In vivo gene regulation in *Salmonella* spp. by a salicylate-dependent control circuit

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Systems allowing tightly regulated expression of prokaryotic genes *in vivo* are important for performing functional studies of bacterial genes in host-pathogen interactions and establishing bacteria-based therapies. We integrated a regulatory control circuit activated by acetyl salicylic acid (ASA) in attenuated *Salmonella enterica* that carries an expression module with a gene of interest under control of the XylS2-dependent *Pm* promoter. This resulted in 20–150-fold induction *ex vivo*. The regulatory circuit was also efficiently induced by ASA when the bacteria resided in eukaryotic cells, both *in vitro* and *in vivo*. To validate the circuit, we administered *Salmonella* spp., carrying an expression module encoding the 5-fluorocytosine–converting enzyme cytosine deaminase in the bacterial chromosome or in a plasmid, to mice with tumors. Induction with ASA before 5-fluorocytosine administration resulted in a significant reduction of tumor growth. These results demonstrate the usefulness of the regulatory control circuit to selectively switch on gene expression during bacterial infection.

Development of systems that allow controlled expression of heterologous genes is critical for the study of gene function. These systems would open new perspectives in the functional analysis of host-pathogen interactions, allowing assessment of the importance of specific virulence genes during the infection process. They would also facilitate the exploitation of recombinant microorganisms for targeted expression of therapeutic molecules. Therefore, effort has been invested in the development of expression systems that allow tightly regulated expression of prokaryotic genes under *in vitro* and *in vivo* conditions[1–3].

Previous expression systems have relied on the use of inducers or on the alteration of environmental conditions. These approaches, however, are not suitable for activation in eukaryotic cells. Promoters derived from virulence genes have permitted the generation of expression systems activated upon bacterial infection[4,5]. These systems, however, are either silent (extracellularly) or activated (within eukaryotic cells), preventing gene expression at will during specific stages of the infection process[1,3,6]. Thus, expression systems have been developed that could be activated in response to external stimuli such as tetracycline, mitomycin or X-rays, even within eukaryotic cells[2,7–9]. Unfortunately, these improved systems are suboptimal, as their implementation is barely compatible with *in vivo* studies because of sub-optimal pharmacokinetics and toxicity of the inducer. Additionally, an ideal system should have negligible basal expression (minimal metabolic burden) and high induction ratio (critical for genes encoding toxic products). Furthermore, none of the existing inducers can breach all biological barriers, reaching bacteria within the endocytic compartment without side effects. Thus, the implementation of a regulatory system permitting tight *in situ* control of bacterial gene expression during microbial transit across different niches during infection is still an elusive target.

ASA is one of the most widely used and best-characterized analgesic and anti-inflammatory drugs on the market[10]. The biological half-life of ASA is only 20 min, as it is rapidly converted into salicylic acid, which has a half-life of 2–4 h. An extensive body of clinical and experimental evidence describes the pharmacological properties of ASA[11,12]. Salicylate-responsive regulatory factors control the naphthalene degradative pathway in *Pseudomonas putida*. The regulatory protein NahR and its target promoters *Psal* or *Pnah* have been used to express heterologous genes[13,14]. The *nahR-Psal* regulatory system is tightly regulated (20–100-fold induction) in response to the natural inducer salicylate[15,16]. Regulatory systems induced by aromatic compounds can also be activated by ASA, such as the mutant *xylS2* regulator of the meta-operon in the toluene-xylene catabolic pathway of *P. putida*[13]. The regulatory capacity of these systems could be amplified 7–20-fold by using a regulatory cascade, in which the regulators (NahR and XylS2) are simultaneously activated by salicylate or ASA[13,16].

Here we implemented and validated an *in vivo* ASA or salicylate-inducible cascade expression system based on a regulatory circuit integrated into the chromosome of an attenuated *Salmonella enterica aroA* (SL7207-4S2 strain). This allows tightly regulated *in vivo* expression of the target gene after bacterial infection in response to salicylate. *In vitro* characterization studies revealed induction ratios of 20–150-fold. *S. enterica* can replicate within solid tumors when delivered systemically, and this has

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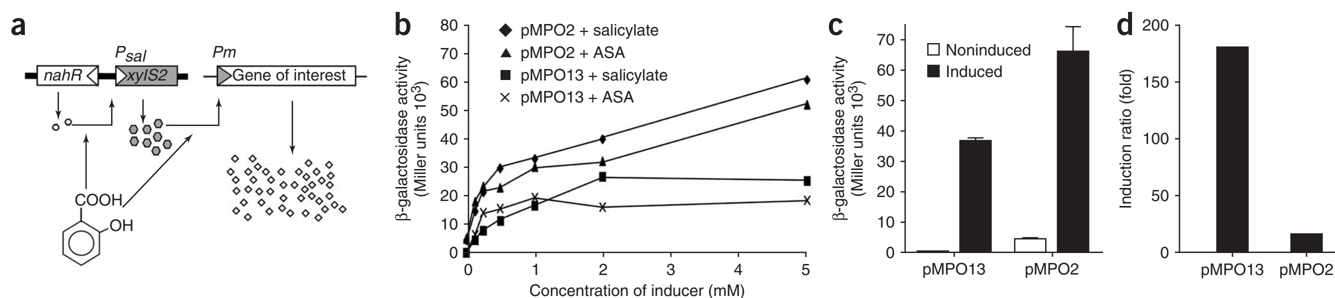

Figure 1 | Tightly regulated expression of *Salmonella* spp. genes by using a circuit based on the regulatory module *nahR/Psal::xylS2*. (**a**) Schematic representation of the regulatory circuit. When ASA or salicylate is present, NahR activates transcription from *Psal*, thereby leading to the expression of XylS2. ASA or salicylate also activates XylS2, prompting high levels of gene expression from the *Pm* promoter owing to a synergic effect. (**b**) Dose-response curve for SL7207-4S2 strains carrying the low (pMPO13) and high (pMPO2) copy number expression vectors encompassing the *Pm-trp′::′lacZ* cassette after 4-h induction with different concentrations of salicylate or ASA. (**c**,**d**) Expression levels and induction ratios obtained using the low (pMPO13) and high (pMPO2) copy number vectors in the presence of 2 mM salicylate for 4 h. Error bars, s.d. ($n = 3$). The values reported in **b** correspond to one representative experiment out of 3, and those presented in **c** and **d** are the average of the independent tests.

previously been exploited for the delivery of therapeutic genes in humans[9,17–21]. Thus, we validated our system *in vivo* in mice challenged with a fibrosarcoma. We administered *S. enterica* carrying an expression module encoding the 5-fluorocytosine–converting enzyme cytosine deaminase to mice bearing tumors. ASA induction before treatment with 5-fluorocytosine resulted in a significant reduction ($P < 0.01$) in tumor growth in comparison to controls and mice receiving bacteria in which cytosine deaminase expression was controlled by a tetracycline-inducible system. These results demonstrate the potential of this approach to achieve tightly regulated expression of prokaryotic genes *in vivo*. We expect that this method will facilitate functional studies to elucidate the role of bacterial genes during different phases of the infection process as well as the implementation of bacteria-based therapies.

RESULTS

Regulated *Salmonella* spp. gene expression in culture medium

We integrated the regulatory circuit for the salicylate-inducible cascade expression system (Active Motif; **Fig. 1a**) into the chromosome of an attenuated *S. enterica aroA* (SL7207-4S2 strain; see **Supplementary Table 1** online for a list of all strains, plasmids and cell lines used in this work). We introduced a target gene (*lacZ*) under the control of the *Pm* promoter using an expression plasmid. When we used either ASA or salicylate to activate the cascade amplification circuit (**Fig. 1a**), we observed a dose-dependent expression of β-galactosidase in clones carrying the *lacZ* expression vector (**Fig. 1b**). Using a low-copy-number plasmid expressing *lacZ* (pMPO13) resulted in 20-fold lower basal expression levels than using a high-copy-number plasmid (pMPO2; 204 versus 4,384 Miller units). After induction with 2 mM salicylate, however, the synergistic amplification effect of the regulatory circuit integrated into the chromosome resulted in much higher and similar enzymatic activities using both vectors (36,792 versus 66,185 Miller units, respectively; **Fig. 1c**). We obtained a tenfold higher induction rate in clones containing pMPO13 compared to those carrying pMPO2 (180 versus 15 Miller units; **Fig. 1d**), thereby suggesting that pMPO13 is the most promising vector for validating the regulatory circuit.

ASA and salicylate induced gene expression in infected cells

To test the performance of the regulatory circuit when *S. enterica* reside in the intracellular compartment of mammalian cells, we infected HeLa cells with *S. enterica* carrying pMPO2 or pMPO13. We detected basal expression only in cells infected with bacteria carrying pMPO2 (**Supplementary Fig. 1** online). In contrast, we observed similar β-galactosidase expression in HeLa cells infected with bacteria bearing pMPO2 or pMPO13 after induction (see **Supplementary Fig. 1**). We observed no differences in protein expression when we used ASA or salicylate as inducers, thereby confirming that both compounds reached intracellular bacteria. We obtained comparable results using the macrophage-like cell line

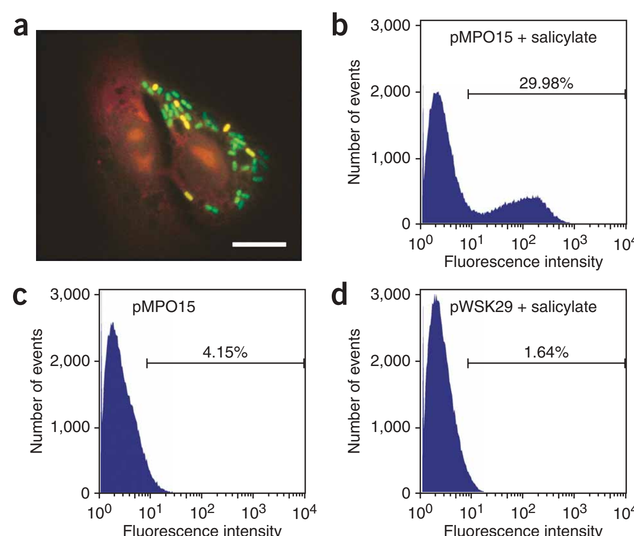

Figure 2 | Intracellular expression of GFP in tumor cells by recombinant *S. enterica* carrying the *gfp* coding gene under control of the regulatory module *nahR/Psal::xylS2*. (**a**) Fluorescence micrograph of F1.A11 cells infected with SL7207-4S2 containing the GFP-encoding vector pMPO15 after induction of protein expression with 2 mM salicylate for 4 h. Scale bar, 10 μm. (**b**–**d**) Results of flow cytometric analysis of cells infected with SL7207-4S2 carrying either the GFP-encoding vector pMPO15 (**b**,**c**) or pWSK29 (**d**) to determine the number of F1.A11 cells containing GFP-producing bacteria. The plots in **b** and **d** correspond to salicylate-induced cells (2 mM for 4 h). The analysis is representative of 3 independent experiments. Percentages indicate eukaryotic cells emitting fluorescence above background.

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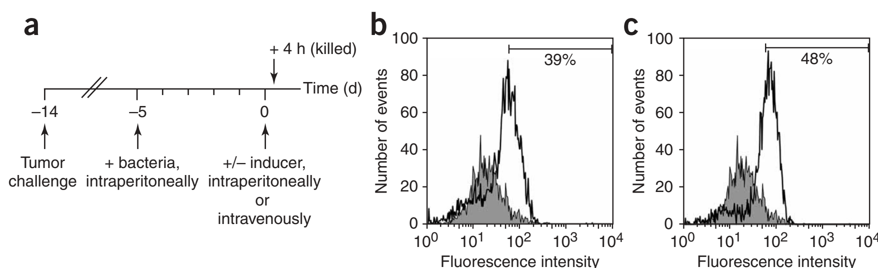

Figure 3 | Tightly regulated *in vivo* expression of prokaryotic genes within tumors using an ASA or salicylate–activated control circuit based on the regulatory module *nahR/Psal::xylS2*. (**a**) Schematic representation of the experimental design. (**b**,**c**) Flow cytometric analysis for bacterial GFP expression in tumor cells recovered from mice infected with SL7207-4S2 carrying pMPO15, 4 h after induction with 150 μl of salicylate (100 mM) intraperitoneally (**b**) or intravenously (**c**), compared to the noninduced controls (gray filled plots). The analysis is representative of two independent experiments.

J774.A1 (data not shown). These results suggested that the inducer can exert its activity on intracellular bacteria, and that the low-copy-number vector was the more appropriate for subsequent studies.

Regulated *Salmonella* spp. gene expression in infected mice

In preliminary studies we evaluated expression of the reporter gene within the cell line to be used for induction of tumors in mice. We infected F1.A11 cells, which derive from a spontaneous murine fibrosarcoma, with an SL7207-4S2 derivative carrying a plasmid that encodes *gfp*, pMPO15, that was derived by replacement of *Pm-lacZ* in pMPO13 with *Pm-gfp* (**Supplementary Table 1**). After 4 h induction with 2 mM salicylate, fluorescence microscopy revealed GFP-expressing bacteria in tumor cells (**Fig. 2a**). Flow cytometry analysis inidcated that 30% of the eukaryotic cells were GFP-positive (**Fig. 2b**). In contrast, only 4% of the cells were GFP-positive in the absence of the inducer, and 1.6% in control cells containing *S. enterica* carrying the empty vector pWSK29 (**Fig. 2c**,**d**).

We also injected mice intraperitoneally with 10^6 c.f.u. of the *S. enterica* strain SL7207-4S2 carrying either the *gfp* encoding plasmid pMPO15 or the empty vector pWSK29. Thirty minutes after infection, mice received salicylate intraperitoneally. After 4 h, we analyzed cells obtained from peritoneal lavages, mesenteric lymph nodes and spleens for GFP expression by flow cytometry. We did not detect GFP-positive cells resulting from infection by *S. enterica* in peritoneal lavages (data not shown). In contrast, 71% of cells in spleens were GFP-positive (**Supplementary Fig. 2** online).

Prokaryotic niche-specific gene expression in mice

To evaluate whether the regulatory control circuit can be exploited to achieve tightly regulated *in situ* expression, we challenged mice with the cell line F1.A11 (**Fig. 3a**). When the F1.A11-derived tumors were palpable (1–1.5 mm in diameter; approximately 0.1–0.2 g), we administered 10^6 c.f.u. of the strain SL7207-4S2 carrying the *gfp* encoding plasmid pMPO15 intraperitoneally. After 4 days, we divided mice into two groups, which we injected with salicylate either intraperitoneally or intravenously. Four hours after induction we killed the mice, excised tumors and lymphoid organs, and prepared and plated cellular suspensions to determine the

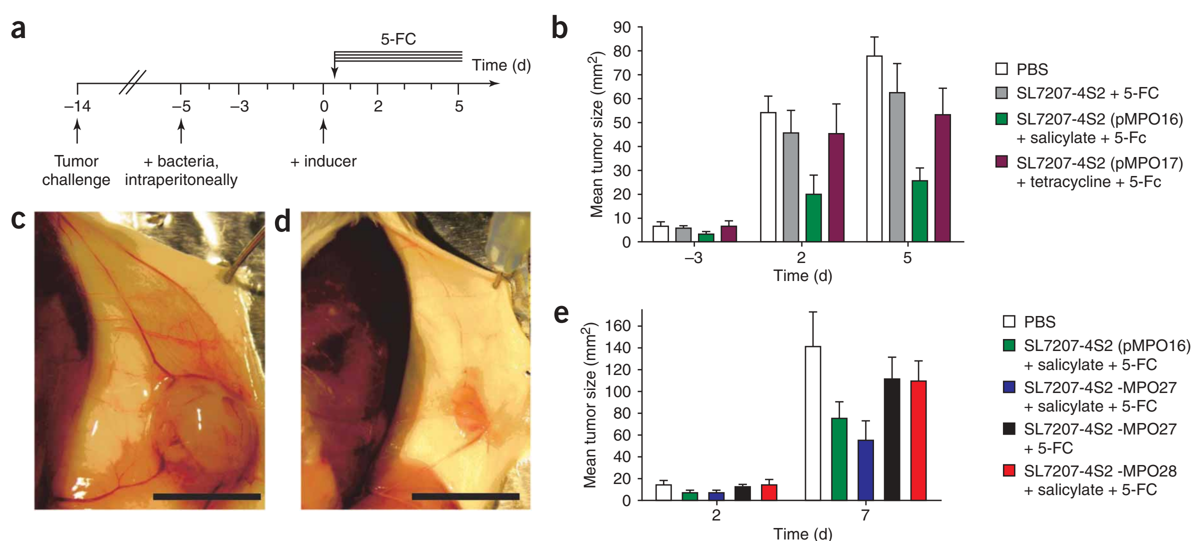

Figure 4 | Salicylate-mediated *in vivo* expression of cytosine deaminase in tumor cells by using the control circuit based on the *nahR/Psal::xylS2* regulatory module. (**a**) Schematic representation of the experimental design. We arbitrarily assigned day 0 to the day when the inducer was administered. (**b**) Tumor growth in untreated mice (PBS), and in mice receiving plasmid-less SL7207-4S2 or bacteria carrying vectors with the *codA* gene under control of either salicylate (pMPO16) or the tetracycline (pMPO17) induced expression systems. After 5 days, we induced protein expression, and initiated 5-fluorocytosine (5-FC) therapy 4 h later. (**c**,**d**) Consistent differences in tumor size were macroscopically evident on day 5 between SL7207-4S2 (pMPO16)–treated (**c**) and PBS-treated (**d**) mice. Scale bar, 10 mm. (**e**) Tumor growth in control mice (PBS), and in mice receiving SL7207-4S2 (pMPO16) or SL7207-4S2 in which the *codA* encoding (SL7207-4S2-MPO27) or control (SL7207-4S2-MPO28) expression modules was integrated into the chromosome. Error bars, s.d. ($n = 6$). The analysis is representative of three independent experiments.

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number of c.f.u./g of tissue. As expected, bacteria were enriched within tumors (an average of 3×10^6, 2×10^6 and 1×10^8 c.f.u./g in spleen, liver and tumors, respectively). We then analyzed tumor cells by flow cytometry to evaluate the presence of GFP-expressing bacteria. Approximately 39 and 48% of the tumor cells were GFP-positive after induction with salicylate by intraperitoneal and intravenous route, respectively (**Fig. 3b,c**). In contrast, only 11% of the F1.A11 cells from noninduced controls contained GFP (**Fig. 3**). We did not observe GFP-positive cells in tumors in samples from mice that received *S. enterica* carrying the empty vector (data not shown).

Expression of a prodrug-converting enzyme in tumor cells

Mammalian cells are resistant to 5-fluorocytosine because they lack cytosine deaminase, an enzyme that converts 5-fluorocytosine into 5-fluorouracil, a cytotoxic compound routinely used in cancer chemotherapy. We evaluated the capacity of the new system to selectively deliver a prodrug-converting enzyme into solid tumors *in vivo*. Thus we compared the effect on tumor growth of infecting *S. enterica* carrying vectors with the *Escherichia coli* cytosine deaminase–encoding gene *codA* under the control of either the salicylate-inducible circuit (pMPO16; **Supplementary Table 1**) or the tetR-tetO promoter and operator (pMPO17; **Supplementary Table 1**). When tumors were palpable, we intraperitoneally administered SL7207-4S2 carrying either pMPO16 or pMPO17 (10^6 c.f.u.). After 5 days, we induced cytosine deaminase expression by a single intraperitoneal injection of salicylate or tetracycline (**Fig. 4a**), and initiated 5-fluorocytosine therapy 4 h after induction. We observed similar tumor growth in mice receiving *S. enterica* in combination with 5-fluorocytosine or phosphate-buffered saline (PBS; **Fig. 4b**). We detected slower tumor progression in mice treated with 5-fluorocytosine that were injected with SL7207-4S2 carrying pMPO17, wherein *codA* expression was induced by tetracycline (**Fig. 4b**). The differences, however, were not statistically significant with respect to controls ($P > 0.05$). In contrast, the size of the tumors from 5-fluorocytosine–treated mice receiving SL7207-4S2 carrying pMPO16, wherein cytosine deaminase expression was induced by salicylate, was significantly smaller than in controls ($P < 0.01$; **Fig. 4b–d**).

These results provide the proof of concept for the usefulness of the regulatory circuit to control prokaryotic gene expression *in vivo*. Broad implementation of this platform, however, might require stabilization of the expression module. Thus, we evaluated the performance of strains in which the expression module was integrated into the chromosome. Tumor size in 5-fluorocytosine–treated mice receiving bacteria expressing *codA* from the chromosome (SL7207-4S2-MPO27; **Supplementary Table 1**) was similar to that observed in mice receiving the strain carrying the expression module in a plasmid (SL7207-4S2 with pMPO16), but only after induction (**Fig. 4e**). In contrast, tumors in mice injected with PBS or with a control strain, where the expression module without *codA* was integrated (SL7207-4S2-MPO28; **Supplementary Table 1**), were significantly larger ($P < 0.05$; **Fig. 4e**).

DISCUSSION

The availability of genomic information from bacterial pathogens now permits essential *in vivo* functional studies on putative virulence genes. This work can be addressed more efficiently by using tightly regulated expression systems. These will allow assessment of the role of specific genes at different stages of the infection process. Presently available systems, however, are suboptimal for *in vivo* studies. The main aim of this work was to validate a system to obtain tightly regulated expression of prokaryotic genes *in vivo* based on inducers exhibiting an adequate pharmacokinetic and safety profile.

Our strategy allows a tightly regulated expression of selected genes under the control of the *Pm* promoter *in vivo*, and presence of the inducers led to activation of protein expression regardless of the topology (intracellular or extracellular). This suggests that the eukaryotic environment does not interfere with gene expression, despite considerable differences in growth conditions. We observed similar expression levels in *S. enterica* carrying low (pMPO13) and high-copy-number (pMPO2) vectors. Although the background expression was 20-fold lower using the low-copy-number vector, more than 50% of the maximal reporter expression was reached after induction. As genetic constructs in low-copy-number vectors are more stable *in vivo*, even without selective pressure[6,22,23], they seem to be the vectors of choice for *in vivo* studies when monocopy gene dosage is not feasible or not required.

The developed approach is extremely flexible, as it is possible to integrate additional expression cassettes under control of *Pm* without titrating out its activator XylS2, which is overexpressed by the upstream regulator *nahR-Psal*. The choice of salicylate as inducer of the regulatory cascade was based on its rapid absorption, broad biological distribution in different tissues, short half-life as well as the lack of toxicity when administered at therapeutic dosages compatible with cascade induction, which would allow transient expression for $\sim$ 9 h after induction. Additional advantages of the system are the minimized metabolic burden resulting from a tight regulation and the high induction levels, which also allow using monocopy gene dosage[16].

In addition to its application in the study of host-pathogen interactions, this approach can be exploited for biomedical interventions in which bacterial vectors are used for expression of heterologous antigens or the targeted delivery of anticancer agents[21,24–26], as the number of viable *S. enterica* start to decline in spleen 4 d after infection to nondetectable levels around day 20 (refs. 6,22). Thus, we evaluated whether the regulatory control circuit can be exploited to deliver the 5-fluorocytosine–converting enzyme cytosine deaminase in tumor cells *in vivo*. Upon induction with salicylate there was a significant reduction in tumor progression in mice treated with strains carrying the expression module either in a plasmid or integrated into the chromosome with respect to controls and mice receiving *S. enterica* in which cytosine deaminase expression was controlled by a tetracycline-inducible system.

Although *S. enterica* gene expression in cell cultures strongly depends on the host cell type[27], suggesting that gene expression in host tissues might be difficult to predict, our data showed that recombinant *S. enterica* bearing the *nahR::Psal/xylS2* module displayed high induction rates within macrophages, epithelial cells and tumor cells. None of the other systems that had been proposed to control *S. enterica* gene expression *in situ* can be compared with the salicylate-dependent regulatory circuit in terms of efficiency, flexibility and safety. Thus, our regulatory control circuit may constitute a cornerstone for functional studies of bacteria-host interactions during the infection process, as well as for the establishment of novel therapeutic interventions.

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METHODS

***In vitro* characterization of clones bearing the expression circuit.** We induced with either sodium salicylate or ASA (Sigma) SL7207-4S2 derivatives carrying β-galactosidase– or GFP-encoding vectors grown in LB broth at 37 °C to an optical density at 600 nm (OD_{600}) of 0.3. After 4 and 6 h, we evaluated the production of β-galactosidase by determining the number of Miller units[28].

***In vitro* infection and intracellular expression studies.** We grew bacteria at 37 °C in LB broth supplemented with 0.3 M sodium chloride and 100 μg/ml ampicillin. We grew HeLa and J774.A1 cells on coverslips placed in 24-well tissue culture plates (Nunc) in Dulbeccós modified Eagle's medium (DMEM; Gibco BRL) supplemented with 2 mM L-glutamine and 10% FCS at 37 °C until they reached 60–80% confluence. We added 10^6 c.f.u. to each well, and incubated cells for an additional 1 or 2 h for J774A.1 and HeLa cells, respectively. We then washed cells, added fresh DMEM supplemented with 50 μg/ml of gentamicin to kill extracellular bacteria, and induced gene expression with sodium salicylate or ASA (2 mM). After a 4-h incubation, we washed, fixed (2% paraformaldehyde) and stained with a β-galactosidase staining kit (Roche) cells infected with the *lacZ* vector–containing *S. enterica*. To evaluate GFP expression in the intracellular compartment of F1.A11 cells, we induced cells as described above and analyzed them by fluorescence microscopy. For quantification, we detached infected cells with trypsin and analyzed them by flow cytometry using a FACScalibur cytometer and the CellquestPro software (Becton-Dickinson).

***In vivo* analysis of clones bearing the regulatory circuit.** We intraperitoneally injected mice with 10^6 c.f.u. of SL7207-4S2 (pMPO15). After 30 min, we injected 150 μl of sodium salicylate (100 mM) intraperitoneally or intravenously, which is compatible with accepted ASA dosages[12]. After 4 h, we killed the mice and analyzed single-cell suspensions obtained from spleens, mesenteric lymph nodes and peritoneal lavages, for the presence of GFP-positive cells by flow cytometry. Animals were treated in accordance with local and European Community guidelines.

To evaluate activation of the regulatory circuit within tumors, mice received a subcutaneous injection into the right flank with 5×10^4 cells from a spontaneous murine fibrosarcoma (F1.A11 cells) resuspended in 100 μl of PBS. When palpable tumors developed, we intraperitoneally injected mice with 10^6 c.f.u. of SL7207-4S2 (pMPO15) . Five days after infection, we induced the expression of *gfp*, as described above. After 4 h, we killed the mice and removed tumors, spleens and livers for flow cytometric analysis and determination of bacterial viable counts.

***In vivo* comparison of salicylate or tetracycline inducible systems.** Mice were challenged with F1.A11 cells, as described above. When tumors were palpable, mice intraperitoneally received 10^6 c.f.u. of SL7207-4S2 carrying *codA* under control of the salicylate-responsive regulatory circuit (pMPO16) or the tetR-tetO promoter-operator (pMPO17), as well as derivatives in which the expression module and control modules were integrated into the chromosome (SL7207-4S2-MPO27 and SL7207-4S2-MPO28). Control mice received PBS or 10^6 c.f.u. of plasmid-less SL7207-4S2. After 5 d, we induced expression of *codA* by intraperitoneal injection of salicylate or tetracycline (100 μg). Four hours later, we initiated 5-fluorocytosine therapy (300 mg/kg every 12 h). We measured tumor growth using calipers at the narrowest and longest surface lengths. We calculated tumor size as the product of the mean of these two lengths per mouse averaged over the total number of mice per group ($n = 6$). We calculated statistical differences in tumor size using the Student's *t*-test. We euthanized mice when tumors were 10 mm long to avoid unnecessary suffering.

Additional methods. Strains, plasmids and cell lines are described in **Supplementary Table 1**. We also provide full description of the integration of the regulatory module *nahR*/*Psal*::*xylS2* and the *codA* expression module into the *S. enterica* chromosome in **Supplementary Methods** online.

Note: Supplementary information is available on the Nature Methods website.

AUTHOR CONTRIBUTIONS

J.L.R. and P.D.B. performed most of the experimental work and analysis of data, and contributed to experimental design. E.M.C. mapped the insertion of the regulatory module and constructed the strains bearing the expression module integrated into the *aroC* locus. A.C. initially designed the cascade expression system for use in eukaryotic cells. C.L. contributed to the *in vivo* work. E.S. and C.A.G. were responsible for experimental design, participated in the analysis of raw data and wrote the paper.

COMPETING INTERESTS STATEMENT

The authors declare competing financial interests: details accompany the full-text HTML version of the paper at http://www.nature.com/naturemethods/.

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