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 coculture 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 antitumor activity in vitro and in vivo, showing the potential for a conditional bacterial-based tumor-specific therapeutic.

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. Cancer Discov; 2(7); 1–14. ©2012 AACR.
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). In addition, tumors undergoing targeted molecular therapy often relapse due to the use 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 antitumor 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 intratumoral colonization in vivo, genetic tractability, as well as the oncolytic capacity of Salmonella typhimurium have made Salmonella strains ideal candidates for anticancer bacterial development (8).

S. typhimurium by itself can illicit an antitumoral 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 (PAMP) of S. typhimurium, such as lipopolysaccharide (LPS) and flagellin, are capable of activating innate immunity by initiating proinflammatory TLR-MyD88/TRIF-NF-κB signaling cascades (10). Third, intracellular Salmonella flagellin can also enhance an antitumor adaptive immune response caused by the associative recognition with cancer cell antigens. The resulting signaling cascades ultimately augment antigen presentation by 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). In addition, 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 RNA interference 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 antitumor effects in vivo (5).
Figure 1. Design and use of a high-throughput screen to identify tumor cell-induced gene activation events in Salmonella. 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 (kan)-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 3 separate coculture conditions: media alone (top), B16F10 melanoma cells (bottom left), and HCT116 colon carcinoma cells (bottom right). Hit 47.74, showing selective activation in coculture with cancer cells, is indicated by the black open arrowhead, whereas the signals in the top and middle wells represent nonspecific 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 cocultured with B16F10 melanoma cells (B) or HCT116 colon carcinoma cells (C). Data are reported as the log2 of the normalized signal for each library clone, where normalized signal is the ratio of the signal in the condition of interest to the signal in media alone.

RESULTS

A High-Throughput Screen to Identify Tumor Cell-Induced Gene Activation Events in Salmonella

To conduct a large-scale, unbiased screen for genes upregulated in the presence of malignant cells, we used a Tn5-based promoterless 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 (Fig. 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 3 conditions: tissue culture media alone, B16F10 melanoma cells and HCT116 colon carcinoma cells, both of the latter in monolayer coculture 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 2 coculture conditions and media alone. After a 2-hour incubation, bioluminescence imaging of plates enabled identification of clones specifically upregulating genes in the context of exposure to melanoma and/or colon carcinoma cells (Fig. 1A). Results of the screen from coculture with melanoma and colon carcinoma cells are shown in

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 queries, we engineered a bioluminescent transposon as the backbone of a 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 (kan)-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 3 separate coculture conditions: media alone (top), B16F10 melanoma cells (bottom left), and HCT116 colon carcinoma cells (bottom right). Hit 47.74, showing selective activation in coculture with cancer cells, is indicated by the black open arrowhead, whereas the signals in the top and middle wells represent nonspecific 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 cocultured with B16F10 melanoma cells (B) or HCT116 colon carcinoma cells (C). Data are reported as the log2 of the normalized signal for each library clone, where normalized signal is the ratio of the signal in the condition of interest to the signal in media alone.
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Fig. 1B and C, respectively. In each case, data are shown as a rank-ordered S-plot of the log₂ 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 (α = 0.0027) identified 5 candidate mutants wherein the transposon reporter was specifically upregulated during coculture with malignant cells.

Verification and Characterization of Salmonella Gene Activation Events in the Context of Tumor Cell Coculture

Following the primary screen, we used 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 upregulated 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 3 of the clones inserted in a cluster in the chromosomal sequence. Mapped to 3 different, but closely linked genes (STM1787, STM1791, and STM1793, respectively), 2 are known hydrogenases, and all 3 genes are likely coregulated 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 acidic 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 coculture-specific gene activation events identified in the primary screen, we first repeated the coculture assay in quadruplicate in at least 3 independent experiments for each clone. Figure 2A shows the data from one representative experiment for clones verified by this assay. Again, all 5 clones showed statistically significant enhancement of bioluminescence in the presence of tumor cells, with a trend toward greater gene upregulation when cocultured with B16F10 melanoma cells. Then, to further characterize tumor cell–induced response of Salmonella, we used the tumor cells in a dose–response assay (Fig. 2B and C). In addition, 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 upregulation of the reporter when exposed to greater numbers of tumor cells in coculture 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 coculture with tumor cells, we used semiquantitative PCR. Following a 3-hour coculture of wild-type (SB3001A1) bacteria with B16F10 cells or in tissue culture media alone, isolated RNA was reverse transcribed to cDNA. Semiquantitative PCR of cDNA showed that coculture with B16F10 melanoma cells enhanced the intensity of target gene transcripts, but not control ribosomal RNA transcripts (rrsH; Fig. 2D). The effect was generalizable, as coculture with HeLa tumor cells produced similar results (Fig. 2D).

Notably, of the genes identified in this screen, at least one, adiY, has previously been reported to be upregulated in acidic pH conditions (16). One characteristic of tumor microenvironments in vivo is an abnormally acidic pH (18). In fact, because of 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 with neutral media. Each of the clones upregulated the reporter gene at pH 6.0 compared with the physiologic pH of normal body tissue (pH 7.5), suggesting that the stimulus Salmonella

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<th>Table 1. Transposon chromosomal insertion locations in Salmonella reporter mutants</th>
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Published OnlineFirst May 3, 2012; DOI: 10.1158/2159-8290.CD-11-0201
B16F10 melanoma

1 x 10⁵ cells/well
2 x 10⁵ cells/well
3 x 10⁵ cells/well

Photon flux (fold media control)

Figure 2. Verification of Salmonella gene activation events in the context of tumor cell coculture. A, Salmonella reporter clones displaying gene activation signals during coculture with tumor cell lines. Salmonella strains luxAB and Tn27.8 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 and C, Salmonella reporter clones display dose-responsive gene activation in coculture with B16F10 and HCT116 cells. Bacteria were cocultured with 1 x 10⁵, 2 x 10⁵, or 3 x 10⁵ B16F10 or HCT116 cells per 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 ≤ 0.06.

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 recombinational deletion strategy, which inserted a chloramphenicol resistance cassette into the targeted genes. The deletion mutants were created from a parental Salmonella strain (luxCDABE mbbB-) containing a chromosomally integrated and constitutively expressed bacterial luciferase operon for imaging bacterial localization in vivo in real-time. The strain also contained an mbbB gene deletion, which causes a less immunogenic LPS structure and minimizes septic shock effects when the strain is administered intravenously (20). On the basis of 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 adY also appeared to be a part of a larger operon of coregulated genes and was therefore targeted along with the adjacent genes adi and yohJ. 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 with the parental strain for localization to and persistence within the tumor using bioluminescence imaging (Supplementary Fig. S1). 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 coculture in vitro, they were not essential for bacterial colonization of the tumor. The experiment was also carried out in an HCT116 colon carcinoma xenograft model with similar results. Supplementary Table S1 summarizes the numbers of mice with colonized tumors on or before day 10 in each experiment. In addition, in pilot competitive infection studies, there was no
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significant difference between the STM1789-1793 mutant and the parental Salmonella strain (luxCDABE mchB-) in tumor colonization [colony-forming units (CFU)/mL; data not shown].

Specificity and Reversibility of the Salmonella STM1787 Promoter In Vivo

We next sought to show the specificity of STM1787 promoter activation in the tumor microenvironment in vivo. We chose this promoter because it displayed the highest acidic pH induction in vitro (Fig. 2A). Here, we used the constitutively bioluminescent Salmonella strain Tn:27.8+luxCDE or the conditionally bioluminescent strain Tn:1787+luxCDE, each of which constitutively express plasmid-encoded luxCDE, but the latter strain will only show bioluminescence 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 2 days to localize and adapt to tumors in vivo. Tumors were then excised, incubated in solutions of various pH values, and imaged periodically for 6 hours. Initially, all tumors showed bioluminescent bacteria *in 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 enhanced in the Tn:1787 *Salmonella*-infected tumor explants incubated in low pH media. In 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 (Fig. 3B). 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. Furthermore, 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 were changed from pH 7.5 to pH 6.0, the signal increased (Fig. 3C). 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, use 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 whether 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 show tumor-mediated transgene activation in vivo. The plasmids pMAAC001 and pLux both encoded constitutively expressed luciferase operons, whereas the pPROMOTERLux 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 (Fig. 4). We chose to use intratumoral injection to directly compare reporter gene activation from 2 different bacterial strains, one inducible and the other constitutive, over time in the same mouse. Although reporter signals from pPROMOTERLux-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-hour exposure to the tumor microenvironment (Fig. 4A). Concurrently, bacteria constitutively expressing pLux- or pMAAC001-luciferase showed less than 20-fold or no reporter activation, respectively, after exposure to the tumor microenvironment (Fig. 4A and B). These data directly showed 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 Antitumor Therapy In Vivo

We used 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 AB2 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 (Gb3; ref. 21). Once inside the host cell, the A subunit cleaves the 285 RNA of the 60S ribosomal subunit, thereby inhibiting peptide elongation and inducing apoptosis. Stx2 subunit B (StxB2) has been extensively studied for its tumor-targeting potential as many invasive tumors display high levels of Gb3 (22).

Using bioluminescence as a reporter of total tumor cell mass, we conducted a coculture experiment with HeLaCMV-Fluc cells in vitro. First, plated HeLa cells were grown to confluence to acidify the media and then cocultured with strain SB300A1 transformed with P1787 (empty vector) or P1787-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 HeLaCMV-Fluc cells in increasing volumes: (i) + media+P1787; (ii) +media+P1787-Stx2; (iii) +HeLa+P1787; and (iv) +HeLa+P1787-Stx2. After 24 hours of treatment, major toxicity was only observed in HeLaCMV-Fluc cells treated with the supernatant of +HeLa+P1787-Stx2 (Fig. 5A); a general concentration–response trend was observed (Fig. 5B). Stx2 expression was verified using mRNA PCR amplification (Fig. 5B inset). No overt cytotoxicity was observed in HeLaCMV-Fluc cells treated with supernatant from any of the other conditioned media groups.

Figure 4. The STM1787 promoter in Salmonella is rapidly activated in vivo by the tumor microenvironment. A, a representative mouse with 2 HCT116 flank tumor xenografts. The left tumor (black arrow) was injected with STM1787 pPROMOTERLux-expressing Salmonella, whereas the right tumor (red arrow) was injected with constitutive pMAAC001-expressing Salmonella, and the mouse imaged at the indicated times postinjection. B, the mean photon flux for each set of Salmonella-injected tumors, normalized to the initial signal in each tumor, and plotted as a function of time. Error bars represent SEM; p = 0.0025.
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A

**Figure 5.** P1787-driven Stx2 cytotoxicity is selectively activated by the cancer cell environment in vitro. A, representative bright-field microscopy of HeLaCMV-FLuc cells treated with 4 different bacteria-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 HeLaCMV-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 with all other treatments. Inset represents PCR amplification of Stx2A/B mRNA from P1787-Stx2 transformed SB300A1 cotreated with HeLa cells.

Given the selective regulation and associated toxicity of P1787-Stx2 in vitro, we next desired to show the tumor-targeting potential in vivo using established s.c. flank HeLaCMV-FLuc xenograft tumors and bioluminescence imaging. In 2 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 5 days after treatment (Fig. 6A). Furthermore, when tumors were treated with a single low dose of SB300A1 transformed with P1787-Stx2, a robust antitumoral effect was observed after 2 weeks (Fig. 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, whereas Luria–Bertani (LB) broth alone showed no inhibitory effect (23). To verify tumor cell death independent of bioluminescence signal, tumor hematoxylin and eosin (H&E) sections from the high-dose treatment were analyzed (Fig. 6C). Sections through LB-treated (control) tumors showed a broad rim of viable tumor cells with focal necrotic regions centrally. In the P1787-treated 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 2 weeks, at which point the experiment was concluded, but each still displayed a significant reduction in tumor size compared with P1787 alone (Fig. 6B).

**DISCUSSION**

S. 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 1800s, 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 shown 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...
targeting studies, including specific amino acid auxotrophs of Salmonella as biotherapeutic 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 TNF-α 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 used 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 4,620 genes at least once. From this library, we identified 5 Salmonella genes specifically upregulated during coculture with cancer cells: STM1787, STM1791, STM1793, adY, and yohB. 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 upregulation 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 conducted a similar in vivo screen using a promoter-trap GFP-based system and identified 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 5 genes identified herein show no overlap with the promoters identified by Leschner and colleagues (33), nor Arrach and colleagues (38). However, the STM1787 promoter located upstream of 3 of our own target genes (STM1787, STM1791, and STM1793) did contain the conserved tusp motif identified by Leschner and colleagues (tattatatataaa). The discrepancy in promoter identification may stem from the different bacterial strains or strategies used for gene identification in the 2 studies. Whereas Arrach and colleagues used 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 upregulated in low oxygen conditions, indicating that hypoxia may serve as a further stimulus for the pH-induced promoters identified in our 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 (K. Flentie, 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). In addition, the low pH environment of the tumor inhibits host defense. Cytotoxic immune cell activity and cytokine secretion have 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 use 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, whereas both conventional therapeutics and host defenses fail.

When using bacteria as a vector for biotherapeutic delivery, tumor-specific colonization and subsequent expression are major concern. The genes identified herein are highly expressed in an acidic tumor environment but are not required for bacterial tumor targeting (Supplementary Fig. S1). Therefore, the promoters regulating these genes and further dissection of the complex regulation of the tusp motif may generate ideal chromosomal insertion sites candidates or synthetic promoter systems for use in therapeutic gene, prodrug, 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 showed this in vivo using bioluminescent imaging. We further applied the STM1787 promoter to conditionally regulate the expression of Stx2 in wild-type 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 coculture 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...
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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 2 independent mechanisms for specifically targeting tumors. First, Salmonella specifically localizes 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 2 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 S. typhimurium strains SB300A1 (14), SB300A1FL6 (luxCDABE, ref. 45), luxAB and AM3 (luxCDABE mbbB) were grown in LB broth with appropriate antibiotics. SB300A1FL6 is modified by chromosomal integration of luxCDABE and is constitutively bioluminescent. The lumAB strain consists of SB300A1FL6 with the integrated luxE gene disrupted. This strain does not show bioluminescence without addition of exogenous decanal substrate. The AM3 strain has the SB300A1FL6 background but also has an mbbB gene disruption, giving it a less immunogenic LPS structure. The Tn278 strain, specifically identified from the screen as a noninducible mutant, phenocopies lumAB with constitutive bioluminescence that requires the addition of exogenous decanal substrate. The pAM3 strain has the pLexCDE promoter and an ampicillin resistance cassette. The plasmid pMAAC001 contains the full bacterial luciferase operon downstream of the luxCDABE promoter and is constitutively bioluminescent. The plasmid contained an R6K origin of replication and therefore was amplified in Echerichia coli DH5α pir, purified, digested with PstI and Bgl II restriction sites in pMOD4 (45). The plasmid contained an R6K origin of replication and therefore was amplified in Echerichia coli DH5α pir, purified, digested with PstI and Bgl II restriction sites in pMOD4 (45).

Tissue Culture Cell Lines and Culture Conditions

B16F10 murine melanoma and HeLa cells were obtained from American Type Culture Collection (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 ccgggtctggaggttcgggtggtgatgaa and the reverse primer ccgggtctggaggttcgggtggtgatgaa and the transposon fragment recovered by gel purification. The purified transposon was combined with transposase (Epipcentre). 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 3 different conditions: coculture with B16F10 mouse melanoma cells, coculture with HCT116 human colon carcinoma cells, and culture in media alone. Each of the 2 tumor cell lines were seeded into 96-well white plates at approximately 70% to 80% confluency in Dulbecco’s Modified Eagle’s Media (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 adherence 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 6 hours after subculturing, 30 µL of bacterial culture was added to 3 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 seconds; binning; 4; filter, <510; f stop, 1; field of view (FOV), 23 cm; ref. 46]. Because white plates were used to maximize signal intensity, images were acquired using 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 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 ImageJ software (48). To identify statistically significant hits from the primary screens, we used a set of statistical requirements. First, a threshold was set to identify active clones. Clones that did not produce photon signals greater than 3 SDs above the signal in the uninoculated, 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 of the fold change of photon flux signal between the condition of interest (coculture with B16F10 or HCT116 cells) and media alone. From these data, we calculated the median (Q2), first (Q1), and third (Q3) quartile values. The boundary for hit selection was calculated as Q3 + 1.7239(Q3−Q1), 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 3 conditions across a 12-well row in a black 96-well plate. Imaging was done with an IVIS 100 imaging system (acquisition time, 60 seconds; 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). PCR was carried out using bacterial chromosomal DNA, 20 pmols of a primer specific to the 5’ end of the transposon (atgctataacacccctg), and 100 pmols of a degenerate primer (cgccctagaattgatg). Reactions were initiated with a 95°C preparation step for 5 minutes, followed by 25 cycles comprising denaturation at 95°C for 30 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 (aatcaatcagatggcttttgcag) before gel purification of products. The cycling conditions and degenerate primer used in the second round of PCR were the same as round one.

Semiquantitative Reverse Transcriptase-PCR

Salmonella strains SB300A1, P1787, or P1787-transformedSB300A1 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 and 50,000 cells per well, respectively. After 3.5 hours of coculture, extracellular media containing bacteria were 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.). 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). Following RT-PCR, samples were treated with RNase H for 25 minutes at 37°C. To conduct semiquantitative PCR, samples were amplified using primers specific to each gene target or to ribosomal RNA: STM1787 (forward: tggtagatgacatgtc, reverse: ggtggtggatacgcttcg), STM1790 (forward: acaacciaggtttacatgtc, reverse: gctgagacatatttctg), STM1791 (forward: tggcagcaggtttgctg, reverse: aagctctttgtgcttc), STM1792 (forward: ggtgcggatctgctt, reverse: aacatcagatggctttttgcag), and STM1793 (forward: ggccacagtccccagtatcgct, reverse: gatactgccgggtggcatgctgg, STM1789-1793 reverse targeting primer: ctaataaagttcatggcctctcatt). 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 InvCDABE mbbB (Ab3), which contains a constitutively active, chromosomally encoded bacterial luciferase operon as well as a mutation in mbbB 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 targeting the genome for each mutant were used (adi forward targeting primer: atgaagttattaattgttgaaagtgagtttctgcatcaggacacctgggt-catacttcgctcaccgcatatgaatatcctgctgtag), reverse targeting primer: atggcagcaggttttgcaggtctcttgctgttaggtcgggttggacctcgtgc, yohB reverse targeting primer: tttgctgcct-ccttgccaccaattgctgcgtgatgatatcttcggctgactagctcatatctggtgctg, and yohB forward targeting primer: atgaaagttattaattgttgaaagtgagtttctgcatcaggacacctgggt-catacttcgctcaccgcatatgaatatcctgctgtag). PCR fragments were then electroporated into AM3 bacteria expressing plasmid-encoded red recombinease. 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 coculture, the assay was conducted 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 coculture with bacteria. Stationary phase bacteria were diluted 1:50 and incubated for 6 hours before identical aliquots were allowed to coculture with the malignant cells. Growth curves conducted for each mutant strain at different pH values showed no significant differences. Imaging was done with an IVIS 100 imaging system (acquisition time, 10 seconds; 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 6 hours after subculturing, 10 µL of bacterial culture was added to 190 µL prewarmed HEPES-buffered media in black 96-well plates adjusted to different pH values and allowed to incubate 3.5 hours. Bacteria were then imaged with an IVIS 100 imaging system (acquisition time, 60 seconds; 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 x 10^6 B16F10 cells or 2.5 x 10^5 HCT116 cells in 100 µL PBS. Tumors were allowed to grow for 2 (B16F10) or 3 (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 x 10^9 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 seconds; binning, 8; filter, open; f stop, 1; FOV, 20 cm). Photon flux data were calculated by using user-determined regions of interest (ROI) 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 x 10^6 HCT116 cells in 100 µL PBS. Tumors were allowed to grow for 1 week. Saturated cultures of *Salmonella* strain SB300A1 containing plasmids pMAAC001, pPROMOTERlux, or pLac were subcultured 1:100 into LB and grown for 3 hours. Twenty microtiter of bacterial culture was injected intratumorally. Mice were imaged as indicated using an IVIS 100 imaging system (acquisition time, 180 or 60 seconds; binning, 8; filter, open; f stop, 1; FOV, 25 cm). Photon flux data were calculated by using software-determined ROIs around bioluminescent tumors with Living Image software.

**Tumor Ex Vivo Imaging**

Six-week-old nu/nu mice (Taconic) were injected subcutaneously in the right flank with 1 x 10^6 B16F10 cells and tumors allowed to grow for 2.5 weeks. Saturated cultures of bacteria were diluted and 5 x 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 seconds; 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 were then separately filtered through a 0.22-µm filter to remove the bacteria and subsequently aliquoted at various volumes onto HeLa^CMV-PLuc^ cells preplated in a 96-well plate in quintuplicate. Twenty-four hours later, bioluminescence of the conditioned media-treated HeLa^CMV-PLuc^ 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**

Six-week-old male homozygous nu/nu (CrTacsNcr-Fax1^tm^) mice (Taconic) were subcutaneously injected in the right flank with of 4.5 x 10^6 HeLa^CMV-FLuc^ cells in 20 µL DMEM. When tumor volumes reached approximately 100 mm^2 (5 days later), mice were injected intraperitoneally (i.p.) with 150 mg/kg of α-luciferin and 10 minutes later imaged using an IVIS 100. Immediately following imaging, mice were injected intratumorally with LB broth or SB300A1-transformed with P1787 or SB300A1-transformed with P1787-Stx2A/B at either 2.5 x 10^8 (low-dose) or 2 x 10^9 (high-dose) CFU/injection. Mice (n = 9 – 14 in each group) were weighed and imaged for bioluminescence every 5 days for 2 weeks. Viable tumor mass is presented as fold initial photon flux (pretreatment/post-treatment).

**Histology**

Tumors were excised and immediately frozen at −80°C. Frozen tumors were fixed in 10% neutral-buffered formalin for 24 hours. Before 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 SE of the linearly regressed data or the SEM where noted.

**Disclosure of Potential Conflicts Interest**

S.T. Gammon is employed by Carestream Molecular Imaging as a Product Manager. No potential conflicts of interest were disclosed by the other authors.

**Authors’ Contributions**

Conception and design: K. Flentie, B. Kocher, J.S. McKinney, D. Piwnica-Worms


Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): K. Flentie, B. Kocher, S.T. Gammon, J.S. McKinney, D. Piwnica-Worms

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): K. Flentie, B. Kocher, S.T. Gammon, D.V. Novack, J.S. McKinney, D. Piwnica-Worms

Writing, review, and/or revision of the manuscript: K. Flentie, B. Kocher, S.T. Gammon, D.V. Novack, J.S. McKinney, D. Piwnica-Worms

Study supervision: D. Piwnica-Worms

Provided histologic images and interpretation: D.V. Novack

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