



Figure 4. Fluorescence microscopy image of F1.A11 tumor cells infected with SL7207-4S2 containing the *gfp*-encoding vector after induction of protein expression (GFP) with 2 mM salicylate.

advantage. The synergic effect that involves the *nahR/P_{sal}::xyIS2* system allows diminishing the basal level (70–90%), maintaining just half of the maximum induction ratios obtained from a multicopy plasmid, but resulting in a relative increase on the induction ratio (from 60 to 250-fold).

Even in the absence of product toxicity and using antibiotic selection to prevent plasmid loss, the overproduction phenotype from a plasmid configuration is unstable, most probably due to the impossibility of the bacterial machinery to replicate the plasmid while keeping high transcription levels.^{29,30} This leads to the appearance of down-expression mutants, unless the expression system is stabilized by insertion into the chromosome. Therefore, in terms of biotechnological production the cascade system in the chromosome configuration combines high levels of expression with great stability of the overproduction phenotype. This in turn results in protein yields that are at least 1 order of magnitude higher than those of expression systems from plasmids, when the time of fermentation is greater than 24 h.³⁰

Use of *nahR/P_{sal}::xyIS2* Expression System to Study Bacteria-Host Interaction in vivo

Most systems used in biotechnology are not suitable for studies involving bacterial behavior inside mammalian cells. In fact, several in vivo prokaryotic inducible expression systems used that respond to external stimuli, such as the tetracycline responsive bacterial tetracycline repressor, are limited for studying host-pathogen interactions by toxicity, side effects of their inducers and leakiness of their expression. Experimental approaches for functional investigation of genes using finely controlled expression systems ('on-off') in vivo are highly desirable, but are not well developed for microbial studies.⁴¹

Acetyl-salicylic acid (ASA) is one of the most widely used and best characterized analgesic and anti-inflammatory drugs in the market.⁴² The biological half-life of ASA is only 20 min, since it is rapidly converted in the stomach and bloodstream into its pharmacological active form, the salicylic acid, which has a half-life of approximately 4 h.⁴² Thus, given the nature of this drug, its putative use as inducer to control heterologous expression in *Salmonella* spp. in vivo was explored. The regulatory module was transposed into an attenuated *aroA* *Salmonella*, thereby generating the SL7207-4S2 strain. The resulting strain was further transformed with a plasmid encoding GFP under control from the P_m promoter. As shown in **Figure 4**, the inducer is capable to exert its activity on intracellular bacteria in mammalian cultured cells.⁴³ In addition, mice bearing tumors were infected with the *S. enterica* strain SL7207-4S2 carrying the P_m -*gfp* encoding plasmid. Then, the system was induced by administering salicylate and after 4 h flow cytometry analysis showed that 30% of the tumor cells were GFP-positive.⁴³ Thus, it could be conceived that the *nahR/P_{sal}::xyIS2* expression system together with *Salmonella* spp. could be used in the context of anticancer therapy, by benefiting from *Salmonella* properties to find a niche in hypoxic and necrotic areas located inside some solid tumors.^{44,45} As a proof-of-principle, the method was validated by engineering attenuated *Salmonella* to express the fluorocytosine-converting enzyme cytosine deaminase that converts the innocuous 5-fluorocytosine (5-FC) into 5-fluorouracil, a cytotoxic compound routinely used in cancer chemotherapy. After infecting mice bearing established tumors with the engineered *Salmonella*, 5-FC was administered. Later, aspirin administration was able to turn on the expression of the converting enzyme in bacteria residing in tumor cells, thereby activating the pro-drug and achieving a significant reduction in tumor growth.⁴³

Concluding Remarks

Simple prokaryotic expression systems are usually enough for small scale production of proteins at the laboratory, but when scaling up, system stability and costs became a major concern. Most expression systems are based on a single promoter. The use of plasmid-based expression systems resulted in instability or appearance of expression-down mutants that rapidly dominate the culture. Moreover, the potential toxicity and high cost of the inducer (e.g., IPTG) might restrict the industrial application of many of these expression systems.^{30,46,47} Thus, the development of cost-efficient approaches to control gene overexpression is a critical issue in biotechnology.⁴⁸

The salicylate inducible cascade expression system allows tightly regulated expression in response to an inexpensive inducer such as salicylate. In addition, the system based on two physically separated regulatory and expression modules provides a great flexibility, allowing the cloning of the expression module in a plasmid (either high or low copy number plasmids) or into the chromosome. Although stability is not always a major concern

in research or small scale production, it is on industrial production or for in vivo applications. Thus, the expression module could be integrated into chromosome, when stable production is desired or the use of antibiotic should be avoided. The induced expression levels using the cascade are so high that the expression module from plasmid (multicopy) to chromosome (monocopy) configuration could be changed without the putative disadvantage of reduced gene dosage. Therefore, this cascade regulatory circuit not only improves the control of heterologous gene expression, but also shows a multilevel regulation operating at different steps of the expression process. This broadens the possibilities with respect to simple control systems in terms of increasing the induction ratios and fine-tuning expression levels.

In addition to its biotechnological use, cascade systems such as the one regulated by salicylate can be exploited to overcome the problem of gene functional studies, both in vitro and in vivo. Currently, the most common approach to study gene function is a gene inactivation approach and then looking for a

gross phenotypic defect. However, this “all-or-none” approach has many limitations, particularly for the analysis of a complex or subtle phenotype. This is the case when the contribution of the gene product should be analyzed in temporal and spatial context. Thus, the exploitation of an inducer of well-known pharmacological properties, such as aspirin, to turn on expression of a bacterial gene inside an infected host provides a new tool not only for developing bacterial-based therapies, but also for studying host-pathogen interactions and the specific role played by putative virulence factors during the course of infection. By the use of this type of circuits it would be possible to switch-on the expression of selected genes at will, thereby assessing their role during the course of the infection process and/or bacterial transit through specific anatomical niches. Thus, the salicylate regulatory control circuit may constitute a cornerstone for biotechnological applications, as well as functional studies of bacteria-host interactions, due to its flexibility and broad-range use.

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