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...
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 pneumonae), 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\textsubscript{3}\textsuperscript{-}) or nitrite (NO\textsubscript{2}\textsuperscript{-}) as the only nitrogen source during aerobic growth by controlling the expression of the nas-FEDCBA operon (20). Upstream and divergent from the nas-FEDCBA 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\textsubscript{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\textsubscript{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 P\textsubscript{m} 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\textsubscript{260}/OD\textsubscript{280}.

**PCRs**

The nasF attenuator was amplified by PCR using K.oxytoca genomic DNA as template and the following primers: TerNasF2, 5'-GGAATTCCAGTAGATAAAAAGTTTGGGCGC GGCTG-3' and TerNasR2, 5'-GGAATTCCGGGCCAAAAGTAGGCCTGGTGTCCTGG-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\textsubscript{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'-ACGGTTATTGGCTGCTGGAGG-3' and NasR1R, 5'-ATGGAGGCTCTCAGTTCDTGGTATCGTACG-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\textsubscript{2}. 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, pMP08, pMP024 or pMP025 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\textsubscript{600} reached 0.2–0.3, cultures were induced using
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.
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 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 pIC552 (28) containing the galK::lacZ gene fusion was inserted into the same sites of pCAS, generating pMP06 (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 pMP06 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 pMP016.

In order to construct a suitable cloning expression vector also bearing the attenuator, a multiple cloning site was introduced downstream of 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 pMP06 digested with SmaI and SacI. The resulting plasmid, designated pMP027, was subcloned into pIZ1016 (29) digested with SmaI and SacI. The resulting plasmid, designated pMP027, contained the lacF4 repressor and expressed nasR under the control of the Plac 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 pMP016, 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).

Table 1. Strains and plasmids

<table>
<thead>
<tr>
<th>Strains</th>
<th>Characteristics</th>
<th>Reference</th>
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<tbody>
<tr>
<td>E. coli DH5α</td>
<td>deoR endA1 gyrA96 recA1 supE44</td>
<td>(35)</td>
</tr>
<tr>
<td>E. coli S17-1λpir</td>
<td>F- recA hsdR R4P-2 (Ts-Mn)(Km::Tn7) lysogenized with λpir phage</td>
<td>(36)</td>
</tr>
<tr>
<td>Klebsiella pneumoniae M5a1</td>
<td>Wild-type strain of K. pneumoniae (pneumoniae)</td>
<td>Laboratory collection</td>
</tr>
<tr>
<td>E. coli CC118 4S2</td>
<td>pheA20 thi-1 rpe rpoB argE (Am) recA1 with a Km mini-Tn5 bearing a nahr/Psal-xylS2 fusion</td>
<td>(9)</td>
</tr>
</tbody>
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<table>
<thead>
<tr>
<th>Plasmids</th>
<th>Characteristics</th>
<th>Reference</th>
</tr>
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<tbody>
<tr>
<td>pB8M</td>
<td>TcR, pVL313 derived plasmid with lacP::lacZ</td>
<td>This work</td>
</tr>
<tr>
<td>pB8M9</td>
<td>TcR, pVL313 derived plasmid with lacP::lacZ</td>
<td>This work</td>
</tr>
<tr>
<td>pCAS</td>
<td>ApR, expression vector with mBT12-Pm::MCS, ColE1 replication origin</td>
<td>Active motif</td>
</tr>
<tr>
<td>pCNB4-S2</td>
<td>ApR, KmR, miniTn5 vector with the nahr/Psal-xylS2 fusion cloned between the I and O sites</td>
<td>(9)</td>
</tr>
<tr>
<td>pIC552</td>
<td>ApR+, promoter-less vector bearing a galK::lacZ fusion</td>
<td>(28)</td>
</tr>
<tr>
<td>pZI016</td>
<td>GmR, broad-host range expression vector bearing lacIq and PlacZ</td>
<td>(29)</td>
</tr>
<tr>
<td>pMP06</td>
<td>ApR, pCAS with mBT12-Pm-galK::lacZ</td>
<td>This work</td>
</tr>
<tr>
<td>pMP016</td>
<td>ApR, pCAS with mBT12-Pm-nasF attenuator-galK::lacZ</td>
<td>This work</td>
</tr>
<tr>
<td>pMP07</td>
<td>ApR, Bluestrip SK+ with nasR cloned in EcoRV</td>
<td>This work</td>
</tr>
<tr>
<td>pMP08</td>
<td>GmR, plasmid derived from pZ1016 with nasR cloned downstream PlacZ</td>
<td>This work</td>
</tr>
<tr>
<td>pMP09</td>
<td>ApR, pMP06 derived plasmid with nasR downstream of Pm</td>
<td>This work</td>
</tr>
<tr>
<td>pMP010</td>
<td>ApR, pMP016 derived plasmid with nasR downstream of the Pm and the nasF attenuator</td>
<td>This work</td>
</tr>
<tr>
<td>pMP024</td>
<td>GmR, plasmid derived from pMP08 with nasR cloned downstream PacI</td>
<td>This work</td>
</tr>
<tr>
<td>pMP025</td>
<td>GmR, plasmid derived from pMP08 with a fusion nasR-nasF attenuator-nasR</td>
<td>This work</td>
</tr>
<tr>
<td>pMP027</td>
<td>ApR, expression vector with mBT12-Pm-nasF attenuator-MCS, ColE1 replication origin</td>
<td>New England Biolabs</td>
</tr>
<tr>
<td>pUC19</td>
<td>ApR, cloning vector, ColE1 replication origin</td>
<td>(37)</td>
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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.

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

When pMPO8, expressing the \textit{nasR} antiterminator gene, was co-transformed together with pMPO16, the basal levels of \(\beta\)-galactosidase activity increased 4-fold; this is possibly due to a residual antitermination activity of the NasR produced owing to the \(P_{\text{lac}}\) 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 \(\beta\)-galactosidase activity additionally increased by 6-fold since the antitermination function

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**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 \textit{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 \textit{lacZ} full transcripts. (C) If \textit{nasR} expression was induced in spite of the absence of nitrate, some antitermination increases the \(\beta\)-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.
Table 2. Summary of expression levels and induction ratios using different combinations of expression and antitermination vectors

<table>
<thead>
<tr>
<th>CC118 4S2</th>
<th>Non-induced (MU)</th>
<th>Induced (MU and fold induction)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>-NO₃</td>
<td>+NO₃</td>
</tr>
<tr>
<td></td>
<td>NasR</td>
<td>Sal</td>
</tr>
<tr>
<td>pMPO6 pIZ1016</td>
<td>1011 ± 200</td>
<td>954 ± 175</td>
</tr>
<tr>
<td>pMPO16</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pIZ1016</td>
<td>84 ± 15</td>
<td>89 ± 10</td>
</tr>
<tr>
<td>pMPO24</td>
<td>81 ± 10</td>
<td>125 ± 50</td>
</tr>
<tr>
<td>pMPO25</td>
<td>75 ± 8</td>
<td>90 ± 10</td>
</tr>
<tr>
<td>pMPO24</td>
<td>310 ± 55</td>
<td>499 ± 125</td>
</tr>
<tr>
<td>pBM8</td>
<td>1592 ± 50</td>
<td>1510 ± 120</td>
</tr>
<tr>
<td>pBM9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pIZ1016</td>
<td>137 ± 25</td>
<td>143 ± 20</td>
</tr>
<tr>
<td>pMPO24</td>
<td>194 ± 20</td>
<td>618 ± 40</td>
</tr>
</tbody>
</table>

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/sal/C0/xyIS2 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 Pm 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 Pm 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 Pn and Pm, respectively. We placed nasR under the control of the Pn 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 lacP³-Pn regulatory region in pMPO8 by a fragment containing the Pn 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 nasF gene preceding 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 Pn (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 level possible is required for bacterial growth.
from the vector lacking the attenuator, thus indicating that induced transcription of nasR from P_{m} 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 S^{2} 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 overlapping 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_{m} 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 expression. Transcription termination at the attenuator significantly increased under non-inducing conditions if the expression of the anti-terminator gene was coupled to the cascade regulatory circuit by placing it downstream of P_{sat}. Providing NasR from this promoter, the circuit displayed the highest induction ratio since P_{sat} is weak enough to produce undetectable levels of NasR in the absence of salicylate, 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^c::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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Conflict of interest statement. None declared.

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