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

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ABSTRACT

Systems enabling 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* 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 ratio *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,* carrying an expression module encoding the 5-fluorocytosine-converting enzyme cytosine deaminase in the bacterial chromosome or in a plasmid, to mice bearing 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.

The development of systems enabling 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-parasite interactions, allowing assessment of the role played by 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 enabling a tightly regulated expression of prokaryotic genes under *in vitro* and *in vivo* conditions[1-3].

Previous expression systems relied on inducers or alteration of the environmental conditions. However, these approaches were not suitable for activation within eukaryotic cells. Promoters derived from virulence genes allowed the generation of expression systems activated upon bacterial infection[4,5]. These systems, however, were 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 were 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, since their implementation is barely compatible with *in vivo* studies due to pharmacokinetics and toxicity of the inducer. In addition, 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 enabling tight *in situ* control of bacterial gene expression during microbial transit across different niches during infection, still remains as an elusive target.

Acetyl salicylic acid (ASA) is one of the most widely used and best-characterized analgesic and anti-inflammatory drugs on the market[10]. The biologic half-life of ASA is only 20 min, since 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 its pharmacological properties[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 further 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 enables tightly regulated *in vivo* expression of the target gene after bacterial infection in response to salicylate. *In vitro* characterization studies showed induction ratios ranging between 20-150 fold. *Salmonella* can replicate within solid tumors when delivered by systemic route, and this has 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 *Salmonella* carrying an expression module encoding the 5-fluorocytosine-converting enzyme cytosine deaminase (CD) to mice bearing tumors. ASA induction before treatment with 5-fluorocytosine (5-FC) resulted in a significant reduction ($P<0.01$) in tumor growth in respect to both controls and mice receiving bacteria in which CD expression was controlled by a tetracycline-induced system. These results showed 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 played by bacterial genes during different phases of the infection process, as well as the implementation of bacterial-based therapies.

RESULTS

Regulated *Salmonella* gene expression in culture medium.

We integrated the regulatory circuit for the salicylate-inducible cascade expression system from Active Motif (**Fig. 1a**) into the chromosome of an attenuated *Salmonella enterica aroA* (SL7207-4S2 strain) (see **Supplementary Table 1** online). A target gene (*lacZ*) under the control of the *Pm* promoter was introduced 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; **Supplementary Table 1**) resulted in 20-fold lower basal expression levels than using a high copy number plasmid (pMPO2; **Supplementary Table 1**) (204 *vs* 4,384 Miller units). However, after induction with 2 mM salicylate, 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 *vs* 66,185, respectively; Fig. 1c). We obtained a ten-fold higher induction rate in clones

harboring pMPO13 with respect to those carrying pMPO2 (180 *vs* 15; Fig. 1d), thereby suggesting that pMPO13 is the most promising vector for validating the regulatory circuit.

ASA and salicylate induced gene expression within infected cells.

To test the performance of the regulatory circuit when *Salmonella* reside in the intracellular compartment, we infected HeLa cells with *Salmonella* carrying pMPO2 or pMPO13. We detected basal expression only in cells infected with *Salmonella* carrying pMPO2 (see Supplementary Fig. 1 online). On the other hand, we observed similar β-galactosidase expression in HeLa cells infected with *Salmonella* bearing pMPO2 or pMPO13 after induction (see Supplementary Fig. 1 online). No differences in protein expression were evident 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 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 further studies.

Regulated *Salmonella* gene expression in infected mice.

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

We also injected mice by intraperitoneal (ip) route with 10^6 colony-forming units (CFU) of the *S. enterica* strain SL7207-4S2 carrying either the *gfp* encoding plasmid pMPO15 or the empty vector pWSK29. Thirty minutes after infection, animals received salicylate by ip route. 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 in peritoneal lavages (data not shown). In contrast, we observed GFP positive cells (71%) in spleens (see 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, mice were challenged with the cell line F1.A11 (Fig. 3a). When the F1.A11-derived tumors were palpable (1-1.5 mm of diameter; approximately 0.1-0.2 g), we administered 10^6 CFU of the strain SL7207-4S2 carrying the *gfp* encoding plasmid pMPO15 by ip route. After 4 days, we divided mice into two groups, which were injected with salicylate by ip or intravenous (iv) route, respectively. Four hours after induction we sacrificed mice, excised tumors and lymphoid organs, and prepared and plated cellular suspensions to determine the number of CFU/g of tissue. As expected, bacteria were enriched within tumors (an average of $3x10^6$, $2x10^6$ and $1x10^8$ CFU/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 ip and iv route, respectively (Fig. 3b and 3c). In contrast, only 11% of the F1.A11 cells from noninduced controls contained GFP (grey-shaded plots in Fig. 3). We did not observe GFP-positive cells in tumors from animals receiving *Salmonella* carrying the empty vector (data not shown).

Expression of a pro-drug converting enzyme within tumor cells.

Mammalian cells are resistant to 5-FC because they lack CD, an enzyme which converts 5-FC into 5-fluorouracil, a cytotoxic compound routinely used in cancer chemotherapy. We evaluated the capacity of the new system to selectively deliver a pro-drug converting enzyme into solid tumors *in vivo.* Thus, we performed a side-by-side comparison between *Salmonella* carrying vectors with the *Escherichia coli* CD encoding gene *codA* under the control of either the salicylate-inducible circuit (pMPO16; **Supplementary Table 1**) or the well-known tetR/tetO promoter/operator (pMPO17; **Supplementary Table 1**). When tumors were palpable, we administered SL7207-4S2 carrying either pMPO16 or pMPO17 by ip route (10^6 CFU). After 5 days, we induced CD expression by a single ip injection of salicylate or tetracycline (Fig. 4a), and initiated 5-FC therapy 4 h following induction. We observed similar tumor growth in mice receiving *Salmonella* in combination with 5-FC or PBS (Fig. 4b). We detected slower tumor progression in animals treated with 5-FC that were injected with SL7207-4S2 carrying pMPO17, wherein *codA* expression was induced by tetracycline (Fig. 4b). However, the differences were not statistically significant with respect to controls ($P>0.05$). In contrast, the size of the tumors from 5-FC-treated animals receiving SL7207-4S2 carrying pMPO16,

wherein CD expression was induced by salicylate, was significantly smaller than in controls (P<0.01; Fig. 4b, 4c and 4d).

These results provide the proof-of-concept for the usefulness of the regulatory circuit to control prokaryotic gene expression *in vivo*. However, a broad implementation of this platform 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-FC treated animals 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 from animals receiving PBS or injected with a control strain, where the expression module without *codA* was integrated (SL7207-4S2-MPO28; **Supplementary Table 1**), were significantly (P<0.05) larger (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 played by specific genes at different stages of the infection process. However, current systems 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.

To this end, we integrated an *in vivo* inducible expression cascade based on a salicylate-dependent regulatory circuit into the chromosome of *S. enterica aroA* serovar Typhimurium. This strategy allows a tightly regulated expression of selected genes under the control of the *Pm* promoter *in vivo*, in response to either salicylate or ASA. The presence of the inducers led to an activation of protein expression regardless of the topology (intracellular or extracellular). This suggests that the eukaryotic environment does not interfere with gene expression, despite significant differences in growth conditions. Similar expression levels were observed in *Salmonella* carrying low (pMPO13) and high copy number (pMPO2) vectors. Despite the fact that 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. Since 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, since it is possible to integrate additional expression cassettes under control of *Pm* without titrating-out its activator XylS2, which is over-expressed by the upstream regulator *nahR/Psal*. The choice of salicylate as inducer of the regulatory cascade was based on its rapid absorption, broad bio-distribution within different tissues and 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 approximately 9 h after induction. In fact, ASA is one of the drugs for which there is vast pharmacological knowledge, and an impressive body of experimental and clinical work. 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], since the number of viable *Salmonella* start to decline in spleen 4 days after infection to nondetectable levels around day 20[6,22]. Thus, we evaluated whether the regulatory control circuit can be exploited to deliver the 5-FC converting enzyme CD within tumor cells *in vivo*. The results showed that upon induction with salicylate there is a significant reduction in tumor progression in animals treated with strains carrying the expression module either in a plasmid or integrated into the chromosome with respect to controls and mice receiving *Salmonella* in which CD expression was controlled by a tetracycline-induced system.

Although *Salmonella* 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 *Salmonella* bearing the *nahR::Psal/xylS2* module displayed high induction rates within macrophages, epithelial cells and tumor cells. Other strategies were proposed to control *Salmonella* gene expression *in situ* such as the use of (i) P_{Bad}-dependent P_{T7} constructs[2], (ii) different *in vivo* inducible promoters[3,5] and (iii) tetracycline-inducible systems[7,8]. However, none of these systems 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.

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 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

Salmonella were grown at 37°C in LB broth supplemented with 0.3 M sodium chloride and ampicillin (100 μg/ml). HeLa and J774.A1 cells were grown onto coverslips placed into 24-well tissue culture plates (Nunc) in Dulbecco´s modified Eagle's medium (DMEM, Gibco BRL) supplemented with 2 mM L-glutamine and 10% fetal calf serum at 37°C, until they reached 60-80% confluence. We added 10^6 CFU to each well, and further incubated cells for 1 or 2 h (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 4 h incubation, we washed, fixed (2% paraformaldehyde) and stained with a β-galactosidase staining kit (Roche) cells infected with the *lac*Z vector-bearing *Salmonella*. To evaluate GFP expression in the intracellular compartment of F1.A11 cells, we performed induction as described above and analyzed cells by fluorescence microscopy. For quantification, we detached infected cells with trypsine and analyzed them by flow cytometry using a FACScalibur cytometer and the CellquestPro software (Becton-Dickinson, USA).

In vivo analysis of clones bearing the regulatory circuit

Mice received 10^6 CFU of SL7207-4S2 [pMPO15] by ip route. After 30 min, animals received 150 μl of sodium salicylate (100 mM) by ip or iv route, which is compatible with accepted ASA dosages[12]. After 4 h, we sacrificed 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

For evaluating the activation of the regulatory circuit within tumors, mice received a subcutaneous injection into the right flank with $5x10^4$ cells from a spontaneous murine fibrosarcoma (F1.A11 cells) resuspended in 100 μl of PBS. When palpable tumors were developed, animals received 10^6 CFU of SL7207-4S2 [pMPO15] by ip route. Five days after

infection, we induced the expression of *gfp*, as described above. After 4 h, we sacrificed 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 received 10^6 CFU of SL7207-4S2 carrying *codA* under control of the salicylate-responsive regulatory circuit (pMPO16) or the tetR/tetO promoter/operator (pMPO17) by ip route, 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 CFU of plasmidless SL7207-4S2. After 5 days, we induced expression of *codA* by ip injection of salicylate or tetracycline (100 µg). Four hours later, we initiated 5-FC 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 animal averaged over the total number of animals 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 online. We also provide full description of the integration of the regulatory module *nahR*/*Psal*::*xylS2* and the *codA* expression module into the *Salmonella* chromosome in Supplementary Methods online.

Author Contributions

JLR and PDB performed most of the experimental work and analysis of data, and also contribute to experimental design. EMC mapped the insertion of the regulatory module and constructed the strains bearing the expression module integrated into the *aroC* locus. AC initially designed the cascade expression system for its use within eukaryotic cells. CL contributed to the *in vivo* work. ES and CAG were the main responsible for experimental design, participate in the analysis of raw data and wrote the paper.

Supplementary items

Supplementary Figure 1. Microscopic analysis of HeLa cells infected with recombinant *Salmonella* carrying the *lacZ* gene under the control of the regulatory module nahR/Psal::xylS2 after induction.

Supplementary Figure 2. Flow cytometric analysis of cells from spleen and mesenteric lymph nodes from mice infected with recombinant *Salmonella* carrying the *gfp* gene under the control of the regulatory module nahR/Psal::xylS2 after induction.

Supplementary Table 1. Strains, plasmids and cell lines used in this work.

Supplementary Methods.

FIGURE LEGENDS

Figure 1. Tightly regulated expression of *Salmonella* 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 due to a synergic effect. (**b**) Dose-response induction curve for SL7207-4S2 strains carrying the low (pMPO13) and high (pMPO2) copy number expression vectors encompassing the *Pm-trp´::´lacZ* cassette. We evaluated expression of β-galactosidase after 4 h induction with different concentrations of salicylate or ASA by determining the number of Miller units. (**c** and **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. Vertical lines indicate the standard deviations (n=3). The values reported in panel **b** correspond to one representative experiment out of 3, whereas those presented in panels **c** and **d** are the average of the independent tests.

Figure 2. Intracellular expression of GFP in tumor cells by recombinant *Salmonella* carrying the *gfp* coding gene under control of the regulatory module *nahR/Psal::xylS2*. (**a**) We infected F1.A11 cells with SL7207-4S2 containing the GFP encoding vector pMPO15. Then, we induced protein expression with 2 mM salicylate during 4 h and observed infected cells under fluorescence microscopy to assess GFP expression (Scale bar: 10 μm). (**b** to **d**) We performed flow cytometric analysis of cells infected with SL7207-4S2 carrying either the GFP encoding vector pMPO15 (**b** and **c**) or pWSK29 (**d**) to determine the number of F1.A11 cells containing GFP-producing bacteria. The plots **b** and **d** correspond to salicylate induced cells (2 mM for 4 h). Plots represent gated cell counts versus the fluorescence obtained in the green channel, which is derived from the GFP. The analysis is representative of 3 independent experiments.

Figure 3. Tightly regulated *in vivo* expression of prokaryotic genes within tumors using an ASA/salicylate-activated control circuit based on the regulatory module *nahR/Psal::xylS2*. (**a**) Schematic representation of the experimental design. (**b** and **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) by ip (**b**) or iv (**c**)

route (empty plots), respect to the noninduced controls (grey filled plots). The analysis is representative of two independent experiments.

Figure 4. Salicylate-mediated *in vivo* expression of the 5-FC converting enzyme CD within 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 wherein the inducer was administered. (**b**) Tumor growth in untreated mice (PBS), and in animals receiving plasmidless 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-FC therapy 4 h later. (**c** and **d**) Consistent differences in tumor size were macroscopically evident on day 5 between PBS and SL7207-4S2 [pMPO16]-treated mice (bar: 10 mm). (**e**) Tumor growth in control mice (PBS), and in animals receiving SL7207-4S2 carrying pMPO16, or SL7207-4S2 in which the *codA* encoding (SL7207-4S2-MPO27) or control (SL7207-4S2-MPO28) expression modules was integrated into the chromosome. Vertical lines indicate the standard deviations (n=6). The analysis is representative of three independent experiments.

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