

Continuous Control of the Flow in Biochemical Pathways through 5' Untranslated Region Sequence Modifications in mRNA Expressed from the Broad-Host-Range Promoter *Pm*^{∇†}

Rahmi Lale,¹ Laila Berg,¹ Friederike Stüttgen,¹ Roman Netzer,² Marit Stafnes,¹ Trygve Brautaset,² Trond Erik Vee Aune,^{1‡} and Svein Valla^{1*}

Department of Biotechnology, Norwegian University of Science and Technology, N-7491 Trondheim, Norway,¹ and
Department of Biotechnology, SINTEF Materials and Chemistry, SINTEF, 7465 Trondheim, Norway²

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The inducible *Pm* promoter integrated into broad-host-range plasmid RK2 replicons can be fine-tuned continuously between the uninduced and maximally induced levels by varying the inducer concentrations. To lower the uninduced background level while still maintaining the inducibility for applications in, for example, metabolic engineering and synthetic (systems) biology, we report here the use of mutations in the *Pm* DNA region corresponding to the 5' untranslated region of mRNA (UTR). Five UTR variants obtained by doped oligonucleotide mutagenesis and selection, apparently reducing the efficiency of translation, were all found to display strongly reduced uninduced expression of three different reporter genes (encoding β -lactamase, luciferase, and phosphoglucomutase) in *Escherichia coli*. The ratio between induced and uninduced expression remained the same or higher compared to cells containing a corresponding plasmid with the wild-type UTR. Interestingly, the UTR variants also displayed similar effects on expression when substituted for the native UTR in another and constitutive promoter, *PI* (*P_{antitet}*), indicating a broad application potential of these UTR variants. Two of the selected variants were used to control the production of the C₅₀ carotenoid sarcinaxanthin in an engineered strain of *E. coli* that produces the precursor lycopene. Sarcinaxanthin is produced in this particular strain by expressing three *Micrococcus luteus* derived genes from the promoter *Pm*. The results indicated that UTR variants can be used to eliminate sarcinaxanthin production under uninduced conditions, whereas cells containing the corresponding plasmid with a wild-type UTR produced ca. 25% of the level observed under induced conditions.

The initially used methods of deleting or overexpressing genes have been demonstrated to be inadequate for many applications in metabolic engineering (21, 31, 32). For example, when the goal is to optimize the expression level of a desired protein by engineering the relevant metabolic pathway, it might be necessary to change the expression of multiple enzymes simultaneously and to different levels (30, 38). Also, reducing the formation of particular by-products can increase the flux of the desired product (29). In addition, low basal expression is critical for applications such as the expression of toxic genes, metabolic engineering, and control analysis (2, 14, 27, 34). This has led to an increased focus on development of genetic tools to fine-tune gene expression to the desired levels. A commonly used strategy is to make so-called promoter libraries of constitutive promoters (1, 15, 17). Such promoters seem to be preferred over the corresponding inducible ones for industrial scale productions because of factors such as inducer costs, sensitivity to inducer concentration, and heterogeneity of expression caused by an all-or-none effect of induction (1, 19). However, the all-or-none induction effect may be eliminated if

the inducer enters the cell interior by passive diffusion (20). Thus, regulatable promoter systems that eliminate as many problems as possible associated with their use are important tools, particularly considering the recent growing interest in systems and synthetic biology.

The broad-host-range *XylS/Pm*-positive regulator/promoter system (originating from the TOL plasmid [pWWO] of *Pseudomonas putida*) was previously in our laboratory inserted into a set of broad-host-range expression vectors (pJBn), together with the RK2 minimal replication elements *oriV* (origin of vegetative replication) and *trfA* (encodes replication initiation and copy number control protein) (6, 7). The expression level from the σ^{32} - and σ^{38} -dependent *Pm* promoter (26) can easily be varied by the specific benzoate derivative used as inducer (enters the cells by passive diffusion) and the concentration of it (33, 42). Further modifications of expression can be obtained by varying the vector copy number via mutations in *trfA*. This vector system has been used successfully in our group for metabolic engineering purposes in *Pseudomonas fluorescens* (4, 13) and also in combination with another newly developed expression system (40). Finally, one of the pJBn expression vectors has been shown to give rise to production of industrial levels of several recombinant proteins in *Escherichia coli* (36, 37). Recently, we have found that the *bla* gene (encoding β -lactamase) expression from the *Pm* promoter can be strongly stimulated in *E. coli* (up to 20-fold at the protein product level) by introducing mutations at the DNA level in its cognate 5' untranslated region (UTR) (5). The inducible promoter phenotype was intact in all of the UTR variants.

* Corresponding author. Mailing address: Department of Biotechnology, Norwegian University of Science and Technology, Sem Sælands vei 6/8, N-7491 Trondheim, Norway. Phone: 47-73598694. Fax: 47-73591283. E-mail: svein.valla@biotech.ntnu.no.

‡ Present address: Vectron Biosolutions AS, P.O. Box 1262, Pirsenteret, 7462 Trondheim, Norway.

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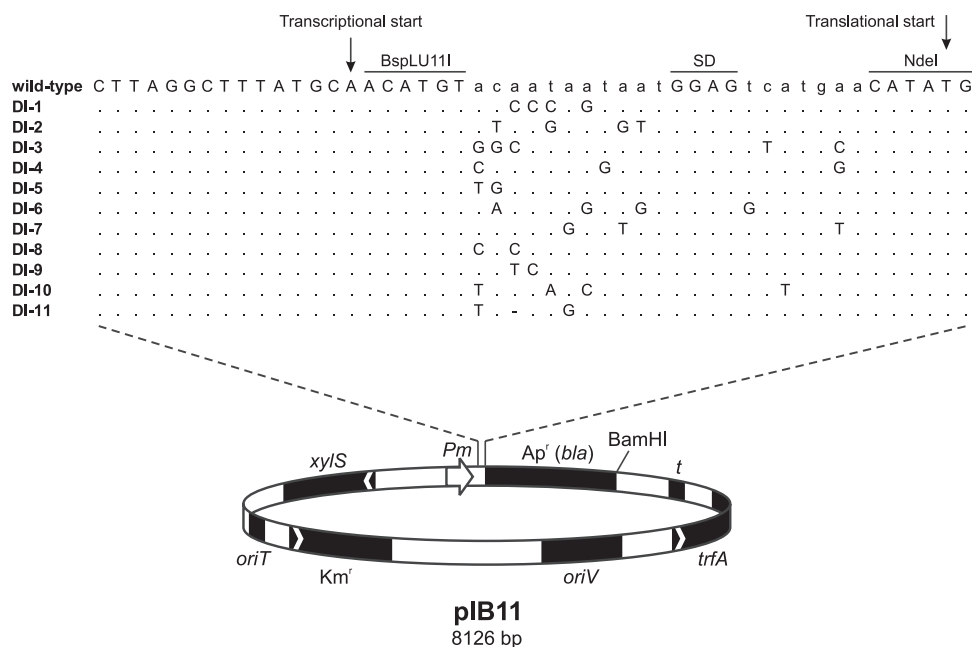


FIG. 1. Downregulating UTR sequences identified in DI library (pIB11), and map of the plasmid pIB11. Putative Shine-Dalgarno sequences are indicated by "SD". Identical bases are indicated by dots, and deletions are indicated by dashes (DI-11). Transcriptional and translational start sites are indicated with arrows, and bases subjected to mutagenesis are shown in lowercase. Features of the plasmid are shown in the map below the sequences. The indicated restriction enzyme sites are unique. *bla*, gene encoding β -lactamase; *Km^r*, kanamycin resistance gene; *trfA*, gene encoding the essential replication protein; *xylS*, gene encoding the transcriptional activator of the *Pm* promoter; *oriV*, origin of vegetative replication; *oriT*, origin of transfer; *t*, bidirectional transcriptional terminator.

The purpose of these earlier studies was to maximize protein expression, but we report here on a study of UTR variants that can be used to downregulate the uninduced gene expression level of different reporter genes both from *Pm* without losing inducibility and also from the constitutive *P_I* promoter. The variants could also, in contrast to wild-type *Pm*, be used to tightly control the metabolic flow in the pathway, leading to synthesis of the carotenoid sarcinaxanthin.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. *E. coli* DH5 α (Bethesda Research Laboratories) was used as a host, except for in the construction and screening of the DI UTR library and for heterologous production of carotenoids *E. coli* Gold (Stratagene) and *E. coli* XL1-Blue were used, respectively. All of the plasmid constructs used in the present study bear the same backbone, consisting of a RK2-based minimal replication system in which the *xylS/Pm* expression cassette has been integrated. The vectors used in the expression studies vary either with respect to the gene to be expressed (*bla* in plasmid pIB11 [5], *celB* in pLB11, and *luc* in pKT1) or to the promoter used (*P_I*, also known as *P_{antiter}* [9]) in pLB9. Details on the growth conditions and plasmid constructions can be found in the supplemental material.

Standard techniques. Plasmid isolation, enzymatic manipulations of DNA, and agarose gel electrophoresis were performed by the methods described elsewhere (35). The QIAquick gel extraction kit and QIAquick PCR purification kit (Qiagen) was used for DNA purifications from agarose gels and enzymatic reactions, respectively. A modified RbCl protocol (Promega) was used for transformations of *E. coli* in cloning experiments. PCR for cloning and amplified fragment length identification was performed by using the Expand High-Fidelity PCR-system (Roche Applied Science) and Dynazyme II (Finnzymes), respectively. Information on the primers used for PCR and DNA sequencing is given in the supplemental material.

Construction and screening of the UTR libraries. The DI library (consisting of about 22,500 transformants) was constructed in plasmid pIB11. The oligonucleotides used to construct the libraries consisted of the wild-type sequence and the complementary strand that was made by synthesis of randomly doped synthetic

oligonucleotide mixture. The doping percentages of the nucleotide mixture were set to 76% of the wild-type nucleotide and 8% for each of the three others. Further details on the library construction can be found elsewhere (3, 5).

RNA isolation, cDNA synthesis, DNase treatment, and two-step qRT-PCR. Transcript amounts were determined by two-step quantitative real-time PCR (qRT-PCR). For further details, see the supplemental material.

Enzyme assays. Luciferase assays were performed by using the Luciferase Assay System (Promega) according to the manufacturer's protocol. Luciferase activity was measured with the GloMax 20/20 Luminometer (Promega). Phosphoglucosylase activities were measured as described previously (12). For all enzyme assays, measurements were carried out at least with two biological and three technical replicates.

Western blotting. SDS-PAGE was carried out according to the method of Laemmli (22), using 12% Tris-HCl Ready Gel (Bio-Rad). For further details, see the supplemental material.

Carotenoid analysis. Carotenoids produced by recombinant *E. coli* strains were extracted from liquid cell cultures and analyzed by liquid chromatography-mass spectrometry (LC-MS) analysis as described elsewhere (28).

RESULTS AND DISCUSSION

The expression level from *Pm* can be strongly reduced by mutating the UTR DNA region flanking the Shine-Dalgarno sequence. It is well established that mutations located near the transcriptional start site may affect expression at the transcriptional level and that the Shine-Dalgarno sequence is important for translational efficiency (8, 23, 24, 41). In our previous studies we found that nucleotide substitutions in the remaining parts of the UTR can also strongly affect expression (5), and for the study reported here we constructed a new UTR mutant library (designated DI, Fig. 1) in which the nucleotides close to the transcriptional start site and the Shine-Dalgarno sequence were not mutagenized. Plasmid pIB11 (Fig. 1) was used for the library construction, and β -lactamase was used as a reporter to

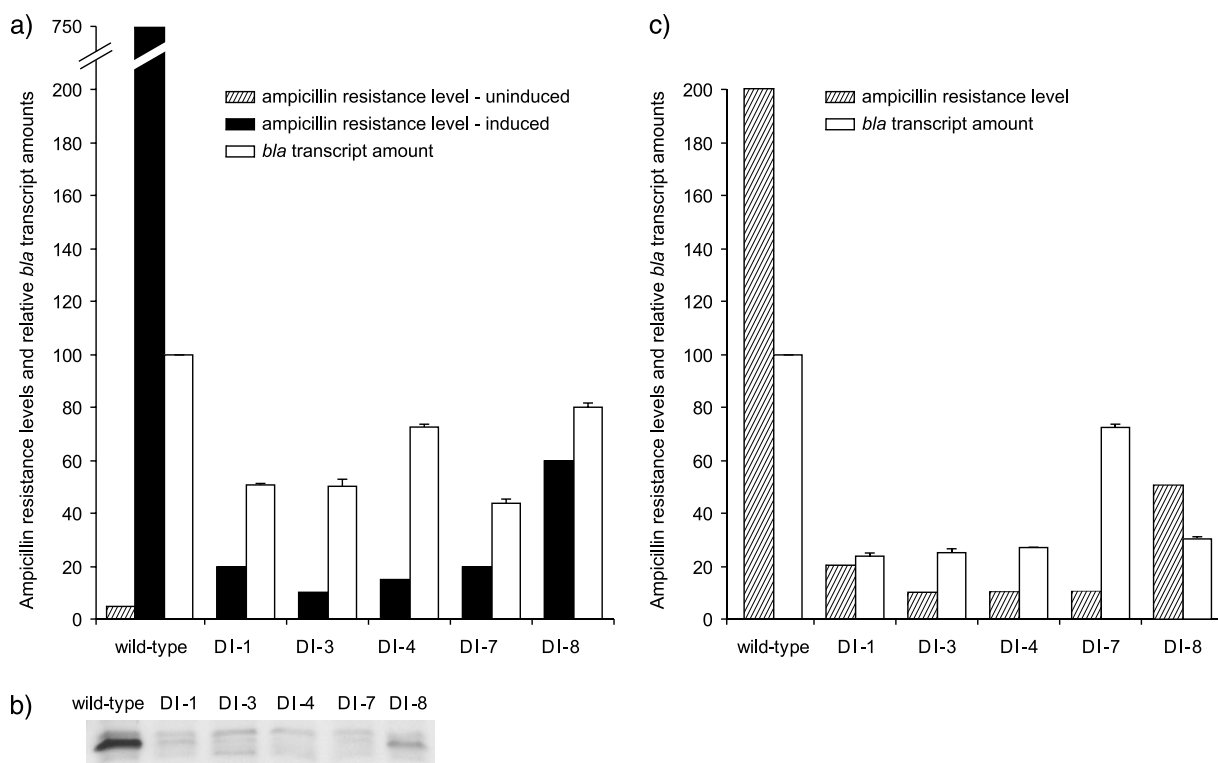


FIG. 2. Ampicillin resistance levels (under uninduced [▨] and induced [■] conditions) and *bla* transcript amounts (□) for the DI UTR variants in plasmid pIB11 (a) and pLB9 (c). (b) Western blot analysis of β -lactamase production from the strains used in panel a. The values in panels a and c are the average of at least two biological replicas, and the error bars indicate the standard deviations. Transcript amounts are normalized against the wild-type value, arbitrarily set to 100.

easily detect variation in expression levels. *E. coli* DH5 α cells containing pIB11 (wild-type UTR) were found to grow on agar medium supplemented with maximally about 750 μ g of ampicillin/ml under induced conditions. From a total of 4,773 randomly picked clones (colonies on agar medium supplied with kanamycin), 11 (DI-1 to -11, Fig. 1) were finally selected, based on their ampicillin resistance phenotypes and DNA sequences (see the supplemental material for further details concerning the selection). The corresponding UTR oligonucleotides were resynthesized, cloned into pIB11, and reconfirmed by DNA sequencing and phenotype analyses. Each variant carried two to five point mutations, and a total of 26 different substitutions were identified, involving 14 of the 18 bases in the wild-type mutagenized sequence.

UTR mutations seem to mostly act by reducing the translational efficiency. Five (DI-1, -3, -4, -7, and -8) of the 11 variants described above were subjected to further detailed studies. The maximum ampicillin tolerance levels of the *E. coli* DH5 α host cells containing the corresponding plasmids were found to vary from 10 (pIB11-DI-3) to 60 μ g/ml (pIB11-DI-6) under induced conditions, and under uninduced conditions none of the clones grew in the presence of 5 μ g of ampicillin/ml on agar media (Fig. 2a), in contrast to cells containing pIB11 (wild-type UTR). Therefore, based on these results, it seemed clear that the variants have kept their inducible phenotypes, even though the exact uninduced maximal ampicillin resistance levels of them could not be determined because the background tolerance of the host cells themselves is too close to 5 μ g/ml. Direct

measurements of the β -lactamase activities were not feasible due to the relatively low sensitivity of the enzyme assay. Therefore, Western analysis was performed with β -lactamase antibody, and the results indicated that the ampicillin resistance results were consistent with the band intensities on the membrane (Fig. 2b).

If the mutations in the UTR are mainly leading to a reduction of the efficiency of translation, one would predict that the amounts of transcript might not be reduced to an extent comparable to that of the protein level. To investigate this, we directly measured the *bla* transcript levels for the selected five UTR variants by qRT-PCR and found that the amounts of accumulated *bla* mRNA varied between 43% (pIB11-DI-7) and 79% (pIB11-DI-8) of the wild-type quantity (Fig. 2a). These results are thus consistent with the hypothesis that the observed reduction of expression at the protein level is mainly the result of reduced translational efficiency.

UTR mutations also downregulate the expression level of two other tested reporter genes. The effects of the mutations in the UTR could be envisioned to display some gene dependency due to interactions with the coding sequence of the gene to be expressed. To monitor this, we substituted the *bla* gene with *luc* (encoding luciferase originating from *Photinus pyralis*, plasmid pKT1) and then measured the corresponding expression levels from plasmids containing each of the UTR variants DI-1, -3, -4, -7, and -8 in *E. coli* DH5 α under induced conditions (Fig. 3). All of the DI variants resulted in a very strong reduction of expression at the protein levels ranging from 0.4%

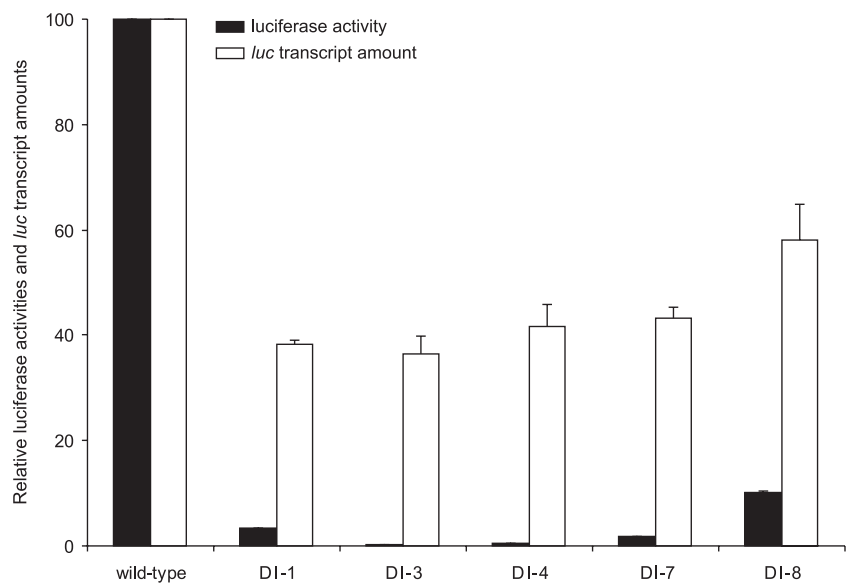


FIG. 3. Luciferase activities (■) and *luc* transcript amounts (□) for the DI UTR variants in plasmid pKT1 under induced conditions. The values are the average of at least two biological replicas, and the error bars are standard deviations. Both enzyme activities and transcript amounts are relative to the wild-type value, arbitrarily set to 100.

(pKT1-DI-3) to 11% (pKT1-DI-8) of the value obtained with pKT1 (wild-type UTR), while the corresponding accumulated transcript levels were found to range from 36% (pKT1-DI-3) to 58% (pKT1-DI-8) relative to pKT1 (wild-type UTR). It could therefore be concluded that the mutations in the UTR give rise to similar effects on the expression level of both *luc* and *bla*, further supporting the hypothesis that the UTR mutations primarily act by negatively affecting the translational efficiency in an apparently gene-independent manner.

Previously, we have found that the phosphoglucomutase gene *celB* (originating from *Acetobacter xylinum*, now *Glu-*

conacetobacter hansenii) can be expressed at unusually high levels from the wild-type *XylS/Pm* system in *E. coli* and other Gram-negative bacteria (7). *celB* was therefore used as a final reporter test gene to study the effect of the UTR variants on expression. The wild-type UTR DNA sequence in pLB11 was substituted by each of the UTR variants DI-1, -3, -4, -7, and -8, and the CelB protein activity levels of the corresponding *E. coli* DH5α host cells under induced *Pm* conditions were measured. Surprisingly, no CelB activity could be detected from any of the recombinant strains. The corresponding *celB* transcript levels were determined by qRT-PCR (Fig. 4a), and they were dra-

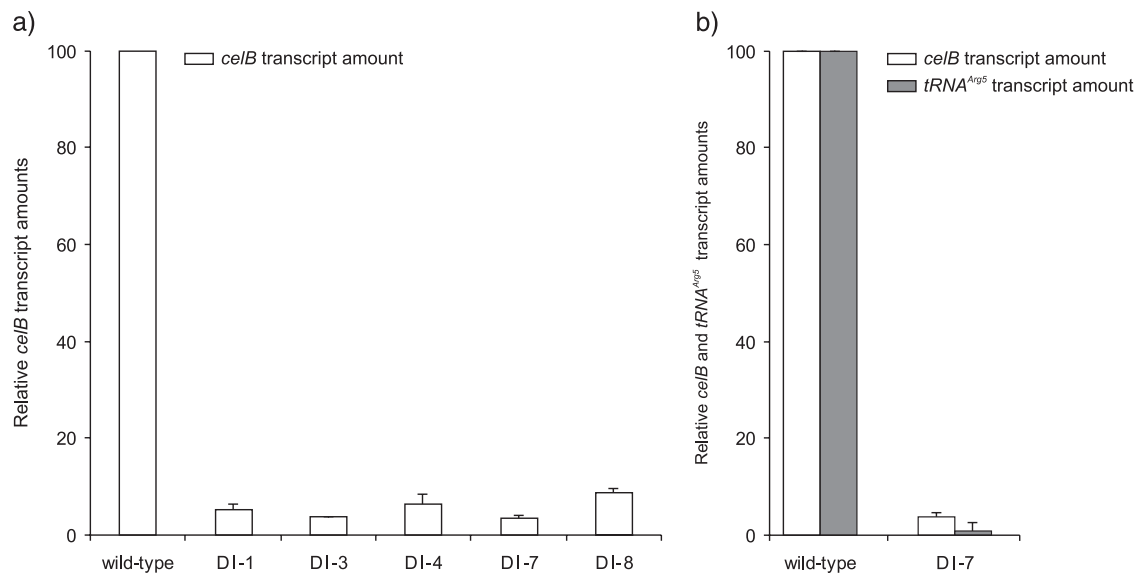


FIG. 4. *celB* transcript amounts (□) for the DI UTR variants in plasmid pLB11 (a) and *celB* (□) and *tRNA*^{Arg5} (■) transcript amounts for the plasmid pLB11Arg5 with the DI-7 sequence (b), relative to the wild type under induced conditions. The wild-type values are arbitrarily set to 100. The values are the average of at least two biological replicas, and the error bars indicate the standard deviations.

matically reduced for all five UTR variants tested, in contrast to what was observed with the *bla* and *luc* reporter genes (see above). *celB* therefore represents an example of a gene responding in a different way (compared to *bla* and *luc*) to the variant UTR sequences, although these gene-specific effects mainly appear to act at the transcriptional level. We also found it intriguing that the effects were similar for all of the UTR variants. If they were somehow caused by an interaction between the UTR and the *celB* gene coding sequence, one would not expect a similar response for all of the UTR variants.

The gene-specific reduction of *celB* transcript accumulation caused by mutations in the UTR is probably the result of enhanced Rho-dependent transcriptional termination. In the absence of translating ribosomes, transcripts are potentially more susceptible to degradation (11), and we therefore reasoned that the proposed drastic reduction of translational efficiency by the UTR mutations might somehow selectively lead to rapid degradation of *celB* mRNA. Since any gene-specific effect of the UTR variants could potentially become important in their future applications, we decided to study the somewhat unexpected specific effects on *celB* transcript accumulation in more detail. For this purpose, we used a method that involves fusion of the gene of interest (in this case *celB*) to a tRNA gene (encoding tRNA^{Arg5}) in such a way that both genes become expressed as one single transcript (generating plasmid pLB11Arg5). This experiment has been used previously by us for a related problem (5) and is based on the assumption that the tRNA^{Arg5} part of the mRNA is essentially stable *in vivo* (25). Thus, even a drastic increase in the *celB* transcript turnover should not negatively affect the accumulation of the tRNA^{Arg5} part of the transcript. The phosphoglucomutase activities and accumulated *celB* transcript levels under induced conditions of *E. coli* DH5 α cells containing the plasmid pLB11Arg5 were found to be similar to those containing pLB11 (data not shown). The wild-type UTR DNA sequence of the plasmid pLB11Arg5 was then substituted with the corresponding DI-7 sequence, and the accumulated *celB* and tRNA^{Arg5} transcript levels were determined by qRT-PCR and compared to the corresponding values from the pLB11Arg5 (Fig. 4b). These experiments clearly demonstrated that the accumulated tRNA^{Arg5} transcript level was also strongly reduced in the presence of the DI-7 mutations (reduced to ca. 2% compared to the wild type), indicating that transcription of *celB* is inefficient or aborted prior to full-length synthesis. If so, one would also conclude that mRNA instability is probably not the primary cause of the selective reduction of transcript accumulation for the *celB* transcripts.

It is known that the absence of translating ribosomes on a transcript might activate mechanisms that prematurely terminate the transcription process (16). We therefore hypothesized that the *celB* transcript may contain a binding site for the transcription termination factor Rho in its coding sequence, thus potentially also explaining why all of the UTR variants act similarly and selectively on the *celB* gene (10). Bicyclomycin (BCM) is a natural inhibitor of Rho (43), and this compound was added to a final concentration of 100 μ g/ml at the time of induction to cell cultures of *E. coli* DH5 α containing pLB11-DI-7 or pLB11. As controls, the same two cultures were grown similarly but without the addition of BCM. The accumulated

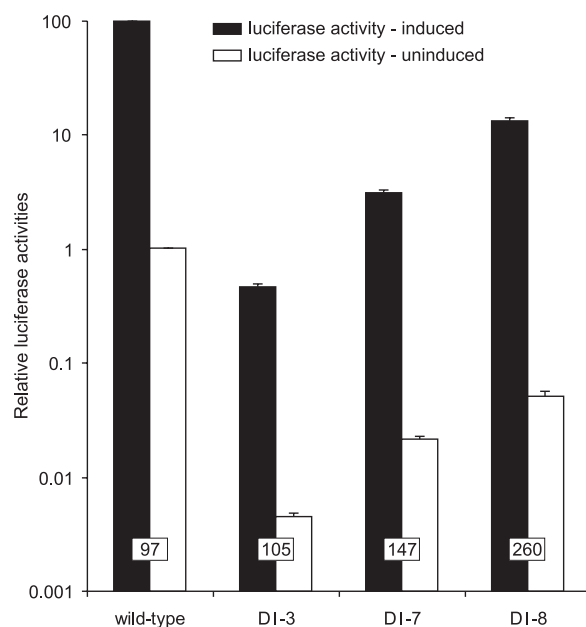


FIG. 5. Luciferase activities under induced (■) and uninduced (□) conditions for selected DI UTR variants in plasmid pKT1. The ratios between the induced and uninduced luciferase activity levels are displayed directly on the bars. Enzyme activities are shown in logarithmic scale. The values are the average of at least two biological replicates, and the error bars indicated the standard deviations. Activity values are relative to the wild type under induced conditions, which is arbitrarily set to 100 for the induced state.

celB transcript levels were then determined in the samples collected 30 min after the time of induction. Interestingly, in the strain with pLB11-DI-7 the accumulated transcript level under induced conditions was ~40-fold higher in the presence of BCM than in its absence (similar to cells containing pLB11 in the absence of BCM, data not shown). BCM also had a stimulatory effect on wild-type transcription, but to a lower extent (~10-fold). This indicates that Rho leads to premature transcription termination of the *celB* mRNA synthesis and that this effect is strongly stimulated when translation is inefficient (in the presence of the DI DNA sequences).

Based on this, it seemed likely that the primary effect of the UTR mutations is to cause reduced translational efficiency of any gene, but that this can lead to drastic reduction also of transcription for genes that are sensitive to early Rho-dependent termination of transcription. Importantly, such effects should not have any obvious negative impact for the generality of application of the UTR variants for control of biochemical pathways (see below).

The inducibility of *Pm* was maintained or improved by the UTR mutations. The luciferase enzyme assay is very sensitive compared to those of β -lactamase and CelB, and activities can be accurately monitored at both very low and high levels of expression. Therefore, we compared these enzyme activities in the host cells containing plasmids with three of the DI UTR variant sequences (pKT1-DI-3, -7, and -8) under induced (the same data are in Fig. 3) and uninduced (Fig. 5) conditions. The ratio between the induced and uninduced level of protein formation for the UTR variants ranged from 105 (pKT1-DI-3) to 260 (pKT1-DI-8), which is similar to or higher than for the wild

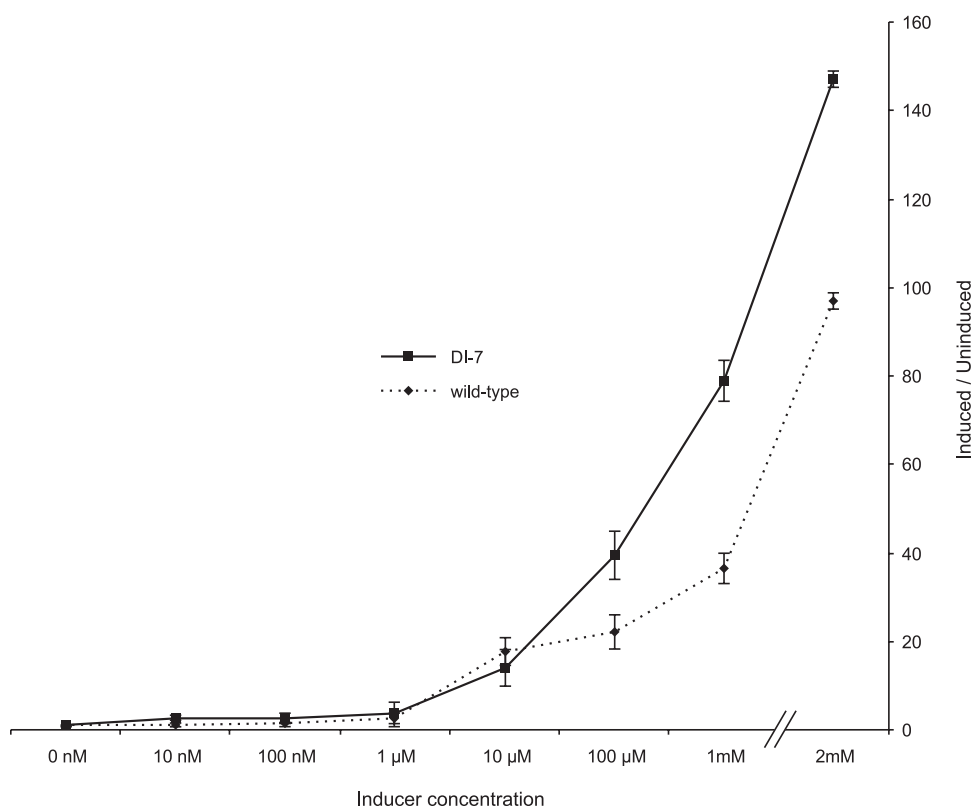


FIG. 6. Luciferase activities under different inducer concentrations for the wild type and UTR variant DI-7 in plasmid pKT1. The ratios between the induced and uninduced luciferase activity levels are plotted against the inducer concentrations. The values are the average of two biological replicas, and the error bars indicate the standard deviations. The activity values of the wild type and DI-7 are relative to their cognate uninduced levels.

type (ratio of 97). Furthermore, the induced expression levels of pKT1-DI-7 and pKT1-DI-8 are higher than the corresponding uninduced level from pKT1.

Previously, it has been demonstrated that the expression level from *Pm* can easily be controlled by varying the type and concentration of the inducer (42). We therefore also characterized the inducibility characteristics of pKT1 and pKT1-DI-7 at a range (0.01, 0.1, 1, 10, 100, 1,000, and 2,000 μ M) of inducer concentrations (Fig. 6). This analysis also showed that the system can be turned on at levels as low as 1 μ M, resulting in induction ratios of 1.9 and 2.6 for pKT1-DI-7 and pKT1, respectively. Furthermore, the induction levels increase gradually and according to a similar pattern for both wild type and variant as the inducer concentrations are increased. These results therefore clearly confirmed that UTR mutations can be applied to reduce the basal expression level without losing the inducibility feature. If we take into account the previously reported UTR variants leading to high expression levels (5), it also follows that expression can be continuously regulated over 5 orders of magnitude in this system.

UTR variants also lead to reduced gene expression when fused to the constitutive *P1* promoter. Although the properties of the UTR variants are clearly very useful in the *XylS/Pm* context they would be even more applicable if they would have a similar function in as many promoter systems as possible. To test this, we chose promoter *P1* (also known as *P_{antitet}*), which differs from *Pm* in that it is constitutive and σ^{70} dependent. It

should therefore represent a good example for evaluation of the generality of the variant UTR functions. The native UTR in *P1* was thus substituted with the *Pm* wild-type UTR and the selected variants, using the same plasmid backbones as in the experiments described above and using *bla* as reporter. Determination of maximum ampicillin tolerance level of the corresponding host cells demonstrated a pattern that was nearly identical to that observed for the corresponding *Pm*-based constructs (Fig. 2c). This clearly indicates that the UTR variants have a much broader application potential than for the *XylS/Pm* system only. We also measured the accumulated transcript levels, and in all cases the amounts of transcript were also reduced, as in the *XylS/Pm* system. The relative patterns were in this case not as strikingly similar as at the protein level (see, for example, the outcomes for the DI-7 and -8 variants), but this is not necessarily surprising, since factors such as transcript formation kinetics from *Pm* and *P1* may not be similar. Note also that while host ampicillin resistance tolerances should allow for direct comparisons between Fig. 2a and c, the same is not the case for the transcript levels.

UTR variants DI-3 and DI-8 can be used to control heterologous production of the C_{50} carotenoid sarcinaxanthin in *E. coli*. Carotenoids represent a diverse class of natural molecules with numerous medical and industrial applications, and it is of interest to be able to produce such compounds in heterologous hosts, including *E. coli*. We recently demonstrated that the *XylS/Pm* promoter system can be used for efficient heterolo-

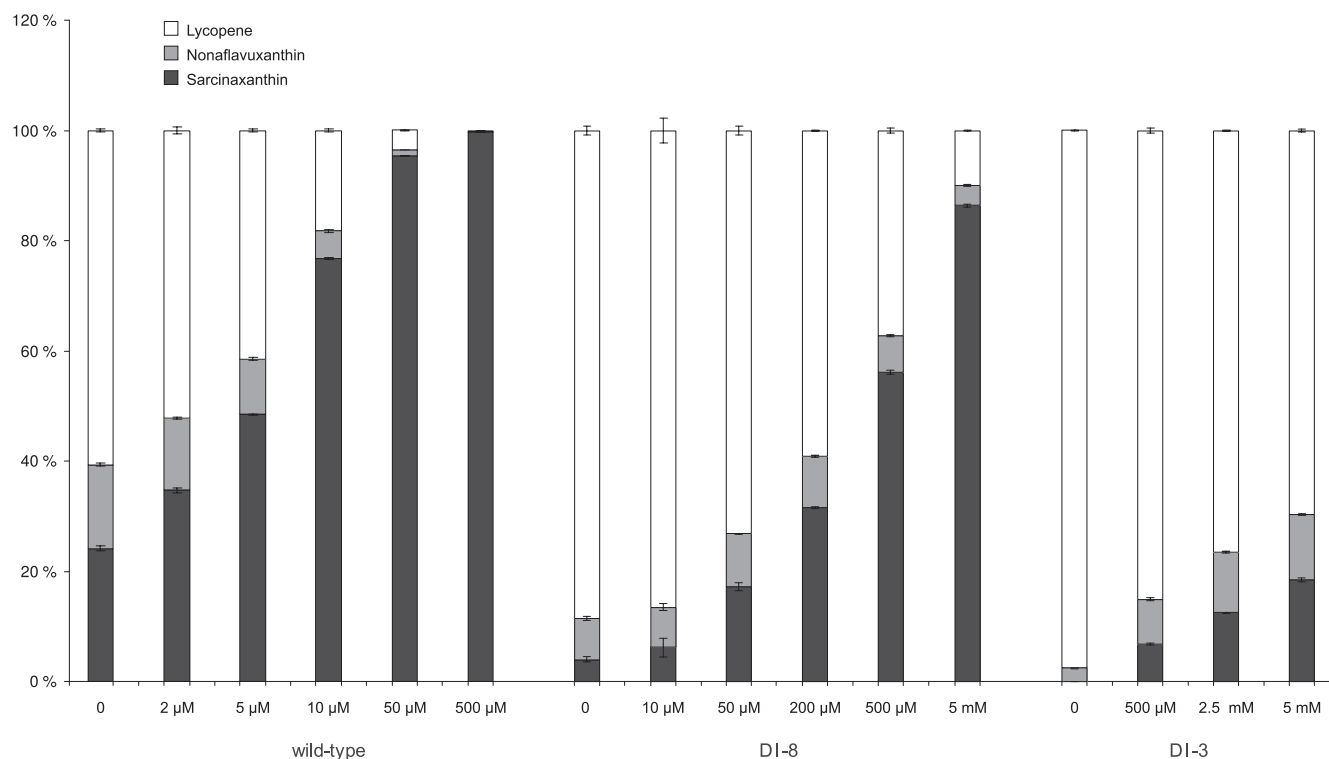


FIG. 7. Relative production levels of sarcinaxanthin in a lycopene-producing *E. coli* host expressing sarcinaxanthin biosynthetic genes from *Pm* and with different UTR variants (wild type, DI-8, and DI-3). Cells were cultivated in liquid cultures, and different concentrations of the *Pm* inducer *m*-toluate were added. Samples were harvested 24 h after induction and analyzed for carotenoid contents (for details see Materials and Methods).

gous production of the C₅₀ carotenoid sarcinaxanthin in a constitutively lycopene (precursor for sarcinaxanthin)-producing strain of *E. coli* (28). Three *M. luteus* genes—*crtE2*, *crtYg*, and *crtYh*—were placed under the control of the *Pm* promoter and were introduced in a lycopene-producing *E. coli* host on a plasmid (28). It was observed that under induced conditions all of the lycopene in the cells was converted into sarcinaxanthin (2.3 mg per g of cell [dry weight]) (28), but substantial amounts of sarcinaxanthin were also produced in the absence of *Pm* induction. This system therefore represented a good test case for investigation of the applicability of the UTR variants to control a metabolic pathway, preferentially at all levels from zero production to the maximum level.

As tools for this purpose, we constructed plasmids pKT1-DI-8-CRT-E2YgYh-2665 and pKT1-DI-8-CRT-E2YgYh-2665 (see the supplemental material) that are both analogous to plasmid pCRT-E2YgYh-2665 (wild-type UTR) (28) but with the *Pm* UTR region substituted with the UTR variants DI-8 and DI-3, respectively. Both plasmids were transformed into the lycopene-producing strain *E. coli* XL1-Blue(pLYC), and the resulting recombinant strains were subjected to sarcinaxanthin production analyses in shake flasks essentially as described previously (28). Cultivations were performed in the presence of different concentrations of the *Pm* inducer, and carotenoid production was investigated by LC-MS analysis of cell extracts (Fig. 7). For the control strain (harboring the plasmid with the wild-type UTR), ca. 25% of the totally extracted carotenoid was identified as sarcinaxanthin in the ab-

sence of induction, and the remaining carotenoid fraction was identified as precursor carotenoids lycopene and nonaflavuxanthin. In the presence of a 500 μM concentration of inducer the entire extracted carotenoid fraction was sarcinaxanthin, and no lycopene or nonaflavuxanthin was detected. Note also that the total extracted carotenoid amount was similar for all analyzed samples (2.3 mg per g of cell [dry weight]).

For strain *E. coli* XL1-Blue(pLYC)(pKT1-DI-8-CRT-E2YgYh-2665) grown under uninduced conditions, only 5% of the total carotenoid fraction was sarcinaxanthin, while 95% remained as C₅₀ carotenoid precursors lycopene and nonaflavuxanthin. The sarcinaxanthin fraction increased gradually with higher inducer concentrations used during cultivation. In the presence of 500 μM inducer, the extracted carotenoid fraction consisted of 53% sarcinaxanthin. A further 10-fold increase in inducer concentration (5 mM) improved sarcinaxanthin production up to 90% of the totally extracted carotenoids. Furthermore, in strain *E. coli* XL1-Blue(pLYC)(pKT1-DI-3-CRT-E2YgYh-2665) no sarcinaxanthin was detected under uninduced conditions, whereas the maximal production (5 mM inducer) was only 17% compared to that of the wild-type UTR control strain under the same conditions. Together, these data demonstrated that the UTR variants can be used to fine-tune the expression of a set of biosynthetic genes, resulting in controlled production of sarcinaxanthin at any level from zero and up to complete conversion of all precursors produced by the cells.

The importance of balanced gene expression has been shown for recombinant lycopene production in *E. coli*. Utilization of a low-copy-number plasmid instead of a high-copy-

number plasmid for arabinose-inducible overexpression of the rate-limiting enzyme DXS (1-deoxy-D-xylulose 5-phosphate synthase) enhanced lycopene production 2- to 3-fold and demonstrated that the overexpression of *dxs* at intracellular concentrations that exceed the availability of its glycolytic precursors pyruvate and G3P causes a significant metabolic burden for the cell and impaired growth significantly (18). This example demonstrates that genetic tools for fine-tuned regulation of gene expression can play an important role for the heterologous high-level production of complex compounds. The idea of using UTR variants to control metabolic pathways is not completely new (39), but the specific approach described here has the advantage that one can select specific UTR features that almost certainly cannot be rationally predicted.

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