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# Exploitation of prokaryotic expression systems based on the salicylate-dependent control circuit encompassing *nahR*/$P_{sal}$::*xylS2* for biotechnological applications

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**Key words:** Salmonella, control circuit, cascade, salicylate, expression system, tumor



Expression vectors appear to be an indispensable tool for both biological studies and biotechnological applications. Controlling gene overexpression becomes a critical issue when protein production is desired. In addition to several aspects regarding toxicity or plasmid instability, tight control of gene expression is an essential factor in biotechnological processes. Thus, the search for better-controlled circuits is an important issue among biotechnologists. Traditionally, expression systems involve a single regulatory protein operating over a target promoter. However, these circuits are limited on their induction ratios (e.g., by their restriction in the maximal expression capacity, by their leakiness under non-induced conditions). Due to these limitations, regulatory cascades, which are far more efficient, are necessary for biotechnological applications. Thus, regulatory circuits with two modules operating in cascade offer a significant advantage. In this review, we describe the regulatory cascade based on two salicylate-responsive transcriptional regulators of *Pseudomonas putida* (*nahR*/$P_{sal}$::*xylS2*), its properties, and contribution to a tighter control over heterologous gene expression in different applications.

Nowadays, heterologous expression has been proven to be an indispensable tool for tackling basic biological questions, as well as for developing biotechnological applications. As the nature of the protein of interest becomes more complex, biotechnologists find that a tight control of gene expression is a key factor which conditions the success of the downstream purification process, as well as the interpretation of the results in other type of studies. Fortunately, different expression systems can be found in the market, each of them with their own pros and cons. In this review we discuss the exploitation of prokaryotic expression systems based on a promising expression system, the salicylate-dependent control circuit encompassing *nahR*/$P_{sal}$::*xylS2*, as well as some of the improvements that have been done on this system to exploit it more efficiently in the context of both biotechnological applications and basic research.

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## Conventional Prokaryotic Expression Systems

In prokaryotes there is only one RNA polymerase (RNAP), which is responsible for transcribing all genes. RNAP is a holoenzyme constituted by a minimum of four subunits ($\alpha_2\beta\beta'$). Specificity for a defined promoter group is given by an additional protein named sigma ($\sigma$), which binds $\alpha_2\beta\beta'$ prior to its assembly to the promoter. There is a plethora of sigma and anti-sigma factors, which in turn represents a first mayor level of regulation.[1,2] The minimal functional promoter recognized by $\sigma^{70}$ is constituted by two hexamers spaced by 17 bp, namely the -35 and -10 boxes. Depending on the similarity between the real promoter and the consensus, the efficiency of the transcription initiation may differ. As a general rule, strong promoters tend to be repressed whilst weak promoters require activators to proceed with transcription initiation.[3] Following the assemblage of the RNAP over the promoter, the complex passes through different stages prior to transcription initiation. First, the initial recognition occurs between the RNAP-sigma and the promoter face, covering from -50 to +20 from the transcription start site. In terms of the status of the double helix, this conformation is known as closed complex and follows a reversible dynamic equilibrium until the promoter is opened between -10 up to +8. This new configuration is known as open complex. Then, RNAP starts a series of short abortive series of elongation (2–8 bases RNA transcripts) in a state called initiation complex. Finally, one of these transcriptions escape from the promoter, the sigma factor is released and the RNAP turn fully processive as an elongation complex.[4] Each of these steps carries a series of conformational changes in the RNAP that can be favored (by activators) or retained (by repressors). Nowadays, we can find several examples in the market of expression systems excised from their natural environments and redesign in order to fulfill the requirements of biotechnologists.

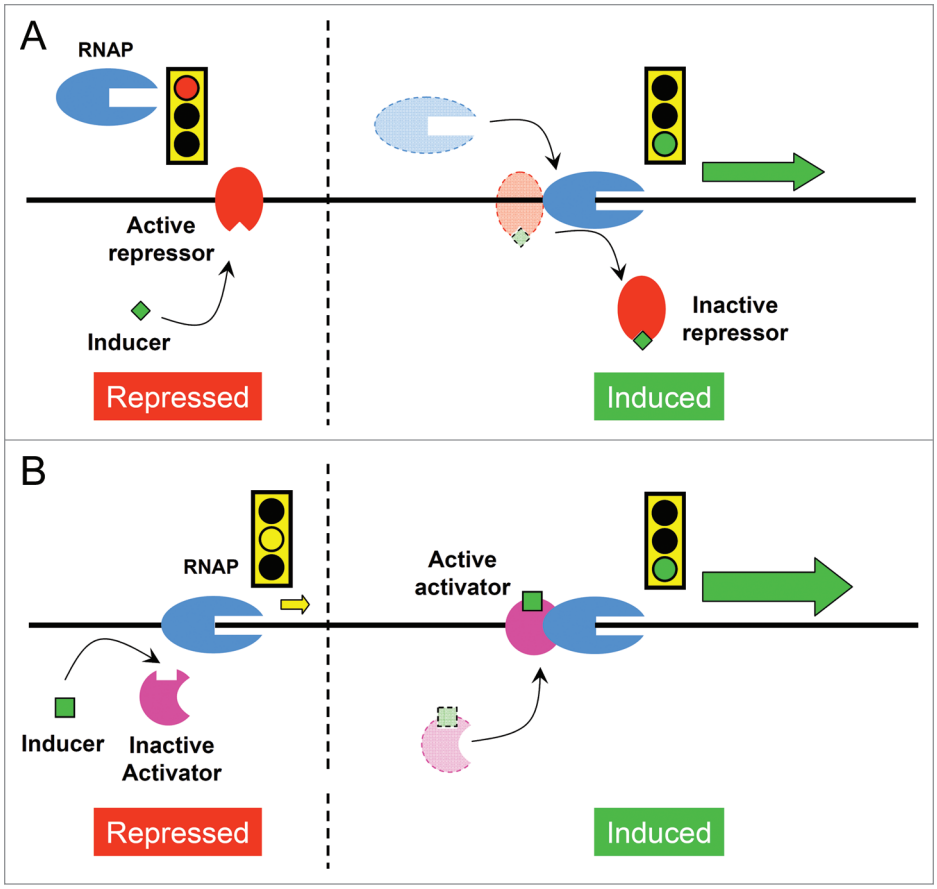




**Figure 1.** Induction can be under positive or negative control. (A) Negative control: induction is achieved when an inducer inactivates a repressor protein allowing the expression of the gene of interest. (B) Positive control: induction is achieved when an inducer activates an activator protein.

## Expression Systems Based on $P_{lac}$ Derivates

Under natural conditions, lactose enters the cell through a permease and binds the LacI repressor, changing its conformation and preventing its action over the lacO site, thereby leaving the RNA polymerase free to access the $P_{lac}$ promoter (**Fig. 1A**). On its biotechnological application, induction takes place through a non-metabolized inducer: isopropyl-β-D-thiogalactoside (IPTG). This also allows the use of the $P_{lac}$ expression system in organisms lacking the lactose permease. However, this promoter is rather weak, limiting the maximum expression rate. Hybrid promoters $P_{tac}$ and $P_{trc}$, exert the strong expression capacity derived from the $P_{trp}$ together with the repression capacity mediated by LacI. $P_{tac}$ consists of the -35 box from the *trp* promoter and the -10 box from the *lac* promoter with a spacer just one bp larger than the wild type promoter. By using this modified system it is possible to accumulate up to 15–30% of the protein of interest of the total dry cell weight.[5] On the other hand, *lacI* has been also subjected to improvement. Certain point mutation placed on the -35 box of the own $P_{lacI}$ generate a higher number of LacI in the cell (from 20 to 100 molecules per cell). This allele named *lacI*$^q$ favors the binding to the lacO region allowing a tighten repression and diminishing the basal levels of expression.[6]

## Expression System Based on $P_{BAD}$

This expression system has an interesting feature that makes it attractive, the regulatory protein AraC acts over the $P_{BAD}$, not only as repressor in the absence of arabinose (**Fig. 1A**), but also as inducer in the presence of the inductor (**Fig. 1B**). The system encompasses two divergent promoters, $P_c$, that controls the expression of *araC* and, $P_{BAD}$, driving the expression of the gene of interest. In the absence of arabinose, AraC forms a loop hiding the $P_{BAD}$ promoter. When arabinose is present, AraC free the loops and actively promote the transcription from $P_{BAD}$.[7]

## Expression System Based on $P_{T7}$

This system consists on a modular system with two elements. The first module comprises the $P_{lacUV5}$ normally inserted in the strain chromosome, driving the expression of the *gene*-1 from phage T7, encoding its own RNA polymerase. The second element is placed in a multicopy plasmid and contains the $P_{T7}$, driving the expression of the gene of interest. This system takes advantage of the tremendous processivity of the T7 RNAP, and controlling its presence by using IPTG. The main problem of this system is basically the high basal transcription. Leakiness from the $P_{lacuv5}$ is

magnified by the fact that T7 polymerase is active per se, and this constrains the induction ratios.[8,9]

## Expression Systems Based on $P_m$ and $P_{sal}$

Both *xylS*/$P_m$ and *nahR*/$P_{sal}$ expression systems have their origin in the degradation pathways of toluene and naphthalene from *Pseudomonas putida*. The catabolism of toluene/xylenes in *P. putida* is encoded in a pathway known as TOL pathway. The catabolic genes of pWW0 (also called TOL plasmid) are organized into 2 operons, referred to as the upper and lower (or meta) pathways. The *xyl* genes of the *P. putida* TOL plasmid that specify catabolism of toluene and xylenes are organized in 4 transcriptional units: the upper-operon (xylUWCAMBN) for conversion of toluene/xylenes into benzoate/alkylbenzoates; the meta-operon (xylXYZLTEGFJQKIH) that encodes the enzymes for further conversion of these compounds into Krebs cycle intermediates; and XylS and XylR proteins which are involved in transcriptional control. The *xylS*/$P_m$ expression system has been fully characterized in different genetic backgrounds.[10,11] The XylS together with AraC define the XylS/AraC family of transcriptional regulators,[12] which is present in more than 47 different genera and 87 species of microorganisms.[13] XylS is naturally expressed in its inactive form at low levels from its cognitive promoter. In the presence of substrates of the meta pathway, such as benzoate or *m*-toluate, XylS activates transcription from the $P_m$ promoter.[14] On the other hand, activation of $P_m$ promoter by XylS can be also triggered by an excess of the regulator (i.e., overexpression of XylS) in the absence of inducers.[15,16] Recently, a molecular model for the mechanism of XylS activation when produced at low and high concentrations was proposed, which explains this apparent contradiction.[17] Many dimeric regulators are in equilibrium between their monomeric and dimeric conformation within the cell. Dimer formation allows XylS binding to DNA, thereby activating transcription from $P_m$. Thus, the overproduction of XylS in a cascade circuit results in high protein concentrations, which shift the equilibrium toward dimerization favoring DNA binding.[18] However, at low basal XylS levels, which are normally found in bacterial cells, the effector (e.g., *m*-toluate) is required to shift the equilibrium toward dimerization.[19] The *m*-toluate favors two important conformational changes: (i) alters the direct interactions between the XylS N-terminal and C-terminal domains promoting protein dimerization and indirectly XylS DNA binding, and (ii) opens the regulator DNA-binding domains favoring contact with DNA.[17] Thus, the presence of an upper pathway substrate alone, such as benzyl alcohol, induces XylS overproduction causing the activation of $P_m$, even without XylS effectors. Thus, a synergistic effect could be obtained when activation of the $P_m$ by XylS overproduction is further augmented in the presence of inducers like *m*-toluate.[20] Induction with *m*-toluate has been used in biotechnological applications such as the production of different enzymes (e.g., the CelB phosphoglucomutase from *Acetobacter xylinum*[21] or XanA from *Xanthomonas campestris*, an enzyme involved in xantano biosynthesis).[10]

On the other hand, the *nahR*/$P_{sal}$ expression system has its origin in the catabolic genes of plasmid NAH7 of *P. putida* for degradation of naphthalene, which is controlled by a single regulatory protein (NahR) that is activated by salicylate, one of the intermediates of the catabolic pathway.[22] Despite the structural similarity between salicylate and benzoate, the transcriptional activator NahR of the NAH pathway belongs to the LysR family, which is unrelated to XylS/AraC family.[23] Members from this family respond to several chemical inducers under natural conditions, being transcribed divergently from its target promoter, in a process which is accomplished by an autoregulatory circuit. LysR-type regulators stay bound to their target sequences even in the absence of inductor, thereby allowing a rapid activation.[24,25] Although these two systems are unrelated, it is important to remark that XylS mutants that respond to salicylate can be isolated, such as XylS2,[26] as well as NahR mutants responding to benzoate.[27]

## Rationale of the Transcriptional Cascade Based on *nahR* and *xylS2*

The simplest regulatory cascades are found in prokaryotic systems and they typically involve at least two different transcriptional activators. These are arranged in such a fashion that a first regulatory component (upstream regulator) controls the expression of a second regulatory gene (downstream regulator) in response to specific signals. In turn, the downstream component acts directly on transcription of the structural genes of the system. Cebolla et al.[28] designed a cascade using two salicylate-responsive transcriptional regulators of *P. putida* and analyzed experimentally the conditions under which two activators could be coupled to each other to multiply the separate response of each activator to the same inducer. Thus, the expression of *xylS2* was coupled to the NahR-dependent $P_{sal}$ promoter in the "regulatory module" *nahR*/$P_{sal}$::*xylS2* fusion, which was inserted into the bacterial chromosome by means of a mini-*Tn5* delivery system (**Fig. 2A**). This allows researchers to clone any protein of interest in a plasmid downstream the $P_m$ promoter in a so-called "expression module" (**Fig. 2A**). In the absence of salicylate basal expression levels are very low, due to the limited concentration of inactive XylS2. However, in presence of salicylate NahR activates transcription from $P_{sal}$, thus producing XylS2 (**Fig. 2B**). Therefore, salicylate activates XylS2, which together with its increased intracellular concentration synergistically amplifies transcription from the $P_m$ promoter present in the expression module.[28-30] It should be highlighted that the expression module can be easily sub-cloned into a mini-*Tn*5 and inserted in monocopy in the bacterial chromosome. Induced expression levels using the cascade are so high that overcome the initial putative disadvantage of changing the expression from multicopy to monocopy. In addition, having the expression module inserted in the chromosome confers an advantage when stable production is desired, or when the use of antibiotics to select the plasmid may be an inconvenience.[29]

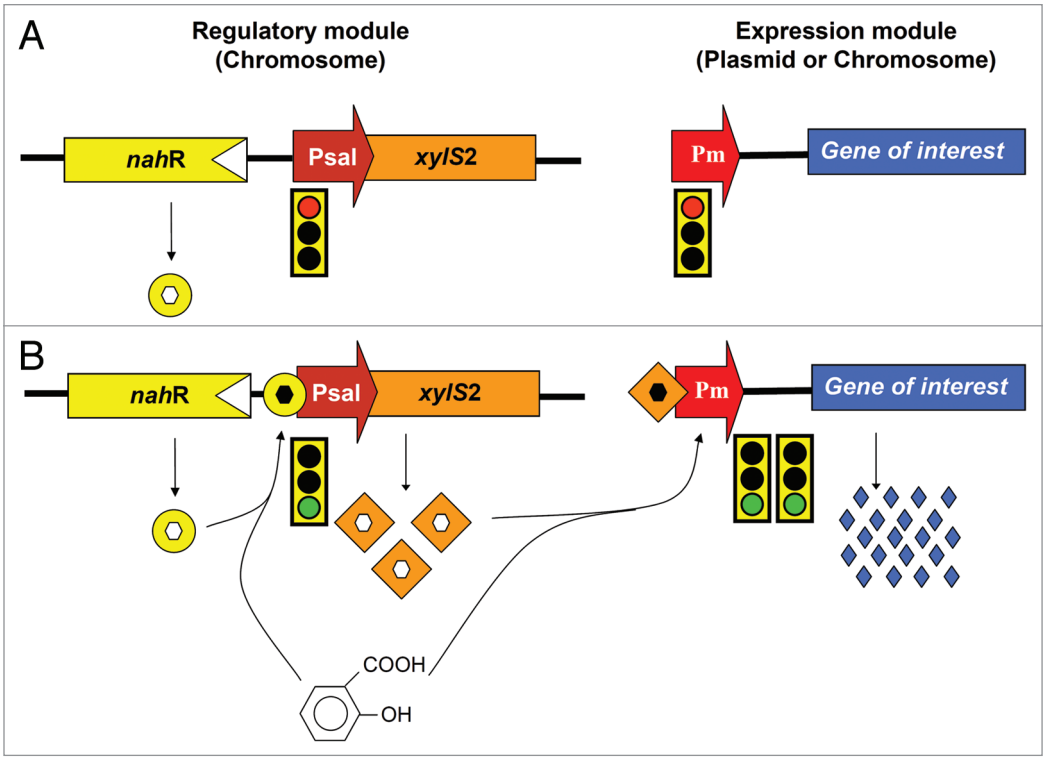


**Figure 2.** Schematic representation of the salicylate-dependent control circuit. (A) The cascade expression system is based on the regulatory module (*nahR*/$P_{sal}$-*XylS2*) and the expression module $P_m$-*gene of interest*. (B) In the presence of salicylate (inducer), NahR activates transcription from $P_{sal}$, producing XylS2. Salicylate concomitantly activates XylS2, which together with its increased intracellular concentration synergistically amplifies transcription from the $P_m$ promoter present in the expression module.



## Over-Imposed Circuit to Improved Salicylate-Dependent Cascade

Most expression systems displaying high induction capacity usually exhibit high basal levels. This leakiness may be a handicap when a potentially toxic protein is expressed, since spontaneous mutants are selected. Thus, in the past few years alternatives such as efficient repressors or combinations of some of them with other strategies have been investigated to create systems with reduced basal levels which maintain high-induction ratios.[31] One of the most helpful strategies in this regard is to include a second regulatory control operating at a different regulatory level. An example of regulation at a step different from transcription initiation is the attenuation of transcription elongation. This regulation has been considered a highly sophisticated and useful strategy,[32] either alone or in combination with repressors or activators, allowing a very tight regulation of gene expression.[33-35] Attenuation comprises a premature transcription stop, due to a RNA conformation that occurs in the nascent transcript. In order to revert this phenomenon, bacteria use a specific RNA-binding regulatory protein to control the formation of a terminator structure.

The main characteristic of an attenuation system that makes it attractive as a complementary regulatory circuit in expression vectors is that it prevents transcription elongation regardless from (1) undesired basal transcription from the regulated promoter of the expression system or (2) spurious initiation of the bacterial RNA polymerase from known or cryptic vector promoters. Thus, attenuation could serve to eliminate most of the unwanted transcription produced under non-inducing conditions. On this regard, the *nasFAD* attenuator and the NasR-dependent anti-termination system from *Klebsiella pneumoniae* was selected to reversibly prevent undesired transcription.[36] The presence of the *nasF* attenuator between the regulated $P_m$ promoter and the gene of interest reduces transcription of the gene by more than one order of magnitude under non-inducing conditions (**Fig. 3A**), which indicates the potential of the attenuator to reduce the basal expression levels. In order to fully control and revert premature termination, NasR should be provided to the circuit. However, NasR is not active per se, since requires $NO_3^-$ for being active, giving the researcher an additional point of control. Thus, transcription anti-termination at the attenuator significantly increased under inducing conditions when the expression of the NasR gene is coupled to the cascade regulatory circuit by placing it downstream of $P_{sal}$ (**Fig. 3B**). By providing NasR from this promoter, the circuit displayed the highest induction ratio (1,700-fold), since $P_{sal}$ is weak enough to produce undetectable levels of NasR in the absence of salicylate and strong enough to allow full anti-termination upon induction.[36]

**Biotechnological applications.** Depending on the flexibility of the plasmid replication origin, the plasmid number can vary between 15–60 copies per cell (such as those derived from pMB1/ColE1 origin from the pBR322 series, or those derived from the

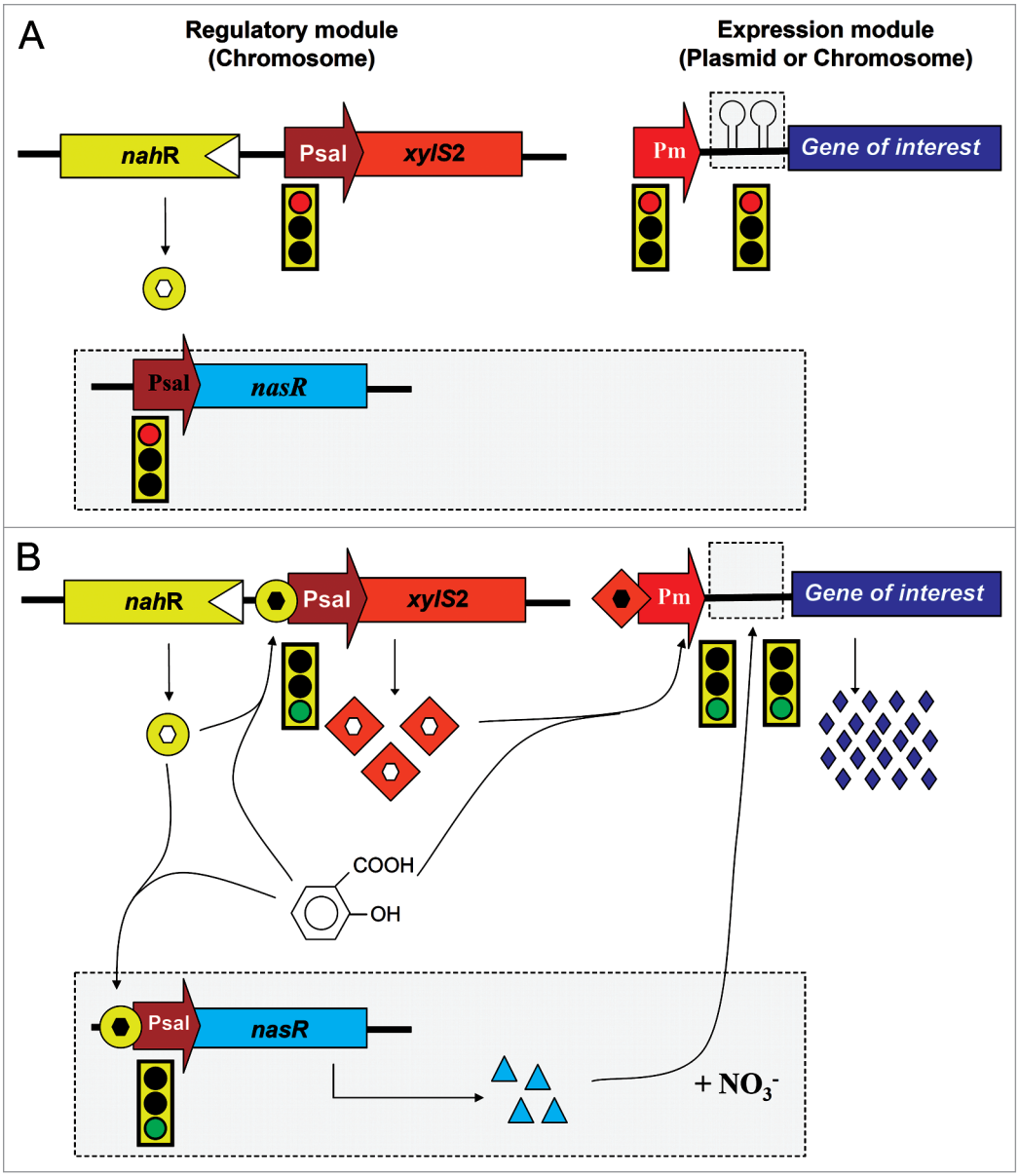


**Figure 3.** Schematic representation of the salicylate-dependent control circuit with the over-imposed attenuation regulatory system. (A) The cascade expression system is based on the regulatory module (*nahR*/$P_{sal}$-*XylS2*) and the expression module $P_m$-gene of interest, but it also contains the $P_{sal}$-nasR regulatory module that targets the nasF attenuator (grey box) that is located between the $P_m$ promoter and the gene of interest (grey boxes). (B) In the presence of salicylate (inducer), NahR activates transcription from $P_{sal}$, producing not only XylS2 but also nasR. In the presence of $NO_3^-$, nasR becomes activated allowing the elongation from the $P_m$ promoter. Thus, the increased intracellular concentration of XylS2 and the concomitantly activation in the presence of salicylate synergistically amplifies transcription from the $P_m$ promoter in the expression module.

p15A of the pACYC series) and several hundreds of copies per cell, such as the pUC series derived from pMB1. Under lab conditions without any selective pressure, these high copy-number plasmids are lost at low rates ($10^{-5}$, $10^{-6}$ per generation). However, this rate dramatically increases under normal induction conditions, or when expressing a toxic product.[5] As previously mentioned, most prokaryotic expression systems are normally placed on a multicopy plasmid to maximize the induced expression levels. These simple systems are very useful for small scale production, however, a large number of heterologous proteins confer instability due to a leaky expression under non-induced conditions. Thus, spurious promoter activity, plasmid read-through or cryptic initiation signals clearly raises basal expression levels of heterologous genes in high-copy number expression vectors,[37] thereby favoring the appearance of expression-down phenotypes in the case of gene expression that decreases the growth rate of the host strain.[29,37,38] To overcome this problem different approaches have been developed to tighten the control of gene expression, such as the reduction of gene dosage either by using low-copy number plasmids or by chromosome integration.[29,39,40] However, these modified systems do not sustain a high level of gene expression. On this regard the cascade expression system offers a significant

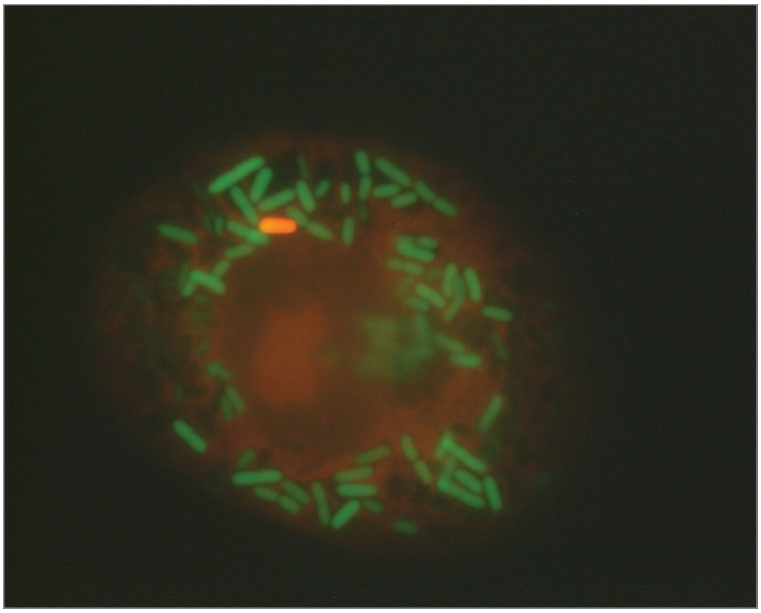

**Figure 4.** Fluorescence microscopy image of F1.A11 tumor cells infected with SL7207-4S2 containing the *gfp*-encoding vector after induction of protein expression (GFP) with 2 mM salicylate.

advantage. The synergic effect that involves the *nahR*/$P_{sal}$::*xylS2* 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*/$P_{sal}$::*xylS2* 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*/$P_{sal}$::*xylS2* 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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