Non-Invasive Analysis of Recombinant mRNA Stability in Escherichia coli by a Combination of Transcriptional Inducer Wash-Out and qRT-PCR

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Abstract
mRNA stability is one among many parameters that can potentially affect the level of recombinant gene expression in bacteria. Blocking of the entire prokaryotic transcription machinery by addition of rifampicin is commonly used in protocols for analysis of mRNA stability. Here we show that such treatment can be effectively replaced by a simple, non-invasive method based on removal of the relevant transcriptional inducers and that the mRNA decay can then be followed by qRT-PCR. To establish the methodology we first used the m-toluate-inducible XylS/Pm expression cassette as a model system and analyzed several examples of DNA modifications causing gene expression stimulation in Escherichia coli. The new method allowed us to clearly discriminate whether an improvement in mRNA stability contributes to observed increases in transcript amounts for each individual case. To support the experimental data a simple mathematical fitting model was developed to calculate relative decay rates. We extended the relevance of the method by demonstrating its application also for an IPTG-inducible expression cassette (LacI/Pm) and by analyzing features of the bacteriophage T7-based expression system. The results suggest that the methodology is useful in elucidating factors controlling mRNA stability as well as other specific features of inducible expression systems. Moreover, as expression systems based on diffusible inducers are almost universally available, the concept can be most likely used to measure mRNA decay for any gene in any cell type that is heavily used in molecular biology research.

Introduction
The use of inducible promoter systems for heterologous protein expression in Escherichia coli is one of the most convenient and actively used methods, accounting for one third of protein production altogether [1–5]. The inducible Pm promoter regulates the expression of the meta-cleavage pathway operon involved in the catabolism of aromatic hydrocarbons in the TOL plasmid (pWWO) of Pseudomonas putida [4]. Together with its positive regulator XylS that is activated by the binding of downstream products in the pathway (alkylbenzoates), the Pm promoter system has been established in our laboratory as a robust model for heterologous expression in various bacterial hosts [5–7]. An important factor controlling the quantity of heterologous protein being produced is the cellular concentration of its mRNA, shaped by the synthesis and degradation rates [8]. The efficiency of transcription has often been one of the first intuitive steps to target in order to stimulate recombinant gene expression [9–11], and for the XylS/Pm expression system this has been accomplished via directed evolution of the Pm promoter region [12] and the coding sequence of the XylS transcriptional regulator [13]. In addition, strong stimulation of the transcript levels has also been achieved by mutating the Pm promoter associated 5’ untranslated region (UTR) or by fusing translocation signal sequence to a heterologous gene [14,15].

The mRNA degradation processes in bacteria are complex and not yet fully understood. The originally proposed mRNA degradation model correctly suggested that the mRNA lifetimes in bacteria are controlled primarily by internal endonucleolytic cleavage followed by 3’–5’ exonuclease attack [16,17], but failed to explain the influence of 5’–terminal ends on mRNA half-lives [18–20]. Two recent studies re-examined the initial events of RNA decay and uncovered that mRNA degradation can be triggered by pyrophosphate removal at the 5’-terminal end [21,22], a modification which makes it a preferred substrate for the essential RNase E [23]. The importance of understanding the factors that affect mRNA stability for applications in biotechnology and metabolic engineering is widely recognized [24–27], and established methods for assessing the stability of mRNAs in prokaryotes commonly involve the use of antibiotic inhibitors of transcription (particularly rifampicin) combined with pulse-chase procedures, Northern-blot analyses and microarrays. The use of rifampicin turns off most or all transcription initiation in the cells, which can lead to serious effects on bacterial cells [28], making it virtually...
impossible to exclude unintended (and possibly also mRNA-specific) effects on transcript decay. Our goal in this work was therefore to develop an alternative, non-invasive procedure that could be used for monitoring mRNA decay in a more controlled manner.

We have previously introduced a quantitative real-time polymerase chain reaction (qRT-PCR) based method to study the kinetics of recombinant transcript accumulation from the XylS/Pm expression system in E. coli, from the point of induction until the transcript reaches its steady-state level [14]. Here we describe how a methodologically related approach can be used to determine the decay kinetics of recombinant transcripts, allowing for evaluation of the mRNA stability. The procedure first involves a rapid wash-out of the transcriptional inducer, which results in a controlled and specific shut off of transcription of the recombinant gene. With the recombinant transcript synthesis stopped, its decay can be followed as a function of time by qRT-PCR. This second gene. With the recombinant transcript synthesis stopped, its decay be followed as a function of time by qRT-PCR. This second

### Materials and Methods

**Growth Conditions and DNA Manipulations**

E. coli strains were generally grown at 37°C in Luria-Bertani (LB) broth (10 g/L tryptone, 5 g/L yeast extract, and 5 g/L NaCl) or on LB agar (LB medium with 15 g/L agar) supplemented with ampicillin (200 mg/L) or kanamycin (50 mg/L) when appropriate. For expression experiments, recombinant cells were grown at 30°C and induction of the XylS/Pm or T7 and LacI/Pm system was done by adding m-toluic acid or IPTG, respectively, to the final concentrations as indicated in the text. When rifampicin was used for inhibiting transcription, it was added to a final concentration of 100 mg/L (90 minutes after induction at 30°C). Standard recombinant DNA procedures were performed as previously described [31]. Custom PCR primers were supplied by Eurofins MWG operon or Sigma-Aldrich Co. Spiked oligonucleotide mixtures used for combinatorial library constructions were supplied by Medprobe AS. DNA sequencing was performed by Eurofins MWG operon.

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**Table 1. Bacterial strains and plasmids.**

| Strain or plasmid | Description | Reference |
|-------------------|-------------|-----------|
| E. coli strain    |             |           |
| DH5α              | General cloning host | Bethesda Research Laboratories |
| NEB Express Iq    | Strain with enhanced expression of LacI repressor | New England Biolabs |
| ER2566            | Strain with intracellular production of T7 DNA polymerase | New England Biolabs |
| **Plasmid**       |             |           |
| plIB11            | RK2-based expression vector containing the XylS/Pm expression cassette, wild type Pm UTR and bla as the reporter gene for Pm; Km; 8.1 kb | [12] |
| plIB11 LV-2       | plIB11 derivative containing mutated 5'-UTR DNA sequence LV2 upstream of the bla gene; Km; 8.1 kb | [14] |
| plIB11 LII-11     | plIB11 derivative containing mutated 5'-UTR DNA sequence LII-11 upstream of the bla gene; Km; 8.1 kb | [14] |
| pGM29             | RK2-based vector expressing GM-CSF-c-myc-his6 fusion protein from the XylS/Pm expression cassette; Ap; 8.7 kb | [15] |
| pGM29ompA         | RK2-based vector expressing ompA-GM-CSF-c-myc-his6 fusion protein from the XylS/Pm expression cassette; Ap; 8.8 kb | [15] |
| pIFN305           | RK2-based vector expressing IFN-α2b-c-myc-his6 fusion protein from the XylS/Pm expression cassette; Ap; 8.8 kb | [15] |
| pIFN305pelB       | RK2-based vector expressing pelB-IFN-α2b-c-myc-his6 fusion protein from the XylS/Pm expression cassette; Ap; 8.9 kb | [15] |
| pBS1bla           | RK2-based expression vector containing the XylS/Pm expression cassette and bla as a reporter gene for Pm; Km; 9.5 kb | [58] |
| pBS1bla           | pBS1bla derivative containing synonymous mutation in the 2nd codon of the bla gene; Km; 9.5 kb | This study |
| pBS1bla-C19       | pBS1bla derivative containing synonymous bla variant C19; Km; 9.5 kb | This study |
| pFLAG-CTC™        | Cloning vector (F1 replicon) for expression of recombinant proteins from the LacI/Pm cassette. Ap; 5.4 kb | Sigma-Aldrich Co. |
| pFLAG-GM          | pFLAG-CTC™ derivative expressing GM-CSF-c-myc-his6 fusion protein; Ap; 5.9 kb | This study |
| pFLAG-OGM         | pFLAG-CTC™ derivative expressing ompA-GM-CSF-c-myc-his6 fusion protein; Ap; 5.9 kb | This study |
| pSB-E2r           | pMB1-based vector expressing IL1-RA protein from the LacI/Pm expression cassette; Km; 6.1 kb | [59] |
| pSB-P2r           | pMB1-based vector expressing IL1-RA protein from the XylS/Pm expression cassette; Km; 5.8 kb | [59] |

1. Ap*: ampicillin resistance gene; Km*: kanamycin resistance gene.

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Biological Materials

The strains and plasmids used in this study are listed in Table 1 and the primers are listed in Table 2. Vectors pBS-P1bla and pBS-P1bla-C19 were made from pBSP1bla, by replacing the NdeI-Ncol fragment containing the bla coding region, with annealed oligonucleotides corresponding either to the original sequence, except for a second codon change from AGT to TCT, or to a synonymous variant C19. Vectors for expression of recombinant proteins under control of the P_tac promoter were based on the commercial pFLAG-CTCTM expression vector. The RBS sequence corresponding either to the original sequence, except for the TCT change in the second codon, was inserted into a pBSP1bla vector, using the synonymous second codon as the new start sequence. SII was constructed from the oligonucleotide mixture defined by: 5′-TATGTC4AT(12)CA5CA8TT6CT4C-3′ and 5′-TATGTC4AT(12)CA5CA8TT6CT4C-3′. The non-coding strand was kept complementary to the RBS sequence to the original sequence, except for the TCT change in the second codon.

Construction of Combinatorial Synonymous Libraries in the 5′ End of the bla Coding Sequence

A synonymous codon library of the bla 5′ coding sequence (SI library) was constructed in the pBS-P1bla plasmid by replacing the sequence corresponding to the first two codons by synthetic oligonucleotide mixtures, followed by screening on solid media according to previously described methods [12,14]. The mixtures were NdeI/KpnI digested, and ligated into corresponding sites of the commercial pFLAG-CTCTM expression vector. The screening for mutated bla coding sequences that result in increased ampicillin-tolerance levels was performed according to a previously described procedure [14].

Table 2. PCR and qRT-PCR primers.

| Name          | Sequence                      | Gene |
|---------------|-------------------------------|------|
| PmUTR.fwd     | 5′-AGAAGAGCGGATACAGGAGTG-3′   | gms-cf/ompA-gm-csf |
| Gol.rev       | 5′-CTTTGATACCTTGTGGCCCGAT-3′  | gms-cf/ompA-gm-csf |
| bla.fwd       | 5′-AGCTTTTCAATGATGAGACCTT-3′  | bla   |
| bla.rev       | 5′-TGCCCAGGGCTCAACAC-3′       | bla   |
| gms-cf37.fwd  | 5′-CCCTGGAGCATTGAAATG-3′      | gms-cf/ompA-gm-csf |
| gms-cf11f.rev | 5′-CATCTCACAGCAGAGCGTTCTACTCT-3′ | gms-cf/ompA-gm-csf |
| gms-cf26fwd   | 5′-GGCCCTCGTGACACATTGATG-3′   | gms-cf/ompA-gm-csf |
| gms-cf301.fwd | 5′-CTGGTTGTCGACAGGAAGTTTT-3′ | gms-cf/ompA-gm-csf |
| ifn-2b324.fwd | 5′-CGGACCCGCGCTGAGAA-3′       | ifn-2b/pelB/ifn-2b |
| ifn-2b396.fwd | 5′-CAGATACAGGTTGATACGCTGAAA-3′ | ifn-2b/pelB/ifn-2b |
| km.fwd        | 5′-TACCTTGGGACCTTGTTAGAAACA-3′ | Km    |
| km.rev        | 5′-AATCAGGTTGCAACATCTACAGA-3′ | Km    |
| il-1a.fwd     | 5′-TCAGACATTTCTCATTCCCATC-3′  | ILIRa5 |
| il-1a.rev     | 5′-ATGATTGTCGAGAGGCTGATAG-3′  | ILIRa5 |
| 16RNA.fwd     | 5′-TTGACGATTTCCGGAGGAAGA-3′   | 16 rRNA |
| 16RNA.rev     | 5′-GTCGCCGACCTCCGTATAC-3′     | 16 rRNA |

1 Km*: kanamycin resistance gene.

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Sequence. Because a synonymous change of the bla second codon (AGT → TCT) was particularly effective for increasing ampicillin tolerance level, a second library (SII library) was generated in the pBS-P1bla vector, using the synonymous second codon as the new start sequence. SII was constructed from the oligonucleotide mixture defined by: 5′-TATGTC4AT(12)CA5CA8TT6CT4C-3′ and 5′-TATGTC4AT(12)CA5CA8TT6CT4C-3′. The non-coding strand was kept complementary to the original sequence, except for the TCT change in the second codon.

Determination of the Kinetics of Recombinant Transcript Accumulation

LB medium was inoculated to final OD₀₅₀ = 0.05 with an overnight culture and grown at 30°C (220 rpm) until mid-log phase. At OD₀₅₀ = 0.5 either 0.5 mM m-toluate (XyS/P or 0.5 mM IPTG (LacIq/P_tac and T7) was added immediately after the first sample (time zero) was collected. Samples for total RNA isolation collected at time points 0, 3, 5, 8, 10, 15, 20 and 60 minutes were processed as described in the qRT-PCR analysis chapter. For immediate stabilization, samples were treated with RNaprotect cell reagent (QIAGEN) prior to freezing.

The Inducer Wash-out Method

20 mL of recombinant cultures were grown and induced as described above. Culture growth was continued for another 90 minutes, allowing the investigated mRNA to reach its steady-state level. 5 mL of the cultures were subsequently concentrated by rapid filtration through a Millipore EZ-Pak® Membrane filter (Millipore) with the use of a vacuum pump. The filter with harvested cells was washed with 10 mL of phosphate buffered saline (137 mmol/L NaCl, 2.7 mmol/L KCl, 8.1 mmol/L Na₂HPO₄, 1.76 mmol/L KH₂PO₄, pH 7.4), followed by resuspension of the cells in 10 mL of fresh 30°C pre-warmed LB medium without inducer and maintaining the culture growth for another 10 to 30 minutes. Samples for qRT-PCR analysis were taken directly after filter transfer and cell resuspension (zero minutes) and at several time points after the filter transfer. Each sample was immediately after collection treated with the stabilizing RNAprotect cell reagent (QIAGEN).

qRT-PCR Analysis

The analysis of transcript levels from different recombinant genes was performed as described previously [31]. A fragment from the 16S rRNA gene was used as a normalizer. All experiments were repeated at least twice, and measurements were carried out with minimum three technical recurrences.

High-performance Liquid Chromatography (HPLC) Analysis of m-toluate

To determine the presence of m-toluate in cell culture samples HPLC analysis was performed as previously described [32], except that the temperature of the Aminex HPX-87-H column was changed to 55°C. Standards of m-toluate in LB media (0.3 and 0.2 mM) were used for calibration. Detection of m-toluate in the cells, recombinant cultures were harvested by centrifugation (3 ml), and cell free extracts were obtained by suspending the cell pellets in 0.9% NaCl and using a Branson Sonifier D5M tip for
cell disruption (sonication for 3 minutes on ice, duty cycle 30% and output control 2.5). Samples were filtered through 0.2 μm Filtrropur S syringe filters (Sarstedt) prior to HPLC analysis.

Gas Chromatography-Mass Spectrometry (GC-MS) Analysis of Isopropyl β-D-1-thiogalactopyranoside (IPTG)

Agilent 7890A GC coupled to Agilent 5975 inert MSD was used for IPTG GC-MS analysis. Aqueous IPTG samples/standards (50 μL) were added to 25 μL stock solution of 11.75 mM Myristic-d-27 acid in solvent mixture water:methanol:isopropanol (2:5:2 (v/v/v)). Myristic acid served as internal standard. Samples/standards were vacuum dried using a Thermo Speed-Vac (v/v/v)). Myristic acid served as internal standard. Samples/standards were vacuum dried using a Thermo Speed-Vac evaporator at 65°C, followed by derivatization according to Agilent Fiehn GC/MS metabolomics RTL library user guide (Agilent Technologies). Analysis was performed according to the guide with a modified temperature program: 60°C for 1 min, then 20°C/min to 325°C, final temperature held for additional 5 min.

β-lactamase Enzymatic Assay and Quantification of Recombinant Proteins by SDS-PAGE/Western Blot Analysis (β-lactamase and GM-CSF)

For analysis of recombinant protein production level, an overnight culture was diluted with LB media to OD600 = 0.05 and grown at 30°C (220 rpm). Exponentially growing cells (OD600 = 0.1) were then induced either with m-toluic acid or IPTG (0.5 mM). Cell growth was continued for 5 hours after which recombinant cultures were harvested by centrifugation (6000 rpm, 5 minutes, 4°C, Eppendorf Centrifuge 5810R). Crude extracts were prepared by sonication 4x90 seconds, with 30 seconds cooling periods (Branson sonifier, 30% duty control, 3 output control). Total protein concentration was determined with the Bio-Rad Detergent-Compatible Protein Assay (Bio-Rad Laboratories, Hercules, CA, USA) as described by the manufactures. β-lactamase assay was performed according to the procedures previously described [33]. All enzyme activity analyses were repeated at least twice, and measurements were carried out with minimum three technical recurrences. Qualitative detection of β-lactamase and GM-CSF was performed using SDS-PAGE and Western blot essentially as previously described [34,35] except that direct detection with HisProbe TM-HRP (Thermo Scientific) was applied for the His-tagged GM-CSF protein. Signals were developed by using Pierce ECL Western blot substrate for chemiluminescent detection according the manufacturer’s instructions. Chemiluminescence was detected by ChemiDoc TM XRS Imaging System and analyzed by Image Lab 4.0 software (Bio-Rad Laboratories.).

Computational Estimation of mRNA Synthesis and Degradation Rate

Similar to the approach in [14], we assume that recombinant transcripts \( M(t) \) are synthesized after induction at a constant rate \( \mu_S \) and degraded at a constant rate \( \delta_S \):

\[
\frac{dM}{dt} = \mu_S - \delta_S M, \tag{1}
\]

with the general solution

\[
M(t) = \frac{\mu_S}{\delta_S} + \frac{\mu_0 - \mu_S}{\delta_S} e^{-\delta_S t}, \tag{2}
\]

where \( \mu_0 \) is the synthesis rate of the non-induced promoter. Assuming constant synthesis and degradation rates after transcriptional shut off, transcript decay is given by:

\[
\frac{dM}{dt} = \mu_1 - \delta_D M, \tag{3}
\]

with general solution

\[
M(t) = \frac{\mu_1}{\delta_D} + \frac{\mu_0 - \mu_1}{\delta_D} e^{-\delta_D t}, \tag{4}
\]

where the rate \( \mu_1 \) corresponds to leakage transcription in the inhibited promoter. We estimated the synthesis rate \( \mu_S \) and the decay rate \( \delta_D \) by employing non-linear least squares fitting using the Levenberg-Marquardt algorithm [36] on Eqn. (2) and Eqn. (4) respectively. All reported rate values are in units of “per minute”.

The robustness of our parameter estimates was analyzed by calculating their 95% confidence intervals (95% CI) and conducting Jackknife sampling.

Results

The Use of UTR Mutants as Models to Study bla mRNA Stability by the Inducer Wash-out Method

Chemical inducers of recombinant gene expression are typically low molecular weight compounds that may or may not diffuse in and out of the cells. The inducer of the XylS/Pm system (m-toluic acid) enters E. coli independently of the cells transport systems and was therefore used as a first test of the wash-out concept. A series of initial experiments showed that a simple filtration procedure can be used to rapidly replace the growth medium of an induced cell culture by fresh medium lacking inducer. We then further confirmed the robustness of the method at different levels, as follows (data not shown): 1) by HPLC it was shown that the inducer is removed from both the media and cells after the filtration; 2) two independent primer pairs targeting the 5’ or the 3’-terminal end, respectively, were found to give corresponding results during qRT-PCR quantifications of the same recombinant mRNA; and 3) it was demonstrated that the amounts of an mRNA produced constitutively (plasmid-borne kanamycin resistance gene) were not affected by the wash-out procedure itself.

To confirm that the wash-out method could be used to analyze mRNA stability we first reinvestigated an earlier study showing strong stimulation of transcript accumulation resulting from specific mutations (LV-2 variant) in the DNA region corresponding to the 5’-UTR mRNA sequence of the bla reporter gene, encoding β-lactamase [14]. In this report it was concluded that the mutations were primarily acting by stimulating the transcription process itself, and not by increasing the mRNA stability. However, these conclusions did not follow from direct measurements of decay rates, and here we wanted to use this case for an initial evaluation of the inducer wash-out method. The bla gene was again used as the reporter (E. coli strains DH5α (pBl11) and DH5α (pBl11 LV-2)), and qRT-PCR quantifications of the mRNA levels at consecutive time points after inducer removal revealed that the decay kinetics is indeed similar for both transcripts (wild type UTR and LV-2 variant), despite a more than 10-fold difference in the accumulated transcript amounts (Figure 1). Subsequent mathematical fitting of the experimental data estimated the relative decay rates (defined by Eq. 4 in Material and Methods) to be 0.14 for the wild type (95% CI = 0.13 – 0.17) and 0.13 for the LV-2 variant (95% CI = 0.12 – 0.16). These results were consistent with the previous findings, and therefore encouraged us to study more cases, involving less predictable outcomes.
In the study in which the LV-2 mutant was identified we also isolated a number of other UTR variants which appeared to stimulate translation more selectively, and we selected such a variant (LII-11) as an example for the next mRNA stability examination. Generally it is known that efficient translation may lead to protection against mRNA degradation, presumably due to a higher ribosomal occupancy of the transcript and steric protection of potential RNase E cleavage sites [37,38]. Interestingly, the kinetics of the LII-11 transcript degradation could not be distinguished from that of the wild type UTR or the LV-2 variant (data not shown). Thus, this result suggested that stimulated translational efficiency does not necessarily lead to increased mRNA stability. Even though the LII-11 UTR variant led to a stronger stimulation at the protein than at the mRNA level (in contrast to LV-2), more transcripts (compared to the wild type) also accumulated when bla was expressed with the LII-11 UTR [14]. The stability analysis reported here may therefore be interpreted to mean that LII-11 lead to a mixture of transcriptional and translational stimulation and that the translational stimulation is not accompanied by a corresponding protection of the transcript from degradation.

Certain Synonymous Codon Changes at the 5’ End of the bla Coding Sequence Stimulate the Gene Transcription without Affecting mRNA Stability

Since complete genes can now be synthesized commercially at a reasonable cost, optimization of codon usage has become a popular strategy to enhance expression of poorly expressed genes [39–41]. The total number of possible synonymous codon variants for a complete gene is enormous, but here we decided to specifically study the effects of codon changes limited to the 5’ end of the bla coding sequence. The advantage of using this particular gene for such analyses is that mutants with stimulated β-lactamase production can be easily selected by the ability of the corresponding host cells to grow on agar medium supplied with elevated levels of ampicillin [12,14]. We constructed and screened a library of such mutants (Materials and Methods) and one of them displayed an approximately 9-fold increase in the host ampicillin tolerance level. This mutant was designated C19 and contained a 2nd codon change from AGT to TCT (Serine) and a 6th codon change from GTC to GTT (Valine). Analysis of bla transcript amounts and β-lactamase activity (strains DH5α (pBSP1bla) and DH5xα (pBSP1bla-C19)) indicated more than 3-fold increase for both, relative to the wild type (Figure 2A). Western blot analysis showed that the increase in enzyme activity is based on increased protein production, as a good correlation between the enzyme amount and corresponding activity level was observed (Figure 2B). The observed increase in accumulated transcripts might potentially be the result of enhanced mRNA stability, but the corresponding bla mRNA decay kinetics showed that this is not the case (Figure 3; relative decay rates: 0.16 for the wild type (95% CI = 0.14–0.18) and 0.19 for the C19 variant (95% CI = 0.15–0.23)). Surprisingly, these results therefore indicated that the mutations in the coding sequence can result in stimulation of transcription, without significantly affecting mRNA stability.

Fusion of a 5’ Translocation Signal Sequence to a Heterologous Gene can Confer Increased mRNA Stability

In a previous study [13], we showed that expression of the gm-csf gene, encoding human granulocyte-macrophage colony-stimulating factor (GM-CSF), is strongly stimulated at the transcript and protein levels, by fusing the ompA translocation signal sequence in-frame with the 5’ end of the cm-csf coding sequence. This therefore represented a different test case for investigation of mRNA stability and its potential role in recombinant protein production. The decay of gm-csf transcript was monitored in parallel for DH5xα (pGM29) and DH5xα (pGM29ompA) strains. The results showed that the addition of ompA 5’ signal sequence leads to a significant increase in the gm-csf mRNA stability (Figure 4), estimated to be a 4-fold decrease in the relative decay rate; 0.31 for gm-csf (95% CI = 0.30–0.33) and 0.08 for ompA-gm-csf (95% CI: 0.08–0.09).

![Figure 1. Decay kinetics of two bla transcripts with different 5’ UTR sequences. The respective genes were expressed from Pm and contained the DNA sequence of either the wild type 5’-UTR or the LV-2 variant. The larger plot describes decay kinetics where the level of the two bla transcripts is arbitrary set to one at time point zero. In the upper right corner the transcript amounts are represented with all values relative to the wild type 5’-UTR; arbitrary set to one at time point zero. Solid lines represent the best fit to the data calculated according to Eqn 4 (Material and Methods). Error bars show the deviation between three biological recurrences. RQ: relative quantification, AU: arbitrary units. doi:10.1371/journal.pone.0066429.g001](image)

![Figure 2. The bla gene expression in DH5xα (pBSP1bla) and DH5xα (pBSP1bla-C19). A. Determination of accumulated transcript levels and enzymatic activity of β-lactamase, with all values relative to the pBSP1bla level (arbitrarily set to one). B. Total β-lactamase production of DH5xα (pBSP1bla) (denoted as wt) and DH5xα (pBSP1bla-C19) (denoted as C19) was visualized by Western blotting and total cell samples were loaded in dilution series of decreasing total protein amount (μg). The size of the mature β-lactamase protein is approximately 29 kDa. doi:10.1371/journal.pone.0066429.g002](image)
In addition, we did a similar test on the *ifn-2b* gene (encoding interferon *2b* cytokine) fused to another translocation signal sequence, *pelB*. The basis for choosing this example was also the previous study [15], in which it was found that expression at the protein level was strongly improved by incorporating the signal sequence. Here we found that transcript amounts also increased up to 3.5-fold for the *pelB-ifn-2b* transcript (20 minutes after the induction), compared to the corresponding *ifn-2b* transcript lacking the *pelB* signal (data not shown). Based on monitoring the mRNA decay (data not shown), the relative decay rates were calculated to be 0.21 for *ifn-2b* (95% CI = 0.19–0.22) and 0.11 for *pelB-ifn-2b* (95% CI = 0.10–0.12). In summary, these results showed that two different 5′ signal sequences, acting on different genes, in both cases lead to enhanced mRNA stability. The corresponding transcripts are therefore inherently more stable than their counterparts lacking the signal sequences, or alternatively the signal leads to stimulation of translation, which in turn protects the transcript from degradation.

The Inducer Wash-out Method can also be Applied to Study Decay Kinetics of mRNAs Produced from an IPTG Inducible Expression System

To broaden the range of possible applications of the inducer wash-out method we have included also an expression cassette of the commonly used IPTG-inducible LacI/Pm expression cassette, using the *gm-csf* gene with and without *ompA* 5′ fusion (constructs pFLAG-GM and pFLAG-OGM, respectively). To reduce background expression from the *Pm* promoter in the absence of inducer to a minimum we used a particular NEB Express Iq strain as host (Table 1). It was confirmed by GC-MS analyses that IPTG was efficiently removed by the filtration step. We further determined (by using SDS PAGE/Western blot analysis) that also in the pFLAG expression vector context the 5′ fusion is necessary for detectable protein production (data not shown).

The NEB Express Iq (pFLAG-GM) and NEB Express Iq (pFLAG-OGM) strains were next used for determination of the corresponding mRNAs accumulation and decay over time. For detectable protein production (data not shown).

Comparison of the *ompA-gm-csf* and *gm-csf* transcript amounts revealed similar finding as when using the XylS/*Pm* system [15]; the *ompA-gm-csf* transcript amount produced from the *Pm* promoter was several-fold increased (about 6-fold 20 minutes after induction) compared to the gm-csf transcript amount (data not shown). Consistent with the initial observation for the XylS/*Pm* system, the presence of *ompA* 5′ fusion led to a significant increase in the *gm-csf* mRNA stability (Figure 5A); the relative decay rates were determined to be 1.27 for *gm-csf* (CI 95% = 1.19–1.35) and 0.43 for *ompA-gm-csf* (CI 95% = 0.38–0.46). The predicted relative decay rates differ by about factor 4 from the values determined for the same transcripts produced from the XylS/*Pm* system (see previous result chapter). However, in both the XylS/*Pm* and the LacI/*Pm* systems, the *ompA* signal sequence was found to contribute to a 3–4-fold decrease in the *gm-csf* relative decay rate. The differences in relative decay rates can be hypothetically caused by the 5′ UTR sequence following either the *Pm* or *Pm* promoter, and/or differences in mRNA degradation machinery of the *E. coli* strains used (NEB Express Iq versus DH5α).

These results therefore clearly demonstrated that the wash-out method can be used also for IPTG-inducible systems, which are probably the most heavily used of all inducible expression systems. We conclude that the methodology reported here is useful for analysis of recombinant mRNA stability in *E. coli* and presumably also in many other microorganisms in which similar inducible systems can be used, and both the XylS/*Pm* and the IPTG-inducible systems are themselves examples of expression cassettes that can be applied in other hosts [42–45].

Selective Inhibition of Transcription by the Inducer Wash-out can be Used to Replace the Approach Based on Global Inhibition of Transcription by Rifampicin

A comparison of the inducer wash out strategy with the conventional method based on rifampicin was performed next. Promoters that are transcribed by the RNA polymerase core enzyme (E) associated with σ70 subunit are known to be more resistant to rifampicin than Eσ70 promoters [46]. Preliminary results with the *Pm* promoter, which is transcribed by Eσ70 in the...
expressed from the LacI pFLAG-OGM) that were used to demonstrate the utilization of rifampicin to minimize the amount of rifampicin necessary to inhibit transcription in the case of the wash-out, which inhibits only transcription of the specie genes by the inducer wash-out (Figure 5B) under the conditions tested. The results showed that global inhibition of transcription in the cells leads to the same outcome as selective stopping of transcription of the ompA-gm-csf and gm-csf genes by the inducer wash-out (Figure 5B) under the conditions tested. The relative rate of ompA-gm-csf mRNA decay was about 2-fold lower than in the wash-out experiment (value of 0.18 with CI 95% = 0.16-0.21) while the decay rate of gm-csf mRNA differed only slightly (value of 1.11 with CI 95% = 0.97-1.25). We could speculate that most likely our simple decay model performs better in the case of the wash-out, which inhibits only transcription of the targeted mRNA. The cell-wide transcription shut-off brought about by rifampicin could introduce unknown effects that a simple mRNA decay model does not accommodate. In conclusion, although rifampicin based methods have proven invaluable for studying global mRNA decay patterns [48,49], the wash out method should allow for detail analysis of decay patterns of individual mRNAs. This can be found especially useful in gene engineering studies and in studying the effects of specific mutations, for example in the UTR or coding sequence regions.

**Analysis of the Bacteriophage T7 RNA Polymerase/promoter System**

Most of the results of the studies reported above were unpredictable beforehand, and to further verify the wash-out methodology we also decided to carry out an analysis with a more predictable outcome. This involved an investigation of the widely used and IPTG-inducible bacteriophage T7 RNA polymerase/promoter expression system (herein referred to as the T7 system). The genetic arrangement of this system implies that induction by IPTG first leads to synthesis of the T7 polymerase and in the second step to transcription of the target gene. In the vector design used here IPTG induction plays a dual role, as the T7 polymerase specific promoter (expressing the target gene) is also regulated by the LacI repressor. This control at two levels keeps expression of the target gene at a very low level in the absence of IPTG. Thus, in this case one would expect detectable target gene transcript only after a short delay representing the time needed to produce sufficient amounts of the polymerase. The effect of the sequential gene activation in the T7 system on recombinant transcript accumulation kinetics could be directly observed and compared to that of XylS/Pm by using E. coli ER2566 strains harboring analogous expression vectors pSB-E2r and pSB-M2r (Table 1). Both vectors express the synthetic codon-optimized gene for human interleukin 1 receptor antagonist (IL1RNS), either from the T7 promoter (pSB-E2r) or from Pm (pSB-M2r). As also previously observed by [14], Figure 6a shows that expression from Pm starts immediately after inducer addition, while for the T7 polymerase dependent construct there is a clear lag in the transcript accumulation during the first minutes after induction. This presumably reflects the time needed to synthesize sufficient amounts of the T7 polymerase, and we therefore conclude that the qRT-PCR method is capable of visualizing this effect.

By similar reasoning we predicted a rather slow decrease in transcript amounts after inducer wash-out, since the T7 polymerase would likely be reasonably stable and would therefore allow continued transcription after IPTG removal. However, the situation is complex since pre-existing T7 polymerase action will be inhibited by the reactivation of LacI. Therefore the apparent decay reflects a mixture of actual decay and some level of new transcript synthesis. Indeed, the apparent decay of IL1RNS transcripts after removal of the IPTG inducer was found to be much slower in the T7 system compared to that of the Pm system. The remaining level of transcripts stayed over 50% of the start level even after 30 minutes of incubation in the absence of IPTG (Figure 6b).

**Discussion**

This study presented a qRT-PCR-based approach that can be used to monitor recombinant mRNA decay in expression systems induced by low-molecular weight molecules whose transport into and out of cells is by diffusion. This rather simple technique only minimally affects the treated cells, in contrast to most other methods for accessing mRNA stability, which involve global...
phenomenon. For all systems, the amounts of transcript were relative to time point 0 (arbitrarily set to one). The amounts of transcripts were generated through the T7 system and increased about 10-fold compared to those of the Pm system (after 60 minutes). Solid lines represent the best fit to the data, calculated according to Eqn 2 (Material and Methods).

Parallel determination of decay for Pm and T7 generated transcripts. For both systems, all transcript amounts are presented relative to the value at time point 60 minutes (arbitrarily set to one). In case of the T7 system, only time points 15, 20, 40 and 60 minutes were included for the generation of the transcript accumulation curve.

The analysis of bla associated 3′-UTR variants directly showed that the stimulation of the transcript level by the LV-2 and LII-11 variants is based on enhanced rate of transcription and not more stable mRNA (Figure 1). As documented by the LII-11 variant, more efficient translation does not always result in better mRNA stability. The reasons for this may be that the adjacent 3′-UTR DNA region can influence the transcriptional process through sequences resembling the −10 promoter element (TATAAT) that are able to induce σ70-dependent transcriptional pausing [55,56]. In case of the tryptophanase operon (tnd), transcriptional pause sites have been described in the 220-nucleotides long spacer region separating the coding regions of TnaC leader peptide and TnaA tryptophanase, and implicated in coupling of translation with transcription [57]. One possibility is therefore that the synonymous mutations within the bla coding region affect pause sites in the wild type bla DNA sequence in a way that is stimulatory for transcription.

To substantiate the general relevance of the new methodology for assessment of mRNA stability, we expanded its use also to an IPTG-inducible promoter. The decay of mRNAs (ompF-gm-csf and gm-csf, Figure 5) generated from the Pm promoter confirmed that the methodology was equally useful for this IPTG-inducible system as for the m-tohulate-inducible XylS/Pm system. One potential limitation of this method might be that it requires the use of an inducible promoter with low background expression level. Any background production of mRNA under study would likely lead to a mixture of actual decay and some level of new transcript synthesis, causing an error in the calculation of the relative decay rate.

The inducer wash-out method was tested with plasmids having both low (e.g. pIB11) and moderately high copy number (e.g. pGM29). Given that the qRT-PCR is generally regarded as a highly specific and sensitive technique, we believe that the method can without difficulty address different expression levels. Moreover, when used together with determination of the transcript levels and the respective protein amount, the inducer wash-out method should be useful for elucidating different factors that might be affecting overall expression level of a recombinant gene. The concept described here can most likely be used to measure mRNA decay for any gene in basically any cell type (even some eukaryotes) that is heavily used in molecular biology research. The reason is that systems based on diffusable inducers are almost universally available. Whatever the gene of interest is, it can simply be PCR-amplified with its own UTR and then inserted downstream of an inducible promoter. This will likely give the same transcript as from the natural environment in which the gene is localized.
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Author Contributions

Conceived and designed the experiments: VK TB SV. Performed the experiments: VK TAS AN. Analyzed the data: VK EA TB SV. Contributed reagents/materials/analysis tools: TB SV. Wrote the paper: VK SV TB and SV wrote the funding application. VK was a PhD student supervised by SV.

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