# Dual expression of CCA-adding enzyme and RNase T in *Escherichia coli* generates a distinct *cca* growth phenotype with diverse applications

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## ABSTRACT

**Correct synthesis and maintenance of functional tRNA 3′-CCA-ends is a crucial prerequisite for aminoacylation and must be achieved by the phylogenetically diverse group of tRNA nucleotidyltransferases. While numerous reports on the *in vitro* characterization exist, robust analysis under *in vivo* conditions is lacking. Here, we utilize *Escherichia coli* RNase T, a tRNA-processing enzyme responsible for the tRNA-CCA-end turnover, to generate an *in vivo* system for the evaluation of A-adding activity. Expression of RNase T results in a prominent growth phenotype that renders the presence of a CCA- or A-adding enzyme essential for cell survival in an *E. coli* ∆*cca* background. The distinct growth fitness allows for both complementation and selection of enzyme variants in a natural environment. We demonstrate the potential of our system via detection of altered catalytic efficiency and temperature sensitivity. Furthermore, we select functional enzyme variants out of a sequence pool carrying a randomized codon for a highly conserved position essential for catalysis. The presented *E. coli*-based approach opens up a wide field of future studies including the investigation of tRNA nucleotidyltransferases from all domains of life and the biological relevance of *in vitro* data concerning their functionality and mode of operation.**

## INTRODUCTION

Due to their essential role in translation, the correct synthesis and maintenance of functional tRNA is indispensable for cell survival. tRNAs are transcribed as precursor molecules that undergo a set of processing events, including the removal of leader and trailer sequences to generate mature 5′ and 3′ ends (1,2).

At the 3′ site, a functional tRNA end includes the invariant CCA triplet that is either already encoded in the tRNA gene or is posttranscriptionally added by ATP(CTP):tRNA nucleotidyltransferase (CCA-adding enzyme; 3–5). Using a single binding pocket, these enzymes have a unique mechanism of polymerization without the involvement of a nucleic acid-based template. Yet, the incorporation of both CTP and ATP occurs at high fidelity (6–10). Within the polymerase β superfamily, tRNA nucleotidyltransferases can be divided into class I (archaeal) and class II (eukaryotic and bacterial) enzymes, depending on the mode of action as well as on the structural organization (5,7,9). Both classes share a common signature motif containing two highly conserved carboxylates (DxD or DxE; 11,12).

Besides the almost error-free CCA-addition, tRNA nucleotidyltransferases show additional surprising features. In a considerable number of bacterial organisms, enzymes with partial activities collaborate to synthesize CCA-ends. One enzyme incorporates the first two CMP residues but fails to add the terminal adenosine (CC-adding enzymes), while the second one exclusively catalyzes the addition of the terminal AMP residue (A-adding enzyme; 13–17). Furthermore, CCA-adding enzymes are involved in tRNA quality control, where they catalyze two rounds of CCA addition on defect and structurally instable tRNAs. The resulting CCACCA stretch is recognized as a degradation tag, leading to a rapid elimination of nonfunctional transcripts from the tRNA pool (18–20). A second quality control mechanism is based on polymerization kinetics, as tRNAs carrying a nick in the sugar-phosphate backbone represent quite inefficient substrates for CCA-addition (21).

Due to the need for continuous CCA restoration as well as their contribution to tRNA quality control, CCA-adding enzymes are also of crucial importance in organisms with encoded CCA-ends (22–24). In *Escherichia coli*, all tRNA genes encode for a CCA sequence (25), yet the disruption of the *cca* gene leads to a mild growth phenotype due to the depleted repair activity of damaged tRNA 3′ ends (25,26). Such a damage can be caused by spontaneous or enzymatic hydrolysis. In *E. coli*, the prominent hydrolytic activ-

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ity against CCA-ends is RNase T, a single-strand specific 3′ to 5′ exoribonuclease involved in both the final steps of short 3′ trailer trimming and tRNA end turnover/quality control (27–30,19,24).

Because of the astonishing functional and evolutionary diversity of tRNA nucleotidyltransferases, it is of great interest to search for novel ways to compare and characterize these enzymes, preferentially under conditions that are close to their native cellular environment. So far, tRNA nucleotidyltransferases have been *in vivo* complemented in an *E. coli*-based approach that is dependent on a multi-layered reporter system involving an introduced suppressor tRNA gene and a chromosomal *lacZ* reporter gene containing the cognate amber-codon for suppression (14,31,32). tRNA nucleotidyltransferases may also be investigated in a temperature-sensitive yeast mutant. However, a background deprived of endogenous CCA-adding enzyme can only be introduced upon incubation at the non-permissive temperature (33,34).

Here, we present a straight-forward *E. coli*-based system that enables the characterization of a broad range of tRNA nucleotidyltransferases under *in vivo* conditions. Utilizing the well-characterized RNase T activity against the 3′ terminal AMP residue of *E. coli* tRNAs, we demonstrate that recombinant RNase T expression prevents *E. coli* growth in the absence of an A-adding activity. Furthermore, we show that the system is applicable for enzymes from all domains of life, including the evaluation of catalytic efficiency, fidelity, temperature sensitivity and protein evolution.

## MATERIALS AND METHODS

### Cloning of vector constructs

The pET28a(+) vector for RNase T overexpression has been previously described (19). In the pETDuet-1 constructs, the coding region of RNase T was inserted between the EcoRV and AvrII restriction sites (in MCS-2) of pETDuet-1 (Novagen). The autonomous expression platform for *cca* was generated via PCR including the native *cca* promotor and flanking restriction sites for EcoRI and NotI. The initial module comprising the coding region of *E. coli* CCA-adding enzyme was introduced in place of MCS-1 via megaprimer mutagenesis using HiFi polymerase (PCR Biosystems) and cloned into the original vector background between the EcoRI and NotI sites. Coding regions of further tRNA nucleotidyltransferases of interest were also introduced via megaprimer mutagenesis, site-specific mutations were introduced via site-directed mutagenesis. Further information on the constitution of the designated CCA gene module is provided in the supplementary section (Supplementary Figure S1).

### Bacterial strains and knockout generation

The bacterial strains used in this study were *E. coli* BL21(DE3) (Novagen) and JM109(DE3) (Promega). Disruption of the *cca* gene (*cca::cam*, Δ*cca*) was achieved using the Quick & Easy *E. coli* gene deletion kit #K006 (Genebridges). *E. coli* TOP10 or NEB 5-alpha were used for cloning and mutagenesis experiments.

### Media and growth conditions

*Escherichia coli* cells were grown at 37°C in LB supplemented with antibiotics at the following concentrations: kanamycin (kan) 30 μg ml$^{-1}$; chloramphenicol (cam) 34 μg ml$^{-1}$; ampicillin (amp) 100 μg ml$^{-1}$. For the analysis of tRNA nucleotidyltransferase complementation in the dual expression system, transformed cells from a 500 μl suspension were pelleted and 450 μl of the supernatant were withdrawn. Cells were resuspended in the remaining volume and 5 μl were streaked in sectors onto LB agar containing ampicillin. For growth curve measurements, overnight cultures were inoculated in 5 ml fresh LB containing kanamycin and incubated to an OD$_{600}$ of approximately 0.5. Subsequently, a 50 ml culture was inoculated to a final OD$_{600}$ of 0.005. Cells were grown at 37°C with agitation and the optical density was assessed at the indicated time points. To determine RNase T tolerance, cells were plated onto agar gradient plates prepared with two layers of sloped LB-amp agar (containing 0.0025 % 2,3,5-triphenyltetrazolium chloride (TTC) to visualize cell viability (35,36)) where only the bottom layer was supplemented with 0.2 mM IPTG for induction of RNase T expression.

### Preparation of semi-randomized plasmid library and *in vivo* selection

A mutagenic pETDuet-1 vector library with a semi-randomization at the highly conserved position D23 in the catalytic DxD motif (D21x22D23; x = any amino acid) of *E. coli* CCA-adding enzyme was generated via site-directed mutagenesis starting from an inactive A23 variant (DxA) in 20 cycles using HiFi polymerase (PCR Biosystems) and the primer pair 5′-AAGACAGAGNNTGGGTGGTGGTCGGCAG-3′ and 5′-ACCACCCANNCTCTGTCTTTGACCGGTAGCC-3′ (HPLC-grade purity, biomers.net). Following DpnI digest, *E. coli* NEB 5-alpha cells (NEB) were transformed with the DNA sample. An overnight culture was prepared to isolate the plasmid library and to introduce the product mix into *E. coli* JM109(DE3) Δ*cca*. Resulting transformants were plated onto LB agar containing ampicillin and sequences from single colonies were analyzed after an overnight incubation at 37°C.

### Drop plate assay

For comparative growth studies on agar plates, the drop plate method was performed (37). 5 ml LB-amp/cam were inoculated with respective *E. coli* cells and incubated at 30°C for 2 days with agitation. OD$_{600}$ was adjusted to 1.0 and 5 μl per drop of a 10-fold dilution series in LB were plated onto LB-amp agar using a multichannel pipette. Agar plates were incubated for two days at the indicated temperatures.

### Preparation of *in vitro* tRNA substrates

Radioactively labelled yeast tRNA$^{Phe(GAA)}$ substrate was synthesized via *in vitro* transcription in the presence of α-$^{32}$P ATP (Hartmann Analytic) and purified as described (38,39).
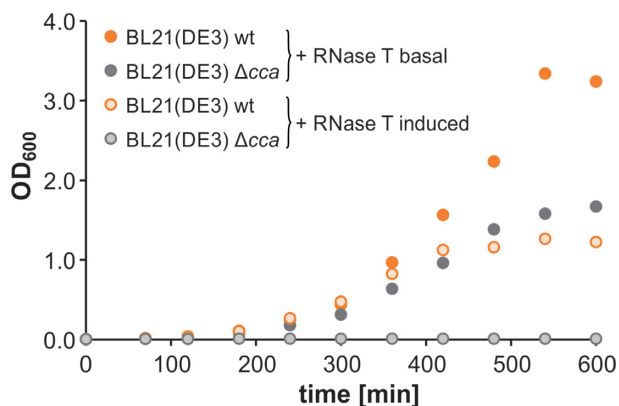
**Figure 1.** Effect of recombinantly expressed RNase T on growth behavior of *E. coli*. Growth was assessed for *E. coli* BL21(DE3) wild type (orange) and Δ*cca* (gray) transformed with pET28a(+)_RNase T at basal (no IPTG) and induced (0.05 mM IPTG) RNase T expression. Growth curves are representative for experiments that were reproducible at least three times.

### *in vitro* nucleotide incorporation assay

For qualitative analysis of crude extract activity, 2 ml of an overnight culture were pelleted. Cells were resuspended in 500 μl 30 mM HEPES/KOH pH 7.6, 30 mM KCl, 6 mM MgCl$_2$, 1 mM DTT and disrupted using a FastPrep-24 homogenizer (MP Biomedicals). The crude extract was cleared from cell debris and kept on ice until further use.

In a total reaction volume of 20 μl, 20 % (v/v) crude extract were incubated at 37°C in 30 mM HEPES/KOH pH 7.6, 30 mM KCl, 6 mM MgCl$_2$, 2 mM DTT in the presence of 1 mM of each NTP and 0.25 μM radioactively labeled yeast tRNA$^{Phe(GAA)}$ or tRNA$^{Phe(GAA)}$-CC, respectively. Prior to addition to the reaction mix, the RNA was heated for 5 min at 65°C and slowly cooled down to room temperature. After 1 h incubation, reactions were ethanol-precipitated, redissolved in 8 μl RNA-loading dye (10 mM Tris–HCl pH 7.6, 80 % formamide, 0.25 % bromophenol blue, 0.25 % xylencyanol) and separated on a 10 % denaturing PAGE. RNA products were visualized on a PhosphorImager (GE Healthcare).

## RESULTS

### Overexpression of RNase T in *E. coli* Δ*cca* impairs growth fitness

tRNA end turnover describes the opposing actions of *E. coli* RNase T and CCA-adding enzyme regarding the removal and re-addition of tRNA 3′-CCA-ends (19,25,26,28,40,41). In the absence of the CCA-adding enzyme, a mild growth defect based on the inability to repair damaged tRNA 3′ ends is observed. However, sporadic revertants can emerge that circumvent this detrimental effect by down-regulating the level of RNase T-mediated hydrolysis (28,42). To further elicit the *cca* knockout phenotype, we introduced a pET28a(+) vector for recombinant expression of RNase T into *E. coli* BL21(DE3) Δ*cca*. The growth curve of the corresponding culture indicated a prominent inhibitory effect on *E. coli* BL21(DE3) Δ*cca* (Figure 1). Even without induction, the basal expression of RNase T resulted in a

clearly visible growth defect. Moreover, RNase T induction by IPTG (0.05 mM) led to complete growth impairment of *E. coli* Δ*cca*, while the corresponding wild type strain was able to compensate for the increased tRNA end turnover to a certain extent.

### Survival under RNase T co-expression requires tRNA 3′ end repair activity

Next, we tested the impact on *E. coli* growth when both RNase T and tRNA nucleotidyltransferase are co-expressed from a single vector construct. The coding region of RNase T under the control of a T7 promotor was inserted into the multiple cloning site 2 (MCS-2) of pETDuet-1, while MCS-1 was replaced by an autonomous expression platform for tRNA nucleotidyltransferases under the control of the native *E. coli cca* promotor (CCA gene module, Figure 2A, see also Supplementary Figure S1). For further analysis, the strain JM109(DE3) was selected, as the Δ*endA1* Δ*recA1* genotype leads to an improved DNA stability and overall plasmid quality. Transformation of *E. coli* JM109(DE3) Δ*cca* with the pETDuet constructs resulted in a selective growth phenotype on agar plates (Figure 2B). In the absence of the two catalytically active carboxylates, *E. coli* CCA-adding enzyme (D21A/D23A, hereinafter referred to as AxA) is unable to repair tRNA 3′ ends which in turn prevented *E. coli* growth. Consistently, growth was not inhibited in a corresponding background of JM109(DE3) wild type, suggesting that the endogenous CCA-adding enzyme sufficiently compensated for the RNase T-mediated tRNA end hydrolysis. Similarly, the single replacement of aspartate at position 23 by alanine (DxA) prevented colony formation. Further, full growth complementation due to an efficient AMP incorporation was obtained by the introduction of the A-adding enzyme from *Bacillus halodurans* (Figure 2B). Intriguingly, the leaky basal expression of RNase T in the uninduced state was sufficient to generate the growth impairment in the absence of A-adding activity. In a corresponding *in vitro* nucleotide incorporation assay, all expected activities could be recovered from the respective crude extract preparations (Figure 2C).

### The dual expression method is suitable as an *in vivo* selection system to recapitulate protein evolution

The generation of a selective growth phenotype in *E. coli* Δ*cca* allows to use the system for *in vivo* selection experiments. We performed a semi-randomization at the highly conserved position D23 in the *E. coli* CCA-adding enzyme. Together with D21, this position is involved in the coordination of the two metal ions essential for catalysis (43). We replaced the original aspartate codon by the partially randomized sequence 'GNN' in the open reading frame for this enzyme, leading to codons for aspartate, glutamate, glycine, valine or alanine, respectively. The randomization was performed on a catalytically inactive variant DxA to exclude selection of residual non-mutagenized original DxD sequences from the starting ORF. This pETDuet-based library was introduced into *E. coli* JM109(DE3) Δ*cca*, and under the RNase T-based selection, all resulting colonies carried vectors with a GAT codon for aspartate at posi-
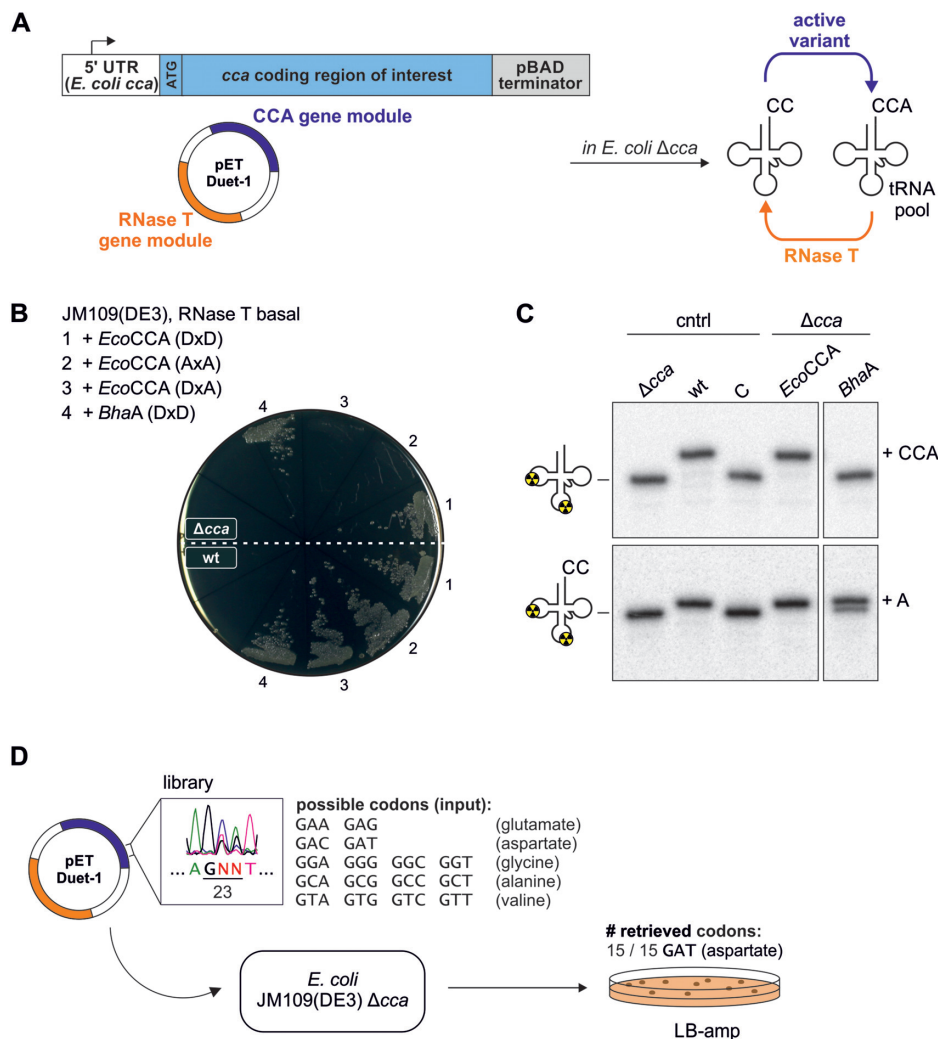
**Figure 2.** Dual expression of RNase T and tRNA nucleotidyltransferase in *E. coli* Δ*cca* enforces selective growth phenotype. (**A**) Schematic presentation of the established *in vivo* system based on the dual expression of RNase T (orange) and tRNA nucleotidyltransferase (blue) from a pETDuet vector construct. In *E. coli*, both activities compete in the turnover of tRNA 3′ ends. (**B**) *in vivo* complementation requires efficient A-adding activity. Streak outs display *E. coli* JM109(DE3) Δ*cca* (top) or wild type (bottom) transformed with the indicated pETDuet construct plated onto LB-amp. *Eco*CCA, *E. coli* CCA-adding enzyme; DxD, wild type enzyme with catalytically active carboxylates; AxA, inactive enzyme variant with catalytically active carboxylates replaced by alanine residues; DxA, inactive enzyme with catalytically active aspartate at position 23 replaced by alanine; *Bha*A, *B. halodurans* A-adding enzyme; Δ*cca*, *cca* gene disruption; wt, wild type. (**C**) Radiolabeled yeast tRNA[Phe(GAA)] (top) or tRNA[Phe(GAA)]-CC (bottom), respectively, was incubated in the presence of 20 % (v/v) crude extract from different *E. coli* JM109(DE3) samples containing the indicated recombinantly expressed enzymes. Reaction products were resolved on a 10 % denaturing PAGE. Δ*cca*, extract prepared from the strain carrying *cca* gene disruption; wt, extract from wild type strain; C, control incubation without extract, *Eco*CCA, extract from *E. coli* expressing recombinant CCA-adding enzyme; *Bha*A, *E. coli* extract expressing *B. halodurans* A-adding enzyme. (**D**) Utilization of dual expression as a selection system. A vector library with a semi-randomized GNN sequence at codon position 23 in the open reading frame for *E. coli* CCA-adding enzyme was generated. Introduction into *E. coli* JM109(DE3) Δ*cca* resulted in colonies carrying vectors with GAT codons.

tion 23, restoring the wild type activity (15 of 15 retrieved codons, Figure 2D, see also Supplementary Figure S2).

### *In vivo* studies on enzyme variants with altered catalytic efficiency

During polymerization, CCA-adding enzymes switch their substrate specificity from CTP to ATP. For class II enzymes, a flexible loop region residing in the catalytic core acts as a lever and readjusts the nucleotide-recognizing amino acids within the nucleotide binding pocket (composition of the catalytic core motifs and the flexible loop element is shown

in Supplementary Figure S3). This rearrangement of the templating residues switches the enzyme's specificity from CTP binding towards ATP (8,13,32,44,45). It was shown that the loop region from a phylogenetically closely related species can be readily exchanged with only minor impact on the enzyme's performance (45). Thus, replacing the loop of the *E. coli* CCA-adding enzyme by the corresponding region of the *Wigglesworthia glossinidia* enzyme (chimera EWEloop) sustained *in vitro* A-adding activity with only a slight reduction in the $k_{cat}$ value by 1.8-fold. In contrast, the replacement of conserved amino acids within the loop, like Y71 (in *E. coli*), by alanine reduced the $k_{cat}$ by 7.5-fold (45).

Based on these facts, we analyzed enzyme versions with different catalytic efficiencies in our *in vivo* approach. We introduced variants of the *E. coli* CCA-adding enzyme with the described alterations in the flexible loop region. On agar plates, both the tested EWEloop chimera as well as the *E. coli* CCA-adding enzyme variant Y71A can complement growth despite their varying catalytic properties (Figure 3A). For a more detailed estimation of the variant's impact on *E. coli* growth efficiency, we utilized the inducible RNase T expression system. The respective transformants were plated on agar containing an IPTG gradient from 0 to 0.2 mM (Figure 3A). The resulting gradient of expressed RNase T led to a gradual increase in the cellular stress level due to the induced tRNA 3′ end hydrolysis. Cells expressing wild type enzyme or EWEloop chimera exhibited a comparable growth behavior with a minimal inhibitory IPTG concentration of ∼0.1 mM. However, expression of the less efficient enzyme variant Y71A led to growth inhibition at ∼0.08 mM IPTG (Figure 3A). These findings are in good agreement with the reported turnover numbers (45) and thus illustrate that catalytic features of various enzyme variants can be easily displayed and compared in our complementation experiments.

As a prerequisite for the remarkable fidelity of CCA addition, the class II enzymes carry a set of highly conserved amino acids EDxxR (x, any residue) in the nucleotide binding pocket (Motif D, Supplementary Figure S3) that form Watson-Crick-like hydrogen bonds to the base moieties of CTP and ATP, respectively (8). The main interaction partner determining nucleotide specificity is the arginine residue, and a replacement by alanine leads to the incorporation of any of the four nucleotides (46,47). Yet, *in vitro* data show that the *E. coli* enzyme carrying the corresponding R134A alteration in the nucleotide binding pocket is able to synthesize proper CCA-ends, as only a tRNA primer carrying correctly added residues is further elongated by the enzyme, while primers ending in misincorporated residues are rejected. This contribution of the growing tRNA 3′ end to the fidelity of the polymerization resulted in a considerable amount of tRNA transcripts and was interpreted as a possible backup mechanism for CCA addition (47). Hence, it is a pertinent question as to whether this backup system for fidelity is also relevant *in vivo*. To address this for the terminal A incorporation, we introduced a plasmid expressing the *E. coli* R134A variant enzyme in our complementation system. However, even after prolonged incubation of the corresponding cells for up to 96 h, no colony growth was visible (Figure 3B). These data clearly indicate that the described backup mechanism is only traceable *in vitro*, while it does not contribute to cell survival *in vivo*.

### tRNA nucleotidyltransferases from various organisms can complement in the *in vivo* system

To further estimate the applicability of the RNase T-based complementation system, we tested tRNA nucleotidyltransferases from all domains of life. Expression of the human CCA-adding enzyme also led to a growth restoration, although at a slightly lower rate (Figure 4A). Representing a member of class II tRNA nucleotidyltransferases, this enzyme has a catalytic mechanism of CCA polymer-

ization very similar to that of the *E. coli* enzyme. Archaea, however, carry class I CCA-adding enzymes of very different shape and mode of action as well as nucleotide recognition, and it was unclear whether these highly different enzymes can complement for the terminal A-addition in an organism that usually carries a class II enzyme. Hence, we introduced the CCA-adding enzyme from the archaeon *Methanosarcina barkeri* in *E. coli* (Figure 4A). This very different enzyme form also led to similar growth behavior as the tested class II enzymes, indicating a successful complementation of the original activity in *E. coli*. Thus, our system has the potential for further investigating a broad phylogenetic range of tRNA nucleotidyltransferases.

For the human CCA-adding enzyme, several mutations in the *cca* gene (*TRNT1*) resulting in partial loss-of-function were identified that correlate with certain hereditary diseases (24,34,48–52). For several of these disease-linked enzyme variants, an increased temperature sensitivity was observed, where L166S showed the most severe effect ($T_m$ of 35.4°C compared to 42.4°C for the wild type enzyme; 53). To investigate whether the decreased thermal stability of this mutant enzyme form can be monitored in our *in vivo* system, temperature-dependent growth of *E. coli* expressing the human L166S variant was tested. In a drop plate approach, *E. coli* Δ*cca* with both the wild type and the L166S version of the human CCA-adding enzyme exhibit a comparable growth behavior at the non-critical incubation temperatures 25 and 30°C (Figure 4B). In contrast and in accordance with reported *in vitro* data (53), the growth ability of *E. coli* Δ*cca* expressing variant L166S is dramatically reduced at 37°C and completely abolished at 42°C (Figure 4B). Hence, our complementation system represents a useful sensor for thermostability of different CCA-adding enzymes within a natural environment, such as the evaluation of pathologically relevant variants from human tRNA nucleotidyltransferase.

## DISCUSSION

Within the past decade, tRNA nucleotidyltransferases have emerged from sole processing enzymes that *de novo* synthesize and maintain a tRNA's CCA-end to crucial scrutinizers in tRNA quality control for all domains of life (10,19,20,23,24,54). The detailed investigation of those highly versatile properties requires reliable assays to further elucidate and evaluate such surprising protein characteristics. In the present study, we established a simple procedure for the *in vivo* analysis of terminal AMP-incorporation based on an inducible, RNase T-mediated deactivation of *E. coli* tRNA 3′-CCA-ends. As RNase T action generates a tRNA-CC end with 3′ OH group, the system provides a tRNA substrate pool that is accessible to all tRNA nucleotidyltransferases of interest with A-adding activity.

Interestingly, tRNA nucleotidyltransferases from different organisms were able to complement for A-addition in our *in vivo* system, and it convincingly shows that the catalytic activity of a class I enzyme efficiently replaces the endogenous class II-mediated A-incorporation, indicating that activity and functionality of these substantially different nucleotidyltransferases are very similar and compatible. The system is also suitable for screening a randomized se-
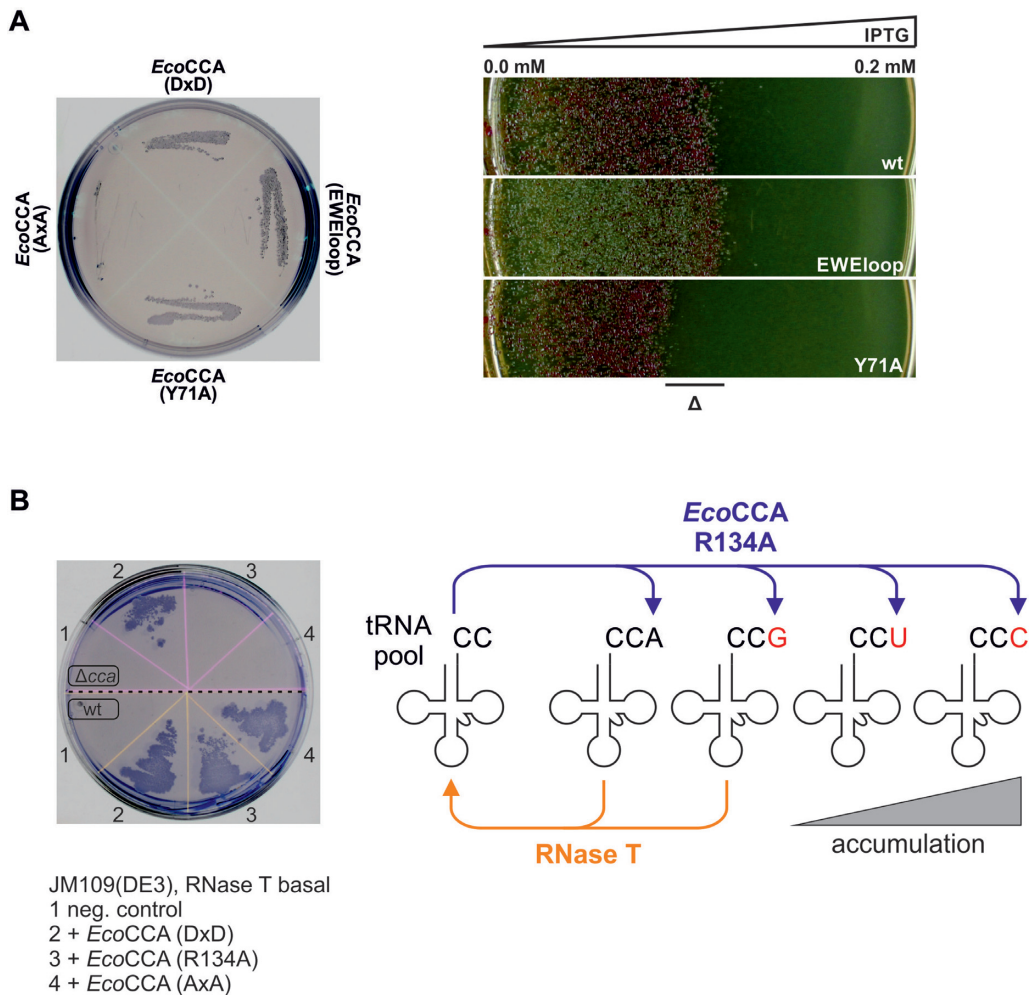
**Figure 3.** Growth defect depends on RNase T activity and CCA-adding enzyme fidelity. (**A**) RNase T expression level affects cell survival. Left: Streak outs of *E. coli* JM109(DE3) Δ*cca* transformed with pETDuet constructs containing the coding regions of indicated enzymes. All introduced variants from *E. coli* CCA-adding enzyme show growth at conditions of RNase T basal expression. Right: *E. coli* growth was examined on LB-amp in an IPTG gradient from 0 to 0.2 mM, resulting in a gradual increase of RNase T-mediated tRNA end hydrolysis. The difference in RNase T tolerance is indicated by 'Δ'. *Eco*CCA, *E. coli* CCA-adding enzyme; DxD, wild type enzyme with catalytically active carboxylates; AxA, inactive enzyme form with catalytically active carboxylates replaced by alanine residues; EWEloop, chimeric *E. coli* CCA-adding enzyme with flexible loop region from the corresponding *W. glossinidia* enzyme; wt, wild type *E. coli* CCA-adding enzyme with catalytically active DxD motif. (**B**) The described backup mechanism for CCA-addition by an error-prone enzyme is not sufficient for cell survival. Left: Streak outs of *E. coli* JM109(DE3) Δ*cca* (top) or wild type (bottom) transformed with indicated constructs on LB-amp. JM109(DE3) Δ*cca* expressing a CCA-adding enzyme with altered amino acid template EDxxA (*Eco*CCA R134A) does not survive in the presence of basally expressed RNase T. Right: Model for the interplay between an erroneous CCA-adding enzyme *Eco*CCA R134A and RNase T in quality control. The erroneous CCA-adding enzyme incorporates the terminal residue of the CCA-end at random, resulting in CCA, CCG, CCU and CCC ends. Due to the substrate preference of RNase T for purine ends, only those tRNAs are trimmed at their 3′ end, while tRNA-CCU and tRNA-CCC are not. The combination of RNase T activity and the erroneous nucleotide incorporation by *Eco*CCA R134A leads to an accumulation of non-functional tRNAs, resulting in a fatal situation for the cell.

quence pool for active enzyme variants, as it was shown for the catalytically important D23 position. Interestingly, we obtained only GAT codons in 15 selected colonies, while the second aspartate codon (GAC) was not observed. A possible explanation for this result is the codon usage in *E. coli* K12 that prefers GAT over GAC (65 % versus 35 %; see http://www.kazusa.or.jp/codon/). In addition, a screen of almost 200 tRNA nucleotidyltransferase sequences in seven genera of Enterobacteria revealed that the first catalytically active aspartate is encoded by GAC or GAT, while the second position (corresponding to D23 in *E. coli*), is exclusively represented by GAT. This indicates that the codon usage at

the second aspartate site is not random but under selective pressure and seems to play an important role in translation so that the synonymous codons are not interchangeable at this position. Such a codon bias might be important for a correct cotranslational folding of the nascent protein, as it is frequently observed in many ORFs in all domains of life (55–57).

The complementation system can be further used to investigate *in vitro* findings concerning A-addition under the more stringent *in vivo* conditions, where the enzyme has to be active in the presence of additional and competing activities acting on tRNA. We addressed the relevance of
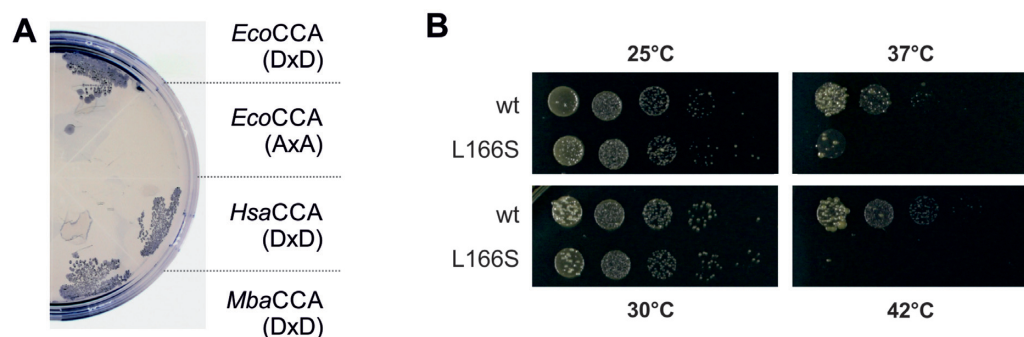
**Figure 4.** The *E. coli* system allows for the *in vivo* complementation of tRNA nucleotidyltransferases from all domains of life. (**A**) Streak outs of *E. coli* JM109(DE3) Δ*cca* transformed with pETDuet constructs containing the coding regions of indicated enzymes. Both eukaryotic and archaeal enzymes can complement the lacking CCA-adding activity in the *E. coli*-based system. *Eco*CCA, *E. coli* CCA-adding enzyme; *Hsa*CCA, *Homo sapiens* CCA-adding enzyme; *Mba*CCA, *Methanosarcina barkeri* CCA-adding enzyme; DxD, catalytically active carboxylates; AxA, catalytically active carboxylates replaced by alanines. (**B**) Drop plate assay to compare the growth of JM109(DE3) Δ*cca* with pETDuet constructs containing coding regions of either wild type or L166S variant from human CCA-adding enzyme at indicated temperatures. Growth of cells with temperature-sensitive variant L166S is inhibited at increased temperatures. wt, wild type.

the *in vitro* observed backup function in an *E. coli* CCA-adding enzyme where the nucleotide-recognizing arginine residue is replaced by alanine in the binding pocket (Supplementary Figure S3). *In vitro*, this variant has a high error rate (46), but is able to synthesize proper CCA-ends to a certain extent, as only primers with correctly added residues (C or CC) are accurately positioned and elongated (47). Our data show that the corresponding transformants are not viable, indicating that under *in vivo* conditions, this mechanism alone is not sufficient for a correct A incorporation (Figure 3B). In tRNA quality control, the terminal A residue is subjected to a frequent turnover, where RNase T removes this residue from the CCA-end (19,28,58). During restoration of this position, the compromised CCA-adding enzyme adds any of the four possible residues, so that at least a portion of the tRNAs should carry a functional CCA-end (46,47). However, RNase T has a strong substrate preference for RNA ending with purines, while it is almost completely inactive on pyrimidine ends, particularly on C residues (30). This enzymatic inhibition probably leads to an accumulation of nonfunctional deleterious tRNAs carrying CCC or CCU-ends, while the amount of tRNAs with correctly restored CCA-ends continuously decreases (Figure 3B). Hence, the RNase T-based 3′ end turnover and tRNA quality control requires a high fidelity CCA-adding enzyme that exclusively restores functional tRNA ends. Thus, although the *in vitro* data are very helpful to understand and clarify the mechanistic basis of proper CCA-addition and substrate selection, the biological relevance of such a backup mechanism can only be tested in a highly selective *in vivo* read-out system where such a limited enzymatic fidelity is not sufficient for tRNA quality control or repair and, consequently, cell survival.

Yet, one limitation of the *E. coli*-based assay is that the tested enzymes must be active within the temperature range of growth of the mesophilic gammaproteobacterium. While we were able to cover temperatures from 25 to 42°C, this range is not sufficient for thermophilic representatives, as the CCA-adding enzyme from *Archaeoglobus fulgidus* (thriving at 60–95°C; 59) did not lead to complementation (and growth) in *E. coli* (Supplementary Figure S4)—a fact

consistent with the observation that the recombinant enzyme is not active at moderate temperatures *in vitro*. Hence, the particular applicability is defined by the temperature range of the host strain's growth ability.

Because of the essential function of the CCA-adding enzyme in eukaryotes (eukaryotic tRNA genes do not encode the CCA-end), it is not possible to generate complete *cca* knockouts for these organisms (33). Instead, a temperature-sensitive yeast mutant could be acquired as a host strain for complementation assays (33,34). Because of the necessity to grow the respective mutant cells at the non-permissive temperature (i.e. 37°C; 33), this approach is not suitable for the evaluation of temperature-dependent protein features. In the *E. coli* complementation system, however, temperature sensitivity can be readily detected. To our knowledge, the presented data demonstrate for the first time the *in vivo* relevance of *in vitro* findings regarding the thermal denaturation of human disease-associated enzyme variants (53). This easy-to-use detection method may prove useful as a rapid test for human disease manifestations associated with a *cca* mutation.

A great advantage of this dual expression system is the convenient and straight-forward procedure. A single transformation step is sufficient to simultaneously introduce both the tRNA nucleotidyltransferase of interest and the RNase T-mediated tRNA 3′ end-directed selective pressure. Moreover, it should be possible to replace the T7-based expression of RNase T by leaky or tunable endogenous *E. coli* promoters (like the lac or the ara promoters; 60,61), rendering the system applicable in almost every *E. coli* strain. In contrast, the previously described amber codon suppression system requires a particular *E. coli* strain CA244 that is equipped with a chromosomal *lacZ* mutant gene with amber codon and, additionally, the recombinant expression of an individual tRNA$^{Tyr}_{su3}$$^+$ (1,31). Moreover, the utilization of a suppressor tRNA does not exclude the potentially detrimental off-target effects caused by occasional read through of endogenous stop codons (1,62). Accordingly, the use of a single-copy plasmid vector is favored for suppressor tRNA transcription to minimize the occurrence of such effects (1,31). Further, the variation of codon sup-

pression efficiency can vary (63), making it difficult to exclude unwanted false-positive events. In fact, previous studies report residual suppressor activity despite the disruption of the *cca* gene (31) which was explained by the side reactions of both poly(A) polymerase I and polynucleotide phosphorylase that are associated with residual tRNA end repair in the absence of CCA-adding enzyme (26,64). In the presented complementation system, however, such residual activities are not sufficient to overcome the increased hydrolysis of tRNA 3′ ends, as in principle the whole tRNA pool is affected by RNase T-mediated CCA degradation. Thus, the introduced selective pressure provides a robust and useful tool that allows for further investigations of tRNA nucleotidyltransferases, like the *in vivo* screening and selection for directed enzyme evolution via randomized, site-directed mutagenesis or the evaluation of pathogenic point mutations described for the human CCA-adding enzyme gene.

## SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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