti-cancer bacterial development (8).

S. typhimurium by itself can illicit an anti-tumoral response through several potentially separate but synergistic mechanisms. First, as a pathogenic and cytotoxic bacterium, *S.* typhimurium can induce apoptosis of cancer cells (9). Second, pathogen-associated molecular patterns (PAMPs) of *S.* typhimurium, such as lipopolysaccharide (LPS) and flagellin, are capable of activating innate immunity by initiating pro-inflammatory TLR-MyD88/TRIF-NF-κB signaling cascades (10). Third, intracellular *Salmonella* flagellin can also enhance an anti-tumor adaptive immune response caused by the associative recognition with cancer cell antigen. The resulting signaling cascades ultimately augment antigen presentation of dendritic cells (DC), thereby promoting T cell clonal expansion and differentiation which leads to an associative recognition of the cancer cell with the PAMPs of *Salmonella* (11, 12). Finally, despite the initial tumor regression, these tumors may eventually relapse, which has spurred the development of *Salmonella* as a delivery vehicle for anti-cancer co-therapies (13). Indeed, *Salmonella* have been used as tumor-specific vectors for gene transfer of RNAi or suicide genes, as well as targeted expression of apoptosis-inducing biologics, such as TRAIL, FASL and the bacterial toxin, Cytolysin A, all of which display pronounced anti-tumor affects *in vivo* (5).

However, few studies have investigated the specific genetic responses of *Salmonella* to tumor cells and bacterial mechanisms regulating these atypical "host" interactions. To address these quires, we engineered a bioluminescent transposon reporter-trap to screen a *S.* typhimurium library for genes specifically regulated by co-culture with malignant cells *in vitro*. Five genes were identified by the screen and their promoter sequences were found to be specifically activated by the acidic microenvironment associated with cancer cells *in vitro* and tumors *in vivo*. Finally, we utilized the most pH-sensitive promoter sequence to demonstrate the utility of tumor-regulated *Salmonella* promoters to conditionally regulate the expression of a toxic tumor transgene *in vitro* and *in vivo*.

Results

A high-throughput screen to identify tumor cell-induced gene activation events in *Salmonella*

To conduct a large-scale, unbiased screen for genes up-regulated by contact with malignant cells, we used a Tn5-based transposon as the backbone of a *luxAB* reporter construct. We chose to use the bacterial luciferase enzyme genes (*luxAB)* only, in contrast to the full bacterial luciferase operon (*luxCDABE*), because the size of the transposon containing the full operon prohibited efficient chromosomal integration, while using only the *luxAB* genes allowed for efficient genomic insertion of the transposon. The transposon was designed to restrict reporter gene expression to only those chromosomal integration sites downstream of an active promoter. A kanamycin resistance cassette with a constitutive promoter was also included to select for integration into the chromosome (Figure 1a). After construction, the purified transposon was electroporated into *S.* typhimurium strain SB300A1(14) for random chromosomal integration, producing a 7,400 clone bacterial library.

Initially, the entire *Salmonella* library was subjected to a primary screen in the context of three conditions: tissue culture media alone, B16F10 melanoma cells and HCT116 colon carcinoma cells, both of the latter in monolayer co-culture with the *Salmonella* reporter library. The tumor cells were grown in 96-well plate format overnight and then bacterial clones added to wells corresponding to each of the two co-culture conditions and media alone. After a two-hour incubation, bioluminescence imaging of plates enabled identification of clones specifically up-regulating genes in the context of exposure to melanoma and/or colon carcinoma cells (Figure 1a). Results of the screen from co-culture with melanoma and colon carcinoma cells are shown in Figures 1b and 1c, respectively. In each case, data are shown as a rank-ordered S-plot of the $\log_2$ of the normalized signal for each clone of the library, where normalized signal was the ratio of the signal in the condition of interest to the signal in media alone. The majority of data points clustered around zero, indicating that most mutants interrogated in the assay did not show tumor-specific gene regulation. However, quartile analysis with a boundary for hit selection corresponding to a high stringency targeted error rate ($\alpha = 0.0027$) identified five candidate mutants wherein the transposon reporter was specifically up-regulated during co-culture with malignant cells.

Verification and characterization of *Salmonella* gene activation events in the context of tumor cell co-culture

Following the primary screen, we utilized inverse touchdown PCR to map the specific location of each transposon in the *Salmonella* genome (15). Table 1 documents the site of chromosomal integration for the transposon and candidate gene up-regulated in each isolate. All genes were novel in that they have not been previously reported to be involved in *Salmonella*-host interactions, nor involved in *Salmonella* colonization of neoplasia. Interestingly, the genomic insertion sites of the transposon in three of the clones inserted in a cluster in the chromosomal sequence. Mapped to three different, but closely linked genes (*STM1787*, *STM1791* and *STM1793*, respectively), two are known hydrogenases, and all three genes are likely co-regulated and involved in the same *Salmonella* function. Sequencing showed that in one high stringency hit, the transposon had inserted into *adiY*, a *Salmonella* gene known to be involved in an acid tolerance response (16). The transposon in the fifth clone was identified to have landed in *yohJ*, a putative membrane protein (17).

To validate cancer cell co-culture-specific gene activation events identified in the primary screen, we first repeated the co-culture assay in quadruplicate in at least three independent experiments for each clone. Figure 2a shows the data from one representative experiment for clones verified by this assay. Again, all five clones showed statistically significant

enhancement of bioluminescence in the presence of tumor cells, with a trend toward greater gene up-regulation when co-cultured with B16F10 melanoma cells. Then, to further characterize tumor cell-induced response of *Salmonella*, we utilized the tumor cells in a dose-response assay (Figures 2b, c). Additionally, to verify that reporter activation seen in the *Salmonella* reporter-trap clones was not an effect of differing substrate permeability due to mutations in bacterial genes, bacteria were generated that contained the original chromosomal *luxAB* insertion as well as a plasmid constitutively expressing *luxCDE*, the biosynthetic genes for the long-chain aldehydes that act as the optical substrates of the bacterial luciferase operon. Therefore, for this assay, it was not necessary to add decanal to the media. Identical inoculations of bacteria showed greater up-regulation of the reporter when exposed to greater numbers of tumor cells in co-culture conditions, indicating that the stimuli from tumor cells instigated a graded response from the bacteria. Because expression of the *lux* operon genes fully complemented the use of exogenous decanal in the system, the data confirmed that the effect was not an artifact of exogenous decanal permeability in the primary screen.

Finally, to verify that the reporters in fact reflected mRNA transcriptional regulation in wild-type *Salmonella* during co-culture with tumor cells, we utilized semi-quantitative PCR. Following a three-hour co-culture of wild-type (SB3001A1) bacteria with B16F10 cells or in tissue culture media alone, isolated RNA was reverse transcribed to cDNA. Semi-quantitative PCR of cDNA showed that co-culture with B16F10 melanoma cells enhanced the intensity of target gene transcripts, but not control ribosomal RNA transcripts (*rrsH*) (Figure 2d). The effect was generalizable, as co-culture with HeLa tumor cells produced similar results (Figure 2d).

Notably, of the genes identified in this screen, at least one, *adiY*, has previously been reported to be up-regulated in acidic pH conditions (16). One characteristic of tumor microenvironments *in vivo* is an abnormally acidic pH (18). In fact, due to the Warburg effect, cancer cells are constitutively glycolytic, even in high oxygen conditions, releasing lactic acid and thereby creating a particularly acidic tumor microenvironment (19). For these reasons, the *Salmonella* transposon insertion mutants were further investigated for reporter signal activation in acidic conditions. Figure 3a shows that reporter signals increased in acidic pH media compared to neutral media. Each of the clones up-regulated the reporter gene at pH 6.0 compared to the physiological pH of normal body tissue (pH 7.5), suggesting that the stimulus *Salmonella* responded to in the context of neoplastic cells was microenvironment acidification.

To determine whether the activated genes were required for localization to tumors or required for colonization and growth within tumors *in vivo*, *Salmonella* strains null for genes identified in the screen were constructed. Selected genes were deleted using a lambda red recombinase insertional deletion strategy, which inserted a chloramphenicol resistance cassette into the targeted genes. The deletion mutants were created from a parental *Salmonella* strain (*luxCDABE msbB-*) containing a chromosomally-integrated and constitutively-expressed bacterial luciferase operon for imaging bacterial localtization *in vivo* in real time. The strain also contained a *msbB* gene deletion, which causes a less immunogenic LPS structure and minimizes septic shock effects when the strain is administered intravenously (20). Based on the analysis that the identified *STM1787*, *STM1791* and *STM1793* genes were contained in a single operon, we targeted a large region of this operon for deletion in a single mutant strain, 1789⁻1793⁻. The gene *adiY* also appeared to be a part of a larger operon of co-regulated genes and was therefore targeted along with the adjacent genes *adi* and *yjdE*. The gene *yohJ* was targeted individually. In a B16F10 melanoma tumor xenograft model, all bacterial strains were injected via mouse tail vein and deletion mutants compared to the parental strain for localization to and persistence

within the tumor using bioluminescence imaging (Supplementary Figure 1). All mutant strains and the parent strain were capable of tumor localization and persistence, indicating that although the identified genes were activated by tumor cell co-culture *in vitro*, they were not essential for bacterial *colonization* of the tumor. The experiment was also performed in an HCT116 colon carcinoma xenograft model with similar results. Supplementary Table 1 summarizes the numbers of mice with colonized tumors on or before day 10 in each experiment. Additionally, in pilot competitive infection studies, there was no significant difference between the *STM1789-1793* mutant and the parental *Salmonella* strain (*luxCDABE msbB-*) in tumor colonization (CFU/ml; data not shown).

Specificity and reversibility of the *Salmonella STM1787* promoter *in vivo*

We next sought to demonstrate the specificity of the STM1787 promoter activation in the tumor microenvironment *in vivo*. We chose this promoter because it displayed the highest acidic pH induction *in vitro* (Figure 2a). Here, we used the constitutively bioluminescent *Salmonella* strain Tn:27.8+p*luxCDE* or the conditionally bioluminescent strain Tn:1787+p*luxCDE*, each of which constitutively express plasmid-encoded *luxCDE*, but the latter strain will only bioluminesce upon activation of the chromosomally-encoded *luxAB* reporter. In a B16F10 melanoma tumor xenograft model, bacteria were injected via mouse tail vein or intratumorally and allowed two days to localize and adapt to tumors *in vivo*. Tumors were then excised, incubated in solutions of various pH values and imaged periodically for six hours. Initially, all tumors showed bioluminescent bacteria *ex vivo*. Over time, constitutive Tn:27.8 *Salmonella* showed a gradual increase in signal consistent with bacterial growth in the tumor explants. This behavior was also observed in the Tn:1787 *Salmonella*-infected tumor explants incubated in low pH media. By contrast, when the Tn:1787 *Salmonella*-infected tumor explants were maintained in basic media conditions throughout, the signal initially increased, but then plateaued around 4 hours and decreased in comparison to the constitutively bioluminescent Tn:27.8 strain (Figure 3b,c). This finding suggested that bacterial gene expression was initially engaged by the low pH conditions of the *in vivo* tumor microenvironment, but after exposure to a higher pH environment *ex vivo*, the promoter driving the reporter was repressed and signal declined. Further, this *ex vivo* effect was reversible. When the medium on the Tn:1787 *Salmonella*-infected tumor explant was changed from pH 6.0 to pH 7.5, the bioluminescent signal decreased. Conversely, when the media was changed from pH 7.5 to pH 6.0, the signal increased (Figure 3b, c). These effects were not seen with the constitutive Tn:27.8 *Salmonella*-infected tumor explants, and provided further evidence in support of the specificity of the trapped *Salmonella* promoter in the Tn:1787 transposon mutant for the tumor microenvironment.

Because the identified promoters were highly activated in the tumor microenvironment *ex vivo*, utilization of these promoters provided a unique opportunity to design tumor-targeting bacterial vectors subject to multiple levels of controlled specificity *in vivo*. Thus, we sought to determine if the acidic pH of the tumor microenvironment could be exploited to specifically activate a target transgene during tumor localization. As proof of principle, we constructed *Salmonella* reporter strains expressing plasmids encoding the bacterial luciferase operon driven by either constitutive promoters or an inducible promoter to demonstrate tumor-mediated transgene activation *in vivo*. The plasmids p*MAAC001* and p*Lux* both encoded constitutively-expressed luciferase operons, while the p*PROMOTERLux* plasmid was engineered to contain the luciferase operon driven by the *Salmonella* candidate promoter (*STM1787*) comprising 500 base pairs upstream of the putative transcription start site of tumor-activated genes *STM1787*, *STM1793* and *STM1791* (which we will now refer to as the *STM1787* promoter). Bacteria expressing these plasmids were identically injected into mice bearing HCT116 tumor xenografts on each flank (Figure 4). We chose to utilize intratumoral injection to directly compare reporter gene activation from two different

bacterial strains, one inducible and the other constitutive, over time in the same mouse. Although reporter signals from p*PROMOTERLux*-expressing bacteria were low immediately after injection into the tumor, the bacteria quickly induced a 90-fold enhanced expression of the reporter after an 8 hr exposure to the tumor microenvironment (Figure 4a). Concurrently, bacteria constitutively expressing p*Lux*- or p*MAAC001*-luciferase showed <20-fold or no reporter activation, respectively, after exposure to the tumor microenvironment (Figures 4a and 4b). These data directly demonstrated tumor-specific induction of a transgene from the *Salmonella STM1787* promoter in an *in vivo* system. Therefore, the *STM1787* promoter could be used as a platform to design tumor-targeting *Salmonella* strains capable of specifically delivering a therapeutic gene or toxin to the site of a tumor *in vivo*.

Selective anti-tumor therapy *in vivo*

We utilized the cancer cell-activated STM1787 promoter to regulate the expression of Shiga toxin 2 (Stx2), a toxic transgene of bacterial origin, in a wild type strain of *S*. typhimurium (SB300A1) to selectively induce tumor cell death *in vitro* and *in vivo.* Stx2 is a secreted AB_5 holotoxin composed of a single N-glycosidase A subunit that is directed to target eukaryotic cell membranes through interaction of the pentameric B subunits and the host receptor, glycosphingolipid globotriaosylceramide (Gb_3) (21). Once inside the host cell, the A subunit cleaves the 28S RNA of the 60S ribosomal subunit, thereby inhibiting peptide elongation and inducing apoptosis. Stx2B has been extensively studied for its tumor targeting potential as many invasive tumors display high levels of Gb_3 (22).

Using bioluminescence as a reporter of total tumor cell mass, we performed a co-culture experiment with HeLa$^{CMV-Fluc}$ cells *in vitro*. First, plated HeLa cells were grown to confluency to acidify the media and then co-cultured with strain SB300A1 transformed with P1787 (empty vector) or P7187-Stx2. Both SB300A1 transformants were also grown in media alone. The supernatant was then filtered from each of the groups and aliquoted onto separately plated HeLa$^{CMV-Fluc}$ cells in increasing volumes: 1) +media+P1787; 2) +media +P1787-Stx2; 3) +HeLa+P1787; and 4) +HeLa+P1787-Stx2. After 24 hours of treatment, major toxicity was only observed in HeLa$^{CMV-Fluc}$ cells treated with the supernatant of +HeLa+P1787-Stx2 (Figure 5a); a general concentration-response trend was observed (Figure 5b). Stx2 expression was verified using mRNA PCR amplification (Figure 5b **inset**). No overt cytotoxicity was observed in HeLa$^{CMV-Fluc}$ cells treated with supernatant from any of the other conditioned media groups.

Given the selective regulation and associated toxicity of P1787-Stx2 *in vitro,* we next desired to demonstrate the tumor-targeting potential *in vivo* using established s.c. flank HeLa$^{CMV-Fluc}$ xenograft tumors and bioluminescence imaging. In two independent proof-of-principle experiments, intratumoral injection of a single high-dose of SB300A1 transformed with P1787-Stx2 resulted in an 80% mean reduction in initial viable tumor mass five days after treatment (Figure 6a). Furthermore, when tumors were treated with a single low-dose of SB300A1 transformed with P1787-Stx2, a robust anti-tumoral effect was observed after two weeks (Figure 6b). We also observed that treatment with P1787 resulted in tumor stasis, consistent with previous reports that *S*. typhimurium alone can block tumor growth *in vivo*, while LB broth alone showed no inhibitory effect (23). To verify tumor cell death independent of bioluminescence signal, tumor H&E sections from the high-dose treatment were analyzed (Figure 6c). Sections through LB-treated (control) tumors showed a broad rim of viable tumor cells with focal necrotic regions centrally. In the P1787 tumors, a thin rim of viable tumor cells was present in most areas, with fibroinflammatory reaction at the periphery of the mass. The central necrotic zone was larger and contained more neutrophils than in the LB-treated tumors. In the P1787-Stx2-treated tumors, viable tumor cells were

difficult to find, and in most sections, only a central necrotic zone surrounded by fibroinflammatory reaction was present. Note that mice treated with high-dose P1787-Stx2 eventually succumbed to the combined bacterial and Stx2 toxin load. However, mice receiving low-dose P1787-Stx2 were healthy for two weeks, at which point the experiment was concluded, but each still displayed a significant reduction in tumor size compared to P1787 alone (Figure 6b).

Discussion

Salmonella typhimurium bacteria are typically classified as human gastrointestinal pathogens and a common cause of modern food-borne illness. However, another noted characteristic of *Salmonella* is the capacity to colonize tumor tissue. In fact, in the 1800's, physicians began to intentionally use bacteria as tumor therapeutics, but due to significant toxicity and lack of consistent, reliable results, these practices were abandoned. However, modern studies using attenuated strains and longitudinal imaging have demonstrated colonization of tumors by *Salmonella* in real time and have sparked a renewed interest in this concept using *Salmonella* (23, 24) as well as various other tumor-localizing microbes as an option for cancer treatment (25–31). These observations along with the current intense focus on developing PAMP/TLR-based anti-cancer immunotherapies offer unique opportunities for combinatorial strategies in tumor targeting.

Both wild-type and genetically engineered *Salmonella* are capable of inducing tumor regression in mouse cancer models (4), as was observed in our experiments (Figure 6). A number of studies utilize bacteria as treatment vectors *per se* or as drug delivery vehicles by exploiting their potentially low toxicity and high genetic tractability to maximize therapeutic efficacy (5). In this regard, various attenuated *Salmonella* strains have been developed for use in tumor-targeting studies, including specific amino acid auxotrophs and LPS mutants (20, 32). However, the greatly reduced toxicity of *Salmonella* LPS mutants (*msbB-*) observed in swine models has not been observed in mouse models (33, 34). In more than one instance, attenuated *Salmonella* have even been used in a clinical trial to treat cancer in humans (35, 36). However, trials so far show relatively low rates of tumor colonization in human hosts, which may be due to excessive attenuation of the bacterial strains (5, 34). Indeed, one study indicates that induction of TNFα by bacteria is necessary for optimal colonization of tumors (37). Nonetheless, few studies have investigated the phenotypic and gene expression patterns of these tumor-targeting bacteria following exposure to tumor cells.

In this study, we utilized an engineered transposon to interrogate the *Salmonella* genome for genes activated during exposure to cancer cells. Toward this objective, we generated a library of greater than 7,400 independent transposon insertions, which, assuming random integration, would predict genomic transposon insertion into each of *Salmonella*'s 4,620 genes at least once. From this library, we identified five *Salmonella* genes specifically up-regulated during co-culture with cancer cells: *STM1787*, *STM1791*, *STM1793*, *adiY* and *yohJ*. Following identification of these tumor cell-activated genes, verification in secondary assays and confirmation in wild-type *Salmonella*, we determined that the common stimulus for up-regulation of target gene expression was acidic pH. In another study aimed at identifying *Salmonella* promoters involved in tumor colonization *in vivo*, *Salmonella* genomic DNA was digested and ligated randomly upstream of a GFP reporter. In this study, the major stimulus identified in reporter activation was hypoxia, but no pH-regulated promoters were identified (38). Another recent study performed a similar *in vivo* screen utilizing a promoter-trap GFP based system and indentified a conserved 'tumor specific' DNA motif (*tusp*) in the promoters of *Salmonella* genes specifically activated in a tumor xenograft model (33). While pH and hypoxia are physiologically linked, the five genes identified herein show no overlap with the promoters identified by Leschner et al. (33) nor

Arrach et al. (38). However, the STM1787 promoter located upstream of three of our own target genes (STM1787, STM1791, and STM1793) did contain the conserved *tusp* motif identified by Leschner et al. (tattttatataaa). The discrepancy in promoter identification may stem from the different bacterial strains or strategies utilized for gene identification in the two studies. Whereas Arrach et. al. utilized a plasmid-based overexpression system, the present study identified genes by chromosomal integration of a transposon. Nonetheless, hydrogenase genes are noted in some cases to be up-regulated in low oxygen conditions, indicating that hypoxia may serve as a further stimulus for the pH-induced promoters identified in the present study (39). However, in pilot studies with an incubation pouch system used for growing anaerobic bacteria, we did not observe any significant changes in transposon reporter activity under hypoxic conditions (KF, unpublished data). While these data do not necessarily rule out oxygen-independence, pH appeared to be the dominant signal inducing responses in the promoters identified by our bioluminescent transposon reporter-trap screen. It will also be of interest in future studies to determine if in addition to hypoxia, pH is another regulator of *Salmonella* promoters that contain the largely uncharacterized *tusp* motif.

In view of the usual pathophysiology of *Salmonella*, it is not surprising that *Salmonella* strains have gained the ability to precisely regulate genes in response to different pH environments. *Salmonella* encounter low pH conditions regularly during human infection, for example, during transit through the stomach, and later during intracellular trafficking through the phagosome (40, 41). Interestingly, the acidic pH of the tumor environment *in vivo* has long been noted as an important microenvironmental condition when designing effective tumor treatments (18, 42). Additionally, the low pH environment of the tumor inhibits host defense. Cytotoxic immune cell activity and cytokine secretion has been shown to be impaired by a low extracellular pH (43). In contrast, with a bacterial-driven tumor therapeutic, low pH may become an exploitable advantage, by adding another level of selectivity to bacterial gene activation. Indeed, the utility of a low pH-activated bacterial therapeutic will avoid toxicity to the liver and spleen which are the other major off-target organ sites of bacterial colonization, but which generally have a neutral pH (33). In this case, a bacterial-based system may succeed, while both conventional therapeutics and host defenses fail.

When using bacteria as a vector for drug delivery studies, tumor-specific colonization and subsequent expression is a major concern. The genes identified herein are highly expressed in an acidic tumor environment, but are not required for bacterial tumor targeting (Supplemental Figure 1). Therefore, the promoters regulating these genes and further dissection of the complex regulation of the *tusp* motif may generate ideal chromosomal insertion site candidates or synthetic promoter systems for utilization in therapeutic gene, pro-drug or toxin delivery studies. We have identified the *STM1787* promoter as an ideal bacterial sequence capable of driving tumor-specific expression of a transgene, and demonstrated this *in vivo* using bioluminescent imaging. We further applied the STM1787 promoter to conditionally regulate the expression of Stx2 in wildtype *S.* typhimurium in tumor targeting toxicity models *in vitro* and *in vivo*. In proof-of-principle studies, we observed dramatic cancer cell death in a co-culture model *in vitro* and dramatic tumor response over a relatively short time scale with a robust therapeutic effect *in vivo*. Future pharmacokinetic studies with P1787-Stx2 will be required to optimize mode of delivery, dose, and efficacy. In addition, it will be of interest to take advantage of the recent discovery that manganese treatment protects the host against lethal levels of Shiga toxin (44). Clearly, other relevant tumor toxins could be explored downstream of STM1787.

In summary, by adapting the *STM1787* promoter in *Salmonella* to drive expression of an appropriate therapeutic transgene, the resulting bacterial vector would provide two

independent mechanisms for specifically targeting tumors. First, *Salmonella* specifically localize to and accumulate in primary tumors and metastases *in vivo*. Second, the *STM1787* promoter is preferentially activated in the acidic tumor microenvironment. The combined effect of these two levels of specificity provides a potential option to design more successful PAMP/TLR-based immunotherapeutic bacterial systems in the future.

Methods

Bacterial strains and culture conditions

The *Salmonella* typhimurium strains SB300A1 (14), SB300A1FL6 (*luxCDABE)* (45), luxAB and AM3 (*luxCDABE msbB-*) were grown in LB broth with appropriate antibiotics. SB300A1FL6 is modified by chromosomal integration of *luxCDABE* and is constitutively bioluminescent. The luxAB strain consists of SB300A1FL6 with the integrated *luxE* gene disrupted. This strain does not bioluminesce without addition of exogenous decanal substrate. The AM3 strain has the SB300A1FL6 background, but also has an *msbB* gene disruption, giving it a less immunogenic LPS structure. The Tn:27.8 strain, specifically identified from the screen as a non-inducible mutant, phenocopies luxAB with constitutive bioluminescence that requires exogenous decanal.

Tissue culture cell lines and culture conditions

B16F10 murine melanoma and HeLa cells were obtained from ATCC and cultured according to ATCC directions. HCT116 human colon carcinoma cells were a gift from Bert Vogelstein and were cultured according to ATCC methods. Cell lines were not further authenticated.

Plasmids

The plasmid pMAAC001 contains the full bacterial luciferase operon *luxCDABE* driven by a T7 promoter and an ampicillin resistance cassette. The plasmid pLuxCDE consists of the pMAAC001 backbone amplified using the forward primer cccgggattggggaggttggtatgtaa and the reverse primer cccgggtgaatgatttgatgagccaaa (*XmaI* sites underlined). This product was then *XmaI* digested and re-ligated to exclude the majority of the *luxA* and *luxB* genes. pLux and pPROMOTERLux plasmids were constructed by inserting the full bacterial luciferase operon between the *KpnI* and *BamHI* restriction sites in the vector pUC19. The pPROMOTERLux plasmid additionally had a 500 base pair promoter region (*STM1787*) from the *Salmonella* genome inserted upstream of the luciferase operon between the *SacI* and *KpnI* restriction enzyme sites. The 500 base pair sequence was amplified from the *Salmonella* genome using the forward primer aaagagctcacgccctctttcaaacagtc and the reverse primer aaaggtaccgcttgataaaaggtctcctcgt (*SacI* and *KpnI* sites underlined). To construct the P1787-based vectors, 500bp of the endogenous SB300A1 *1787* promoter was cloned into the *BglII* and *NdeI* sites of pET3a (Novagen) using the following primers: forward-gagagagaagatct gggacgccctctttcaaacagtctc, reverse-ccttcctgcccatatgaacgcgtattttttctcctttttgcacc. This cloning strategy conserved the endogenous 1787 Shine-Dalgarno sequence and removed the *T7* promoter and synthetic *RBS* of pET3a. The P1787-Stx2A/B vector was constructed by inserting the *Stx2A/B* operon downstream of the *1787* promoter using *NdeI* and *BamHI* with the following primers: forward-gagagagacatatgaagtgtatattatttaaatgggtactgtgcctgttactgggttttttcttcggtatcc, reverse-ccttccttccggatccttatcaatggtgatggtgatggtgg.

Construction of a Salmonella typhimurium reporter-trap library

Salmonella strain SB300A1 was used to construct a bacterial library comprising approximately 7,400 clones of unique chromosomal integrations of our reporter transposon

(14). The custom *Tn5*-based transposon was designed with the EZ-Tn5 system (Epicentre, Madison, WI) using the pMOD4 transposon construction vector. A kanamycin-resistance cassette and promoter from EZ-Tn5<KAN-2> was amplified using the forward primer acgaca*aagctt*ggacgcgatggatatgttct and the reverse primer agcttt*tctagagg*tggaccagttggtgattt (*HindIII* and *XbaI* restriction sites underlined) and inserted into the *HindIII* and *XbaI* restriction sites of pMOD4. The luciferase enzyme genes *luxAB* from *Photorhabdus luminescens* were amplified with the forward primer acagtc*gaattc*cgccgaatgagaattgagat and the reverse primer aagctg*ggtacct*gttggctgctttcactcac (*EcoRI* and *KpnI* sites underlined) and inserted between the *EcoRI* and *KpnI* sites in pMOD4 (45). The plasmid contained an R6Kγ origin of replication and therefore was amplified in *E. coli* DH5α λpir, purified, digested with *PvuII*, and the transposon fragment recovered by gel purification. The purified transposon was combined with transposase (Epicentre). After bench top incubation for 30 minutes, followed by 48 hours at 4°C, the transposon DNA was electroporated into bacteria as per the vendor's instructions. Bacteria were plated on LB kanamycin plates to select for transformants containing the chromosomally-integrated transposon. Each clone was expanded and stored in 60% glycerol in 96-well plates at −80°C.

Screening the library

To screen for gene activation events occurring in the context of malignant cells, *Salmonella* library clones were cultured under three different conditions: co-culture with B16F10 mouse melanoma cells, co-culture with HCT116 human colon carcinoma cells and culture in media alone. Each of the two tumor cell lines were seeded into 96-well white plates at approximately 70–80% confluency in DMEM with 10% FBS. In the plate containing media alone, each well contained 100 µl of DMEM with 10% FBS only. Plates were incubated overnight to allow tumor cell adhesion to the 96-well white plates. Independently, bacterial clones were grown overnight in LB broth with kanamycin in 96-well plates and subcultured the following day 1:10 into LB broth. Five to six hours after subculturing, 30 µl of bacterial culture were added to three replicate plates, each corresponding to a separate culture condition. Bacteria were allowed to co-incubate with the malignant cells or media alone for 2 hours. Subsequently, bacteria were imaged by adding 30 µl of decanal solution, waiting 10 minutes, and imaging with an IVIS 100 imaging system (Caliper; acquisition time, 60 sec; binning, 4; filter, < 510; f stop, 1; FOV, 23 cm) (46). Because white plates were used to maximize signal intensity, images were aquired utilizing a <510 filter to reduce phosphorescence from the plates. Three control wells were included on every plate comprising: *luxCDABE Salmonella* (SB300A1FL6), which contain the full luciferase operon inserted into the chromosome; *luxAB* strain, which contains the luciferase enzyme genes only and therefore requires addition of exogenous substrate to image reporter activity in the assay; and a blank well, which contained media, but was not inoculated with bacteria, to serve as a control for background luminescence. Imaged plates were analyzed with Living Image (Caliper) and Igor (Wavemetric) analysis software packages as described (47). Data were normalized by dividing the photon flux of experimental wells by media alone wells and presented as the $\log_2$ of the normalized photon flux data.

Identification of hits

Library screening data representing photon flux from each well of a library plate were analyzed with Image J software (48). To identify statistically significant hits from the primary screens, we utilized a set of statistical requirements. First, a threshold was set to identify active clones. Clones that did not produce photon signals greater than three standard deviations above the signal in the un-inoculated, media alone wells were not further analyzed. A quartile method of statistical analysis was then applied to the remaining clonal data (49). For quartile analysis, plates of clones were grouped by assay date into sets for data analysis. For each set, we normalized data by calculating the $\log_2$ of the fold-change of

photon flux signal between the condition of interest (co-culture with B16F10 or HCT116 cells) and media alone. From this data, we calculated the median (Q2), first (Q1), and third (Q3) quartile values. The boundary for hit selection was calculated as Q3 + c(ICQ), where ICQ = Q3-Q1 and c = 1.7239, corresponding to a high stringency targeted error rate of α = 0.0027 (49).

Verification of primary screen hits

To verify hits identified by the primary screen, clones were tested again in a similar manner, in quadruplicate. The assay followed the same steps as those in the primary screen, except each clone was tested in 4 wells under each of three conditions across a 12-well row in a black 96-well plate. Imaging was done with an IVIS 100 imaging system (acquisition time, 60 sec; binning, 4; filter, open; f stop, 1; FOV, 23 cm).

Identification of transposon insertion site

To map sites of transposon integration in the chromosome of clones of interest, an inverse touchdown PCR strategy was used (15). Genomic DNA was isolated from bacteria using DNAzol (Molecular Research Center, Cincinatti, Ohio). PCR was performed using bacterial chromosomal DNA, 20 pmols of a primer specific to the $5'$ end of the transposon (atggctcataacacccttg), and 100 pmols of a degenerate primer (cggaatccggatngayksnggntc). Reactions were initiated with a 95°C preparation step for 5 minutes, followed by 25 cycles comprising denaturation at 95°C for 45 seconds, annealing at various temperatures for 45 seconds and extension at 72°C for 2 minutes. The annealing temperature started at 60°C and decreased 0.5°C per cycle for the subsequent 24 cycles. Then PCR proceeded with 25 cycles of 95°C for 45 seconds, 50°C for 45 seconds and 72°C for 2 minutes. PCR reaction products were fractionated on a 1% agarose gel, and the most prominent bands in each lane were excised and gel purified (Qiagen kit). For some reactions, PCR products were purified (Qiagen) and the resulting purified PCR product was used as a template for a second round of PCR using a different transposon-specific primer (aacatcagagattttgagacacc) before gel purification of products. The cycling conditions and degenerate primer used in the second round of PCR were the same as round one.

Semi-quantitative RTPCR

Salmonella strains SB300A1, P1787 or P1787 transformed SB300A1 were subcultured from a stationary phase culture 1:10 and grown for 6 hours. Bacteria were then diluted 1:20 and 30 µl added to 96-well plates containing tissue culture media alone, B16F10 melanoma cells or HeLa cells, seeded 24 hours previously at 100,000 cells/well and 50,000 cells/well, respectively. After three and a half hours of co-culture, extracellular media containing bacteria was removed from the 96-well plates and triplicates pooled. Media were centrifuged to pellet bacteria and pellets were frozen at −80°C. After thawing, pellets were resuspended in 200 µl water with 5 mg/ml lysozyme and incubated at room temperature for 5 minutes. Then, 700 µl of RLT buffer was added and bacterial RNA was purified using the Qiagen RNeasy kit (Qiagen Inc, Valencia, CA). Samples were then treated with DNase I at room temperature for 15 minutes, after which EDTA was added and samples were incubated for 10 minutes at 65°C to inactivate the DNase. Samples were then ethanol precipitated and resuspended in 30 µl water. For reverse transcriptase PCR, 1 µg of total RNA was used as a template and reverse transcribed using Superscript II Reverse Transcriptase and 300 ng random primers as per the manufacturer's instructions (Invitrogen, Carlsbad, CA). Following RTPCR, samples were treated with RNase H for 25 minutes at 37°C. To perform semi-quantitative PCR, samples were amplified using primers specific to each gene target or to ribosomal RNA: STM1787 (forward: tcggtagatcgcatgatgtc, reverse: ggttggtcataagcctgtcg), STM1791 (forward: acacgggaacatccagattc, reverse: cggcaaaggacaaatctcat), STM1793(forward: ttcggcaacctgtttttagg, reverse:

acgcctccttgcataatcac), *adiY* (forward: ccttattgaccgccaactgt, reverse: gtggtcaagaaagcgggata), *yohJ* (forward: caggcatttttcttgcatca, reverse: cgccatataacgaatcagca), *rrsH* (forward: cagccacactggaactgaga, reverse: gttagccggtgcttcttctg), Stx2A (forward: atgacgccgggagacgtgga, reverse: ggccacagtccccagtatcgct) and Stx2B (forward: gcaatggcggcggattgtgc, reverse: acaatccgccgccattgcat). PCR cycling conditions were: 95°C for 5 minutes, 30 cycles (or 20 cycles for *rrsH* reactions) of denaturation at 95°C for 45 seconds, annealing at 50°C for 45 seconds and extension at 72°C for 1 minute. PCR products were fractionated on a 1% agarose gel.

Construction of deletion mutants

Mutant strains deficient for the identified target genes were constructed in *Salmonella* strain *luxCDABE msbB-* (AM3), which contains a constitutively active, chromosomally-encoded bacterial luciferase operon as well as a mutation in *msbB* to create a less immunogenic LPS structure. Mutants were constructed using a lambda red recombinase strategy (50). First, primers were designed to amplify the chloramphenicol-resistance cassette in pKD3 with tails flanking the targeted locus of the *Salmonella* genome to be deleted. Primer sequences specifically targetting the genome for each mutant were used (*adi* forward targetting primer: atgaaagtattaattgttgaaagtgagtttctgcatcaggacacctgggtgtgtaggctggag-ctgcttc, *adi* reverse targetting primer: atcctgtttaaccggcgcatccagcggatacgggtttttgtgaatgc-ggtcatatgaatatcctccttag; *yohJ* forward targetting primer: agtaagtcactgaatattatctg-gcaatatatacgcgcttgtgtaggctggagctgcttc, *yohJ* reverse targetting primer: tttttttcgttcc-cttctgcccaaccactttacgctcaccgcatatgaatatcctccttag; STM1789-1793 forward targetting primer: atgaatgcgcaacgcgtagtggtgatggggttaggaaaccgtgtaggctggagctgcttc, STM1789-1793 reverse targetting primer: ctaataaagttcatgatcgttgcggcggagggtccccaggcatatgaa-tatcctccttag). PCR fragments were then electroporated into AM3 bacteria expressing plasmid-encoded red recombinase. Following electroporation, growth on chloramphenicol plates at 37°C selected for strains that had lost the temperature-sensitive recombinase plasmid and inserted the chloramphenicol-resistance cassette into the targeted genomic loci. Deletion of the genes was confirmed by PCR.

Dose-response to tumor cells

To test the dose-response of hits from the screen to tumor cell co-culture, the assay was performed as described, except that either B16F10 or HCT116 cells were plated at 1×10^5; 2×10^5; or 3×10^5 cells per well 24 hours before co-culture with bacteria. Stationary phase bacteria were diluted 1:50 and incubated for 6 hours before identical aliquots were allowed to co-culture with the malignant cells. Growth curves performed for each mutant strain at different pH values showed no significant differences. Imaging was done with an IVIS 100 imaging system (acquisition time, 10 sec; binning, 8; filter, open; f stop, 1; FOV, 20 cm). Imaged plates were analyzed with Living Image (Caliper) and Igor (Wavemetrics) analysis software packages as described (47).

Assaying promoter activation in different pH media

Stationary phase bacteria were subcultured 1:100 into LB broth. Five to six hrs after subculturing, 10 µl of bacterial culture were added to 190 µl pre-warmed HEPES-buffered media in black 96-well plates adjusted to different pH values, and allowed to incubate for three and a half hours. Bacteria were then imaged with an IVIS 100 imaging system (acquisition time, 60 sec; binning, 8; filter, open; f stop, 1; FOV, 20 cm).

Mouse imaging studies

To generate tumor xenografts, 6-week old *nu/nu* mice (Taconic) were injected subcutaneously in the right flank with 1×10^6 B16F10 cells or 2.5×10^6 HCT116 cells in

100 μl PBS. Tumors were allowed to grow for two (B16F10) or three (HCT116) weeks before bacterial challenge. Saturated cultures of strain AM3 and deletion mutant bacteria were subcultured 1:100 into LB and grown for 3 hours. Bacteria were then diluted to 1×10^6 bacteria/ml (based on OD_{600} readings) and 100 μl were injected via tail vein. Mice were imaged as indicated using an IVIS 100 imaging system (acquisition time, 60 sec; binning, 8; filter, open; f stop, 1; FOV, 20 cm). Photon flux data were calculated by utilizing user-determined regions of interest (ROIs) around bioluminescent tumors with Living Image software.

For *in vivo* promoter inducibility experiments, 6-week old *nu/nu* mice (Taconic) were injected subcutaneously in the right and left flanks with 1×10^7 HCT116 cells in 100 μl PBS. Tumors were allowed to grow for one week. Saturated cultures of *Salmonella* strain SB300A1 containing plasmids pMAAC001, pPROMOTERLux, or pLux were subcultured 1:100 into LB and grown for 3 hours. Twenty microliters of bacterial culture were injected intratumorally. Mice were imaged as indicated using an IVIS 100 imaging system (acquisition time, 180 or 60 sec; binning, 8; filter, open; f stop, 1; FOV, 25 cm). Photon flux data were calculated by utilizing software-determined regions of interest (ROIs) around bioluminescent tumors with Living Image software.

Tumor ex vivo imaging

6-week old *nu/nu* mice (Taconic) were injected subcutaneously in the right flank with 1×10^5 B16F10 cells and tumors allowed to grow for two and a half weeks. Saturated cultures of bacteria were diluted and 5×10^5 bacteria (based on OD_{600} readings) were injected intratumorally. At 24 and 48 hours following bacterial injections, mice were sacrificed, and tumors excised and dissected into 4 sections each. The bacterial-colonized tumor sections were incubated in HEPES/Tris-buffered media at the indicated pH values and imaged using an IVIS 100 imaging system at the indicated times (acquisition time, 180 sec; binning, 8; filter, open; f stop, 1; FOV, 12 cm).

In vitro toxicity assays

Confluent HeLa cells or mock media alone (DMEM + 10% FBS) were inoculated at 1:100 with a stationary culture of SB300A1 transformed with P1787 or P1787-Stx2A/B and cultured at 37 C for 18 hours. The cultured media was then separately filtered through a 0.22 μm filter to remove the bacteria and subsequently aliquoted at various volumes onto HeLa[CMV-FLuc] cells pre-plated in a 96 well plate in quintuplicate. 24 hours later, bioluminescence of the conditioned media-treated HeLa[CMV-FLuc] cells was imaged using an IVIS 100. Phase contrast microscopy (TMS-F, Nikon) was used in parallel to qualitatively confirm loss of cell viability.

In vivo toxicity assays

6 week old male homozygous CrTac:NCr-*Foxn1[nu]* mice (Taconic) were subcutaneously injected in the right flank with of 4.5×10^6 HeLa[CMV-FLuc] cells in 20 μL DMEM. When tumor volumes reached approximately 100 mm^2 (5 days later), mice were injected i.p. with 150mg/kg of D-luciferin and 10 minutes later imaged using an IVIS 100. Immediately following imaging, mice were injected intratumorly with LB broth, or SB300A1 transformed with P1787, or SB300A1 transformed with P1787-STx2A/B at either 2.5×10^5 (low-dose) or 2×10^6 (high-dose) CFU/injection. Mice (n= 9–14 in each group) were weighed and imaged for bioluminescence every five days for 2 weeks. Viable tumor mass is presented as fold-initial photon flux (pre-treatment/post-treatment).

Histology

Tumors were excised and immediately frozen at −80°C. Frozen tumors were fixed in 10% neutral buffered formalin for 24 hours. Prior to paraffin embedding, histology sectioning and H&E staining, fixed tumors were washed with 30%, 50% and then 70% ethanol for 5 minutes each.

Statistics

Error bars represent the standard error of the linearly regressed data or the standard error of the mean where noted.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Statement of significance

Salmonella, which often encounter acidic environments during classical host infection, may co-opt evolutionarily conserved pathways for tumor colonization in response to the acidic tumor microenvironment. We identified specific promoter sequences that provide a platform for targeted *Salmonella*-based tumor therapy *in vivo*.
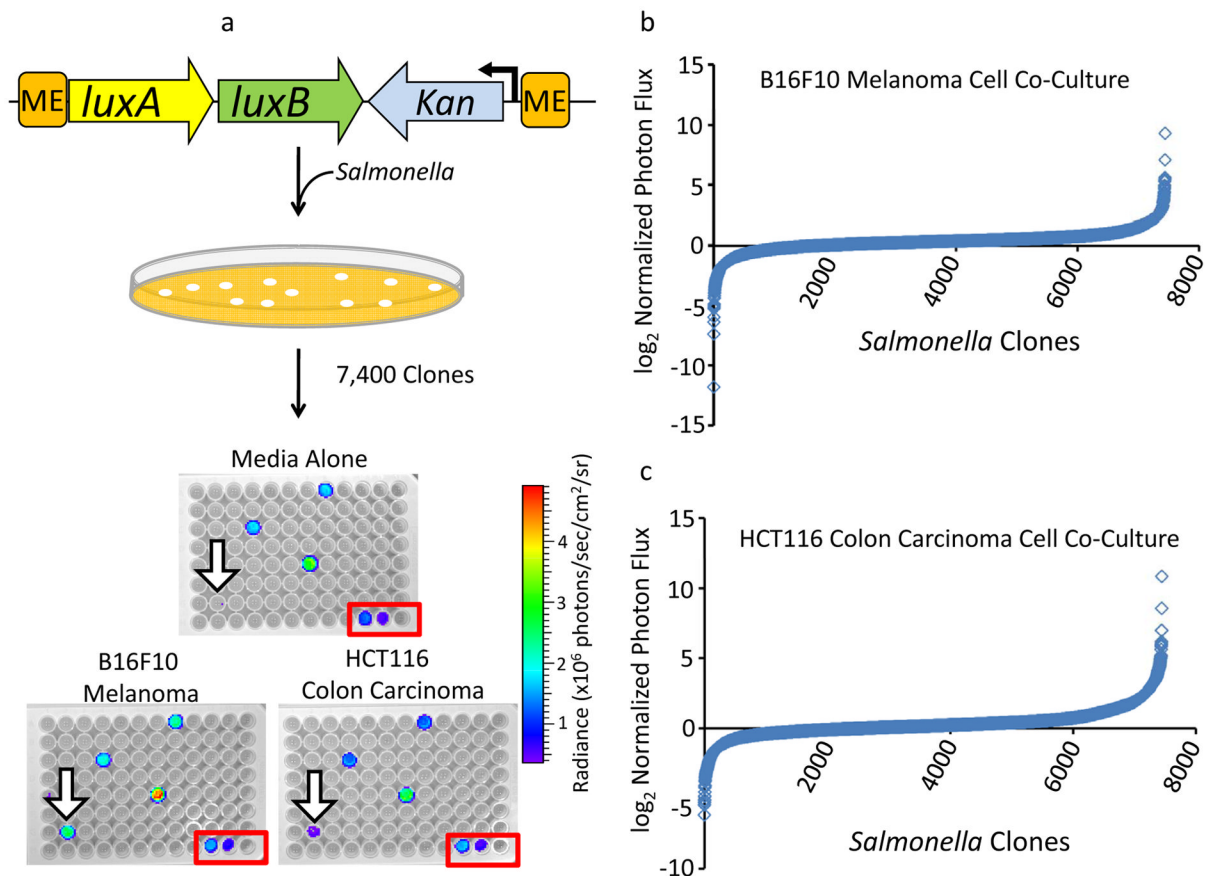
Figure 1. Design and utilization of a high throughput screen to identify tumor cell-induced gene activation events in *Salmonella*

(a) A schematic of the promoter trap system using Tn5-based *luxAB* chromosomal integration. Expression of the promoterless *luxAB* reporter vector, and resulting *Salmonella* bioluminescence, is dependent on "trapping" an active promoter upstream of the chromosomal integration site. The transposon was randomly integrated into strain SB300A1, and kanamycin-resistant colonies were selected and arrayed into 96-well plates for library screening. Representative primary screening plates in triplicate show responses of *Salmonella* library strains to three separate co-culture conditions: media alone (top), B16F10 melanoma cells (bottom left), HCT116 colon carcinoma cells (bottom right). Hit 47.74, showing selective activation in co-culture with cancer cells, is indicated by the black open arrowhead, while the signals in the upper and central wells represent non-selective activation of clones. In each plate, wells H10, H11, and H12 (red box) contain media and bacteria constitutively expressing *luxCDABE*, bacteria constitutively expressing *luxAB*, and no bacteria, respectively, as controls. Primary library screening data from *Salmonella* promoter trap clones co-cultured with B16F10 melanoma cells (b) or HCT116 colon carcinoma cells (c). Data are reported as the $\log_2$ of the normalized signal for each library clone, where normalized signal was the ratio of the signal in the condition of interest to the signal in media alone.
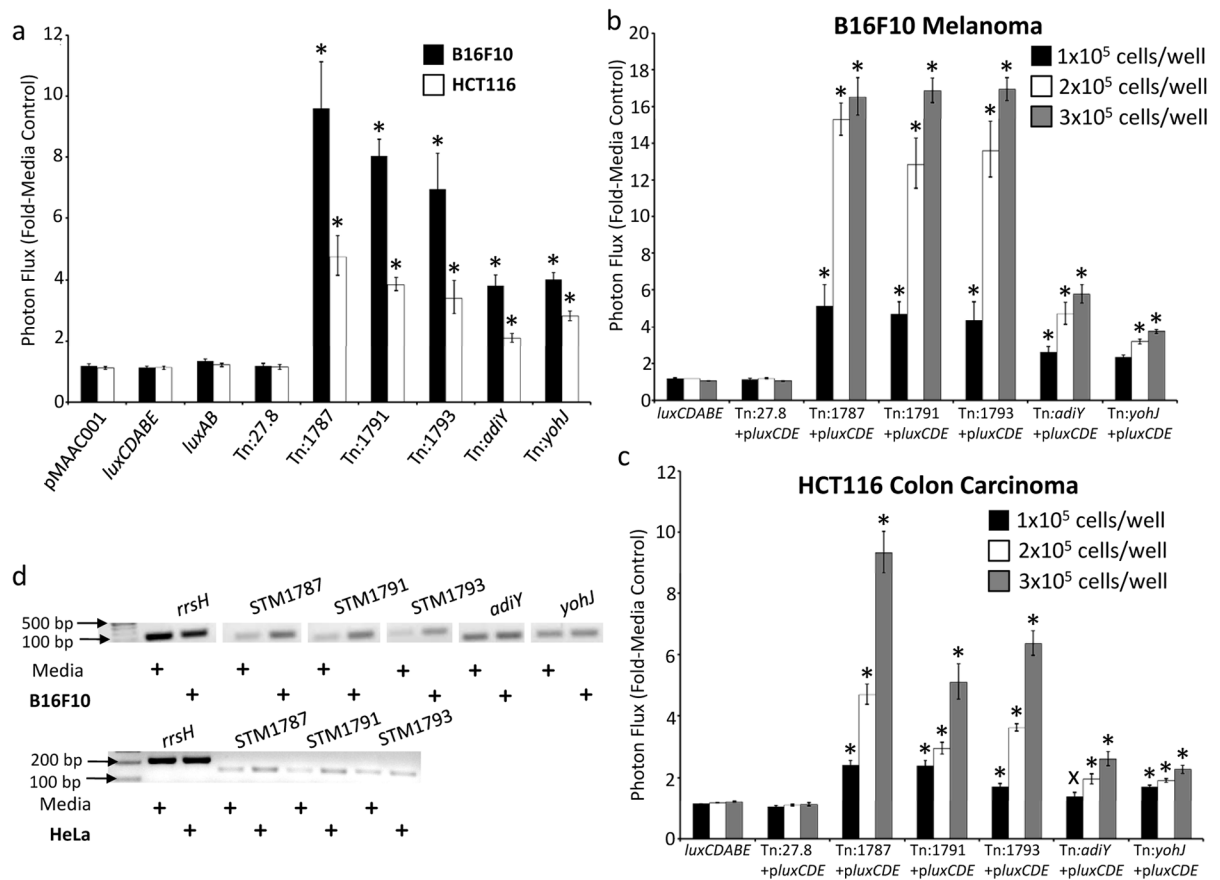
Figure 2. Verification of *Salmonella* gene activation events in the context of tumor cell co-culture
(a) *Salmonella* reporter clones displaying gene activation signals during co-culture with tumor cell lines (black bars, B16F10 cells; open bars, HCT116 cells). *Salmonella* strains *luxAB* and Tn:27.8 contain chromosomal *luxAB* genes under constitutive promoter control; *luxCDABE Salmonella* contain the full luciferase operon inserted into the chromosome; pMAAC001 constitutively expresses plasmid-encoded *luxCDABE*. (b, c) *Salmonella* reporter clones display dose-responsive gene activation in co-culture with B16F10 and HCT116 cells. Bacteria were co-cultured with 1×10^5, 2×10^5, or 3×10^5 B16F10 or HCT116 cells/well. Data were normalized as the ratio of the signal in the condition of interest to signal in media alone. Error bars correspond to SEM. All *p* value calculations are between *luxCDABE* and the group indicated by the symbol: * $p < 1\times10^{-7};$ x$p < 0.06$. (d) Semi-quantitative reverse transcriptase PCR with wild-type SB300A1 bacteria verifies that genes identified by the reporter transposon screen in *Salmonella* are activated during co-culture with B16F10 melanoma and HeLa tumor cells. *rrsH* = ribosomal RNA.
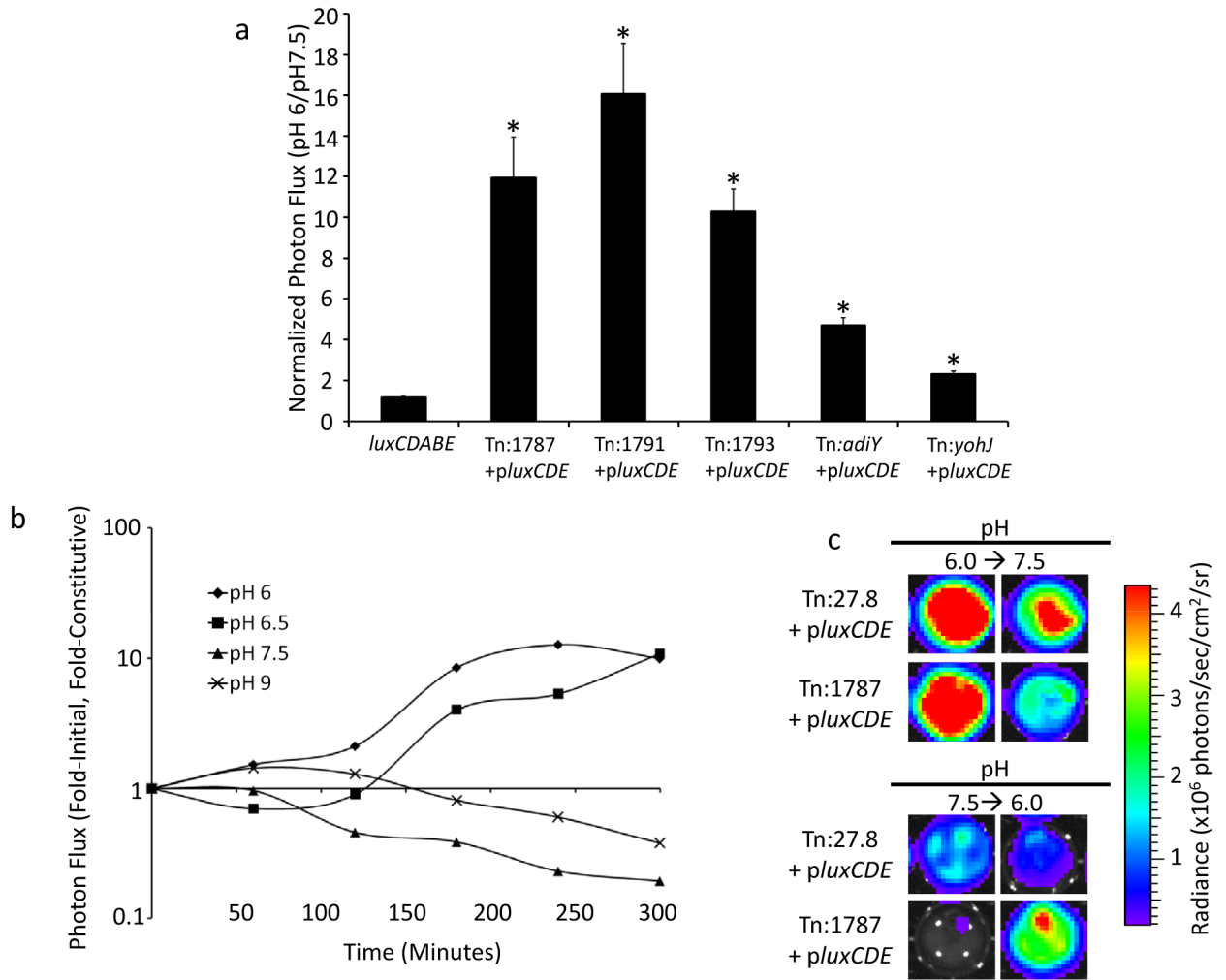
**Figure 3. Acidic pH specifically and reversibly stimulates the Tn:1787 trapped promoter**
(a) Bacteria were cultured in media of different pH values and reporter activation by
Salmonella library clones in low pH media (pH 6) were compared to reporter activation in
normal pH (7.5). Genes identified in the tumor cell co-culture screen were activated in the
context of acidic pH compared to pH 7.5. pMAAC001 and *luxCDABE* constitutively
express plasmid-encoded and chromosomally-encoded *luxCDABE*, respectively. Data were
normalized as the ratio of the signal in media pH 6.0 to signal in media pH 7.5. Error bars
correspond to standard error. The data show one representative experiment with 4 replicates
per condition tested. All *p*-value calculations are between *luxCDABE* and the group
indicated by the asterisk, $*p \leq 2\times10^{-14}$. (b) Mice bearing B16F10 flank tumor xenografts
were injected intratumorally with tumor-activated (Tn:1787+p*luxCDE*) or constitutively
bioluminescent (Tn:27.8+p*luxCDE*) *Salmonella*. The excised tumors were imaged hourly
and data are presented as the normalized signal at each time point. The normalized signal
represents the ratio of the mean of the fold-initial signal of two Tn:1787+p*luxCDE*-
colonized tumors to the mean of the fold-initial signal of two constitutive Tn:
27.8+p*luxCDE*-colonized tumors. The data presented are from a representative experiment;
the experiment was performed independently two times, each with two mice per bacterial
treatment group. (c) Representative *ex vivo* tumor imaging shows reversibility of the
bioluminescent signal in the tumor-activated *Salmonella*. Images on the left show
Salmonella-infected tumor explants after 6 hours of incubation at the indicated pH (pH 6.0,

top; pH 7.5, bottom). Two hours later (8 hours total), media was removed and replaced with media of the indicated pH (pH 7.5, top; pH 6.0, bottom). Images on the right show *Salmonella*-infected tumor explants 4 hours after the pH of the media was changed.
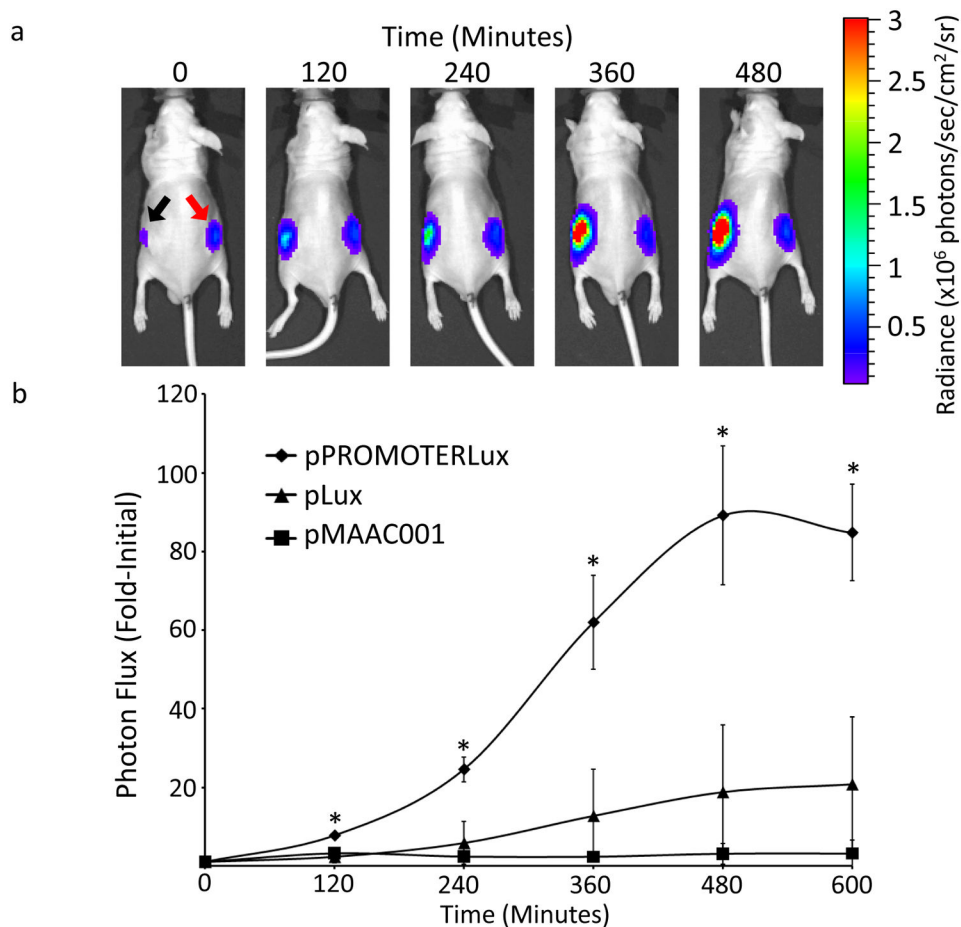
Figure 4. The *STM1787* promoter in *Salmonella* is rapidly activated *in vivo* by the tumor microenvironment

(a) A representative mouse with two HCT116 flank tumor xenografts. The left tumor (black arrow) was injected with *STM1787* p*PROMOTERLux*-expressing *Salmonella*, while the right tumor (red arrow) was injected with constitutive p*MAAC001*-expressing *Salmonella*, and the mouse imaged at the indicated times post-injection. (b) The mean photon flux for each set of *Salmonella*-injected tumors, normalized to the initial signal in each tumor, plotted as a function of time. Error bars represent SEM; p*PROMOTERLux* (n=6); p*Lux* (n=3); p*MAAC001* (n=3), *p<0.025
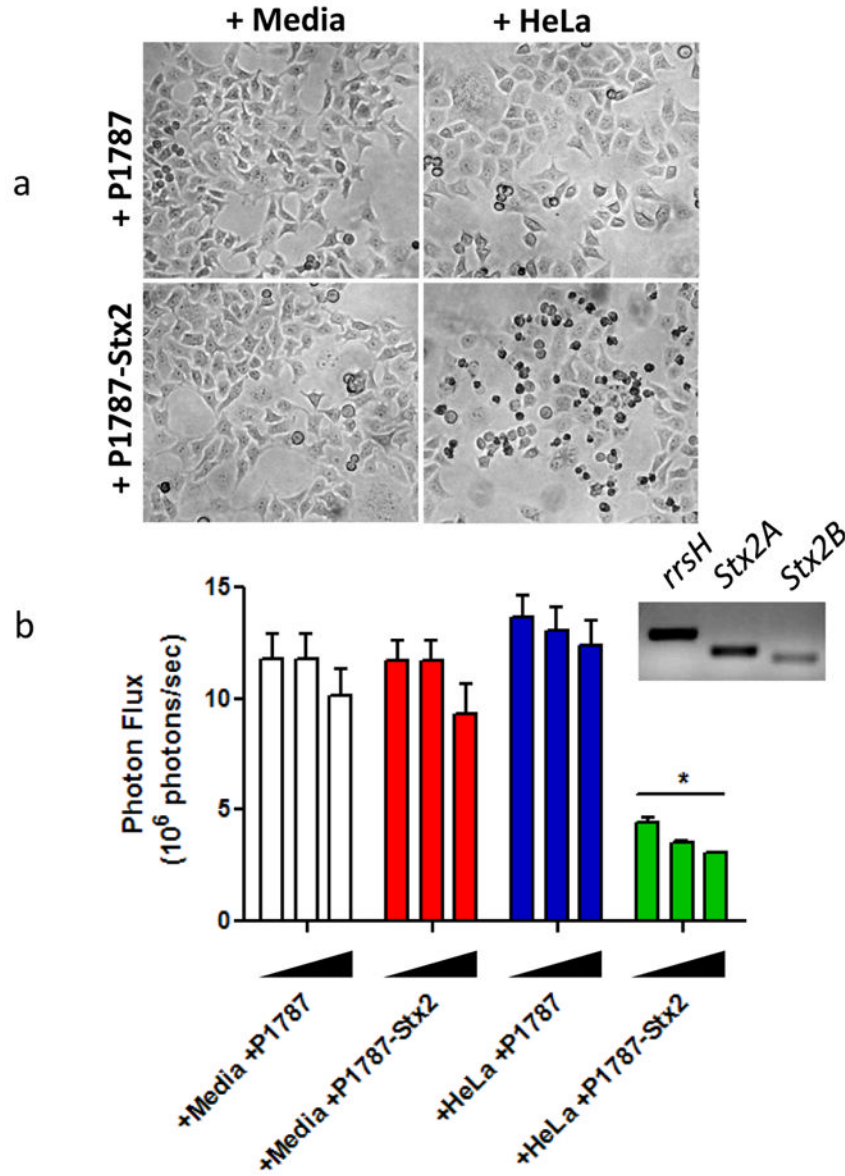
Figure 5. P1787-driven Stx2 cytoxicity is selectively activated by the cancer cell environment *in vitro*

(a) Representative brightfield microscopy of HeLa[CMV-FLuc] cells treated with 4 different conditioned, filtered media for 24 hours (+media+P1787; +media+P1787-Stx2; +HeLa +P1787; and +HeLa+P1787-Stx2). Note the dramatic membrane blebbing and apoptotic morphology of +HeLa+P1787-Stx2 conditioned media-treated cells. (b) Bioluminescence imaging of HeLa[CMV-FLuc] cells treated with increasing amounts of 4 different conditioned, filtered media for 24 hours (bar groups, left to right: 17%, 29%, 44% of total volume per well). *p<0.0005 compared to all other treatments. *Inset* represents PCR amplification of *Stx2A/B* mRNA from P1787-Stx2 transformed SB300A1 co-cultured with HeLa cells.
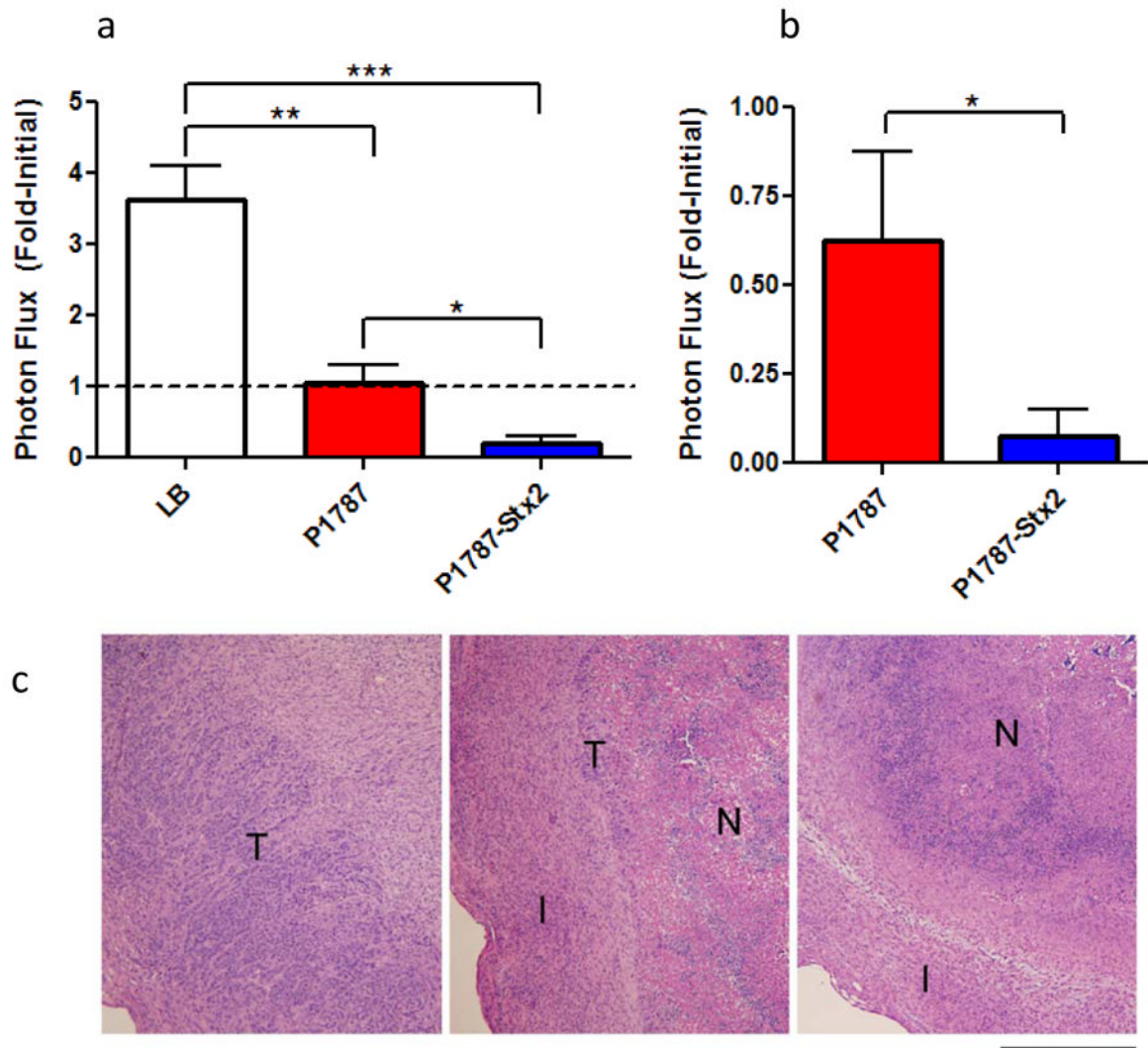
Figure 6. Enhanced anti-tumor response with P1787-Stx2 *in vivo*.
(a) Viable cell mass of HeLa$^{CMV-FLuc}$ tumors from mice treated with LB (n = 14), or high-dose SB300A1 transformed with P1787 (n= 12) or P1787-Stx2 (n = 9) at five days post treatment. Results are combined from two independent experiments and presented as fold-initial photon flux. Dotted line demarks lack of fold-change in tumor bioluminescence. Error bars indicate standard error of the mean ***p<0.0002,**p<0.0003, *p<0.007). (b) Fold-initialized photon flux of HeLa$^{CMV-FLuc}$ tumors from mice treated with low-dose SB300A1 transformed with P1787 (n= 7) or P1787-Stx2 (n = 7) at 14 days post treatment. Error bars indicate standard error of the mean.*p <0.04. (c) H&E staining of HeLa$^{CMV-FLuc}$ tumors from mice treated with LB (left), or high-dose SB300A1 transformed with P1787 (middle) or SB300A1 transformed with P1787-Stx2 (right) after five days. Regions of tumor are denoted as tumor (T), fibroinflammatory reaction (I), and necrotic zone (N). Scale bar, 500 μm.

Table 1

Transposon chromosomal insertion locations in *Salmonella* reporter mutants.

Strain Name	Transposon Insertion Location	Base pairs Downstream of Start Codon	Function (Putative) (17)
Tn:1787	*STM1787*	1,189	Hydrogenase
Tn:1791	*STM1791*	505	Hydrogenase
Tn:1793	*STM1793*	661	Cytochrome oxidase
Tn:adiY	*adiY*	439	araC-like transcriptional activator; arginine-dependent acid tolerance
Tn:yohJ	*yohJ*	205	Hypothetical membrane protein