Nucleic Acids Research, 2001, Vol. 29, No. 3 759–766

Rational design of a bacterial transcriptional cascade for amplifying gene expression capacity

Angel Cebolla*, Carolina Sousa[1] and Víctor de Lorenzo[2]

Departamento de Genética and [1]Departamento de Microbiología y Parasitología, Universidad de Sevilla, 41080 Seville, Spain and [2]Centro Nacional de Biotecnología CSIC, Madrid, Spain

Received September 7, 2000; Revised and Accepted November 23, 2000

ABSTRACT

Cascade regulatory circuits have been described that control numerous cell processes, and may provide models for the design of artificial circuits with novel properties. Here we describe the design of a transcriptional regulatory cascade to amplify the cell response to a given signal. We used the salicylate-responsive activators of *Pseudomonas putida* NahR of the naphthalene degradation plasmid NAH7 and XylS2, a mutant regulator of the TOL plasmid for catabolism of *m*-xylene and their respective cognate promoters *Psal* and *Pm.* Control of the expression of *xylS2* with the *nahR*/*Psal* system permitted either their selective activation with specific effectors for each protein or the simultaneous activation of both of them with salicylate. When cells face the common effector of the two regulators, both the increase in XylS2 concentration and the stimulation of its activity act synergistically on the *Pm* promoter, amplifying the gene expression capacity by at least one order of magnitude with respect to the individual systems. By changing the hierarchy of regulators, we showed that the specific features of the downstream regulator were crucial for the amplification effect. Directed changes in the effector profile of the regulators allowed the extension of the amplifying system to other molecular signals.

INTRODUCTION

Transcriptional control circuits involving cascades of regulatory proteins are known to determine the functioning of numerous cellular processes both in prokaryotic and eukaryotic systems (1,2). Such cascades channel specific environmental signals into changes in gene expression, so that multiple simultaneous stimuli are processed and translated to bring about activation or repression of specific genes or groups of genes. 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. Such an arrangement of regulatory genes may serve to control different cellular functions, i.e. to integrate different signals, for the sequential timing of different regulatory events or to amplify the gene expression capacity (ratio of induced:uninduced activity) in order to maximize cell responses. Although examples of transcriptional cascades can be found in prokaryotic systems (3,4), experimental analysis determinating the requisites for the amplifying properties of such a regulatory device remain scarce. The TOL operons for catabolism of toluene/xylenes in *Pseudomonas putida* via benzoate/toluate and catechol/methylcatechol intermediates (5), provide a suitable example of regulation in cascade. Transcription of the *meta*-operon of the TOL plasmid of *P.putida*, which determines the degradation of benzoate/toluates to tricarboxilic acid cycle intermediates, originates at the *Pm* promoter. *Pm* is activated by the XylS protein, which belongs to the AraC family of transcriptional regulators (6), in the presence of substrates of the *meta* pathway such as benzoate or *m*-toluate (7). Transcription of the *xylS* gene is in turn controlled by another regulator, XylR (8,9), in combination with the σ^{54}-containing form of RNA polymerase (10) and inducers of the *upper* pathway. Within this scheme, XylR and XylS act as upstream and downstream regulators, respectively. One key feature of this circuit is that activation of *Pm* promoter by XylS can also be triggered by an excess of the regulator in the absence of inducers (11,12). This means that the presence of an *upper* pathway substrate such as benzyl alcohol alone causes *Pm* to be activated through XylS overproduction even without XylS effectors. Such an activation can then be further augmented in the presence of inducers like *m*-toluate (13). Furthermore, when the XylS level is artificially increased through different expression systems and gene copy numbers, *Pm* strength increases through an almost 1000-fold range maintaining its inducibility by benzoate (12). This wide window of intracellular activity indicates that the maximal potentiality of XylS-mediated regulation may require to concert intracellular XylS concentration to the presence of the aromatic inducer. The last work also showed that for a given configuration of the *xylS/Pm* expression system, the gene expression capacity could not be higher than 100-fold.

In contrast to the TOL system, the catabolic genes of plasmid NAH7 of *P.putida* for degradation of naphthalene are controlled by a single regulatory protein that is activated by

*To whom correspondence should be addressed at present address: Biomedal, CE Pabellón de Italia, Isaac Newton P3ª NO, 41092 Seville, Spain. Tel: +34 954 557106; Fax: +34 954 557104; Email: acebolla@cica.es

Table 1. Bacteria and plasmids

Strains and plasmids	Description	Reference/source
E.coli CC118	*phoA20 thi-1 rspE rpoB argE* (Am) *recA1*	(27)
CC118 (λ*pir*)	CC118 lysogenized with λ*pir* phage	(27)
S17-1 (λ*pir*)	F$^-$, *recA*, *hsdR*, RP4-2 (Tc::Mu) (Km::Tn*7*) lysogenized with λ*pir* phage	(36)
CC118FH26	CC118 with mini-Tn*5 xylS2*/*Pm*→*lacZ* inserted in the chromosome, Km^R	(24)
CC118RSL9	CC118 with mini-Tn*5 nahR*/*Psal*→*lacZ* inserted in the chromosome, Km^R	This work
CC1184S2	CC118 with mini-Tn*5 nahR*/*Psal*→*xylS2* inserted in the chromosome, Km^R	This work
CC1184S2PT32	CC1184S2 with mini-Tn*5 Pm*→*lacZ* inserted in the chromosome, Km^R, Tc^R	This work
CC118SAL1	CC118 with mini-Tn*5 nahG′::′lacZ* inserted in the chromosome, Sp/Sm^R	This work
CC1182NRSL7	CC118SAL1 with mini-Tn*5 xylS2/Pm*→*nahR Psal*→*lacZ* inserted in the chromosome, Km^R, Sp/Sm^R	This work
P.putida KT2442	Prototrophic, Rif^R derivative of KT2440	(27)
Plasmids		
pMS15	Ap^R, *nahR/Psal* sequences clones as a 1.6 kb *Hin*dIII–*Pst*I insert in pUC8	(25)
pNM482	Ap^R, ′*lacZ* promoter probe plasmid	(37)
pUJ9	Ap^R, ′*lacZ* promoter probe plasmid	(38)
pUT/mini-Tn*5* Sp/Sm	Ap^R Sp/Sm^R, R6K*oriV*, RP4*oriT*, delivery vector	(38)
pTSAL1	Ap^R Sp/Sm^R, *nahG′::′lacZ* inserted as a *Not*I fragment in pUT/mini-Tn*5* Sp/Sm	This work
pFH2	Ap^R, similar to pBKT7-0 (23) but with *Not*I sites flanking the MCS	Lab stock
pFH28	Ap^R Km^R, pUC18Sfi-Km^R-*xylS-Pm*→*Sfi*I	(24)
pS2	Ap^R, *xylS2* with an engineered *Nco*I site in the first codon inserted as an *Eco*RI–*Hin*dIII fragment in pGC1	This work
pNS2	Ap^R, *xylS2* cloned as a *Nco*I–*Hin*dIII fragment in pFH2	This work
pNS	Ap^R, *xylS* cloned as a *Nco*I–*Hin*dIII fragment in pFH2	This work
pNNR	Ap^R, *nahR* cloned as a *Nco*I–*Hin*dIII fragment in pFH2	This work
pCNB2	Ap^R Km^R, pUT/mini-Tn*5 xylS2*/*Pm*→*Not*I	(24)
pCNB1-*lacZ*	Ap^R Km^R, pUT/mini-Tn*5 xylS*/*Pm*→*trp′::′lacZ*	(24)
pCNB2-*lacZ*	Ap^R Km^R, pCNB2 with *trp′::′lacZ* reporter downstream of *Psal*	(24)
pCNB2-NR	Ap^R Km^R, pUT/mini-Tn*5 xylS2*/*Pm*→*nahR*	This work
pCNB4	Ap^R Km^R, pUT/mini-Tn*5 nahR*/*Psal*	(24)
pCNB4-*lacZ*	Ap^R Km^R, pCNB4 with *trp′::′lacZ* reporter downstream of *Psal*	(24)
pCNB43-*lacZ*	Ap^R Km^R, pUT/mini-Tn*5 nahR3*/*Psal*→*trp′::′lacZ*	(17)
pCNB44-*lacZ*	Ap^R Km^R, pUT/mini-Tn*5 nahR4*/*Psal*→*trp′::′lacZ*	(17)
pCNB43-S2	Ap^R Km^R, pUT/mini-Tn*5 nahR3*/*Psal*→*xyS2*	This work
pCNB44-S2	Ap^R Km^R, pUT/mini-Tn*5 nahR4*/*Psal*→*xylS2*	This work
pCNB44-S	Ap^R Km^R, pUT/mini-Tn*5 nahR4*/*Psal*→*xylS*	This work
pUT/Tc Pm-*lacZ*	Ap^R Tc^R, pUT/mini-Tn*5 Pm*→*xylX′::′lacZ*	This work
pTPmlac	Ap^R Tc^R, pUT/mini-Tn*5 Pm*→*trp′::′lacZ*	This work

salicylate, one of the intermediates of the catabolic pathway (14). In spite of the structural similarity between salicylate and benzoate, the transcriptional activator NahR of the NAH pathway, which mediates the response to salicylate, is unrelated to XylS since it belongs to the LysR family (15). Interestingly, it is possible to isolate XylS mutants that respond to salicylate (16) with similar ease as for the selection of NahR mutants responding to benzoate (17).

The availability of two different regulators (NahR and XylS2) which respond to the same inducer, and the existence of several effector mutants has allowed us to investigate the performance of the concerted action of the two activators and their cognate promoters in artificial cascade circuits. One of the possible arrangements merges the two systems into a regulatory cascade that amplifies synergistically the promoter output in response to salicylate, starting from a very low level of basal activity. Such an amplification relies on the properties of the downstream regulator–promoter pair (XylS and *Pm*) and permitted us to draw some general conclusions on the assembly of regulatory cascades designed for the amplification of signals.

MATERIALS AND METHODS

Strains, plasmids, media and general procedures

All plasmids and bacterial strains used in this work are listed in Table 1 and Figure 5. Cultures were grown in LB medium

supplemented, where necessary, with ampicillin (150 μg/ml), kanamycin (50 μg/ml), streptomycin (25 μg/ml), tetracyclin (3–10 μg/ml) or piperacillin (40 μg/ml). All manipulations of DNA were made following standard protocols (18). Restriction enzymes were from New England Biolabs, while other modification enzymes (T4 DNA ligase, alkaline phosphatase) were from Boehringer Mannheim. Aromatic inducers were purchased from Aldrich Quimica. Promoter activity was measured by monitoring accumulation of β-galactosidase by cells grown under different conditions. To this end, overnight inoculations of the *Escherichia coli* strains under examination were diluted 1:100 and incubated for 2 h at 37°C (i.e., up to an OD_{600} of 0.1). The cultures were then added to the aromatic inducer at the final concentrations indicated in each case, and were further incubated for 5 h at 30°C. The same procedure was employed for the *P.putida* strains, except that the length of exposure to the inducers was limited to 3 h. In either case, β-galactosidase levels were measured according to Miller (19) in cells permeabilized with chloroform and SDS. Each enzymatic measurement was repeated at least twice in duplicate samples.

Assembly of DNA segments bearing regulatory elements

A mobile DNA fragment bearing the *xylS2* gene under the control of NahR and its cognate promoter *Psal* was constructed as follows. First, the *xylS2* gene was excised from plasmid pERD2 (20) as a 1.5 kb *Bam*HI fragment and cloned in vector pTZ18 to give rise to pVLT5, in which *xylS2* transcription is codirectional with the *Plac* promoter of the vector. The insert was then recloned as an *Eco*RI–*Hin*dIII fragment in phagemid vector pCG1 (21) and further subjected to site-directed mutagenesis (22) to insert a new *Nco*I site overlapping the first ATG of its coding sequence. The resulting plasmid (pS2) was then digested to completion with *Hin*dIII and partially with *Nco*I to release a 1.2 kb *Nco*I–*Hin*dIII fragment spanning the entire *xylS2* sequence but lacking any sequence upstream of the first structural codon. This fragment was then cloned in plasmid pFH2 (kindly provided by S. Fernández), which coupled the leading ATG of the *xylS2* gene with the optimized translation initiation region of the *ner* gene of phage Mu and flanked the whole promoterless gene sequence with *Not*I sites (23). Such a DNA segment was excised from the resulting plasmid (pNS2), and inserted in the unique *Not*I site of pCNB4 (24), to yield plasmid pCNB4-S2. This construct placed expression of *xylS2* under the control of the *Psal* promoter and its cognate salicylate-responsive regulator NahR, all assembled in a mini-Tn*5* Km transposon vector.

Plasmids equivalent to pCNB4-S2, but with the wild-type *nahR* gene replaced by its variants *nahR3* and *nahR4* (encoding benzoate-responsive mutants; 17), were made by cloning the *Not*I insert of pNS2 (which carries the promoterless *xylS2* as mentioned above) in the single *Not*I sites of plasmids pCNB43 and pCNB44, described previously. These derived plasmids, pCNB43-S2 and pCNB44-S2, are the delivery plasmids for mini-transposon mini-Tn*5* *nahR3Psal→xylS2* and mini-Tn*5* *nahR4Psal→xylS2*, respectively. Finally, mini-transposon mini-Tn*5* *nahR4/Psal→xylS*, equivalent to mini-Tn*5* *nahR4/Psal→xylS2* but expressing the wild-type *xylS* gene under the control of the *Psal* promoter, was produced by first exchanging *Nco*I inserts between pNS2 and pCNB1 (originating plasmid pNS) and then excising and cloning the resulting *Not*I segment into the *Not*I site of pCNB44. This gave rise to delivery plasmid pCNB44-S. Plasmid pCNB44-*lacZ*, bearing the mobile elements mini-Tn*5* *nahR4/Psal→lacZ*, has been described previously (17).

A XylS-responsive and XylS2-responsive reporter segment bearing a transcriptional fusion *Pm→lacZ* was also constructed by exchanging the 2.3 kb *Xba*I fragment of plasmid pUT/Tc*Pm→xylX′::′lacZ* (12) for the 3.7 kb *Xba*I fragment of pCNB1-*lacZ* (24). This exchange gives rise to a delivery plasmid (pTPmlac) for a Tc^R mini-Tn*5* element which bears the exact same *Pm→lacZ* transcriptional fusion to a reporter *trp′::′lacZ* gene as that of pCNB2-*lacZ*. As shown below, this allowed us to compare faithfully the activity of the *Pm* promoter controlled by either a simple regulator or by two regulators coupled in a cascade.

For the construction of a mobile DNA fragment bearing the *nahR* gene under the control of XylS2 and its cognate promoter *Pm*, the *nahR* gene was excised from plasmid pMS15 (25) as a 1.2 kb *Nco*I–*Hin*dIII fragment and cloned in pFH2. The resulting plasmid was digested with *Not*I to yield a fragment containing the promoterless *nahR* gene preceded by an optimized TIR. This fragment was cloned at the unique *Not*I site of pCNB2 (24), to yield plasmid pCNB2-NR. This construct placed expression of *nahR* under the control of the *Pm* promoter and its salicylate-responsive regulator XylS2, assembled in a mini-Tn*5* Km transposon vector. The matching NahR-responsive element was produced by the mini-Tn*5* Sm *Psal→nahG′::′lacZ* of pTSAL1.

Mobilization and transposition

Each of the DNA segments produced by the plasmids described above and assembled in mini-Tn*5* transposon vectors were targeted to the chromosome of *E.coli* CC118 or *P.putida* KT2442 with the delivery system described in detail elsewhere (26), generally known as the pUT system. Donor plasmids (Table 1) were transformed into a λ*pir* lysogen of the *E.coli* S17-1 strain, which contains a chromosomally-integrated RP4 derivative providing conjugal transfer functions (27). Biparental matings between *E.coli* S17-1λ*pir* derivatives and the recipient strain were set on nitrocellulose filters as described elsewhere (26). To discriminate authentic transposition from cointegration, exconjugant colonies were replica-plated on media with ampicillin or piperacillin to screen for the loss of the *bla* gene present in the delivery plasmid (27). Strains bearing insertions arising from different transposition events did not differ significantly with regard to the inducibility or absolute values of β-galactosidase activity and, therefore, only one of them (Table 1) was selected in each case for studies on gene expression.

Protein techniques

Western blot assays to detect the XylS product were performed as described elsewhere (28). To this end, equal amounts of whole *E.coli* or *Pseudomonas* cells (typically 10^8) were lysed in a sample buffer with 2% SDS and 5% β-mercapthoethanol and run in denaturing 12% polyacrylamide gels. These were subsequently blotted and probed with a 1:1000 dilution of a pre-adsorbed rabbit serum raised against purified inclusion bodies of the XylS protein (kindly provided by G. Bertoni). The bands in the blots corresponding to XylS were developed with 1 μg/ml of Protein A coupled to alkaline phosphatase 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (BCIP/NBT, Sigma).

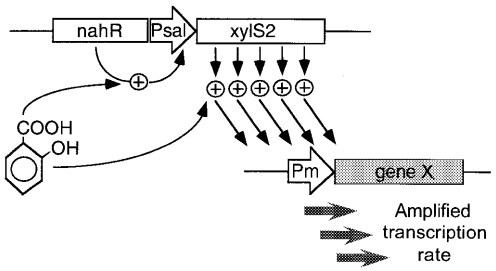

Figure 1. Schematic representation of the cascade regulatory circuit. An expression cassette containing the *nahR* gene and the *Psal* promoter controls the expression of *xylS2*. In the presence of a common inducer, NahR activates the expression of *xylS2*. Simultaneous high intracellular levels of XylS2 and activation of XylS2 intrinsic transcriptional activity could achieve amplified expression levels of a particular gene(s) under the control of the *Pm* promoter.

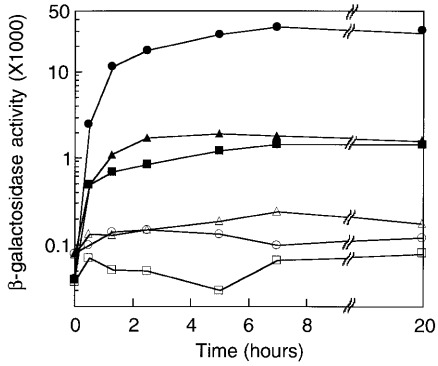

Figure 2. Kinetics of β-galactosidase production by the three expression systems. Salicylate (2 mM) was added to the cultures (OD_{600} = 0.2), and β-galactosidase activity was monitored at different intervals. Open symbols, cultures without salicylate. Closed symbols, cultures with 2 mM salicylate. *nahR/Psal::trp′::′lacZ* (squares), *xylS2/Pm:trp′::′lacZ* (triangles) and *nahR/Psal→xylS2/Pm→trp′::′lacZ* (circles).

RESULTS

Rationale for the design of a transcriptional cascade based on *nahR* and *xylS2*

We tested the possibility of amplifying the promoter/cell response to a specific compound by connecting the activity of two regulators, in response to the same signal, through the design of a cascade regulatory circuit. We employed two salicylate-responsive transcriptional regulators of *P.putida* to analyze experimentally the conditions under which two activators could be coupled to each other to multiply the separate response of each activator to a given inducer. These regulators, NahR and XylS2, belong to different families of prokaryotic regulators (LysR and AraC, respectively) and the mechanisms of transcriptional activation are different, yet the effector profile of each regulator overlaps by several compounds, including salicylate (16,17). One key feature of XylS and its effector-specificity variants (such as XylS2) is that its cognate promoter *Pm* can be triggered by either a low level of the protein in the presence of an inducer or by high levels of the regulator without inducer (11). For a fixed XylS concentration, the activity of *Pm* can be induced up to 100-fold in *P.putida*. However, the range of *Pm in vivo* activity seemed to be even broader, since varying the XylS intracellular concentration using different constructs could achieve a range of 1000-fold of *Pm* activity (12). We reasoned that maximal XylS/*Pm* transcriptional capacity could be obtained when *xylS* expression is inducible, as part of the secondary regulator in a transcriptional cascade. On this basis, we reasoned that coupling expression of *xylS2* to the NahR-dependent *Psal* promoter should result in a cascade with a potential to multiply the activity of *Pm* in the presence of salicylate (Fig. 1). This is because the inducer should both lead to the overproduction and activation of XylS2, thus the two effects could cooperatively contribute to the activation of transcription of the same promoter. These notions were put to the test by placing the relevant regulatory elements of *P.putida* into the chromosome of *E.coli* hosts, where all the features of the transcriptional control can be faithfully reproduced.

Amplified gene expression capacity resulting from a coupled NahR–XylS2 system inducible by salicylate

To ascertain whether NahR and XylS2 could coordinately function to expand the responsiveness of *Pm* to salicylate we produced a set of isogenic *E.coli* strains, differing only in the nature and arrangement of the regulatory elements controlling *lacZ* expression, and inserted them into the chromosome with mini-Tn*5* transposon vectors. To this end, the accumulation of β-galactosidase in response to inducer addition was examined in single-inserted *E.coli* strains RSL9 (*nahR/Psal→lacZ*) and FH26 (*xylS2/Pm→lacZ*) as well as in the double-inserted strain *E.coli* 4S2PT32 (*nahR/Psal→xylS2*, *Pm→lacZ*). The results shown in Figure 2 demonstrate the multiplicative effect of coupling the two regulators. While β-galactosidase accumulation by the single-inserted strains ranged between 65 (average of the dynamic uninduced values) and 2100 U (induced) in strain RSL9 (*nahR/Psal→lacZ*) and between 150 (non-induced) and 2300 U (induced) in FH26 (*xylS2/Pm→lacZ*), the induced levels of the reporter enzyme increased by almost 20-fold in the double-inserted strain *E.coli* 4S2PT32 (*nahR/Psal→xylS2*, *Pm→lacZ*), while maintaining equivalent basal levels [~130 Miller Units (MU) average of dynamic values during the 24 h incubation]. This caused the overall induction ratio to be 180–240-fold. In fact, the accumulation over a few hours of about 40 000 β-galactosidase units raised from a single chromosomal promoter revealed the extraordinary strength of the *Pm* promoter when properly activated. Another finding from the experiment shown in Figure 2 was the instant coupling of the two regulators that showed the augmented effect at the earliest time of measurements, even 10 min after salicylate addition (data not shown). It thus became apparent that the gene expression capacity (ratio of induced level to basal level) of the *Pm* promoter in response to salicylate underwent a dramatic upshift when it was subjected to an activation cascade, but without any significant qualitative effect on the induction kinetics.

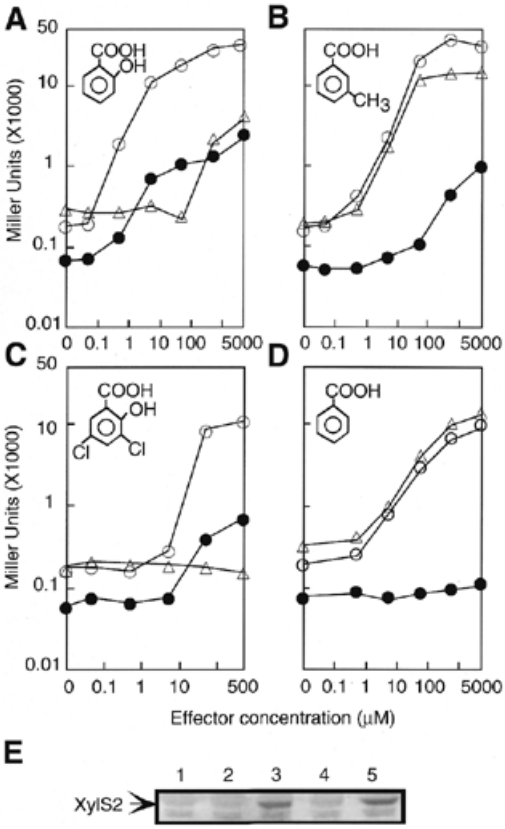

Figure 3. Gene expression analysis of the simple and cascade systems with different effectors. β-galactosidase activity from the three systems at different concentrations of salicylate (**A**), 3-methylbenzoate (**B**), 3,5-dichlorosalicylate (**C**) and benzoate (**D**). Closed circles, *nahR/Psal→trp′::′lacZ*; triangles, *xylS2/Pm→trp′::′lacZ*; open circles, *nahR/Psal→xylS2/Pm→trp′::′lacZ.* Note that the maximun concentration used for 3,5-dichlorosalicylate in the β-galactosidase assays was 0.5 mM because concentrations >2 mM resulted in growth inhibition. (**E**) Immunodetection of the XylS2 product in a control culture of CC118 (lane 1), and in the cascade-containing strain CC1184S2PT32 upon incubation without inducer (lane 2), or with 1 mM 3,5-dichlorosalicylate (lane 3), benzoate (lane 4) and salicylate (lane 5).

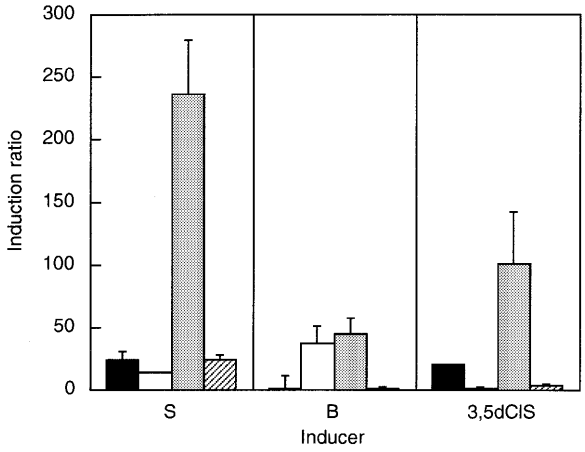

Figure 4. Capacity for regulation of simple circuits and with two cascades using a different hierarchy of upstream and downstream regulators. S, 2 mM salicylate; B, 2 mM benzoate; 3,5dClS, 2 mM 3,5-dichlorosalicylate. Basal values of β-galactosidase activity from each circuit established in *E.coli* were the following: *nahR/Psal*, 65 MU (black bars); *xylS2/Pm*, 192 MU (open bars); *nahR/Psal→xylS2/Pm*, 169 MU (gray bars); *xylS2/Pm→nahR/Psal*, 69 MU (hatched bars). Data are the mean values of three independent experiments. The corresponding standard deviations are indicated with error bars.

Dissection of the synergic coupling of NahR and XylS2

To analyze the specific contribution of each regulator to the steps involved in the cascade, we exploited the fact that both regulators can be induced independently by specific effectors to each regulator, with little response in the other (Fig. 3). When β-galactosidase production from each system was measured in the absence or presence of different compounds that were effectors of both regulators, the induction ratio obtained with the cascade system, with respect to basal level, was always superior to the simple circuits (Fig. 3A and B). Figure 3C and D illustrates the results of subjecting *E.coli* strains RSL9, FH26 and 4S2PT32 to induction experiments with the XylS2-only inducer benzoate as well as with the NahR-only effector 3,5-dichlorosalicylate. Although only the addition of a shared inducer (i.e., salicylate) leads to maximal augmentation effect on promoter output, the combined system still showed amplification when the upstream regulator (NahR) could be activated. As shown in Figure 3C, 500 μM of 3,5-dichlorosalicylate, which had no effect on XylS2 activity, could induce the NahR-mediated expression of *Psal::lacZ* 19-fold. The same concentration of inducer caused *Pm* activity to increase ~76-fold in the combined system of strain 4S2PT32. The same trend was observed when the coupled system was exposed to other NahR-exclusive inducers such as 4-chlorosalicylate, which at 2 mM increased *Pm* activity up to 190-fold, as compared with the 30-fold increase observed for *nahR/Psal→lacZ* (data not shown). These data reflected the ability of XylS2 to activate *Pm* in an inducer-independent manner. Such a feature seemed sufficient to have a substantial amplification effect (~4-fold), indicating that at certain XylS2 concentrations, a cooperative effect during the transcriptional activation of *Pm* may occur. In contrast, benzoate, which has no effect on NahR, promoted a 40-fold induction of *Pm* regardless of whether the strain used had the single *xylS2/Pm→lacZ* assembly or the coupled *nahR/Psal→xylS2* and *Pm→lacZ* system. Immunoblots of the *E.coli* strain 4S2PT32 confirmed that XylS2 production was induced when using an effector molecule for NahR but not when using benzoate (Fig. 3E). These results suggest that the mere addition of an upstream regulator results in a greater amplification.

The performance of the XylS2/NahR cascade relies on a determined hierarchy of regulators

To ascertain the requirement of regulatory hierarchy in the cascade amplification, we studied the effect of swapping the upstream and downstream regulators in the XylS2/NahR-based cascade by constructing *E.coli* 2NRSL7. The chromosome of this strain bears the DNA elements *xylS2/Pm→nahR* and *Psal→lacZ* carried by specialized transposons (see above) and is thus equivalent to *E.coli* strain 4S2PT32, apart from the order of the regulators in the coupled system. As shown in Figure 4, the capacity of the reverse-coupled system responding to salicylate (24-fold induction) did not increase the capacity over the single *nahR/Psal→lacZ* element. Along

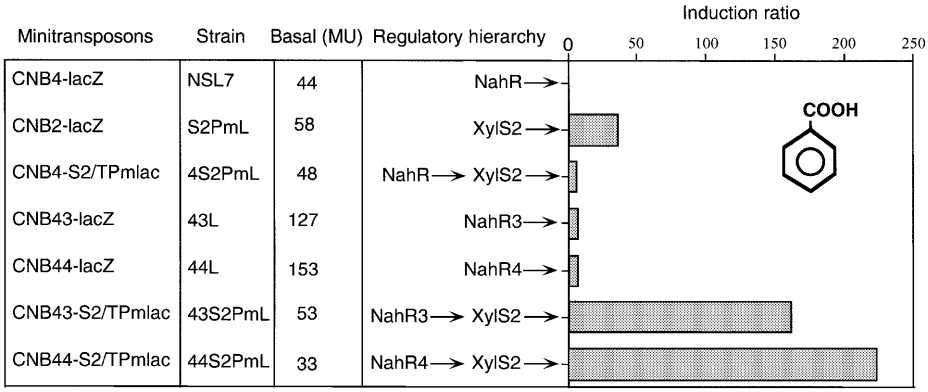

Figure 5. Capacity for regulation of single and cascade circuits in *P.putida*. Strains in the chromosome bearing different minitransposons (first column) containing the regulatory system described in the text were assayed for their β-galactosidase accumulation in response to benzoate 2 mM. The fourth column displayed the regulators and sequential order in the corresponding strain. Data are mean values of three independent experiments. Basal values are indicated in MU.

the same lines, the reversed-coupled system of *E.coli* 2NRSL7 was completely insensitive to the XylS2-only effector benzoate. These observations indicated that overexpression of *nahR* did not result in a parallel increase in *Psal* activity, but in a non-productive excess of the second regulator because the same gene expression capacity from *Psal* could be achieved at a relatively low concentration of NahR. Studies on the mechanism of *Psal* activation indicated that the target site for this activator is occupied regardless of the induction conditions (29). Thus, the *Psal* promoter appears to depend exclusively on the presence or absence of salicylate and overexpression of *nahR* does not produce higher promoter activity. Nevertheless, the basal expression of *nahR* from the heterologous *xylS2/Pm* system seemed to limit the available NahR protein necessary to activate *Psal* promoter, since the *xylS2/Pm→nahR/Psal→lacZ* cascade could only be induced 4-fold after the NahR-unique inducer 3,5-dichlorosalicylate. In contrast, the same compound induced 20-fold *lacZ* expression from *nahR/Psal*.

Taken together, these results indicate that the efficiency of the amplification of the regulatory cascade requires specific properties of the downstream regulator/promoter which include, at least, the activation of the final target promoter in a dose-dependent fashion for a broad range of regulator concentrations. This is true for XylS2 but not for NahR. Thus, the mere sensitivity of the two regulators to the same effector did not result in an augmentation effect unless the adequate hierarchy, based on the appropriate mechanisms of the second regulatory system, was present.

Redesigning a cascade circuit for benzoate hyper-response in *P.putida*

In view of the results above, and given the ease of obtaining both XylS and NahR mutants responsive to a large number of structural homologs of the natural effectors (16,17), it became possible to construct regulatory cascades, as required, to amplify some cell responses to predetermined inducers. In this context we pursued the design of a specialized *P.putida* strain bearing a benzoate cascade control circuit. Since the downstream regulator (XylS2) already responded to this inducer (Fig. 5), the design of a new cascade involved mainly the modification of the upstream regulatory system. To this end, we employed two *nahR* mutants encoding benzoate-responsive variants *nahR3* and *nahR4* (17). They were assembled in the coupled system *nahR3/Psal→xylS2* and *nahR4Psal→xylS2* along with the reporter segment *Pm→lacZ* and then inserted into the chromosome of *P.putida* strain KT2442 to yield *P.putida* 43S2PmL and *P.putida* 44S2PmL. The induction of these strains by benzoate was compared to that of strains bearing either the simple elements *P.putida* 43L (*nahR3/Psal→lacZ*), *P.putida* 44L (*nahR4/Psal→lacZ*) and *P.putida* S2PmL (*xylS2/Pm→lacZ*), or the cascade with the wild-type benzoate-insensitive *nahR* variant (Fig. 5). The cascade with the benzoate-responsive *nahR* mutants increased the gene expression capacity from 6- to 35-fold in response to benzoate, compared with the single expression systems with *nahR3*, *nahR4* or *xylS2*. In contrast, the cascade circuit with the wild-type *nahR* in 4S2PmL (*nahR/Psal→xylS2*, *Pm→lacZ*) in *Pseudomonas* showed reduced induction capacity by benzoate (~7-fold), due to the absence of response of the upstream regulator to benzoate. These values matched the predictions raised from the simultaneous induction of each regulator upon acquisition of the ability to respond to benzoate by NahR.

To eliminate the possibility that the amplification property was only a particular feature of the mutant form of XylS (XylS2) and not of the wild type, we constructed a regulatory circuit with the *nahR4* mutant as a first regulator and *xylS/Pm* as a second regulator. We tested the inducibility of the circuits in response to the common effector benzoate and salicylate that is not recognized by the XylS. Augmentation of gene expression capacity was produced when the common effector was added but not when salicylate was present (Fig. 6). The regulatory scheme *nahR4/Psal→xylS* plus *Pm→lacZ* showed at least 4-fold more induction capacity in response to benzoate than the single circuits, *nahR4/Psal→lacZ* (Fig. 5) or *xylS/Pm→lacZ* (Fig. 6). Western blotting of *P.putida nahR/Psal→xylS* and *Pm→lacZ* cells exposed to each of the inducers revealed that the increase in XylS production brought about by salicylate was insufficient to match the amplification effect caused by benzoate even at lower intracellular concentrations of the regulator (Fig. 6). Therefore, the simultaneous response of the first and second regulators was also required in the natural host for the amplification effect using a wild-type *xylS*.

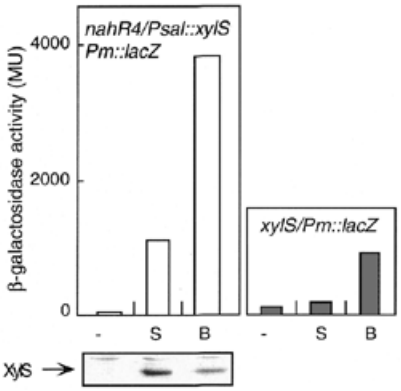

Figure 6. Analysis of a cascade circuit with *nahR4/Psal* leading to the expression of wild-type *xylS* in *P.putida*. β-galactosidase accumulation after addition of no effector (–), NahR4-specific effector (S, salicylate) or NahR4/XylS common effector (B, benzoate) at 2 mM in the cascade system (left) or a *xylS/Pm→trp′::′lacZ* simple circuit (right). A western blot for detection of XylS production in *P.putida* (*nahR4/Psal→xylS/Pm→trp′::′lacZ*) cultures after addition of the different effectors is shown under the corresponding graph.

DISCUSSION

In this work, we have shown how two regulators can amplify the response to a specific signal by combining the regulatory elements in a cascade circuit. Optimal coupling between the XylS and NahR regulators for the synergistic effect in gene expression required that (i) the downstream regulator activates the target promoter in a dose-dependent manner over a broad range of intracellular concentration, and (ii) both activators respond positively to the same inducer.

The effectiveness of the amplification by the analyzed cascade depends on a previous sensing of the signal molecule to lead the expression of the downstream regulator. The natural cascade regulatory circuit in which XylS is involved, implies the control of *xylS* expression through the XylR regulatory protein. This protein induces *xylS* expression and the upper TOL operon in response to *m*-xylene or toluene, which are the substrates of the upper pathway. The products of catabolism of the upper pathway (benzoate or *m*-toluate) are effectors of the XylS protein. This regulator induces the expression of the *meta*-operon, whose products degrade benzoate to three carboxylic acids. It is then expected that for the efficient regulation, the upstream regulator (XylR) senses first the signal that triggers all the cascade event through the downstream regulator, XylS. The XylR regulator may prepare the *meta*-operon for a high induction to the upper-pathway benzoate products by increasing the levels of the XylS regulator, and thus, increasing the response to the catabolytes that will be produced by the upper operon products. In fact, results of Marqués *et al.* (13) showed that mRNA produced from the *Pm* promoter, including the *upper*-pathway inducer-substrate, was 2-fold higher than with a *meta*-pathway substrate-effector.

Although maximal amplification of gene expression capacity occurred when the signal inducer was positive for both regulators, the inducer-independent activitation of *Pm* by XylS2 can also augment gene expression capacity from the upstream regulator in *E.coli*. Increment of XylS2 concentration due to the presence of a NahR-only inducer, multiply the gene expression capacity reached by *nahR/Psal* ~4-fold. An increment over the unit may indicate cooperative effect in the activation of *Pm* with the inducer independent XylS2 activity. However, in *P.putida* the constitutive transcriptional activity of XylS did not show such amplification of gene expression capacity since induction of β-galactosidase activity from the *nahR/Psal→xylS/Pm→lacZ* cascade by the NahR4-only inducer salicylate did not result in augmentation of gene expression capacity with respect to the single system. This might be explained by the superior constitutive activity of XylS2 with respect to XylS or because of different specific activity of these regulators in *E.coli*, with respect to *P.putida*, or both. It is noteworthy that the basal value of the *Pm* promoter is considerably lower in *P.putida* than in *E.coli*, indicating that there are differences in the specific activities of the regulatory elements in each background.

The comparison of 30 different prokaryotic regulatory circuits (30) revealed that the rhamnose-responsive system of *E.coli* presents the maximum gene expression capacity known in a natural system. Interestingly, this system works in a similar manner to the artificial cascade described in this work. Rhamnose-responsive promoters are induced by this sugar through the action of two activators of the AraC/XylS family called RhaR and RhaS. Each activator can separately stimulate transcription of their cognate promoters by 300-fold in the presence of the carbohydrate. Interestingly, RhaR controls the expression of the *rhaS* gene, whose product triggers rhamnose catabolism (31). The coupling of RhaR and RhaS allows a 30 000-fold induction of the rhamnose operon, which results from the cooperative stimulation with the same signal.

The efficiency of the amplification we observed in the NahR/XylS2 combination might not be that different to the fact that several naturally occurring regulatory cascades include an activator of the AraC family as a downstream regulator. For instance, a part of XylS itself in the TOL system, the VirF product of *Vibrio cholerae*, as well as the RhaS and SoxS regulators of *E.coli*, act as the second regulator in different circuits (32,33). The occurrence of XylS-related regulators as downstream regulatory factors described above may suggest the existence of a type of regulator whose expression is eventually controlled by a first or 'master' regulator and that might act as an amplifier in different control circuits. It is conceivable that at least part of the AraC/XylS family of regulators might share the signal amplifier potential shown for XylS and RhaR/S.

The degree of precision in the knowledge of the mechanisms and factors for gene expression control is allowing the design of artificial regulatory circuits to engineer new functions (34,35). The possibility of engineering the amplification of gene expression capacity in response to environmental signals by cascade circuits with the positive regulators shown might be used for the design of new expression systems, whole cell biosensors or control circuits for gene therapy requiring reduced basal levels and high induced levels.

ACKNOWLEDGEMENTS

The authors are indebted to G. Bertoni (University of Milan) for anti-XylS antibodies and gratefully acknowledge E. Santero, J. Pérez-Martín and S. Marqués for critical reading of the manuscript. This work was supported by contracts ENV4-CT95-0141

and BIO4-CT97-2040 from the EU and by grant BIO98-0808 from the Plan Nacional de Investigación (CICYT).

REFERENCES

1. Stragier,P. and Losick,R. (1990) Cascades of sigma factors revisited. *Mol. Microbiol.*, **4**, 1801–1806.
2. Banuett,F. (1998) Signalling in the yeasts: an informational cascade with links to the filamentous fungi. *Microbiol. Mol. Biol. Rev.*, **62**, 249–274.
3. Fischer,H.M. (1994) Genetic regulation of nitrogen fixation in rhizobia. *Microbiol. Rev.*, **58**, 352–386.
4. Dorman,C.J. and Porter,M.E. (1998) The *Shigella* virulence gene regulatory cascade: a paradigm of bacterial gene control mechanisms. *Mol. Microbiol.*, **29**, 677–684.
5. Harayama,S. and Timmis,K.N. (1989) Catabolism of aromatic hydrocarbons by *Pseudomonas*. In Hopwood,D. and Chater,K. (eds), *Genetics of Bacterial Diversity.* Academic Press, London, UK, pp. 151–174.
6. Gallegos,M.T., Schleif,R., Bairoch,A., Hofmann,K. and Ramos,J.L. (1997) AraC/XylS family of transcriptional regulators. *Microbiol. Mol. Biol. Rev.*, **61**, 393–410.
7. Mermod,N., Lehrbach,P.L., Reineke,W. and Timmis,K.N. (1984) Transcription of the TOL plasmid toluate catabolic pathway operon of *Pseudomonas putida* is determined by a pair of co-ordinately and positively regulated overlapping promoters. *EMBO J.*, **3**, 2461–2466.
8. Ramos,J.L., Mermod,N. and Timmis,K.N. (1987) Regulatory circuits controlling transcription of TOL plasmid operon encoding meta-cleavage pathway for degradation of alkylbenzoates by *Pseudomonas*. *Mol. Microbiol.*, **1**, 293–300.
9. Ramos,J.L., Marqués,S. and Timmis,K.N. (1997) Transcriptional control of the *Pseudomonas* TOL plasmid catabolic operons is achieved through an interplay of host factors and plasmid-encoded regulators. *Annu. Rev. Microbiol.*, **51**, 341–373.
10. Dixon,R. (1986) The *xylABC* promoter from the *Pseudomonas putida* TOL plasmid is activated by nitrogen regulatory genes in *Escherichia coli*. *Mol. Gen. Genet.*, **203**, 129–136.
11. Marqués,S. and Ramos,J.L. (1993) Transcriptional control of the *Pseudomonas putida* TOL plasmid catabolic pathways. *Mol. Microbiol.*, **9**, 923–929.
12. Kessler,B., Herrero,M., Timmis,K.N. and de Lorenzo,V. (1994) Genetic evidence that the XylS regulator of the TOL *meta*-operon of *Pseudomonas* controls *Pm* promoter through weak DNA-protein interaction. *J. Bacteriol.*, **176**, 3171–3176.
13. Marqués,S., Holtel,A., Timmis,K. and Ramos,J.L. (1994) Transcriptional induction kinetics from the promoters of the catabolic pathways of TOL plasmid pWW0 of *Pseudomonas putida* for metabolism of aromatics. *J. Bacteriol.*, **176**, 2517–2524.
14. Yen,K.-M. and Serdar,C.M. (1988) Genetics of naphtalene metabolism in pseudomonads. *Crit. Rev. Microbiol.*, **15**, 247–268.
15. Schell,M.A. (1993) Molecular biology of the LysR family of transcriptional regulators. *Annu. Rev. Microbiol.*, **47**, 597–626.
16. Ramos,J.L., Stolz,A., Reineke,W. and Timmis,K.N. (1986) Altered effector specificities in regulators of gene expression: TOL plasmid *xylS* mutants and their use to engineer expansion of the range of aromatics degraded by bacteria. *Proc. Natl Acad. Sci. USA*, **83**, 8467–8471.
17. Cebolla,A., Sousa,C. and de Lorenzo,V. (1997) Effector specificity mutants of the transcriptional activator NahR of naphtalene degrading *Pseudomonas* define protein sites involved in binding of aromatic inducers. *J. Biol. Chem.*, **272**, 3986–3992.
18. Sambrook,J., Fritsch,E.F. and Maniatis,T. (1989) *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
19. Miller,J.H. (1972) *Experiments in Molecular Genetics.* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
20. Ramos,J.L., Carrero,M. and Timmis,K.N. (1988) Broad host range expression vectors containing manipulated meta-cleavage pathway regulatory elements of the TOL plasmid. *FEBS Lett.*, **226**, 241–246.
21. Myers,R.M., Lerman,L.S. and Maniatis,T. (1985) A general method for saturation mutagenesis of cloned DNA fragments. *Science*, **229**, 242–247.
22. Kunkel,T.A., Roberts,J.D. and Zakour,R.A (1987) Rapid and efficient site-specific mutagenesis without phenotypic selection. *Methods Enzymol.*, **154**, 367–382.
23. Kessler,B., Timmis,K.N. and de Lorenzo,V. (1994) The organization of the *Pm* promoter of the TOL plasmid reflects the structure of its cognate activator protein XylS. *Mol. Gen. Genet.*, **244**, 596–605.
24. de Lorenzo,V., Fernández,S., Herrero,M., Jakubzik,U. and Timmis,K.N. (1993) Engineering of alkyl- and haloaromatic-responsive gene expression with mini-transposons containing regulated promoters of biodegradative pathways of *Pseudomonas*. *Gene*, **130**, 41–46.
25. Schell,M. and Sukordhaman,M. (1989) Evidence that the transcriptional activator encoded by the *Pseudomonas putida nahR* gene is evolutionarily related to the transcriptional activators encoded by the *Rhizobium meliloti nodD* genes. *J. Bacteriol.*, **171**, 1952–1959.
26. de Lorenzo,V. and Timmis,K.N. (1994) Analysis and construction of stable phenotypes in gram-negative bacteria with Tn*5*- and Tn*10*-derived minitransposons. *Methods Enzymol.*, **235**, 386–405.
27. Herrero,M., de Lorenzo,V. and Timmis,K.N. (1990) Transposon vectors containing non-antibiotic resistance selection markers for cloning and stable chromosomal insertion of foreign genes in gram negative bacteria. *J. Bacteriol.*, **172**, 6557–6567.
28. Cebolla,A., Guzman,C. and de Lorenzo,V. (1996) Non-disruptive detection of activity of catabolic promoters of *Pseudomonas* with an antigenic surface reporter system. *Appl. Environ. Microbiol.*, **62**, 214–220.
29. Huang,J.Z. and Schell,M.A. (1991) In vivo interactions of the NahR transcriptional activator with its target sequences. Inducer-mediated changes resulting in transcription activation. *J. Biol. Chem.*, **266**, 10830–10838.
30. Hlavacek,W.S. and Savageau,M.A. (1996) Rules for coupled expression of regulator and effector genes in inducible circuits. *J. Mol. Biol.*, **255**, 121–139.
31. Egan,S.M. and Schleif,R.F. (1993) A regulatory cascade in the induction of *rhaBAD*. *J. Mol. Biol.*, **234**, 87–98.
32. DiRita,V.J., Parsot,C., Jander,G. and Mekalanos,J.J. (1991) Regulatory cascade controls virulence in *Vibrio cholerae*. *Proc. Natl Acad. Sci. USA*, **88**, 5403–5407.
33. Gallegos,M.T., Michán,C. and Ramos,J.L. (1993) The XylS/AraC family of regulators. *Nucleic Acids Res.*, **21**, 807–810.
34. Elowitz,M.B. and Leibler,S. (2000) A synthetic oscillatory network of transcriptional regulators. *Nature*, **403**, 335–338.
35. Gardner,T.S., Cantor,C.R. and Collins,J.J. (2000) Construction of a genetic toggle switch in *Escherichia coli*. *Nature*, **403**, 339–342.
36. de Lorenzo,V., Eltis,L., Kessler,B. and Timmis,K.N. (1993) Analysis of the *Pseudomonas* gene products using *lacI*^{q}/*Ptrp-lac* plasmids and transposons that confer conditional phenotypes. *Gene*, **123**, 17–24.
37. Minton,N.P. (1984) Improved plasmid vectors for the isolation of translational *lac* gene fusions. *Gene*, **31**, 269–273.
38. de Lorenzo,V., Herrero,M., Jakubzik,U. and Timmis,K.N. (1990) Mini-Tn*5* transposon derivatives for insertion mutagenesis, promoter probing and chromosomal insertion of cloned DNA in gram negative eubacteria. *J. Bacteriol.*, **172**, 6568–6572.