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A new generation of vectors with increased induction ratios by overimposing a second regulatory level by attenuation

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ABSTRACT

A major drawback of regulated gene expression from vectors bearing strong promoters is the associated high basal expression level. Simple regulatory systems have an intrinsic limitation in the range of induction, and attempts to mutate promoters to reduce basal expression usually result in concomitant reduction of induced levels. We have explored the possibility of reducing basal levels of gene expression while keeping induced levels intact by incorporating an additional regulatory circuit controlling a different step of the expression process. We have integrated the *nasFEDCBA* transcriptional attenuation system of *Klebsiella oxytoca* into a cascade expression circuit based on different regulatory elements of *Pseudomonas putida*, and also into a system based on the *tac* promoter, to expand their regulatory capacity. Basal expression from the promoters of these circuits was reduced by more than 10-fold by the *nasF* attenuator sequence while keeping the induced levels intact in the presence of the antiterminator protein, thus increasing the induction ratio by up to 1700-fold. In addition, using different combinations of regulatory elements and inducing conditions, we were able to obtain a broad range of expression levels. These vectors and the concept of their design will be very useful in regulating overproduction of heterologous proteins both at laboratory and industrial scales.

INTRODUCTION

Most prokaryotic expression systems consist of a strong transcriptional initiation signal for bacterial or viral RNA polymerases placed on a multicopy plasmid. Spurious promoter escape from the selected transcriptional promoter, plasmid read-through or cryptic initiation signals clearly raises basal expression levels of heterologous genes in high-copy number expression vectors (1). A large number of heterologous sequences, particularly those coding for membrane proteins (2), are often recalcitrant to cloning or confer instability owing to a leaky expression under non-induced conditions. Any gene whose expression decreases the growth rate of the host strain will favor the appearance of expression-down phenotypes, which will dominate cell cultures when fermentation reaches high cell densities (1,3,4).

Different approaches have been developed to tighten the control of gene expression. Some involve the reduction of gene dosage either by low-copy number plasmids or by chromosome integration (4–6); others decrease the basal level of the promoter by means of point mutations. However, these modified systems do not sustain a high level of gene expression. A general strategy also used in the past few years has been the search for more efficient repressors, or even combinations of some of them to reduce basal levels while maintaining high-induction ratios (7).

Natural regulatory systems may have additional control levels to modulate gene expression. A supplementary control step in expression vectors may help to coordinate the expression of different proteins or to improve the heterologous recombinant protein yield (8,9). Several alternative regulatory levels to transcription initiation control have also been described. These include mRNA stability (10,11), translational efficiency (12,13) and protein stability (14). Although these additional regulatory levels may provide advantages to silence protein expression under non-induced conditions, few of them have been employed in expression.

An example of regulation at a step different from transcription initiation is the attenuation of transcription elongation. Among the different mechanisms to control gene expression, attenuation has always been considered a highly sophisticated

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and useful strategy (15). Either alone or in combination with repressors or activators, attenuation-dependent expression allows very tight regulation of gene expression (16–18). Besides the attenuation exerted by the ribosomes, several attenuation systems, which involve the use of a specific RNA-binding regulatory protein to control the formation of a terminator structure, have been studied in different bacteria, such as *Escherichia coli*, *Klebsiella oxytoca* (formerly *pneumoniae*), *Salmonella typhimurium* and *Bacillus subtilis* (19).

In this work we have focused our interest on the regulatory elements controlling the genes of nitrate assimilation of *K.oxytoca*. When necessary, *K.oxytoca* can use nitrate (NO_3^-) or nitrite (NO_2^-) as the only nitrogen source during aerobic growth by controlling the expression of the *nasFEDCBA* operon (20). Upstream and divergent from the *nasFEDCBA* operon (abbreviated as *nasF*) lies *nasR*, which encodes an antiterminator protein (21). While growing under nitrogen sufficient conditions, expression of the *nasF* operon remains silenced owing to transcription termination at an attenuator located upstream of the *nasF* coding region. Upon nitrogen starvation, NasR is expressed from its own promoter. In the presence of nitrate or nitrite NasR is activated, thus preventing transcription termination at the attenuator and, consequently, allowing expression of the *nas* genes required for nitrate and nitrite assimilation (22–24).

We have recently developed a novel expression circuit based on different elements of *Pseudomonas putida*, which involve both a regulatory module and an expression module working in cascade. In this system, the regulatory module *nahR*/P_{sal}–*xylS2* fusion is inserted in the bacterial chromosome by means of a mini-*Tn5* delivery system. When salicylate is present in the culture medium, NahR activates transcription from P_{sal}, thus producing XylS2 (Figure 1A). XylS2 is a mutated form of the transcriptional activator XylS, which responds to salicylate. Therefore, salicylate concomitantly activates XylS2, which together with its increased intracellular concentration synergistically amplifies transcription from the Pm promoter present in the expression module (4,9,25). In the absence of salicylate, basal expression levels are very low, owing to the limited concentration of inactive XylS2. However, basal levels may not be sufficiently low to clone toxic proteins in this kind of cascade regulatory circuit.

In this work, we have used the *nasF* attenuator and the NasR-dependent antitermination system to construct a novel expression circuit that could conditionally prevent undesired transcription from any transcriptional initiation signal. Additionally, we show that the antitermination system may also be functional when combined with other expression systems based on promoters recognized by the bacterial RNA polymerase, such as the *tac* promoter. To our knowledge, this is the first expression vector with an attenuation mechanism, which avoids undesired transcription of the cloned gene under non-inducing conditions.

MATERIALS AND METHODS

Strains and growth conditions

Plasmids and strains are described in Table 1. Luria–Bertani (LB) medium contained 10 g/l tryptone, 5 g/l NaCl and 5 g/l yeast extract. When necessary, LB was supplemented with 0.2 g/l sodium nitrate to induce NasR-dependent antitermination. Ampicillin was used at final concentration of 100 μg/l while gentamicin was used at 7.5 μg/l. Cultures were grown aerobically at 150 r.p.m. and 37°C; upon induction, cultures were incubated at 30°C.

Genomic DNA isolation

Isolation of genomic DNA from *K.oxytoca* M5a1 was performed as described previously by Silberstein and Cohen (26) with some variations. Briefly, 5 ml of a saturated culture of *Klebsiella* was centrifuged and the pellet was frozen at −20°C. The pellet was then resuspended in 0.4 ml of lysis buffer (50 mM Tris–HCl, pH 8, 10 mM EDTA, 100 mM NaCl, 0.2% SDS and 100 mg/l RNase) and incubated at 37°C. After 30 min, 20 μl of proteinase K (20 g/l) was added and further incubated at 65°C for 2 h. The sample was extracted with phenol and precipitated with ethanol. Genomic DNA was resuspended in 0.5 ml of sterile miliQ water; concentration and purity of DNA were determined by OD_{260}/OD_{280}.

PCRs

The *nasF* attenuator was amplified by PCR using *K.oxytoca* genomic DNA as template and the following primers: TerNasF2, 5′-GGAATTC GAG TGA ATA AAA GGT TTT GGG CAG CGC-3′; and TerNasR2, 5′-GGAATTC GCG CAA AAA AAA AGC GCC CGG CGG TGC-3′. Underlined nucleotides are EcoRI restriction sites. PCR was performed at a final volume of 25 μl containing 25 ng of *K.oxytoca* chromosomal DNA, 10 pg of each primer and 2.5 mM $MgCl_2$. Initial 5 min denaturation at 95°C was followed by 35 cycles of amplification (95°C for 30 s and 72°C for 2 min), and a final extension of 5 min at 72°C. It should be noticed that some differences were found between the *nasF* attenuator sequence described by Lin and co-workers (GenBank accession no. AF038047) and others derived from the annotated genome of *K.oxytoca* (KPU34073). They could be simply due to different ecotypes of the strains from which sequences were obtained rather than annotation mistakes. In fact, these polymorphisms did not change the secondary structure of the attenuator (data not shown). Primers were designed according to the sequence with the GenBank accession no. AF038047.

The regulatory gene *nasR* was amplified using the following primers: NasR1F, 5′-ACG GTT ATT GCT TGG CTG AAG-3′; and NasR1R, 5′-ATGAGCTC CTA CTC CTT TGG GGT TAC G-3′. Underlined nucleotides are SacI restriction sites. PCR contained again 25 ng of *K.oxytoca* chromosomal DNA as template, 10 pg of each primer and 2.5 mM MgCl. Initial 5 min denaturation at 95°C was followed by 35 cycles of amplification (95°C for 30 s, 62°C for 30 s and 72°C for 45 s), and a final extension of 5 min at 72°C.

β-Galactosidase activity determination

The plasmids pMPO6, pMPO16, pBM8 and pBM9 were used to transform either alone or together with pIZ1016, pMPO8, pMPO24 or pMPO25 into the strain CC118 4S2. Cultures inoculated with the transformed strains were grown aerobically overnight in LB with ampicillin and/or gentamicin when required. Grown cultures were diluted 50-fold and incubated at 37°C. When OD_{600} reached 0.2–0.3, cultures were induced

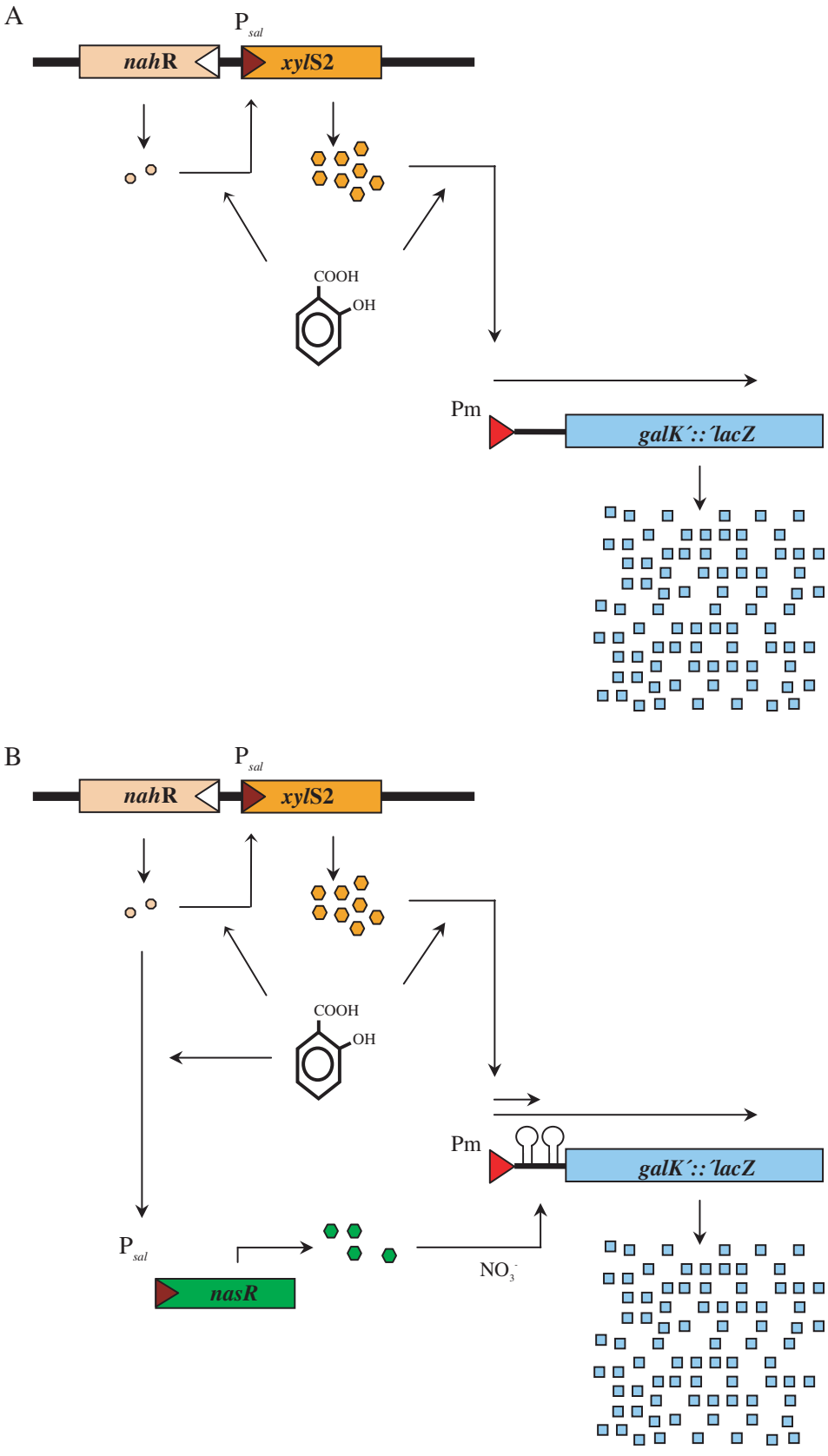

Figure 1. (**A**) Design of the cascade expression system consisting of the regulatory module *nahR*/P_{sal}–*xylS2* and the expression module Pm-*galK′::′lacZ*. (**B**) Modified design of the expression circuit which contains the *nahR*/P_{sal}–*xylS2* and the P_{sal}-*nasR* regulatory modules, and their target the Pm-*nasF* attenuator expression module.

Table 1. Strains and plasmids

	Characteristics	Reference
Strains		
Escherichia coli DH5α	*deoR endA1 gyrA96 recA1 supE44*	(35)
E.coli S171-λpir	F^- *recA hsdR* RP4-2 (Tc::Mu)(Km::Tn7) lysogenized with λpir phage	(36)
Klebsiella oxytoca M5a1	Wild-type strain of *K.oxytoca* (*pneumoniae*)	Laboratory collection
E.coli CC118 4S2	*phoA20 thi-1 rspE rpoB argE* (Am) *recA1* with a Km mini-*Tn*5 bearing a *nahR*/P*sal–xylS2* fusion	(9)
Plasmids		
pBM8	Tc^R, pVLT31 derived plasmid with *lacI*^{q}-P_{tac}-*galK′::′lacZ*	This work
pBM9	Tc^R, pVLT31 derived plasmid with *lacI*^{q}-P_{tac}-*nasF* attenuator-*galK′::′lacZ*	This work
pCAS	Ap^R, expression vector with rrnBT1T2-Pm::MCS, ColE1 replication origin	Active motif
pCNB4-S2	Ap^R, Km^R, miniTn5 vector with the *nahR*/P_{sal}–*xylS2* fusion cloned between the I and O sites	(9)
pIC552	Ap^R, promoter-less vector bearing a *galk′::′lacZ* fusion	(28)
pIZ1016	Gm^R, broad-host range expression vector bearing lacI^q and P_{tac}	(29)
pMPO6	Ap^R, pCAS with rrnBT1T2-Pm-*galK′::′lacZ*	This work
pMPO16	Ap^R, pCAS with rrnBT1T2-Pm-*nasF* attenuator-*galK′::′lacZ*	This work
pMPO7	Ap^R, Bluescript SK^+ with *nasR* cloned in EcoRV	This work
pMPO8	Gm^R, plasmid derived from pIZ1016 with *nasR* cloned downstream P_{tac}	This work
pMPO9	Ap^R, pMPO6 derived plasmid with *nasR* downstream of Pm	This work
pMPO10	Ap^R, pMPO16 derived plasmid with *nasR* downstream of the Pm and the *nasF* attenuator	This work
pMPO24	Gm^R, plasmid derived from pMPO8 with *nasR* cloned downstream P_{sal}	This work
pMPO25	Gm^R, plasmid derived from pMPO8 with a fusion P_{sal}-*nasF* attenuator-*nasR*	This work
pMPO27	Ap^R, expression vector with rrnBT1T2-Pm-*nasF* attenuator::MCS, ColE1 replication origin	This work
pUC19	Ap^R, cloning vector, ColE1 replication origin	New England Biolabs
pVLT31	Tc^R, expression vector bearing *lacI*^{q}-P_{tac}	(37)

with 2 mM salicylate or 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) and incubated at 30°C. If necessary, LB was supplemented with 0.2 g/l sodium nitrate. Induced and non-induced cultures were incubated at 30°C and 150 r.p.m., and β-galactosidase activities were assayed 5 h after induction as described previously (27).

RESULTS

Construction of cascade expression vectors bearing the *nasF* attenuator

To explore the potential use of the *nasF* transcriptional attenuator as an element to reduce undesired basal transcription, we analyzed both non-induced and induced expression from the cascade expression vector pCAS bearing the regulated Pm promoter, in which the attenuator was inserted downstream of the promoter. To report expression levels, we used a *galK′::′lacZ* gene fusion that provides a linear correlation between the level of transcription and the protein yield because of its low mRNA stability (A. Cebolla, unpublished data).

An EcoRI–HindIII fragment from pIC554 (28) containing the *galK′::′slacZ* gene fusion was inserted into the same sites of pCAS, generating pMPO6 (Figure 2). This plasmid contained a unique EcoRI restriction site between the transcription initiation (+1) and the Shine–Dalgarno of *galK*. The 120 bp corresponding to the *nasF* attenuator was amplified as described in Materials and Methods, digested and cloned into pMPO6 already linearized with EcoRI, thus placing the attenuator between the Pm promoter and the SD of the *galK′::′lacZ* gene. The correct orientation of the insertion was checked by PCR (data not shown). The resulting plasmid was named pMPO16.

In order to construct a suitable cloning expression vector also bearing the attenuator, a multiple cloning site was introduced downstream the Pm promoter and the *nasF* attenuator. For this, pUC19 was digested with EcoRI, filled in and subsequently digested with HindIII. The resulting 50 bp fragment containing the polylinker was isolated and inserted in pMPO16 digested with SmaI–HindIII, which eliminated the fragment bearing the *galK′::′lacZ* gene fusion. The resulting plasmid named pMPO27 allows cloning of any gene downstream of the regulatory Pm-*nasF* promoter–attenuator region, as described in Figure 2.

To provide the system with the antiterminator protein, *nasR* (GenBank accession no. L27824) was amplified as described in Material and Methods and the 1.3 kb amplicon was cloned into pBluescript SK^+ linearized with EcoRV, thus yielding pMPO7. A HindIII(filled in)–SacI fragment from pMPO7, containing *nasR*, was subcloned into pIZ1016 (29) digested with SmaI and SacI. The resulting plasmid, designated pMPO8, contained the *lacI*^{q} repressor and expressed *nasR* under the control of the P_{tac} promoter. Compatibility of its replication origin with ColE1 replicons allowed the coexistence of this modulator plasmid and the expression vector.

We characterized the functionality of the expression module present in pMPO16, using the strain CC118 4S2, which bears the regulatory module of the cascade system in the chromosome. Some of the different configurations of the expression system together with their expected expressions are illustrated in Figure 3. When neither active XylS2 nor NasR were present in the cytoplasm, *nasF* attenuator should reduce the non-induced transcription levels (Figure 3A). When salicylate was added to the culture medium, active XylS2 would bind to the Pm upstream target sequence, thus prompting a high-transcription initiation rate; however, the attenuator should provoke termination of most of the potential *lacZ* full transcripts (Figure 3B) since the absence of nitrate would prevent NasR antitermination function although NasR was produced; alternatively, some residual antitermination activity would increase the levels of β-galactosidase activity (Figure 3C).

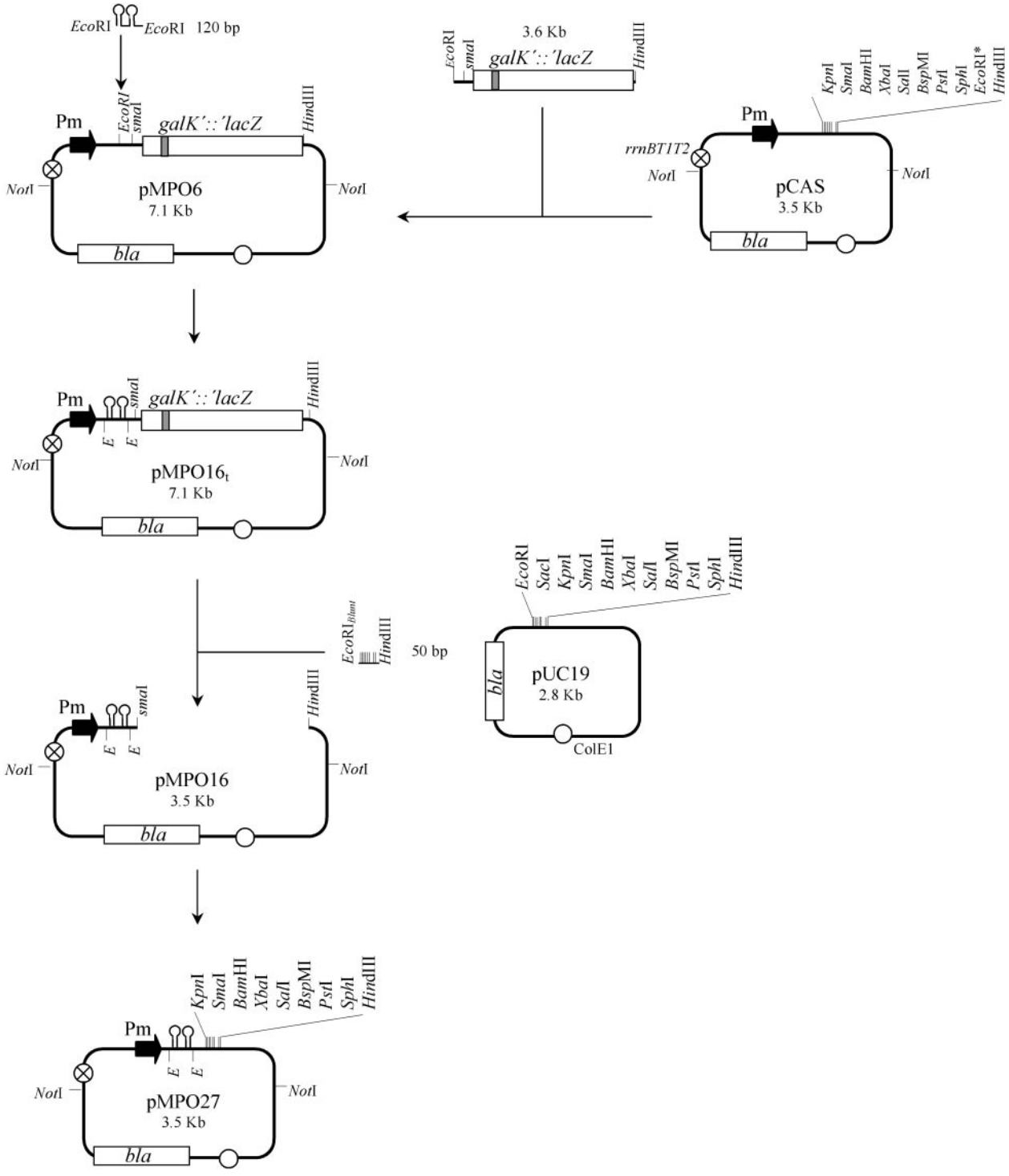

Figure 2. Schematic diagram of the plasmid constructions. Relevant restriction sites are indicated. *bla* corresponds to β-lactamase resistance gene, hairpin loops represent the *nasF* attenuator, crossed circles represent transcription terminators while open circles represent the *ori*V.

The system would be fully induced only when all the inducers were added to the culture medium. This way NasR activity would be maximal, thus enabling maximum antitermination and, therefore, highest expression levels (Figure 3D). These two overimposed circuits controlling both transcription initiation and premature termination should allow a fine tuning of gene expression.

Effect of the *nasF* attenuator on the basal levels of expression

To quantify the effect of the *nasF* attenuator over the basal transcription levels, pMPO16 was used to transform *E.coli* CC118 4S2. In the absence of any inducer, strains bearing pMPO16 showed >10-fold lower β-galactosidase activity

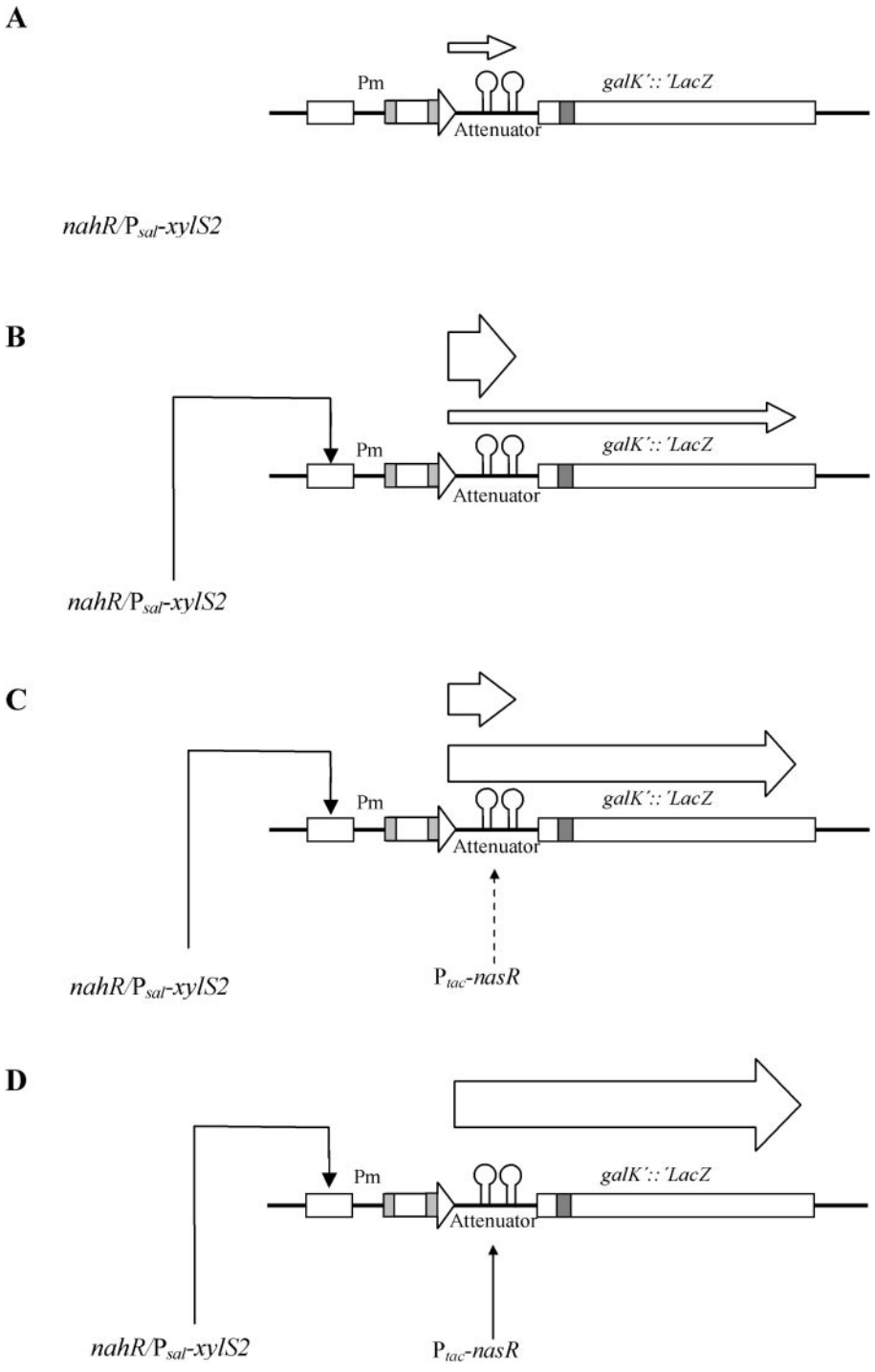

Figure 3. Schematic diagram of the different rates of transcription of the modular expression system. (**A**) When neither active XylS2 nor NasR is present in the cytoplasm, the *nasF* attenuator terminates unspecific transcription. (**B**) When salicylate is added to the culture medium, active XylS2 binds to Pm prompting a high-transcription initiation; however, the attenuator still reduced most of the potential *lacZ* full transcripts. (**C**) If *nasR* expression was induced in spite of the absence of nitrate, some antitermination increases the β-galactosidase levels. (**D**) The system is fully induced when both IPTG and salicylate are added to the culture medium together with nitrate in order to activate NasR.

levels than those bearing pMPO6, thus indicating that more than 90% of the undesired transcripts were prematurely terminated at the *nasF* attenuator (Table 2).

When pMPO8, expressing the *nasR* antiterminator gene, was co-transformed together with pMPO16, the basal levels of β-galactosidase activity increased 4-fold; this is possibly due to a residual antitermination activity of the NasR produced owing to the P_{tac} transcriptional escape. Therefore, the termination capacity of the system in this configuration was reduced from 90 to 35%. When nitrate was present in the culture medium, basal levels of β-galactosidase activity additionally increased by 6-fold since the antitermination function

Table 2. Summary of expression levels and induction ratios using different combinations of expression and antitermination vectors

CC118 4S2	Non-induced (MU)		Induced (MU and fold induction)				
	$-NO_3$	$+NO_3$	Inducer	$-NO_3$		$+NO_3$	
pMPO6							
pIZ1016	1011 ± 200	954 ± 175	Sal	144 800 ± 6500	143-Fold	142 500 ± 15 200	141-Fold
pMPO16							
pIZ1016	84 ± 15	89 ± 10	Sal	24 500 ± 5000	292-Fold	24 200 ± 3000	287-Fold
pMPO24	81 ± 10	125 ± 50	Sal	108 300 ± 2400	1337-Fold	138 600 ± 14 500	1711-Fold
pMPO25	75 ± 8	90 ± 10	Sal	35 400 ± 3000	472-Fold	63 600 ± 1000	848-Fold
pMPO8	310 ± 35	499 ± 125	Sal	23 500 ± 1800	76-Fold	51 100 ± 12 400	165-Fold
			Sal + IPTG	85 700 ± 21 000	276-Fold	147 100 ± 21 800	475-Fold
pBM8							
pIZ1016	1592 ± 50	1510 ± 120	IPTG	20 000 ± 1500	13-Fold	21 200 ± 600	13-Fold
pBM9							
pIZ1016	137 ± 25	143 ± 20	IPTG	1800 ± 80	13-Fold	2000 ± 50	14-Fold
pMPO24	194 ± 20	618 ± 40	IPTG + Sal	3900 ± 100	20-Fold	18 000 ± 350	93-Fold

of NasR was fully activated. Induction of *nasR* expression by adding 1 mM IPTG to the culture medium also resulted in basal expression levels increasing by 8-fold. The presence of nitrate together with IPTG allowed basal expression levels from pMPO16 similar to those obtained from the plasmid without attenuator, since antitermination should happen with maximum efficiency under these conditions.

Capacity of gene expression control using combinations of NasR production and *nahR*/P_{sal}–*xylS2* activation

An important aspect to test is whether this regulation by the attenuation system could be reproduced when maximal transcription initiation rates were achieved from the Pm promoter. In the total absence of NasR (pMPO16 and pIZ1016), the fully induced system displayed a 292-fold induction (Table 2). This configuration did not allow maximum induction levels of the system since there was no antitermination. However, the basal expression levels were minimal, so this configuration may be useful in some circumstances, especially when the minimum expression level possible is required for bacterial growth. When pMPO8, which bears *nasR*, was present, the low levels of inactive NasR produced by basal transcription from the P_{tac} promoter allowed the expression system to achieve a 76-fold induction. Even if NasR was activated by the addition of nitrate, the induced levels were just doubled (164-fold induction). Thus, under these conditions, the residual NasR was insufficient to fully prevent transcription termination at the attenuator even when NasR was activated by nitrate. However, when NasR production was induced by the addition of 1 mM IPTG, 60% of the fully induced level was obtained even in the absence of nitrate. Finally, if nitrate, salicylate and IPTG were present, the induced levels from pMPO16 were completely recovered, since they were similar to those from the vector lacking the *nasF* attenuator (Table 2).

Therefore, using different combinations of plasmids bearing the *nasF* attenuator and the antiterminator *nasR*, together with the combinatorial addition of IPTG, salicylate and nitrate at fixed concentrations, it is possible to achieve a wide range of discrete induction ratios with different absolute expression levels, and to increase the regulation capacity of the cascade expression system from 150- to 480-fold induction.

In spite of these improvements, two disadvantages of the system were still evident. First, although escape from the P_{tac} promoter resulted in poorly active NasR, its residual antitermination activity still prevented full termination at the *nasF* attenuator under non-inducing conditions. Second, three different inducers were required for maximal expression. Particularly, the need of IPTG for full induction of the system may not be convenient if production is scaled up. These two negative aspects could be simultaneously eliminated if the system is re-designed so that transcription of *nasR* can also be driven from one of the promoters that are part of the cascade expression system.

Engineering coordinated expression of *nasR* with the cascade system

In an attempt to increase the regulation capacity of the system by using the *nasF* attenuation system, we coupled the expression of the *nasR* gene to the expression of the transcriptional activator XylS2 of the cascade expression system. This way, expression of *nasR* is minimized under non-induced conditions but it is co-induced with the second transcriptional activator upon addition of salicylate. The cascade amplification system involved two regulators: NahR and XylS2, and their target promoters P_{sal} and Pm, respectively. We placed *nasR* under the control of the P_{sal} promoter, thus allowing synchronous co-expression of XylS2 and the antiterminator protein (Figure 1B). For that purpose, we substituted the NcoI–SalI fragment containing the *lacI*^{q}-P_{tac} regulatory region in pMPO8 by a fragment containing the P_{sal} promoter, thus generating pMPO24. Additionally, to further prevent basal expression of *nasR* in case it was still significant, a derivative of pMPO24 was generated by replacing *nasR* with a fragment containing the *nasR* gene preceded by the *nasF* attenuator, which resulted in plasmid pMPO25.

We used either pMPO24 or pMPO25 together with pMPO16 to transform the CC118 4S2 strain and analyzed basal and induced expression levels. The lower promoter basal activity of P_{sal} (pMPO24) prevented significant antitermination at the attenuator present in pMPO16, thus resulting in a non-induced level undistinguishable from that shown by the configuration lacking *nasR* (Table 2). As expected, no lower basal level was obtained when using pMPO25, since it was already minimal. When induced with salicylate and nitrate, expression from pMPO16 in this new configuration using pMPO24 reached a level similar to the maximal expression

from the vector lacking the attenuator, thus indicating that induced transcription of *nasR* from P_{sal} provides sufficient antiterminator to fully prevent termination at the attenuator. Therefore, this configuration resulted in the highest induction ratio (1711-fold induction; Table 2). In contrast, the configuration using pMPO25 resulted in lower induction ratios (848-fold induction; Table 2). It appears that this configuration cannot generate enough NasR to fully antiterminate at both attenuators located in pMPO25 and pMPO16.

Attenuation/antitermination functions in other expression systems

In order to test whether the *nasF* transcriptional attenuator may also be useful to improve regulated transcription of other commonly used expression systems, we tested the functionality of the attenuator in a vector bearing the P_{tac} promoter (30), a negatively controlled very strong promoter, which shows significantly high leaky transcription even in the presence of the $lacI^q$ repressor allele. For this, XbaI–HindIII fragments from pMPO6 and pMPO16, containing the reporter *galK–lacZ* gene fusion preceded by the 5′-untranslated region, were cloned in the P_{tac} containing pVLT31 vector, thus generating pBM8 and pBM9 plasmids, respectively.

We introduced each plasmid, either alone or together with pMPO24, into the CC118 4S2 strain, and analyzed basal and induced expression levels using different combinations of IPTG, salicylate and nitrate. As shown in Table 2, basal expression from pBM8 in the absence of any inducer was significantly high. However, expression from the plasmid bearing the attenuator (pBM9) was >10-fold lower, thus indicating that the attenuation is similarly efficient in eliminating unwanted transcription from this completely different expression system. The presence of the plasmid bearing *nasR* did not significantly affect this basal level, suggesting that antitermination activity is quite low in the absence of any inducer. The presence of salicylate, which induces production of NasR, increased the expression level from pBM9, and full antitermination was achieved by the additional presence of nitrate, which activates the antiterminator protein.

When transcription from the P_{tac} promoter was induced by the presence of IPTG the effect of the attenuator is clearly visible if neither salicylate nor nitrate is present. However, expression levels from pBM9 similar to those shown from pBM8 were obtained if salicylate and nitrate were added to the culture medium.

Taken together, these data clearly show that adding the attenuation control to the P_{tac} regulatory circuit clearly increases the induction ratio by drastically reducing the basal level of expression while keeping the induced levels intact. Nevertheless, comparison of these expression levels to those obtained from the combined cascade-attenuation system indicates that performance of this latter regulatory system is superior (Table 2).

DISCUSSION

When designing the strategy for cloning and expressing a protein, it is necessary to decide which expression system may be the most appropriate to use. When maximal production is desired, high-copy number plasmid vectors bearing strong regulated promoters are the systems of choice. However, non-induced expression from these vectors is quite high and may lead to problems in the growth of the recombinant strains if the protein is sufficiently toxic. This is the case for proteases, proteins involved in the redox balance, and many membrane proteins. Unfortunately, failed attempts to clone and express many genes are not reported in standard publications but it is a common experience to most of the molecular biologists. Some authors have isolated different mutations along the regulated promoters in an attempt to obtain different expression levels. In the case of the Pm promoter, these point mutations moved the induction window up and down, but rarely increased the induction ratio. Therefore, polymorphisms that reduced promoter basal activity also compromised the induced level, and could not broaden the induction window (A. Cebolla, unpublished data) (31,32). In a previous work, we placed the expression module of the cascade system in the bacterial chromosome using a mini-*Tn5* delivery system. By diminishing gene dosage, we reduced non-induced levels while keeping induced levels at just half of the levels obtained from a multicopy plasmidic configuration. However, cloning in a mini-*Tn5* requires additional steps, and construction of the overproducing strain is worth only if it is going to be repeatedly used in long-term industrial processes (4,25).

In this work we have tested the idea that the induction window of an expression system may be widened by introducing an additional regulatory circuit, which operates at a different step of the expression process. We have addressed this question by adding a new modular element, the *nasF* attenuator, to a cascade expression system based on the Pm promoter. The main characteristic of an attenuation system that makes it attractive as a complementary regulatory circuit in expression vectors, is that it prevents elongation of the transcription regardless it comes from undesired basal transcription from the regulated promoter of the expression system or from spurious initiation of the bacterial RNA polymerase from cryptic or other known promoters in the vectors. Thus, attenuation could serve to eliminate most of the unwanted transcription produced under non-inducing conditions.

The presence of the *nasF* attenuator between the regulated Pm promoter and the gene of interest reduces transcription of the gene by more than one order of magnitude under non-inducing conditions, which indicates the potential of the attenuator to reduce the basal expression levels (Table 2). Similar results were obtained when the attenuator was located downstream of the *tac* promoter in a completely different expression system. Of course, this additional element would only be useful if transcription termination at the attenuator may be efficiently prevented when production is desired. Full rescue of the expression levels from the Pm or from the P_{tac} promoters was achieved by providing an inducible source of the antiterminator NasR, thus showing that overimposing different regulatory elements allows a tighter control of protein production. These results also show that the additional attenuation control is functional regardless of the regulatory circuit controlling transcription initiation, thus suggesting that the use of attenuation to improve gene regulation may be extended to other expression systems not tested in this study.

Intracellular levels of the antiterminator NasR are important for the regulation efficiency of the combined systems. When *nasR* is transcribed from the strong P_{tac} promoter, the levels of

NasR are apparently too high, and transcription termination under non-inducing conditions is prevented to some extent, thus resulting in higher basal levels of gene expresson. Transcription termination at the attenuator significantly increased under non-inducing conditions if the expression of the antiterminator gene was coupled to the cascade regulatory circuit by placing it downstream of P_{sal}. Providing NasR from this promoter, the circuit displayed the highest induction ratio since P_{sal} is weak enough to produce undetectable levels of NasR in the absence of salycilate, while strong enough to allow a full antitermination upon induction (Table 2). Using this configuration, we obtained an induction ratio of 1711-fold, which is one of the highest regulation capability described for a multicopy plasmid expression vector (33), and a production level under maximal induction conditions that represents up to 15% of the total protein without maximizing the translation initiation signal of the *galK′::′lacZ* reporter gene (data not shown). It should be noted that the use of reporter proteins more unstable than β-galactosidase, i.e. luciferase, and the use of lower copy number vector may even increase this apparent induction ratio (A. Cebolla, data not shown).

However, when soluble functional protein overproduction is desired, optimal expression levels are not necessarily achieved using maximal induction conditions, since high-production rates may lead to products aggregated in inclusion bodies (1,34). By combining different biological parameters, such as the disposition of the *nasF* attenuator and physical parameters, such as temperature, together with the presence and the amount of the different inducers, salicylate and nitrate, it is possible to modulate the induction level that will be adequate for the experimental requirement (Table 2). In addition, this combined attenuated-cascade system may allow coordinated regulation of multiple genes, which require the same or different stoichiometries of their products. This is an interesting additional feature to the potential for cloning of genes encoding toxic proteins using the lowest possible basal expression.

In summary, this work presents a new cascade regulatory circuit that improves control of heterologous gene expression, and shows that multilevel regulation of expression circuits broadens the possibilities of simple control systems for increasing the induction ratio and fine tuning the expression level, thus expanding the number of proteins whose production can be efficiently regulated from different expression vectors without affecting maximal gene expression.

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