# Ribozyme-mediated expression of tRNA-derived small RNAs in bacteria

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## Abstract

Transfer RNA-derived RNAs (tDRs) have emerged as important regulatory molecules found across all three domains of life. Despite their discovery over four decades ago, their biological significance has only recently begun to be elucidated. However, studying bacterial tDRs poses challenges due to technical limitations in assessing their *in vivo* functionality. To address this, we established a novel approach utilizing a self-cleaving Twister ribozyme to express tDRs in *Escherichia coli*. Specifically, we employed the type P1 Sva1–1 Twister ribozyme, to generate tDRs with genuine 3′ ends. Our method involves the inducible expression of tDRs by incorporating the desired tDR sequence into a plasmid construct downstream of two lac operators and

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upstream of the Twister ribozyme. Upon induction with IPTG and transcription of the construct, the Twister ribozyme undergoes self-cleavage, thus producing tDRs with defined 3′ ends. As a proof of principle, we demonstrated the *in vivo* application of our novel method by expressing and analyzing two stress-induced tRNA halves in *E. coli*. Overall, our method offers a valuable tool for studying tDRs in bacteria to shed light on their regulatory roles in cellular processes.

## 1. Introduction

Small non-coding RNAs (sRNAs) play pivotal roles in regulating various cellular processes, including stress response, cell morphology, virulence, and metabolism at the post-transcriptional level (Chao & Vogel, 2016; Miyakoshi, Matera, Maki, Sone, & Vogel, 2019; Murashko & Lin-Chao, 2017; Sy & Tree, 2021). In *Escherichia coli* sRNAs predominantly act *in trans* by base-pairing with target mRNAs, thereby altering their structure and/or stability to modulate gene expression within the cell. Remarkably, a single sRNA can target multiple mRNAs, enabling the regulation of complex cellular expression networks. RNA chaperones such as Hfq and ProQ stabilize many bacterial sRNAs and facilitate the interactions between sRNAs and their targets (Wagner & Romby, 2015).

Among the diverse classes of regulatory sRNAs, transfer RNA-derived RNAs (tDRs) have emerged as significant players, found across all three domains of life (Cristodero & Polacek, 2017; Holmes, et al., 2023). First described in 1977, tDRs originate from cleavage events of mature tRNAs (Borek, et al., 1977), or pre-tRNA transcripts (Lalaouna, et al., 2015). Notably, tDRs retain tRNA modifications, suggesting that processing occurs at least after near-complete tRNA modification (Diebel, Zhou, Clarke, & Bemis, 2016). Despite the initial discovery of stable tRNA cleavage products, it was not until over three decades later that their biological significance began to be elucidated (Gebetsberger, Zywicki, Künzi, & Polacek, 2012; Yamasaki, Ivanov, Hu, & Anderson, 2009). For example, in *Trypanosoma brucei* during stress recovery, the $tRNA^{Thr}$-3′-half (AGU) associates with mitochondrial ribosomes and promotes translation, which enhances mitochondrial activity and the ability of cells to produce energy. As an instant stress recovery reaction, this tDR improves translational control, which increases the protozoan's energy metabolism (Brogli, Cristodero, Schneider, & Polacek, 2023).

Although multiple functions have been described for eukaryotic tDRs, much less is known about the roles played by bacterial tDRs. 5′-tDRs

ending in 2′,3′ cyclic phosphates and nicked tRNAs from *Salmonella Typhimurium* have been found to bind the transcriptional activator RtcR and activate the expression of an RNA repair operon (Chen & Wolin, 2023; Hughes, Xinguo, Burroughs, Aravind, & Wolin, 2020). The 3′ external transcribed spacer of the pre-$tRNA^{LeuZ}$ was found to work as an sRNA sponge in *E. coli*, repressing transcriptional noise (Lalaouna, et al., 2015). Microbial tDRs have also been found highly enriched in outer membrane vesicles (OMVs), suggesting that tDRs could function not only endogenously, but also intercellularly *via* extracellular vesicle trafficking, affecting host biology (Ghosal, et al., 2015). In one such study looking at host-pathogen interactions, the OMV-enriched $tRNA^{Met}$-derived fragment from *Pseudomonas aeruginosa* was indeed found to downregulate cytokine secretion in human primary airway epithelial cells (Koeppen, et al., 2016).

One of the main challenges in studying tDRs in bacteria is the absence of experimental tools for assessing *in vivo* functionality. The inability to use knock-out approaches for tDR precursors (genuine tRNAs) and the inefficiency of introducing synthetic RNA fragments into bacterial cells (*e.g. via* electroporation) (Volkov et al., 2018) impose limits on the comprehensive *in vivo* characterization of bacterial tDRs. To address these limitations, we established the application of a novel self-cleaving ribozyme approach to express tDRs in *E. coli* (Fig. 1).

Twister ribozymes (TwR) are a class of catalytic RNA molecules that can catalyze self-cleavage (Roth, et al., 2014). They consist of three

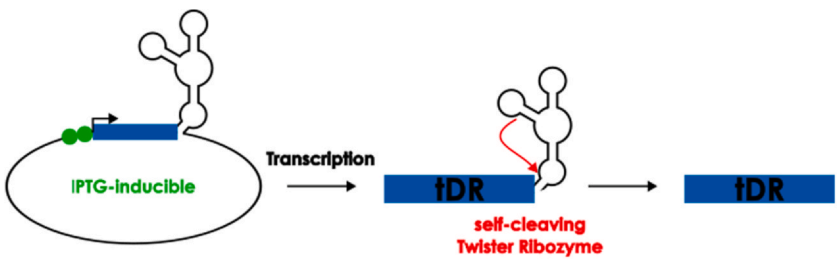


**Fig. 1 Schematic representation of the developed method for the inducible expression of tRNA-derived RNAs in *E. coli*.** A plasmid construct containing two lac operators (green dots) followed by the desired tRNA-derived RNA (tDR) sequence, a modified self-cleaving Twister ribozyme, and a transcription terminator. Upon addition of IPTG and subsequent transcription of the construct, the modified Twister ribozyme undergoes self-cleavage (red arrow), producing the tDR with solely two extra nucleotides at its 3′ end.

essential stems (P1, P2, and P4) and can contain up to three additional stems (P0, P3, and P5). TwRs can be classified into three groups named type P1, P3 or P5 (corresponding to stems P1, P3, P5) (Gebetsberger & Micura, 2017). The Sva1–1 TwR belongs to the type P1 and contains an additional P0 stem. It was identified in the genome of *Subdoligranulum variable,* a Gram-negative bacterium. Since the cleavage efficiency of Sva1–1 was shown to be only slightly affected when removing the P0-stem, in this study, we worked with Sva1–1 lacking P0 (Felletti, Bieber, & Hartig, 2017).

Here we present a novel method to express tDRs in bacteria by fusing the Sva1–1 Twister ribozyme downstream of a tDR sequence to inducibly express tRNA halves with genuine 3′ ends. We showed that the minimum length requisite for the TwR generates tDRs with only 2 extra nucleotides added and validated the *in vivo* application of this method by expressing not only an *E. coli* tDR (tDR-1:35-Trp-CCA-1) but also the well-characterized *T. brucei* TbtRNA$^{Thr}$-3′-half(AGU) (tDR-37:73-Thr-AGU-1) (for tDR nomenclature we follow Holmes, et al., 2023). This method offers a valuable tool for studying the functional role not only of tDRs but any sRNA in bacteria.

## 2. Materials

### 2.1 Bacterial growth

Bacterial cultures were incubated in LB media supplemented with antibiotic, at 37 °C shaking 220 rpm, under standard conditions. LB media was prepared with the following compounds: 1 % (w/v) tryptone, 0.5 % (w/v) yeast extract, 170 mM NaCl. Antibiotic used: 1000X Kanamycin stock solution (25 μg/mL). For induction of desired constructs 1 M IPTG stock solution was used which resulted in 1 mM IPTG final concentration.

### 2.2 General cloning

- LB media
- 1000X Kanamycin stock solution
- Plasmid pBbE6k containing the tRNA$^{Trp}$-5′-half sequence
- Gradient Thermal Cycler
- Wizard® *Plus* SV Minipreps DNA Purification System
- Phusion® High-Fidelity DNA Polymerase (NEB), 10 μL 5X Phusion buffer (NEB)

- 10 μM Primers (forward, reverse)
- 10 mM dNTPs
- *Dpn*I (2000 unit/mL, NEB)
- XL1-Blue *Escherichia coli* chemically competent, 50 μL aliquots
- MG1655 *Escherichia coli* chemically competent, 50 μL aliquots
- Shaking incubator
- Water bath

## 2.3 RNA extraction

- Acidic phenol (pH 4.0) + 1 % SDS
- 10X TEN (100 mM TRIS/HCl pH 7.5, 10 mM EDTA pH 8.0, 1 M NaCl)
- ROTI® Aqua-Phenol
- Chloroform
- 3 M NaOAc, pH 5.5
- EtOH

## 2.4 Northern blot analysis

- 12 % polyacrylamide gel solution containing 7 M Urea (12 % Acrylamide/Bis-acrylamide, 1X TBE, 7 M Urea, 8 μL/mL 10 % APS, 0.57 μL/mL TEMED)
- 10X TBE (890 mM Tris, 890 mM boric acid, 20 mM EDTA)
- EtBr stock solution (10 mg/mL)
- 0.5x TBE + EtBr (0.5X TBE, 1 μg/mL EtBr)
- 2x Thick Whatman paper, Hybond Nylon H+
- 2X RNA loading dye (95 % (v/v) formamide 0.025 % (w/v) bromophenol blue, 0.025 % (w/v) xylene cyanol)
- $\gamma$–$^{32}$P-ATP (10 mCi/mL)
- 10X PNK buffer, T4 PNK (10′000 U/mL)
- Hybridization buffer (178 mM $Na_2HPO_4$ pH 6.2, 822 mM $NaH_2PO_4$, 7 % SDS)
- Washing buffer 1 (2X SSC, 0.1 % SDS)
- Washing buffer 2 (0.1X SSC, 0.1 % SDS)

## 2.5 Growth competition assay

- Wizard® Plus SV Minipreps DNA Purification System
- Phusion® High-Fidelity DNA Polymerase (NEB), 5X Phusion buffer (NEB)
- 10 μM Primers (CE024, MC55)
- 10 mM dNTPs

- 1X TBE
- 6X DNA loading dye (60 % (v/v) glycerol, 0.03 % (w/v) bromophenol blue, 0.03 % (w/v) xylene cyanol, 60 mM EDTA, 10 mM Tris pH 7.6)
- 10 % Polyacrylamide gel (10 % Acrylamide/Bis-acrylamide, 1X TBE, 8 μL/mL 10 % APS, 0.57 μL/mL TEMED)

### 2.6 SDS-PAGE preparation

- 12 % separating gel solution (375 mM TRIS/HCl pH 8.8, 0.1 % (w/v) SDS, 12 % Acrylamide/Bis-acrylamide, 5.5 μL/mL 10 % APS, 1.5 μL/mL TEMED)
- 4 % stacking gel solution (125 mM TRIS/HCl pH 6.8, 0.1 % (w/v) SDS, 4 % Acrylamide/Bis-acrylamide, 3 μL/mL 10 % APS, 1.5 μL/mL TEMED)
- Isopropanol

### 2.7 Metabolic labeling after starvation with MOPS

- $^{35}$S-methionine (10 mCi/mL)
- Vacuum filtration pump and filter
- 1X MOPS (10X MOPS, 1.32 mM $K_2HPO_4$, 9.52 mM $NH_4Cl$)
- 4x Laemmli buffer (400 mM TRIS/HCl pH 6.8, 40 % Glycerol, 8 % SDS, 0.04 % bromophenol blue, 20 % (v/v) β-mercaptoethanol)
- Pierce™ Universal Nuclease for Cell Lysis
- 12 % SDS-PAGE
- Colloidal Coomassie (2 % phosphoric acid, 756.6 mM $(NH_4)_2SO_4$, 1.2 mM Coomassie G250)
- 100 % methanol

## 3. Methods

### 3.1 Overview

One of the main hurdles for studying tDRs is the impossibility to apply classical knockout approaches, since their precursors are genuine tRNAs. In bacteria, expression of sRNA molecules with defined 3′ ends is also challenging as transcription termination requires a ~30 nucleotide long stem-loop structure thus generating transcripts containing 3′ end extensions, sometimes larger than the desired sRNA. Moreover, the introduction of synthetic RNA fragments (*e.g. via* electroporation) has proven to be very inefficient in bacteria (Volkov et al., 2018). Therefore, we developed a method (Fig. 1) to inducibly express tDRs in *E. coli*, generating genuine 3′ ends. Firstly, we generated a construct consisting of two lac operators

and the desired tDR sequence, immediately followed by a modified self-cleaving TwR and a terminator. Upon addition of IPTG and subsequent transcription of the construct, the modified TwR undergoes self-cleavage, producing the tDR.

In the first variant utilizing the originally described TwR, 5 nucleotides from the TwR's 5′ end were appended to the tDR's 3′ end. To minimize the number of nucleotides added without significantly affecting the efficiency of the TwR self-cleavage, three additional constructs were generated, each carrying plasmids with mutations in the TwR aimed at adding either 0, 1, or 2 nucleotides to the tDR's 3′ end. Northern blot analysis (Fig. 2A) illustrates variations in the self-cleavage efficiency among the four constructs. Quantification (Fig. 2B) revealed that the + 2 nucleotides variant was only 1.32 times less efficiently processed than the original + 5 nucleotides variant. The addition of only two extra residues represents a significant advantage compared to the typically utilized transcription terminator helix-based approaches, which add approximately 26–30 nucleotides to the 3′ ends of transcripts (Roberts, 2019). Given the closer resemblance to the endogenous tDR with the 2 nucleotides addition, this variant was adopted as the standard in subsequent experiments.

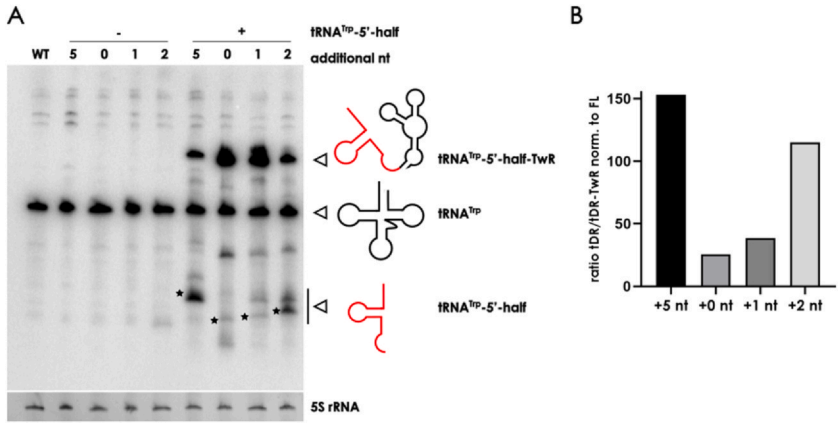


**Fig. 2 Analysis of self-cleavage efficiency of different Twister ribozyme variants.** (A) Northern blot analysis of total RNA extracted from cells expressing plasmid constructs containing different Twister ribozyme mutants. The Twister ribozyme mutants are designed to either add 2, 1 or 0 nucleotides to the 3′ end of the tDR after self-cleavage (asterisks). Signals for the induced, but uncleaved precursor transcript (tRNA$^{Trp}$-5′-half-TwR), and the endogenous tRNA$^{Trp}$ are indicated as well. 5S rRNA served as loading control. (B) Analysis of the northern blot results in (A). The efficiency of self-cleavage for each construct was normalized to the full-length tRNA content. The + 5 nucleotides variant served as the reference.

### 3.1.1 General cloning

The design and protocol follow the principles of one-step cloning PCR (Qi & Scholthof, 2008). One-step cloning PCR allows sequence insertions, deletions and substitutions using only a plasmid and a set of overlapping primers in a single PCR reaction. The vector backbone pBbE6k (Lee, et al., 2011) already harbors the $tRNA^{Trp}$-5′-half(CCA), hence the primer pair used to clone the TwR sequence anneal both to the tDR and to the pBbE6k backbone. Following the PCR reaction, the TwR sequence was inserted between the tDR's 3′ end and the plasmid transcription terminator sequence.

1. The Master mix contained 15 ng of vector (pBbE6k-TrpT-tDR), 1.5 μL 10 μM forward primer (AM037), 1.5 μL 10 μM reverse primer (AM038), 1.8 μL 10 μM dNTPs, 1 μL Phusion® High-Fidelity DNA Polymerase, 10 μL 5X Phusion buffer, $mqH_2O$ to 60 μL.
2. The Master mix was split into six tubes containing 10 μL each. All tubes corresponded to a PCR reaction carried out at a different annealing temperature.
3. Gradient thermal cycling was performed with the following settings: initial denaturation: 98 °C for 5 min; 30 cycles: denaturing 98 °C 30 s, annealing gradient 48 °C, 50 °C, 52 °C, 54 °C, 56 °C, 58 °C, extension 72 °C for 15 s; final extension 72 °C for 5 min
4. To degrade the template plasmid, 1 μL *Dpn*I was added to the pooled PCR reactions and incubated at 37 °C for 2 h and then purified using the Wizard® *Plus* SV Minipreps Kit.
5. Bacterial transformation was performed as follows: 15 μL of *Dpn*I treated PCR reaction was added to 50 μL XL 1-Blue chemically competent cells and incubated for 30 min on ice.
6. The bacteria-PCR sample was heat shocked at 42 °C for 45 s in a water bath, then placed on ice for 2 min.
7. 300 μL LB media without antibiotics was added and incubated at 37 °C, 220 rpm, for 1 h. The bacterial suspension was plated on LB agar plates containing Kanamycin and incubated at 37 °C, 220 rpm, overnight.
8. Individual colonies were picked with a sterile loop and inoculated in 5 mL LB + Kanamycin media, then incubated overnight at 37 °C, 220 rpm.
9. The cultures were pelleted by centrifugation (10′000 × g, 2 min) and plasmids purified using the Wizard® *Plus* SV Minipreps Kit. Correct insertion was confirmed by sequencing and plasmids were stored at −20 °C.

10. After validation of the construct, the plasmid was transformed into wild type MG1655 *E. coli* cells for further experiments. Approximately 10 ng of plasmid was transformed into chemically competent MG1655 cells according to the aforementioned protocol.

Subsequent cloning steps, such as nucleotide deletions or the exchange of the tDR sequence, were performed using one-step PCR cloning, but under slightly different conditions. Each PCR reaction had a total volume of 50 μL and contained the following components: 0.5 μL plasmid, 2.5 μL 10 μM forward primer, 2.5 μL 10 μM reverse primer, 1 μL 10 μM dNTPs, 0.5 μL Phusion® High-Fidelity DNA Polymerase, 10 μL 5X Phusion buffer, 33 μL $mqH_2O$. The annealing temperature was calculated according to NEB guidelines, and 25 cycles were performed.

Primers used for cloning are shown in Table 1.

### 3.1.2 RNA extraction

In the following protocol, all centrifugation steps were performed in an Eppendorf tabletop centrifuge at 4 °C and full speed.

1. 4 mL overnight cultures in LB + Kanamycin media were prepared the day before.
2. The overnight cultures were diluted 1:100 in 15 mL fresh LB + Kanamycin media.
3. The cultures were incubated at 37 °C, 220 rpm, until $OD_{600} \sim 0.2$ was reached.
4. 15 μL 1 M IPTG was added to induce expression of the desired tDR-TwR construct.
5. 2 mL samples of the culture were taken at desired time points, pelleted by centrifugation for 2 min, and the supernatant was discarded. From this step on, all work was done on ice.
6. In a new tube, 500 μL of acidic phenol solution containing 1 % SDS was heated to 67 °C.
7. The pellets were resuspended in 500 μL of cold 1X TEN, added to the hot phenol-SDS mixture, and incubated at 67 °C for 5 min.
8. The samples were centrifuged (10 min), and 450 μL of the aqueous phases were transferred into new tubes. 450 μL ROTI® Aqua-Phenol was added and samples vortexed for 1 min.
9. The samples were centrifuged (10 min), and 400 μL of the aqueous phases were transferred into new tubes. 400 μL of chloroform was added and samples were vortexed for 1 min.

**Table 1** Oligonucleotides used in this study.

| Name | Sequence 5′ → 3′ | Construct |
|---|---|---|
| CE001 | CGGTCTCCAATGCAGCCA GGGCGTAG | Fw TrpT-tDR + 0 nt |
| CE002 | CTGCATTGGAGACCGGT GCTCTACC | Rv TrpT-tDR + 0 nt |
| CE013 | CGGTCTCCTAATGCAGC CAGGGCGTAGC | Fw TrpT-tDR + 1 nt |
| CE014 | TGCATTAGGAGACCGGTG CTCTACC | Rv TrpT-tDR + 1 nt |
| CE015 | CGGTCTCCTTAATGCAGC CAGGGCGT | Fw TrpT-tDR + 2 nt |
| CE016 | GCATTAAGGAGACCGGTG CTCTACCA | Rv TrpT-tDR + 2 nt |
| CE038 | AGTGGCCTAATGCAGCCA GGGCGTAG | Fw Thr-tDR + 0 nt |
| CE039 | CTGCATTAGGCCACT GGGGGGATC | Rv Thr-tDR + 0 nt |
| CE024 | TTACCGCCTTTGAGT GAGCT | Rv Comp. assay |
| MC55 | GCGTCCGGCGTAGAGG | Fw Comp. assay |
| AM037 | TGCCGGTCCCAAGCCCGG AATGATGCAGAGGGAGGT CAAATAAAACGAAAGGCT CAGTCG | Fw Sva1-1 |
| AM038 | GCTTGGGACCGGCAGG GCTACGCCCTGGCTGCAT TAAGGCGGAGACCGGTGC TCTACCAA | Rv Sva1-1 |
| AM022 | GTGCTCTACCAATTGAAC TACGCC | NB TrpT probe |
| Thr10 | ATCGAACCCCCGACCTCC GTCTT | NB Thr probe |

10. The samples were centrifuged (10 min) and 350 μL of the aqueous phases were transferred into new tubes. To precipitate RNA, 35 μL 3 M NaOAc and 875 μL 100 % EtOH (4 °C) were added. RNA was precipitated for at least 1 h at −80 °C. Afterwards, the samples were centrifuged (40 min) and the supernatants were removed. The samples were washed with 700 μL of 70 % EtOH (4 °C), the supernatant was removed, and the pellets air-dried.
11. RNA was resuspended in mq$H_2O$ and stored at −80 °C.

### 3.1.3 Northern blot analysis

1. 5 μg of RNA were loaded onto a 20 × 20 cm 12 % polyacrylamide gel containing Urea and run with 1X TBE for 2-3 h at 250 V constant.
2. Before blotting, the gel was stained with EtBr and imaged under UV light.
3. A blotting sandwich was constructed consisting of one piece of thick Whatman paper on the bottom, a nylon membrane (Hybond N + ) on top of it, the gel on top of the nylon membrane, and one piece of thick Whatman paper on top. The nylon membrane and the Whatman papers were soaked beforehand in 0.5X TBE.
4. Transfer was done for 45 min at 400 mA, 10-15 V, using a semi-dry blotter.
5. The membrane was cross-linked under UV-light (120 mJ, 50 s) after blotting.
6. A $^{32}$P 5′-end labeled oligonucleotide was generated for probing the blot. Each oligonucleotide labeling reaction consisted of: 6 μL mq$H_2O$, 1.2 μL oligonucleotide (10 μM), 1 μL 10X PNK buffer, 0.8 μL T4 PNK (10′000 U/mL), 1 μL $\gamma$-$^{32}$P-ATP. The reaction was incubated for 30 min at 37 °C.
7. While labeling the oligo, the membrane was added to a hybridization bottle with 20-30 mL hybridization buffer and incubated for at least 15 min at 42 °C in a hybridization oven. The radiolabeled oligonucleotide was heated for 1 min at 95 °C, added to the bottle containing the membrane and incubated over night at 42 °C.
8. The hybridization buffer was discarded, and the membrane was washed twice for 10 min at room temperature, once with washing buffer 1 and once with washing buffer 2. Then, the membrane was rinsed with water and air-dried.
9. The membrane was wrapped in saran wrap and exposed to a phosphorimager screen.

10. The screen was scanned with a phosphorimager after the desired exposure time.

Oligonucleotides used for labeling are shown in Table 1.

## 3.2 Methods results

### 3.2.1 Growth curve analysis

1. 4 mL overnight cultures in LB + Kanamycin media were prepared the day before.
2. The overnight cultures were diluted 1:100 in 15 mL fresh LB + Kanamycin media.
3. The cultures were incubated at 37 °C, 220 rpm, until $OD_{600}$ ~ 0.2 was reached.
4. The cultures were re-diluted to $OD_{600}$ = 0.1, and 400 μL per well were plated into a 48-well plate. 0.4 μL 1 M IPTG was added to each well. $OD_{600}$ was measured in a plate reader (in this study used: Tecan M1000) 37 °Cat 220 rpm. To ensure constant expression of tDRs, IPTG was re-added after 2 h and 4 h of growth curve analysis.

Note: For conducting growth curve analysis at 42 °C, cells were pelleted when $OD_{600}$ ~ 0.2 was reached and subjected to heat shock with prewarmed LB media (42 °C). The settings in the Tecan plate reader were adjusted to 42 °C.

### 3.2.2 Growth competition assay

1. 4 mL overnight cultures in LB + Kanamycin media were prepared the day before.
2. The overnight cultures were diluted 1:100 in 30 mL fresh LB + Kanamycin media.
3. The cultures were incubated at 37 °C, 220 rpm, until $OD_{600}$ ~ 0.3 was reached.
4. The control ribozyme culture and the culture expressing the desired tDR were mixed in a 1:1 ratio, achieving a total volume of 30 mL with a total $OD_{600}$ of 0.3
5. 15 mL of the starting culture was taken as the 0 h sample. 15 μL of 1 M IPTG was added to the starting culture and incubated for 24 h at 37 °C, 220 rpm.
6. The 0 h sample was pelleted by centrifugation (10′000 × g, 2 min) and the plasmids purified using the Wizard® *Plus* SV Miniprep Kit.
7. After 24 h, a 5 mL sample of the culture was taken and processed as in step 6. Additionally, the culture was diluted 1:100 in 15 mL fresh media

and 15 μL of 1 M IPTG was added. IPTG was re-added after 5 h of incubation. This step was repeated for as many days as desired.

8. A PCR of the purified plasmids from all time points was performed using the forward primer MC55 and reverse primer CE024 (Table 1). PCR reactions were performed using the Phusion DNA polymerase in a 50 μL total volume consisting of: 32.5 μL mq$H_2O$, 10 μL 5X Phusion buffer, 1 μL 10 mM dNTPs, 2.5 μL 10 μM MC55, 2.5 μL 10 μM CE024, 1.0 μL plasmid (10 ng/μL), 0.5 μL Phusion DNA polymerase.
9. 10 μL of the PCR reactions were loaded onto a 10 % polyacrylamide gel (without Urea) and run with 1x TBE at 180 V for 3-4 h. The PCR products were visualized using EtBr staining.

### 3.2.3 Preparation of SDS-PAGE gels

1. 5 mL of 12 % separating gel solution and 2.5 mL 4 % stacking solution were prepared for one gel. 10 % APS and TEMED were added directly before pouring the gel.
2. The gel chamber was assembled, and the separating gel solution was poured into the chamber. The gel was overlayed with approximately 300 μL of 100 % isopropanol and allowed to polymerize.
3. After polymerization, the isopropanol was removed, and the stacking gel mixture was poured on top of the polymerized separating gel. The comb was carefully introduced, and the stacking gel was allowed to polymerize.

### 3.2.4 Metabolic labeling after starvation with MOPS

1. 4 mL overnight cultures in LB + Kanamycin media were prepared the day before.
2. The overnight cultures were diluted 1:100 in 40 mL of fresh LB + Kanamycin media.
3. The cultures were incubated at 37 °C, 220 rpm, until $OD_{600}$ ~ 0.2 was reached, and 40 μL of 1 M IPTG was added. The cultures were incubated at 37 °C, 220 rpm, until $OD_{600}$~ 0.5–0.6 was reached (approximately 1 h, total 2 h).
4. The cultures were filtered with a vacuum filtration pump and the filtered bacteria were resuspended in 40 mL MOPS without glucose. A 1 mL sample was taken, pelleted, and resuspend in 1 mL LB + Kanamycin media. 1 μL of $^{35}$S-methionine was added and incubated at 37 °C, 220 rpm for 10 min. The unstressed, filtered sample was pelleted and resuspended in 1X Laemmli buffer (approx. 50 μL per 0.5 $OD_{600}$).

The sample was heated for 3 min at 95 °C and stored at −20 °C. The bacterial MOPS cultures were incubated for 30 min, 220 rpm, at 37 °C.

5. After 30 min, the cultures were centrifugated (4000 × g, 3 min, RT) and resuspended in 40 mL LB + Kanamycin media. The culture was split into two Erlenmeyer flasks, one to measure $OD_{600}$ to determine the amount of 1X Laemmli buffer needed for each sample. To the second one, 1 μL of $^{35}$S-methionine per mL culture was added to monitor translation recovery.
6. 1 mL samples were taken at chosen time points, pelleted, and resuspended in 1X Laemmli buffer as in step 4. Additionally, $OD_{600}$ was measured to determine the amount of 1X Laemmli buffer needed for each sample.
7. 1 μL of Pierce™ Universal Nuclease for Cell Lysis was added to all samples and incubated for 5 min at room temperature. The nuclease should reduce sample viscosity due to genomic DNA.
8. 10 μL of sample was loaded per lane onto a 12 % SDS-PAGE gel. The gel was run at 150 V constant for 1 h. After gel disassembly, it was stained with Colloidal Coomassie overnight. For that, 45 mL Colloidal Coomassie solution was mixed with 15 mL 100 % methanol.
9. The staining solution was discarded, the gel rinsed with $mqH_2O$, and vacuum dried for 1 h 15 min at 65 °C. Before drying, an image of the stained gel was captured for use as a loading control. The dried gel was wrapped in plastic wrap, exposed to a phosphorimager screen, and scanned with a phosphorimager.

## 4. Results and concluding remarks

Our previous analysis showed the selective enrichment of $tRNA^{Trp}$-5′-half during bacterial growth in stationary phase (Raad, Luidalepp, Fasnacht, & Polacek, 2021). To investigate the possible role of the tDR in cellular fitness, we used a series of growth and competition assays (Fig. 3A–E). As a control, we used bacteria solely expressing the TwR, to exclude possible inferences in our assays. Growth analysis under standard conditions (LB media, 37 °C) showed no difference between *E. coli* cells overexpressing $tRNA^{Trp}$-5′-half and control cells that expressed only the TwR (Fig. 3A). Nevertheless, in a growth competition assay (Fig. 3B/C), we observed that after 4 days of analysis cells expressing the $tRNA^{Trp}$-5′-half

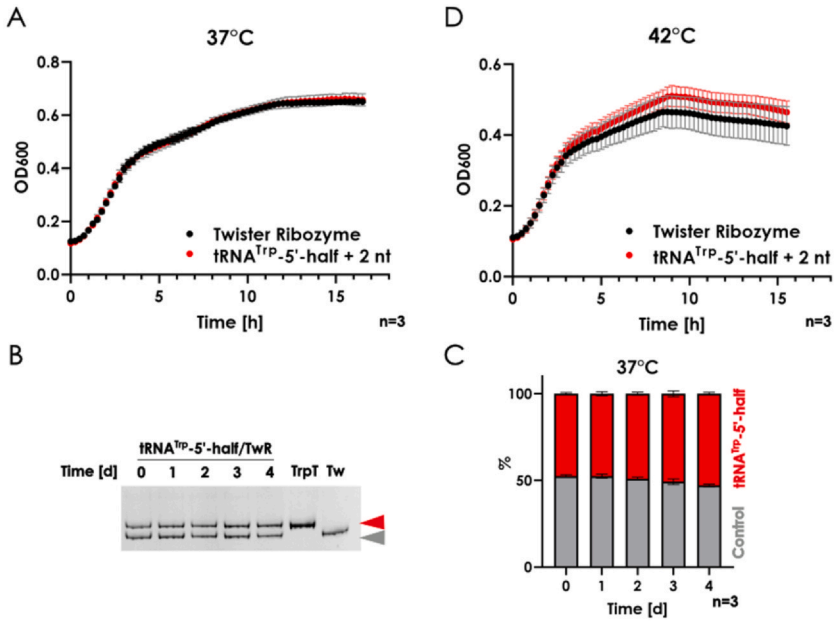


**Fig. 3 Growth and competition assays of *E. coli* expressing tRNA$^{Trp}$-5′-half under standard and heat stress conditions**. (A) Growth curves of *E. coli* at 37 °C. Cells inducible expressing tRNA$^{Trp}$-5′-half (red) were compared to a control cell line expressing only the Twister ribozyme (black). Experiment was performed in triplicates and standard deviation is shown. (B) Competition assay between cells expressing the tRNA$^{Trp}$-5′-half and the Twister ribozyme at 37 °C. The relative abundance of each cell line was monitored over 4 days and was assessed *via* PCR on a polyacrylamide gel. The arrows indicate the PCR products for the tRNA$^{Trp}$-5′-half plasmid (red) and for the Twister ribozyme plasmid (gray). The experiment was performed in triplicates. (C) Quantification of the competition assay performed at 37 °C by comparing the band intensity of tRNA$^{Trp}$-5′-half product and Twister ribozyme product. (D) Growth curves of *E. coli* at 42 °C. Cells inducibly expressing tRNA$^{Trp}$-5′-half (red) were compared to a control cell line expressing only the Twister ribozyme (black). Experiment was performed in triplicates and standard deviation is shown.

started overgrowing control cells. This data suggests that the expression of tRNA$^{Trp}$-5′-half may provide a competitive advantage under standard conditions. Growth curves (Fig. 3D) conducted under heat stress (LB media, 42 °C) revealed a modest growth advantage for tRNA$^{Trp}$-5′-half over-expressing cells starting at 4 h of induction, as cells start entering stationary phase. Overall, these results suggested that ectopic expression of the tRNA$^{Trp}$-5′-half provides a competitive edge under standard conditions and during the initial adaptation phase of heat stress.

Our newly developed method allowed us to express endogenous tDRs in *E. coli*. Recent publications have shown that tDRs can also be secreted in extracellular vesicles, reaching other cells, and acting in intercellular communication. To study this aspect of tDR functionality we tested whether our method also allows the expression of tDRs from foreign species in *E. coli*. For this purpose, we made use of the well-characterized TbtRNA$^{Thr}$-3′-half. This tDR is known to enhance mitochondrial translation in *T. brucei* during starvation recovery (Brogli, et al., 2023; Fricker, et al., 2019), and was shown to stimulate translation *in vivo* in *S. cerevisiae* and *H. volcanii*, as well as *in vitro* in HeLa cell extracts (Fricker, et al., 2019). Fig. 4A shows that we were able to express the TbtRNA$^{Thr}$-3′-half with our TwR system efficiently in *E. coli*. Notably, we successfully generated a version of the TbtRNA$^{Thr}$-3′-half-TwR plasmid, which did not add any extra nucleotides to the TbtRNA$^{Thr}$-3′-half 3′ end and still allowed efficient self-cleavage. Next, we analyzed if the TbtRNA$^{Thr}$-3′-half can stimulate translation *in vivo* also in *E. coli*. For this purpose, we performed metabolic labeling of cells expressing the TbtRNA$^{Thr}$-3′-half during recovery from glucose starvation (Fig. 4B). Interestingly, and as observed in the other species analyzed, cells expressing the TbtRNA$^{Thr}$-3′-half were able to translate more efficiently during stress recovery. All these data showed that our method can be used not only for the expression and study of endogenous *E. coli* tDRs but also for heterologous tDRs. Furthermore, we could show that TbtRNA$^{Thr}$-3′-half does not only stimulate translation in *T. brucei*, *S. cerevisiae,* and *H. volcanii in vivo* (Fricker, et al., 2019), but also in *E. coli*, suggesting a highly conserved mechanism of action.

## 5. Limitations of the method

Overall, we were able to show the *in vivo* application of our newly developed method of expressing endogenous and exogenous tDRs in *E. coli*. Our attempts to extend the capabilities of the methods to other organisms were, unfortunately, unsuccessful. Neither the expression of an *E. coli* tDR in *S. cerevisiae* and *T. brucei* nor of the tRNA$^{Thr}$-3′-half in *T. brucei* (data not shown) could be achieved. However, with more dedicated optimizations it is possible that our method can as well be applied in other organisms.

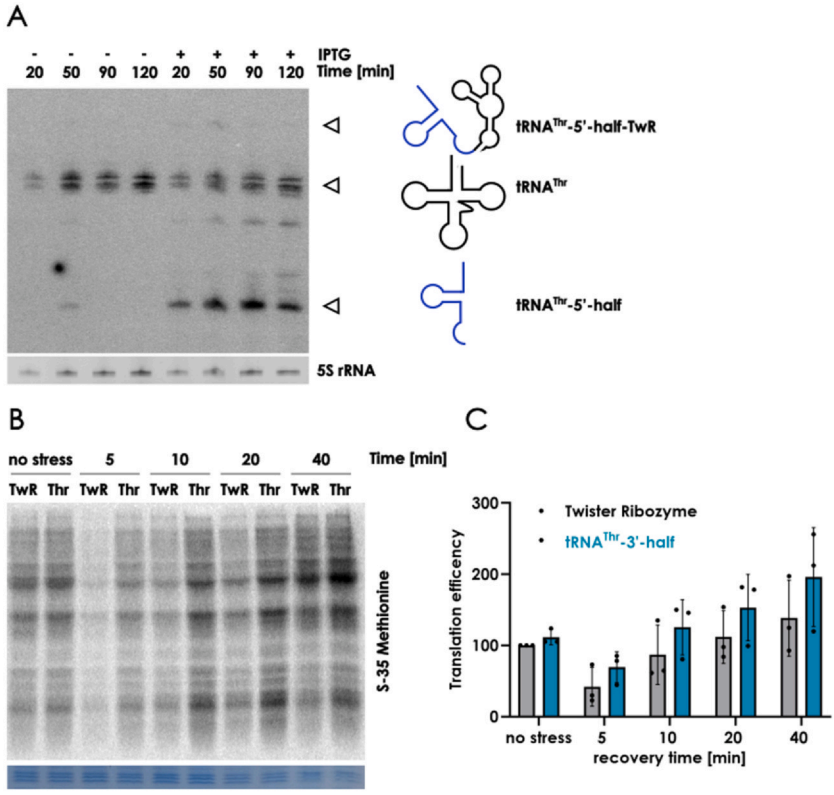


**Fig. 4 Efficient expression of exogenous *T. brucei* tRNA$^{Thr}$-3′-half in *E. coli* and its role in enhancing translation after glucose starvation**. (A) Northern blot analysis of total RNA extracted from cells expressing exogenous TbtRNA$^{Thr}$-3′-half using the developed Twister ribozyme system in *E. coli*. 5S rRNA served as loading control. (B, C) Metabolic labeling experiment with $S^{35}$-methionine, and its quantification, in cells expressing the TbtRNA$^{Thr}$-3′-half (Thr) or solely the Twister ribozyme (TwR). The recovery time after starvation is shown in minutes (min). The experiment was performed in triplicates.

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