Salmonella Promoters Preferentially Activated Inside Tumors

Nabil Arrach,[1] Ming Zhao,[2] Steffen Porwollik,[1] Robert M. Hoffman,[2,3] and Michael McClelland[1]

[1]Sidney Kimmel Cancer Center, [2]AntiCancer, Inc., and [3]Department of Surgery, University of California, San Diego, San Diego, California

Abstract

Salmonella enterica **and avirulent derivatives prefer solid tumors over normal tissue in animal models. The identification of endogenous *Salmonella* promoters that are preferentially activated in tumors could further our understanding of this phenomenon. Toward this goal, a random library of *S. enterica typhimurium* 14028 genomic DNA was cloned upstream of a promoterless gene encoding the green fluorescent protein (GFP) TurboGFP. A population of *Salmonella* containing this library was injected i.v. into tumor-free nude mice and into human PC3 prostate tumors growing subcutaneously in nude mice. After 2 days, fluorescence-activated cell sorting was used to enrich for bacterial clones expressing GFP from spleens or tumors. The resulting libraries were hybridized to an oligonucleotide tiling array of the *Salmonella* genome. Eighty-six intergenic regions were found to be enriched in tumor samples but not in spleen. Twenty of these candidate promoters were also detected in the sequences of 100 random clones from a library enriched for expression in bacteria growing in tumors. Three candidate promoter clones were individually tested *in vivo*, and enhanced GFP expression in bacteria growing in tumor relative to spleen was confirmed. Two of the three clones (*pflE* and *ansB* promoter regions) are known to be induced in hypoxic conditions that pertain to many tumors. For many of the other candidate promoters preferentially induced in bacteria growing in tumors, regulatory mechanisms may not be related to hypoxia. The expression of therapeutics in *Salmonella* under the regulation of one or more promoters that are activated preferentially in tumors has the potential to improve the targeting of drug delivery.** [Cancer Res 2008;68(12):4827–32]

Introduction

Salmonella enterica serovar *typhimurium* is a facultative anaerobic bacterium that naturally accumulates in a wide variety of solid tumors as opposed to normal tissue (1–10). Avirulent mutants of this bacterium prefer tumors over normal tissue at ratios that range between 250:1 and 9,000:1 (1, 11), and can lead to tumor reduction or cures in animal models (2). Necrotic regions of the tumor are hypoxic and relatively acidic compared with normal tissues (12, 13). The ability of *Salmonella* to accumulate in tumors may be due to such differences (14), and perhaps other mechanisms not yet established. To investigate this phenomenon, a high-throughput method was used to screen for *Salmonella* promoters that are preferentially expressed in tumors versus spleen. A random library of *Salmonella* DNA fragments was cloned upstream of a promoterless green fluorescent protein (GFP) to monitor *Salmonella* promoter activation in human-PC3 tumors in nude mice. Differential fluorescence induction (15, 16) was captured using fluorescence-activated cell sorting (FACS) to enrich for promoters active in tumors, and separately, for promoters active in spleen. Libraries enriched for active promoters in bacteria growing in tumor and spleen were then compared using an oligonucleotide tiling array of the *Salmonella* genome. The behavior of three bacterial promoters active in tumors but not in spleen was subsequently individually confirmed *in vivo*.

Materials and Methods

Vector construction. Promoter trap plasmids with TurboGFP were generated by PCR from the pTurboGFP plasmid.[4] The constructions, plasmids, bacterial strains, and primers used in this work are described in Supplementary Table S1.

Promoter library construction. *S. enterica* serovar *typhimurium* 14028 genomic DNA was sonicated and separated on a 1% agarose gel. Three hundred to 500 bp fragments were recovered from the gel and DNA ends were repaired by T4 DNA polymerase. Repaired fragments were cloned in a dephosphorylated pTurboGFP vector. Two libraries were constructed upstream of a promoterless TurboGFP, one stable and one destabilized. The two libraries combined (designated library-0) contained ~180,000 independent Typhimurium fragments, representing ~15-fold coverage of the 4.8 Mb genome, with clone spacing averaging every 25 bases. Hybridization to a *Salmonella* array showed that library-0 included sequences from almost the entire genome (deposited at GEO, GSE9998).[5]

NimbleGen array design. A high-resolution array was designed that contained 387,000 46-mer to 50-mer oligos, with the length adjusted to generate a similar predicted Tm. 377,230 of these probes were designed based on the Typhimurium LT2 genome (NC_003197; ref. 17). Oligonucleotides tiled the genome every 12 bases, on alternating strands. Thus, each base pair in the genome was represented in four to six oligonucleotides, with two to three oligos on each strand. Probes representing the three LT2 regions not present in the genome of the very closely related 14028s strain (phages Fels-1 and Fels-2, STM3255–3260) and >9,000 other oligos were included as controls for hybridization performance, synthesis performance, and grid alignment. The oligos were distributed in random positions across the array.

FACS analysis. Bacteria harboring the constitutive pTurboGFP plasmid were used as a positive control for the Becton Dickinson FACSAria FACS system. Side scatter ssc-w (*X*-axis) and ssc-H (*Y*-axis) were used to gate on single bacterial cells. GFP-fluorescence (GFP-A) on the *X*-axis and autofluorescence (PE) on the *Y*-axis permitted discrimination between green *Salmonella* cells and other fluorescent particles of different sizes. Fluorescent particles tended to be distributed on the diagonal of the GFP-A/PE plot, and had a fluorescence/autofluorescence ratio close to 1, whereas individual GFP-positive *Salmonella* cells had a higher ratio of fluorescence/autofluorescence and tended to be distributed close to the *X*-axis of the GFP-A/PE plot. Putative GFP-positive events in the window

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

Requests for reprints: Michael McClelland, Sidney Kimmel Cancer Center, 10905 Road to the Cure, San Diego, CA 92121. Phone: 858-450-5990; Fax: 858-450-3251; E-mail: mmcclelland@skcc.org.

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[4] http://www.evrogen.com/

[5] http://www.ncbi.nlm.nih.gov/geo/

enriched for GFP-expressing *Salmonella* were sorted at a speed of ~5,000 total events per second.

Results and Discussion

Enrichment of active promoters in spleen. To identify active *Salmonella* promoters in the spleen, five tumor-free nude mice were i.v. injected with 10^7 cfu of *Salmonella* carrying a promoter library. This library, designated "library-0" consisted of ~180,000 plasmid clones each containing a fragment of the *Salmonella* genome upstream of a promoterless GFP (see experimental procedures). Two days after injection, spleens were combined, homogenized on ice, and treated thrice with PBS containing 0.1% Triton X-100. An aliquot of the final homogenized sample was plated on Luria-Bertani (LB) medium with 50 μg/mL of ampicillin (Amp) to determine the number of bacterial colony-forming units (cfu). The remainder of the bacteria in the sample was immediately separated by FACS. Fifty thousand potentially GFP-positive events were sorted and this sublibrary was grown overnight in LB+Amp and designated "library-1." The spleen was chosen because it is the primary site of *Salmonella* accumulation in normal mice (18).

Enrichment of active promoters in tumor. The experimental design for tumor samples is described in Fig. 1. Five nude mice bearing human-PC3 prostate tumors, between 0.5 and 1 cm^3 in size, were injected intratumorally with 10^7 cfu of *Salmonella* promoter library-0. Two days after injection, tumors were combined, homogenized on ice and washed, as above. An aliquot was plated to determine the number of bacterial colony-forming units. The remainder of the sample was immediately separated by FACS. Fifty thousand GFP-positive events were recovered and grown overnight in LB+Amp containing ampicillin (library-2). A small aliquot of these bacteria were then pelleted and resuspended in PBS (10^6 cfu/mL) and FACS sorted. GFP-negative events (10^6) were collected, grown in LB overnight, washed in PBS and reinjected into five human-PC3 tumors in nude mice. After 2 days, bacteria were extracted from tumors and 50,000 GFP-positive events were FACS sorted and expanded in LB+Amp (library-3). A biological replicate of library-3 was obtained by repeating the experiment from the beginning using library-0. This was designated library-4.

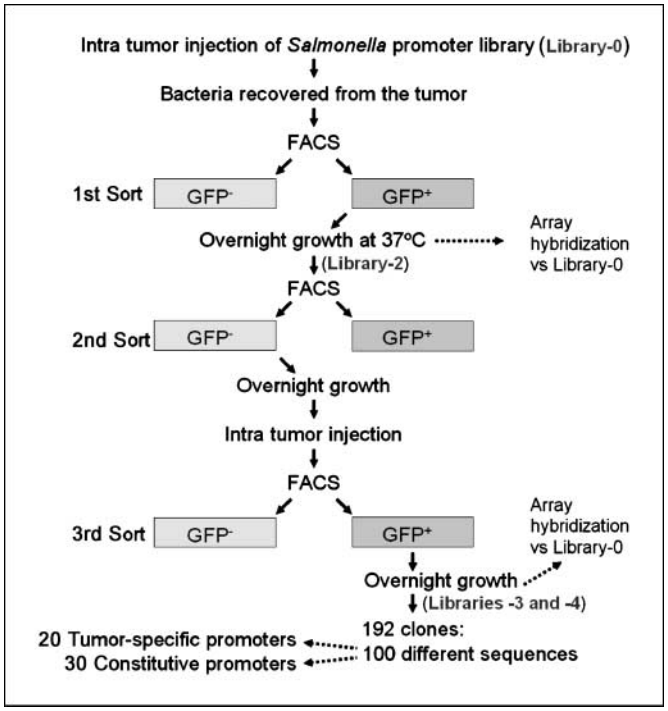

Figure 1. Enrichment of promoters that are active in tumor using differential fluorescence induction. Putative promoters activated in tumors were obtained in three enrichment steps: five nude mice bearing human PC3 prostate tumors were injected intratumor with 10^7 cfu of the *Salmonella* promoter library mixture. Two days after injection, tumors were combined and GFP-positive bacteria were recovered by FACS and grown overnight in LB+Amp medium (library-2). Subsequently, GFP-negative events were collected; the resulting pool was expanded overnight in LB+Amp, and injected into five tumor-bearing mice. Finally, library-3 was obtained after FACS sorting of GFP-positive bacteria, and overnight expansion in LB+Amp. A biological replicate (library-4) was obtained by repeating this entire experiment.

Table 1. Microarray-based classification of promoter clones in *Salmonella* growing in mouse spleen and tumors

Genome location	Promoter status		
	Not detected	Active in spleen and tumor	Preferentially active in tumor
Intragenic sequences	27	10	6
Intergenic sequences	7	30	20

NOTE: Sequencing of 192 clones from library-3 yielded 100 different sequences. The activation status of each of these 100 putative promoter sequences could be inferred from results of microarray hybridizations of spleen and tumor samples (library-1, library-2, library-3, and library-4).

Genomewide survey on tumor-activated promoters using NimbleGen arrays. Plasmid DNA was extracted from the original promoter library (library-0), from clones activated in spleen (library-1), and from clones activated in subcutaneous PC3 tumors in nude mice after one (library-2) or two passages (library-3 and library-4) in tumors. Promoter sequences were recovered by PCR using primers Turbo-4F and Turbo-1R (Supplementary Table S1), and the PCR product was labeled by CY5 (library-0) and CY3 (library-1, library-2, library-3, library-4). The resulting products were then hybridized to an array of 387,000 oligonucleotide sequences positioned at 12-base intervals around the Typhimurium genome (using the manufacturer's protocol).[6] Spot intensities were normalized based on total signal in each channel. The enrichment of genomic regions was measured by the intensity ratio of the tumor or the spleen sample versus the input library (library-0). A moving median of the ratio of tumor versus input library from 10 data points (~170 bases) was calculated across the genome. The highest median of each intergenic and intragenic region was chosen to represent the most highly overrepresented region of that promoter or gene in the tested library. Using a threshold of (exp / control) $\geq$ 2, and enrichment in both replicates of the experiment (library-4, plus at least one of library-2 or library-3), there were 86 intergenic regions enriched in tumors but not in the spleen (Supplementary Table S2), and 154 intergenic regions enriched in both tumor and spleen (Supplementary Table S3).

[6] http://www.nimblegen.com/

Table 2. Cloned candidate intergenic tumor-specific *Salmonella* promoters

Intergenic regions	Genome position of peak signal	Arbitrary clone number	Median ratio of experiment versus input			
			Spleen	Tumor (+)	Tumor (+)(−)(+)	Tumor (+)(−)(+)
			Library-1	Library-2	Library-3	Library-4
STM0468 - STM0469	526177	85	0.9	**2.3**	**5.5**	**9.5**
STM0474 - STM0475	529126	**86**	1.9	1.7	**3.2**	**2.6**
STM0580 - STM0581	638735	**87**	0.9	**3.2**	0.3	**8.5**
STM0844 - STM0845	914762	**10**	0.8	1.9	**5.8**	0.4
STM0937 - STM0938	1014704	**11**	0.7	**4.2**	**6.5**	**10.3**
STM1382 - STM1383	1466034	**16**	0.7	**4.6**	**7.4**	**13.9**
STM1529 - STM1530	1606103	**20**	1.9	**5.5**	**2.8**	**13.0**
STM1807 - STM1808	1909051	**26**	1.2	1.6	**6.5**	**9.7**
STM1914 - STM1915	2011503	**28**	0.9	**3.9**	**7.2**	**7.5**
STM1996 - STM1997	2079476	**30**	1.2	**2.9**	**7.4**	**4.0**
STM2035 - STM2036	2114187	**31**	1.3	**5.9**	**4.7**	**8.0**
STM2261 - STM2262	2359663	**34**	0.6	**2.1**	**3.5**	**4.8**
STM2309 - STM2310	2417301	**36**	0.6	**2.7**	**6.5**	**6.3**
STM3070 - STM3071	3233025	**44**	0.8	1.4	**2.8**	**3.1**
STM3106 - STM3107	3266543	**45**	1.1	**3.5**	**4.6**	**4.6**
STM3525 - STM3526	3688646	**55**	0.8	**3.8**	1.8	**5.6**
STM3880 - STM3881	4091492	**61**	0.9	**5.4**	0.1	**13.8**
STM4289 - STM4290	4530650	**71**	0.9	**2.0**	**8.3**	**10.0**
STM4418 - STM4419	4661108	**77**	0.8	**3.4**	**8.3**	**6.0**
STM4430 - STM4431	4674477	**78**	1.3	**6.1**	**5.6**	**8.0**

NOTE: Intergenic tumor-specific candidate promoters that were found in the sequence analysis of 100 clones from library-3 and by microarray hybridizations are listed. Sequences enriched more than 2-fold relative to the input library are shown in bold. Genes that are expected to be expressed from these 20 promoters, based on GFP orientation, are shown in boldface. Genes known to be anaerobically induced in *E. coli* and/or *Salmonella* are indicated with a "Yes." The last column indicates whether the clone was from a library with stable GFP or with destabilized GFP.

There were at least 30 regions enriched in spleen alone (Supplementary Table S4).

Sequencing of promoters. One hundred and ninety-two clones from a library that underwent two rounds of enrichment in tumor (library-3) were picked at random and sequenced, yielding 100 different sequences. These were mapped to the genome and their potential regulation (tumor-specific activation, or activation in both spleen and tumor) was determined by comparison with the microarray data (Table 1). The clones included 26 that were preferentially activated in tumors, and 40 that were activated both in tumor and spleen. 77% of the tumor-enriched clones (20 of 26) and 75% of the clones induced in both tumor and spleen (30 of 40) mapped at least partly to intergenic regions. As expected, none of these 100 clones were spleen-specific. The 20 intergenic clones supported by both biological replicates on array experiments are presented in Table 2.

Some possible tumor-specific promoters mapped inside annotated genes; 23% of the sequenced clones (6 of 26) and 18% of candidates identified by microarray (19 of 105; Supplementary Table S5). Some "promoters" may be artifacts that could arise from a variety of effects such as the inherent high copy number of the plasmid clone, or mutations that cause the copy number to increase or a new promoter to be created. However, based on data from *Escherichia coli*, a close relative of *Salmonella*, intragenic regions might indeed contain promoters, based on evidence from transcription start sites, binding sites for RNA polymerase (19, 20), and sigma factors (21) as well as motif finders (22). Further work may provide confirmatory evidence of promoter activity in some cases.

Some weaker promoters may generate detectable GFP in the stable, but not the destabilized, GFP plasmid library. Fifty clones sequenced after FACS selection could be assigned to either the stabilized or destabilized library. Forty of these were of the stable GFP variety versus an expected 25 of each type if there had been no bias. Therefore, the destabilized library is, as expected, underrepresented following FACS.

Confirmation of tumor specificity of individual clones *in vivo*. Five cloned promoters potentially activated in bacteria growing in tumor but not in the spleen were selected to be individually confirmed *in vivo*. A group of tumor-bearing mice and normal mice were injected i.v. with bacteria containing the cloned promoters. Tumors and spleens were imaged after 2 days, at low and high resolution using the Olympus OV100 small animal imaging system. Three of the five tumor-specific candidates (clones 10, 28, and 45) were induced much more in tumor than in spleen. Clone 44 produced low signals and clone 84 was highly expressed in tumor but was detectable in the spleen.

Among the most likely promoters to be uncovered in this study are those induced by hypoxia, which is thought to be an important contributor to *Salmonella* targeting of tumors (14). *Salmonella* promoters induced by hypoxia include those controlled directly or indirectly by the two global regulators of anaerobic metabolism, Fnr and ArcA (23). Clone 45 contains the promoter region of *ansB*, which encodes part of asparaginase. In *E. coli, ansB* is positively

Table 2. Cloned candidate intergenic tumor-specific promoters (Cont'd)

Arbitrary clone number	Cloned promoter	5′ gene	5′ gene orientation	3′ gene	3′ gene orientation	Anaerobically induced	Stable/Unstable GFP
85	+	*ylaB*	−	***rpmE2***	+		Unstable
86	−	***ybaJ***	−	*acrB*	−		Stable
87	−	**STM0580**	−	STM0581	+		Stable
10	−	***pflE***	−	*moeB*	−	Yes	Unstable
11	−	***hcp***	−	*ybjE*	−	Yes	Unstable
16	−	**orf408**	−	*ttrA*	−		Stable
20	−	STM1529	+	STM1530	+		Stable
26	+	*dsbB*	+	**STM1808**	+		Stable
28	−	***flhB***	−	*cheZ*	−		Unstable
30	−	***cspB***	−	*umuC*	−		Stable
31	−	***cbiA***	−	*pocR*	−		Stable
34	−	***napF***	−	*eco*	+	Yes	Stable
36	−	***menD***	−	*menF*	−		Stable
44	−	***epd***	−	STM3071	+		Unstable
45	−	***ansB***	−	*yggN*	−	Yes	Stable
55	+	*glpE*	+	***glpD***	+		Stable
61	+	*kup*	+	***rbsD***	+		Stable
71	−	***phnA***	−	*proP*	+		Unstable
77	+	STM4418	−	**STM4419**	+		Stable
78	+	STM4430	−	**STM4431**	+		Stable

coregulated by Fnr and by CRP (cyclic AMP receptor protein), a carbon source utilization regulator (24). In *S. enterica*, the anaerobic regulation of *ansB* may require only CRP (25, 26). Clone 10 is the promoter region of a putative pyruvate-formate-lyase activating enzyme (*pflE*). This clone was only observed in library-3, but enrichment was considerable in that library (Table 2). This clone was pursued further because the operon is coregulated in *E. coli* by both ArcA and Fnr (27, 28). Finally, clone 28 contains the promoter region of *flhB*, a gene that is required for the formation of the flagellar apparatus (29) and is not known to be regulated in anaerobic metabolism.

Further screening was performed on these three clones. Bacteria containing these clones were i.v. injected at 5×10^6, 5×10^7, and 5×10^7 cfu into tumor- and non–tumor-bearing nude mice. One or 2 days postinjection, spleens and tumors were imaged using the OV100 imaging system, homogenized, and the bacterial titer was quantified on LB+Amp. Spleens from normal mice were compared with tumors that had a similar number of colony-forming units, so that any difference in fluorescence would be attributable to increased GFP expression rather than bacterial numbers. Figure 2 confirms that tumors are much more fluorescent than spleens infected with the same number of bacteria for each of the three clones. A positive control that constitutively expresses TurboGFP resulted in strong fluorescence in spleen even with doses as low as 2×10^5 cfu.

The *Salmonella* endogenous promoter for *pepT* is regulated by CRP and Fnr (14). In previous studies, the TATA and the Fnr binding sites of this promoter were modified to engineer a hypoxia-inducible promoter that drives reporter gene expression under both acute and chronic hypoxia *in vitro* (14). Induction of the engineered hypoxia-inducible promoter *in vivo* became detectable in mice 12 hours after death, when the mouse was globally hypoxic (14). In our experiments, the wild-type *pepT* intergenic region did not pass the threshold to be included in the tumor-specific promoter group. Perhaps the appropriate clone is not represented in the library, or induction (i.e., level of hypoxia in the PC3 tumors) was not enough for this particular promoter.

In summary, *Salmonella* thrives in the hypoxic conditions found in solid tumors (14). There are four promoters known to be regulated by hypoxia among the 20 sequenced intergenic clones (Table 2), of which two (clones 10 and 45) were tested and shown to be induced in tumors (Fig. 2). Many candidate promoters that seem to be preferentially activated within tumors may be unrelated to hypoxia, including clone 28 (Fig. 2). Any promoters that are later proven to respond in their natural context in the genome may illuminate conditions within tumors, other than hypoxia, that are sensed by *Salmonella*.

Attenuated *Salmonella* strains with tumor-targeting abilities (1–10) could be used to deliver therapeutics under the control of promoters preferentially induced in tumors. Such promoters are technically useful whether or not they are regulated in the same way in their natural context in the genome. These promoters would be tools to reduce the expression of the therapeutic in bacteria outside the tumor, and thus reduce side effects, thereby producing a highly selective and effective therapy for metastatic cancer. Further sophistications are also possible. For example, combinations of two or more promoters that are preferentially induced in tumors by different regulatory mechanisms would allow the delivery of two or more separate protein components of a therapeutic system under different regulatory pathways. In addition, new promoter systems induced by external agents such as arabinose (30) or salicylic acid (31) allow promoters in *Salmonella* to be induced throughout the body at the time of choice. Such inducible regulation could be combined with tumor-specific *Salmonella* promoters to express useful products in

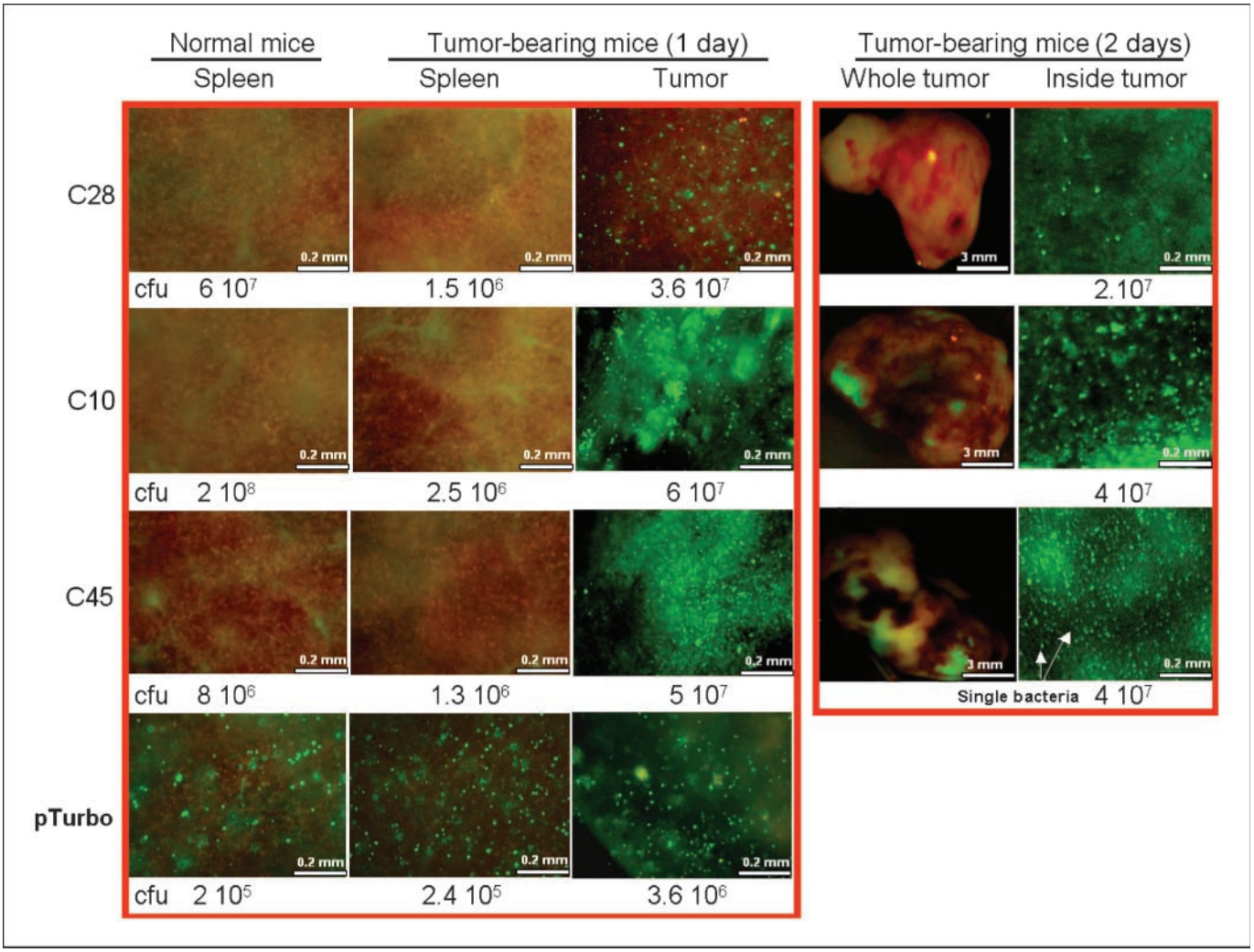

Figure 2. OV100 microscopic imaging of fluorescent bacteria in mouse spleen and tumors. *Left,* clones; *C28, flhB* upstream intergenic region (IR); *C10, pefL* IR; *C45, ansB* IR; *pTurbo,* control with a constitutive promoter (pTurboGFP). Each clone was i.v. injected into nude mice. The number of colony-forming units of *Salmonella* recovered from each sample is shown. Images were chosen so that the colony-forming units recovered were approximately equal between spleen and tumor for any particular clone, so that fluorescence differences could be attributed to changes in protein expression rather than differences in the number of bacteria.

the tumor only when the exogenous activator is added; therapy delivery would be exquisitely controlled both in time and space.

Disclosure of Potential Conflicts of Interest

The authors are pursuing the commercial application of engineered infectious agents to cancer therapeutics.

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Accession numbers: microarray hybridization data are accessible as GSE9998 at the GEO depository of the NCBI (http://www.ncbi.nlm.nih.gov/geo/).

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