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tRNA 5′-end repair activities of tRNA^{His} guanylyltransferase (Thg1)-like proteins from Bacteria and Archaea

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ABSTRACT

The tRNA^{His} guanylyltransferase (Thg1) family comprises a set of unique 3′–5′ nucleotide addition enzymes found ubiquitously in Eukaryotes, where they function in the critical G_{-1} addition reaction required for tRNA^{His} maturation. However, in most Bacteria and Archaea, G_{-1} is genomically encoded; thus post-transcriptional addition of G_{-1} to tRNA^{His} is not necessarily required. The presence of highly conserved Thg1-like proteins (TLPs) in more than 40 bacteria and archaea therefore suggests unappreciated roles for TLP-catalyzed 3′–5′ nucleotide addition. Here, we report that TLPs from *Bacillus thuringiensis* (BtTLP) and *Methanosarcina acetivorans* (MaTLP) display biochemical properties consistent with a prominent role in tRNA 5′-end repair. Unlike yeast Thg1, BtTLP strongly prefers addition of missing N_{+1} nucleotides to 5′-truncated tRNAs over analogous additions to full-length tRNA (k_{cat}/K_M enhanced 5–160-fold). Moreover, unlike for −1 addition, BtTLP-catalyzed additions to truncated tRNAs are not biased toward addition of G, and occur with tRNAs other than tRNA^{His}. Based on these distinct biochemical properties, we propose that rather than functioning solely in tRNA^{His} maturation, bacterial and archaeal TLPs are well-suited to participate in tRNA quality control pathways. These data support more widespread roles for 3′–5′ nucleotide addition reactions in biology than previously expected.

INTRODUCTION

The tRNA^{His} guanylyltransferase (Thg1), originally identified in yeast, adds a single essential G residue (G_{-1}) to the 5′-end of tRNA^{His} in eukaryotes (1). The presence of G_{-1} is a nearly universal feature of tRNA^{His} in all three domains of life, since G_{-1} is an important recognition element for aminoacylation of tRNA^{His} by its cognate histidyl-tRNA synthetase (HisRS) (2–5). In *Escherichia coli* and chloroplast, G_{-1} is incorporated into tRNA^{His} by an alternative pathway; the G_{-1} residue is genomically encoded, incorporated into the precursor tRNA during transcription, and retained in the mature tRNA^{His} following processing by ribonuclease P (RNase P) (6,7). A G_{-1} residue is similarly encoded in the genome of some archaea, and all bacteria, with the exception of 20 α-proteobacteria that are the only species known to lack a requirement for G_{-1} on tRNA^{His} (8). Thus G_{-1} could be incorporated during transcription in these species, as in *E. coli* (5). In other archaea and in metazoan mitochondria, a G residue is not present at the −1 position of tRNA^{His} genes, and G_{-1} is presumably added post-transcriptionally by Thg1 family members present in these species, consistent with the recent demonstration that archaeal Thg1 enzymes catalyze a G_{-1} addition reaction similar to yeast Thg1 (9,10). Recent results suggest that, even in organisms that contain a genomically encoded G_{-1}, the post-transcriptional pathway for incorporation of G_{-1} into tRNA^{His} may be used, since RNase P-catalyzed removal of a genomically encoded G_{-1} from tRNA^{His} in plant mitochondria has been reported (11).

Yeast Thg1 adds G_{-1} to tRNA^{His} using an unusual 3′–5′ nucleotide (nt) addition reaction, employing a three-step chemical mechanism for nucleotidyltransfer (1) that proceeds via formation of a 5′-adenylylated tRNA intermediate (Figure 1A). The first crystal structure of a Thg1 family enzyme revealed unexpected structural similarity between Thg1 and DNA polymerases, suggesting that Thg1 uses a two-metal ion active site for catalysis, albeit to add nucleotides in the opposite (3′–5′) direction to canonical 5′–3′ nt polymerases (12). In eukaryotes, G_{-1} addition to cytoplasmic tRNA^{His} occurs opposite a universally conserved A_{73} residue, however yeast Thg1 also catalyzes Watson–Crick template-dependent 3′–5′

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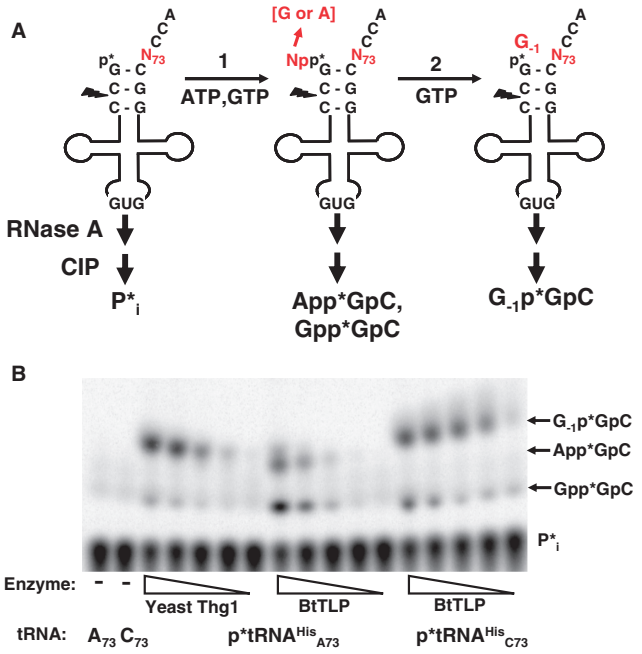


Figure 1. BtTLP catalyzes templated, but not non-templated addition of G_{-1} to $tRNA^{His}$. (**A**) Schematic of p^*tRNA^{His} G_{-1} addition assay (24); products expected from RNase A/CIP treatment are indicated below each tRNA. The site of RNase A cleavage is indicated on each tRNA. (**B**) G_{-1} addition to A_{73}- or C_{73}-containing 5′-^{32}P-$tRNA^{His}$ substrates was tested using the phosphatase protection assay with serial dilutions of enzymes, as indicated, in the presence of 0.1 mM ATP and 1.0 mM GTP.

polymerization of nucleotides *in vitro* and *in vivo* (13,14). While archaeal Thg1 family members share the ability to catalyze 3′–5′ nt addition, they do not efficiently catalyze the non-templated addition reaction observed in yeast (addition of G_{-1} to A_{73}-containing $tRNA^{His}$), but preferentially add Watson–Crick base paired nucleotides to $tRNA^{His}$ substrates (9). Thus, the template-dependent reaction is a shared property of eukaryal and archaeal enzymes, and is likely to represent an ancestral activity of the earliest Thg1 family enzymes. In contrast, addition of non-templated G_{-1} appears to be a specialized evolution of Thg1 activity that is so far unique to Eukarya.

The Thg1 enzyme family is comprised of related protein sequences (Pfam PF04446/InterPro IPR007537) whose members, as expected due to the requirement for post-transcriptional G_{-1} addition, are widely distributed throughout eukarya, and are also present in archaeal species that lack a genomically encoded G_{-1} residue (1). However, Thg1 family members are also found in bacteria and archaea that contain G_{-1} in their $tRNA^{His}$ genes, and thus a role for these proteins in $tRNA^{His}$ maturation is not necessarily required. The overall similarity between diverse Thg1 family members is relatively high (~40–45% pairwise sequence similarity between yeast Thg1 and archaeal/bacterial family members), including many highly conserved residues that are required for yeast Thg1-catalyzed 3′–5′ addition activity (15). Nonetheless, phylogenetic analysis indicates a distinct lineage for the archaeal/bacterial genes in the Thg1 enzyme family (16), and this, combined with the uncertainty regarding physiological function of at least some of the prokaryotic enzymes has led us to employ the designation Thg1-like proteins (TLPs) to distinguish the archaeal and bacterial enzymes from the eukaryal Thg1 enzymes that were the founding members of the Thg1/TLP superfamily.

The occurrence of highly conserved TLPs in bacterial and archaeal species that do not inherently require Thg1 activity for $tRNA^{His}$ maturation suggests the possibility of alternative roles for 3′–5′ addition. To uncover such functions for Thg1/TLP family members, we have investigated the biochemical activities of a bacterial TLP from the Gram-positive soil bacterium *Bacillus thuringiensis* (BtTLP). Like archaeal TLPs investigated previously, BtTLP preferentially catalyzes template-dependent 3′–5′ addition of nucleotides at the −1 position of various $tRNA^{His}$ substrates. Surprisingly, we also find that BtTLP exhibits substantial activity with truncated tRNA substrates lacking their mature 5′-end. In each case, the k_{cat}/K_M for templated N_{+1} addition is dramatically greater than for the analogous addition at the −1 position of $tRNA^{His}$. Since BtTLP catalyzes the same reaction with 5′-truncated $tRNA^{Phe}$, the ability to add nucleotides to restore a complete aminoacyl-acceptor stem and thus repair the 5′-end of the tRNA is not restricted to $tRNA^{His}$. In addition, we find that archaeal TLPs catalyze similar reactions. Taken together, our data suggest an alternative role for bacterial and archaeal TLPs in tRNA 5′-end repair. This activity bears striking similarities to the 5′-tRNA repair component of a mitochondrial 5′-tRNA editing activity that occurs in several lower eukaryotes (17–23), although the enzyme(s) that catalyze the 5′-tRNA editing reaction remain unknown.

MATERIALS AND METHODS

TLP and tRNA plasmid constructs

The *B. thuringiensis* TLP was cloned following PCR from *B. thuringiensis* serovar *israelensis* genomic DNA (kindly provided by Dr Don Dean, Ohio State University) into a pET15-derived vector for the expression of an N-terminal His_6-tagged protein in *E. coli*. tRNA constructs were derived from previously described yeast $tRNA^{His}$ and yeast $tRNA^{Phe}$ plasmids for T7 RNA polymerase-dependent *in vitro* transcription (24); alterations to N_{73} or N_{72} and/or removal of the G_{+1} residue were accomplished by Quik-Change Mutagenesis (Stratagene) according to the manufacturer's instructions. All NTPs and dNTPs for cloning, substrate preparation and assays were obtained from Roche.

Protein expression and purification

Plasmids encoding yeast Thg1 (1), BtTLP (this work) or MaTLP (9) were transformed into *E. coli* strain BL21(DE3) pLysS and cultures were grown and proteins were purified using immobilized metal-ion affinity chromatography (IMAC), as previously described (9). All proteins were >95% pure as judged by SDS-PAGE and

stored at −20°C. Purified protein concentrations were determined by BioRad protein assay.

3′–5′ nt addition assays

Nucleotide addition assays were performed using tRNA substrates prepared by *in vitro* transcription followed by 5′-end labeling with ^{32}P using T4 polynucleotide kinase and [γ-^{32}P]-ATP (24). Activity assays contained ∼10–30 nM 5′-^{32}P-tRNA (specific activity 6000 Ci/mmol) in Thg1 assay buffer [25 mM HEPES pH 7.5, 10 mM $MgCl_2$, 3 mM DTT, 125 mM NaCl, 0.2 mg/ml bovine serum albumin (BSA)]. Reactions to test G, U or C addition contained 0.1 mM ATP in addition to 1 mM NTP; A addition reactions contained only 1 mM ATP. For GTP competition assays, 1 mM GTP was added along with 1 mM NTP, as indicated.

Reactions (5 µl each) were initiated using 1 µl enzyme (undiluted or serial dilutions, ∼0.01-15 µg of each purified protein) and were incubated at room temperature for 2-3 h. ATP and GTP addition reactions were quenched by adding 1 mg/ml RNase A (Ambion) and 50 mM EDTA and incubating at 50°C for 10–20 min, whereas UTP and CTP addition reactions were quenched with 1 U RNase T1 (Ambion) in 20 mM NaOAc pH 5.2, 1 mM EDTA, 2 µg Yeast RNA (Ambion), followed by incubation at 37°C for 30 min. RNase digested samples were treated with 0.5 U calf intestinal alkaline phosphatase (CIP) (Invitrogen) and incubated at 37°C for 30 min; reactions were resolved using silica thin-layer chromatography (TLC) in an 1-propanol:NH_4OH:H_2O (55:35:10) solvent system. TLC plates were visualized using a Typhoon Trio and results quantified using ImageQuant software (GE Healthcare).

Steady-state kinetic parameters for N_{-1} and N_{+1} addition were measured as described previously, using triphosphorylated tRNA transcripts (24). To improve resolution of the labeled pyrophosphate product (which is released from the 5′-end of the tRNA following 3′–5′ nt addition) from unreacted labeled substrate tRNA, samples taken at each time point were first treated with 1 mg/ml RNase A and 50 mM EDTA for 10 min at 50°C, and precipitated with 10% (v/v) trichloro-acetic acid (TCA) for 10 min on ice prior to spotting on the PEI-cellulose TLC plates.

Primer extension analysis

$tRNA^{Phe}$ substrates lacking G_{+1} only, or lacking both G_{+1} and G_{+2}, were generated by *in vitro* transcription, and used as the substrate for TLP-catalyzed 3′-5′ nt addition, followed by 5′-end analysis using primer extension, according to (13). Addition reactions contained 2–4 µM unlabeled tRNA, 0.1 mM ATP, 1 mM GTP and 48 µM BtTLP or 25 µM MaTLP in Thg1 assay buffer, and were carried out at room temperature for 2–3 h. The resulting tRNAs (3–4 pmol) were purified by phenol extraction followed by ethanol precipitation and used as the template for primer extension with ∼1 pmol 5′-^{32}P-labeled $tRNA^{Phe}$-specific DNA primer (5′-GCTCT CCCAACTGAGCTAAA-3′).

Bulk tRNA was isolated from yeast to test the presence of a −1 nt on $tRNA^{His}$ using hot phenol extraction and ethanol precipitation (1). 5′-^{32}P-labeled $tRNA^{His}$-specific DNA primer (5′- ACTAACCACTATACTAAGA-3′) was used for the primer extension assays.

In vivo genetic complementation of Thg1 function by BtTLP

In vivo complementation was tested using the previously described yeast strain (JJY20: relevant genotype, *Matα thg1Δ::kanMX his3-1 leu2Δ met15Δ ura3* [*CEN URA3* P_{THG1}-*THG1*]) (9). Drop tests were performed with strains transformed with plasmids for galactose-inducible expression of yeast *THG1* or BtTLP [*CEN LEU2* P_{GAL}-*THG1*/*TLP*], or with empty vector. To test the effect of tRNAs on complementation, drop tests were also performed with strains containing a second plasmid [*CEN HIS3*] expressing either yeast wild-type A_{73}-$tRNA^{His}$, C_{73}-$tRNA^{His}$, or empty vector (14).

RESULTS

BtTLP catalyzes template dependent N_{-1} addition to $tRNA^{His}$

The recombinantly expressed and purified TLP from the bacterium *B. thuringiensis* serovar *israelensis* (BtTLP) was tested for its ability to catalyze the prototypical Thg1 reaction, G_{-1} addition to yeast $tRNA^{His}$ (24). Addition to the 5′-end of 5′-^{32}P labeled monophosphorylated yeast $tRNA^{His}$ (p*A_{73}-$tRNA^{His}$) results in protection of the labeled phosphate from removal by phosphatase, and reaction products, such as G_{-1}p*GpC (Figure 1A), can be resolved from $^{32}P_i$ generated from unreacted substrate using TLC. BtTLP only weakly catalyzes addition of a non-templated G_{-1} to A_{73}-$tRNA^{His}$, as evidenced by the relatively small amount of G_{-1}p*GpC product (the G_{-1} product spot migrates only slightly higher than the major product, described below, and is apparent only in the reactions with the highest concentration of BtTLP) (Figure 1B). However, BtTLP efficiently adds a Watson–Crick base paired G_{-1} residue to C_{73}-$tRNA^{His}$ (Figure 1B). The preferential addition of the Watson–Crick paired G_{-1} over non-templated G_{-1} to yeast $tRNA^{His}$ is the same pattern of reactivity previously observed with archaeal TLPs (9).

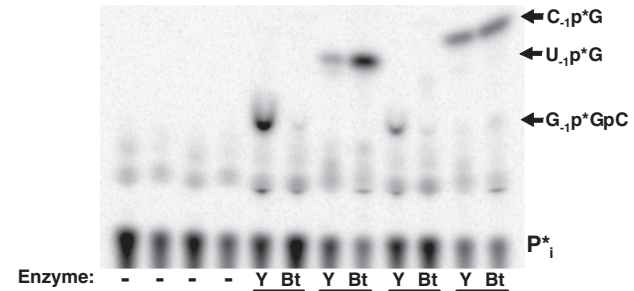
In assays with A_{73}-$tRNA^{His}$ substrate in the presence of ATP and GTP, BtTLP accumulates two different lower migrating products, both of which correspond to activated $tRNA^{His}$ intermediates (Figure 1). The first of these two products (App*GpC) migrates slightly below the G_{-1} addition product and corresponds to 5′-adenylylated $tRNA^{His}$, which is also produced by yeast Thg1 when GTP is omitted from the reaction (9). The second, more slowly migrating product corresponds to 5′-guanylylated $tRNA^{His}$ (Gpp*GpC) resulting from activation of the 5′-monophosphorylated tRNA with GTP instead of ATP, as evidenced by resistance of this isolated product to RNase T2 digestion and sensitivity to snake venom pyrophosphatase treatment (data not shown). The observation of roughly equivalent amounts of these two

activated $tRNA^{His}$ species suggests that BtTLP exhibits greater flexibility than yeast Thg1 with respect to the identity of the nucleotide (ATP or GTP) used for the activation step at the 5′ end of the tRNA substrate. The direct observation of activated 5′-tRNA intermediates in these assays indicates that BtTLP, like archaeal TLPs (9), uses the same basic mechanism for catalysis of 3′–5′ nt addition as yeast Thg1 (1).

To further probe the preference of BtTLP for templated versus non-templated nucleotide addition, we constructed $tRNA^{His}$ variant substrates with each of the four possible nucleotides at position 73 (N_{73}-$tRNA^{His}$). Using 5′-^{32}P-labeled tRNAs, we developed assays to test addition of each of the four possible NTPs that form Watson–Crick base pairs with the indicated N_{73} residue (Figure 2). For these assays, the identity of the nuclease used to treat the reactions was altered; to detect purine addition, RNase A was used to generate R_{-1}p*GpC products (where R = A or G) and to detect pyrimidine addition, RNase T1 was used to generate Y_{-1}p*G products (where Y = U or C). In each case, the identities of products were further confirmed by RNase T2 digestion to yield the expected N_{-1}p* nt (data not shown).

BtTLP, like yeast Thg1, can add any of the 4 nts at the −1 position of $tRNA^{His}$ (Figure 2). However, BtTLP is distinct from yeast Thg1 in its selective preference for Watson–Crick templated N_{-1} addition, as demonstrated using a competition experiment. For the competition assay, equimolar amounts of GTP and a competing Watson–Crick pairing nucleotide were provided simultaneously, and then nuclease digestions were performed separately in parallel, to compare the relative amounts of G_{-1} addition products (RNase A) versus U_{-1} or C_{-1} addition products (RNase T1) from the same assay (Figure 3). While yeast Thg1 added ∼5-fold higher amounts of G_{-1} than U_{-1} to A_{73}-$tRNA^{His}$ in the presence of equimolar GTP and UTP, the nucleotide preference for BtTLP was reversed, with ∼40-fold higher amounts of U_{-1} added over G_{-1}. A similarly enhanced preference of BtTLP for templated C_{-1} addition was observed (Figure 3).

To quantify these biochemical differences, steady-state kinetic parameters were determined. In agreement with the competition assay results, the catalytic efficiency of BtTLP-catalyzed G_{-1} addition to C_{73}-$tRNA^{His}$ was ∼50-fold greater than for addition of G_{-1} to the A_{73}-$tRNA^{His}$ substrate, whereas the k_{cat}/K_M values exhibited by yeast Thg1 for G_{-1} addition these two



Ratio:	Up*G/Gp*GpC	Cp*G/Gp*GpC
YThg1	0.2	2.3
BtTLP	38.4	20.6

Figure 3. GTP does not compete effectively with Watson–Crick base pair forming nucleotides for N_{-1} addition catalyzed by BtTLP. GTP competition assays were conducted using 5′-^{32}P labeled A_{73}-$tRNA^{His}$ or G_{73}-$tRNA^{His}$ substrates in the presence of equimolar amounts (1 mM each) of GTP and the correct Watson–Crick base pairing NTP (either UTP or CTP, as indicated). ATP (0.1 mM) was present in all reactions for 5′-monophosphate activation. Reactions were initiated with 1 µl enzyme and digested as indicated, to separately visualize purine and pyrimidine nucleotide addition products derived from the same assay, so that the ratio of templated addition to non-templated addition could be calculated for each enzyme/substrate combination.

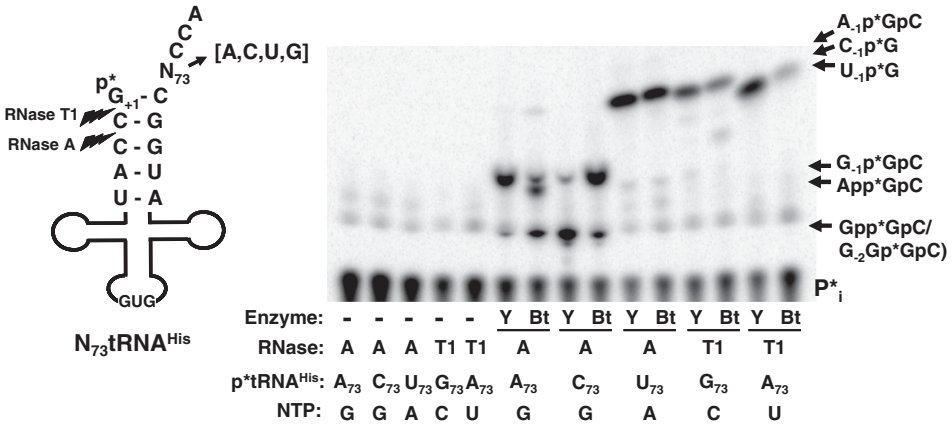


Figure 2. BtTLP catalyzes template-dependent 3′–5′ nucleotide additions to N_{73}-$tRNA^{His}$ variants. Assays for N_{-1} nucleotide additions contained $tRNA^{His}$ variants with each of the four possible N_{73} discriminator nucleotides, as indicated; the tRNA diagram shows the expected positions of RNase A or RNase T1 cleavage to yield the various labeled oligonucleotide products, as indicated to the right of the figure. Reactions contained 5′-^{32}P-labeled tRNA, 1 mM NTP (either G, A, U or C as indicated) and 0.1 mM ATP (unless ATP was already present in the assay) for activation of the 5′-monophosphorylated tRNA, and were initiated by addition of 1 µl (∼15 µg) yeast Thg1 or BtTLP. Gpp*GpC, formed by BtTLP with A_{73} or C_{73}-$tRNA^{His}$, and G_{-2}pGp*GpC, formed by yeast Thg1 with C_{73}-$tRNA^{His}$ are not resolved from each other using this TLC solvent system, but have each been verified by further digestion. Lanes dash: buffer control reactions for each N_{73}-$tRNA^{His}$ variant.

substrates are nearly identical (Table 1). While k_{cat}/K_M values measured for templated G_{-1} and C_{-1} addition were similar, rates of U_{-1} and A_{-1} addition were significantly lower. The competition assays and kinetic data demonstrate that BtTLP preferentially catalyzes templated, but not non-templated, N_{-1} addition reactions. Template-dependent 3′–5′ nt addition, previously shown to be a property of archaeal and eukaryal Thg1/TLP enzymes (9), is therefore an enzymatic activity common to family members from all three domains of life.

BtTLP catalyzes template dependent N_{+1} addition to 5′-truncated $tRNA^{His}$

Although BtTLP adds N_{-1} nucleotides to $tRNA^{His}$, albeit with varying catalytic efficiencies (Table 1), a role for the enzyme in $tRNA^{His}$ maturation in *B. thuringiensis* is not necessarily required. Thus, we hypothesized that the biochemical characteristics of BtTLP could be exploited for an alternate function *in vivo*.

Based on a previously described mitochondrial tRNA editing activity catalyzed by unknown enzymes (17–23,25), we tested whether BtTLP could add nucleotides to 5′-truncated tRNA substrates, thus restoring a completely base paired aminoacyl acceptor stem. We used a $tRNA^{His}$ substrate previously constructed to test 5′-end repair activity (13); the G_{+1} nucleotide has been removed from this tRNA leaving an unpaired C_{72} residue in the aminoacyl acceptor stem ($C_{72}tRNA^{His}{}_{\Delta G+1}$, Figure 4). Yeast Thg1 has little detectable ability to add the missing G_{+1} nucleotide to the monophosphorylated 5′-truncated tRNA substrate.

Using the phosphatase protection assay with 5′-^{32}P labeled monophosphorylated $C_{72}tRNA^{His}{}_{\Delta G+1}$, BtTLP, unlike yeast Thg1, displayed robust G_{+1} addition even at the lowest concentration of enzyme in the assay (Figure 4). Since addition of the missing G_{+1} restores a full-length $tRNA^{His}$, which is essentially the same molecule as the A_{73}-$tRNA^{His}$ tested previously (Figure 1), we observed additional reaction products at high concentrations of BtTLP (Figure 4). The identity of the lower migrating products can not be unambiguously assigned due to the position of the labeled phosphate between G_{+1} and G_{+2}, outside of the bond linking the additional nucleotides to the tRNA. Nonetheless, digestions with RNase T2 and snake venom pyrophosphatase (data not shown) suggest that these lower migrating products include a mixture of species derived from the G_{+1}-containing tRNA. These products likely include both activated ($NppG_{+1}p$*GpC) and G_{-1}-containing ($G_{-1}pG_{+1}p$*GpC) species, consistent with products seen previously (Figure 1B).

We constructed a set of truncated $tRNA^{His}$ variants with various N_{72} residues (N_{72}-$tRNA^{His}{}_{\Delta N+1}$), similar to the set of N_{73}-$tRNA^{His}$ variants, to examine each of the four possible templated N_{+1} addition reactions. Using 5′-end labeled tRNA substrates and varied nuclease digestions to detect each of the four N_{+1} addition products, we observed addition of each of the four N_{+1} nucleotides (Figure 5A), as evidenced by further digestion of the reactions with RNase T2 to generate each Np*, as expected (Figure 5B). As with N_{-1}-addition, BtTLP prefers to add the correct Watson–Crick base pairing N_{+1} nucleotide over adding a non-templated G_{+1} (Supplementary Figure S1). In the competition assay (Supplementary Figure S1), only RNase T1-dependent C_{+1} and U_{+1} addition products were detected, and little, if any, G_{+1} addition was detected in the parallel RNase A digestions.

As seen with G_{+1} addition above, restoration of the +1–72 base pair allowed formation of additional products with each substrate (starred products, Figure 5A). Although the exact identity of these lower migrating products cannot be unequivocally assigned due to the absence of an appropriately labeled phosphate, RNase T2 digestion of the same reactions shown in Figure 5A revealed that these are a mixture of activation and/or addition products, depending on the substrate used in each assay (Figure 5B).

Table 1. Steady-state kinetic parameters for N_{-1} addition to $tRNA^{His}$

Enzyme	$tRNA^{His}$	N_{-1}	k_{cat} (h^{-1})	K_M (μM)	k_{cat}/K_M ($M^{-1}s^{-1}$)
yThg1	A_{73}	G	8.4 ± 0.9[a]	0.42 ± 0.13[a]	5500 ± 1200[a]
yThg1	C_{73}	G	20.4 ± 2.4[a]	0.99 ± 0.29[a]	5670 ± 1200[a]
BtTLP	A_{73}	G	≥3.9[b]	≥10[b]	108[b]
BtTLP	C_{73}	G	23 ± 2	1.2 ± 0.3	5500 ± 1260
BtTLP	G_{73}	C	2.9 ± 0.3	0.6 ± 0.2	1400 ± 400
BtTLP	U_{73}	A	4.2 ± 0.7	12 ± 4	94 ± 13
BtTLP	A_{73}	U	1–2[c]	∼1[c]	230[c]

[a]Values reproduced from ref. (9).
[b]k_{cat}/K_M was obtained from the linear slope of the initial rate versus [tRNA] plot, which did not reach saturation even at the highest concentration of tRNA achievable in the assays (10 μM). The lower limit for k_{cat} and K_M were extrapolated from this value.
[c]Due to slow rates of U_{-1} addition observed in the assays, estimates for k_{cat} and K_M were made based on the apparent saturation of the initial rate of the reaction at >1 μM A_{73}-$tRNA^{His}$ and average observed rates of reactions performed at 2, 5 and 10 μM tRNA (ranging from 1 to 2 h^{-1}). The estimate for k_{cat}/K_M was subsequently calculated using these values.

3′–5′ addition of nucleotides to truncated tRNA substrates is kinetically preferred

To determine the efficiency with which BtTLP adds missing nucleotides to 5′-truncated tRNAs, we measured steady-state kinetic parameters for N_{+1} addition to each of the $tRNA^{His}{}_{\Delta N+1}$ substrates. These assays revealed significant (from 5- to 160- fold) enhancements of k_{cat}/K_M for addition of each missing N_{+1} nucleotide over the analogous N_{-1} addition reactions measured with full-length $tRNA^{His}$ (Supplementary Table S1, Figure 6). Moreover, k_{cat}/K_M values measured for each of the four templated N_{+1} additions are quite similar, particularly for G_{+1}, C_{+1} and U_{+1}, with only 5-fold lower efficiency observed for A_{+1} (Supplementary Table S1), as compared with the more than 50-fold variation observed in k_{cat}/K_M for the corresponding −1 additions (Table 1). These results suggest that 5′-truncated tRNAs are more optimal substrates than full-length tRNAs for 3′–5′ nt addition catalyzed by BtTLP, and suggest that BtTLP is well-suited to function in 5′-end repair of tRNA.

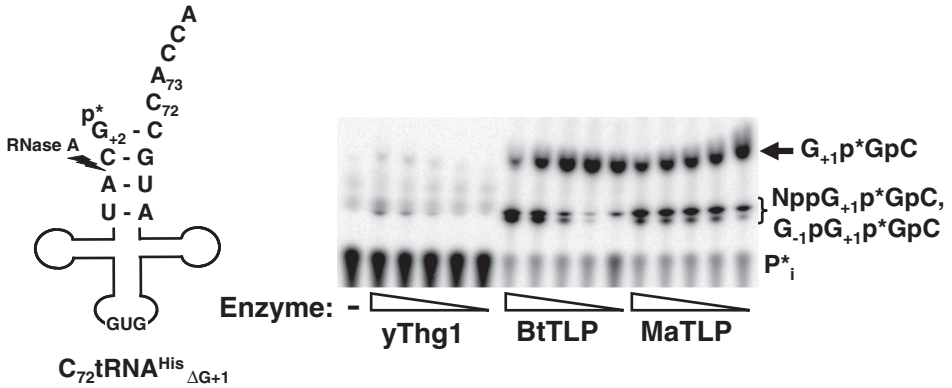


Figure 4. BtTLP catalyzes robust G_{+1} addition to 5′-truncated C_{72}-$tRNA^{His}_{\Delta G+1}$. G_{+1} addition to 5′-^{32}P labeled-$C_{72}tRNA^{His}_{\Delta G+1}$ was performed as described for G_{-1} addition assay above, using serial dilutions of yeast Thg1 (yThg1), BtTLP or MaTLP, as indicated, in the presence of 0.1 mM ATP and 1.0 mM GTP. The identity of the G_{+1}p*GpC product was verified by migration with standards and RNase T2 digestion to release 3′-GMP (data not shown). The lower migrating products indicated by the bracket cannot be unequivocally identified due to the remote position of the labeled phosphate from the added nucleotide, but further digestions and comparison to known standards suggests that these are a mixture of further activation and addition products following G_{+1} addition.

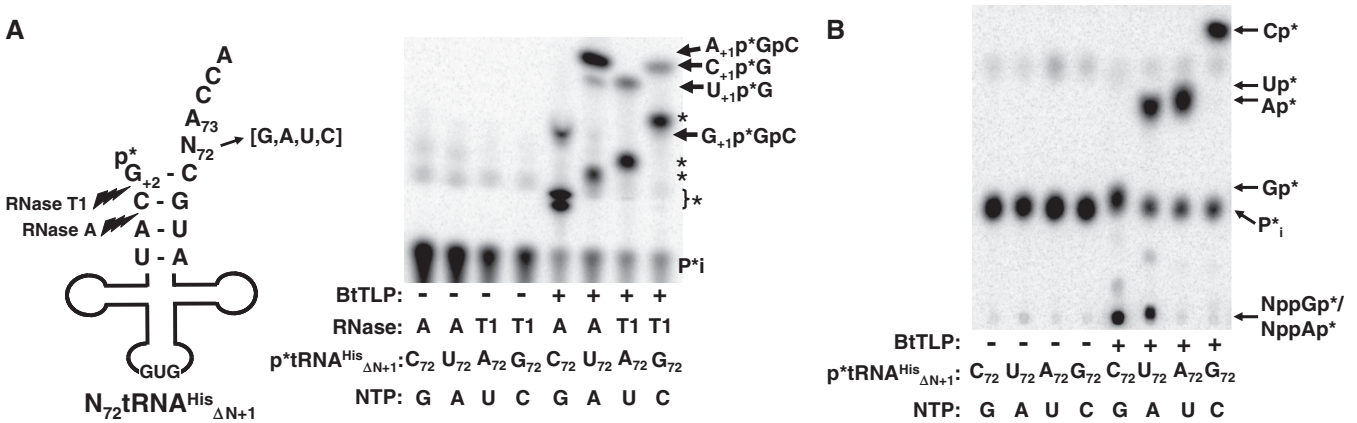


Figure 5. BtTLP adds all four possible templated N_{+1} nucleotides to 5′-truncated $tRNA^{His}$ variants (N_{72}-$tRNA^{His}_{\Delta N+1}$). (**A**) N_{+1} addition assays were performed using the same assay described in Figure 2, but with 5′-^{32}P labeled $tRNA^{His}$ variants missing a +1 nt and containing each of the four possible N_{72} nucleotides to serve as the template for +1 nt addition (see tRNA diagram). The single N_{+1} addition products produced by the relevant nuclease treatment are indicated by arrows. The full-length tRNAs generated following N_{+1} addition are each substrates for further activation and/or N_{-1} addition reactions; products of these reactions are indicated by asterisks to the right of the image, but these products are not further identified since the remote position of the labeled phosphate (between N_{+1} and G_{+2} nucleotides) does not readily permit identification by RNase T2 digestion. (**B**) RNase T2 digestion of reactions from (A) confirms each of the four N_{+1} nucleotides added to 5′-truncated $tRNA^{His}_{\Delta N+1}$ substrates. RNase T2 products were resolved by PEI-cellulose TLC in 0.5 M formate, pH 3.5; positions of each 3′-^{32}P labeled mononucleotide products (Cp*, Up*, Ap* and Gp*) were identified based on the migration of cold NMP standards. 5′-activated N_{+1} addition products generated from G_{+1} and A_{+1} addition reactions are indicated by NppGp* and NppAp*, respectively.

5′-end repair of truncated $tRNA^{His}$ is also catalyzed by archaeal TLPs

Members of the Thg1/TLP enzyme family are found in some Archaea that, as with *B. thuringiensis*, do not necessarily require post-transcriptional addition of G_{-1} to $tRNA^{His}$. We tested the TLP from *Methanosarcina acetivorans*, a methanogenic archaeon in which G_{-1} is genomically encoded, for its ability to add nucleotides to truncated $tRNA^{His}_{\Delta N+1}$ variants, using the same assays described above. The *M. acetivorans* TLP (MaTLP) catalyzed robust addition of G_{+1} to C_{72}-$tRNA^{His}_{\Delta G+1}$ (Figure 4), exhibited the same pattern of all four N_{+1} additions to the various N_{72}-containing truncated tRNA substrates that we observed previously with BtTLP (Supplementary Figure S2), and is similarly selective for addition of the Watson–Crick base pairing nucleotide over non-templated G-addition (Supplementary Figure S3). Finally, as with BtTLP, addition of G_{+1} to C_{72}-$tRNA^{His}_{\Delta G+1}$ occurs more efficiently than the corresponding G_{-1} addition reaction (Supplementary Table S1). Thus, the tRNA 5′-end repair reaction is also catalyzed with high efficiency by archaeal members of the Thg1/TLP enzyme family.

TLP-catalyzed N_{+1} addition is not limited to $tRNA^{His}$

Although eukaryal Thg1 enzymes that function in G_{-1} addition exhibit rigorous specificity for $tRNA^{His}$ (24), 5′-tRNA repair could be a more generalized process. We tested whether BtTLP could add nucleotides to the 5′-ends of other truncated tRNA substrates. To this end, we

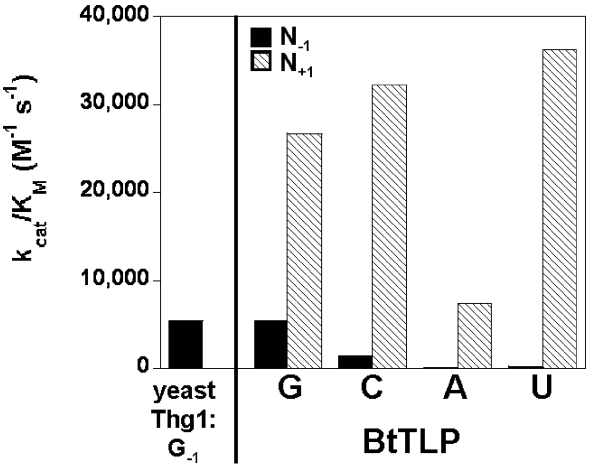


Figure 6. BtTLP catalyzes N_{+1} nucleotide addition to 5′-truncated $tRNA^{His}$ with enhanced catalytic efficiency over N_{-1} addition reactions. k_{cat}/K_M values are shown for BtTLP-catalyzed addition of each of the four possible Watson–Crick templated N_{-1} (solid bars) or N_{+1} (hatched bars) nucleotides to full-length $tRNA^{His}$ or 5′-truncated $tRNA^{His}_{\Delta N+1}$ substrates, respectively. For each of the four nucleotides (G, C, A or U, as indicated below the figure), kinetic parameters were measured using a tRNA substrate with the appropriate N_{73} or N_{72} residue to allow Watson–Crick base paired 3′–5′ addition, as in Table 1 and Supplementary Table S1. For comparison, the k_{cat}/K_M value measured previously for yeast Thg1-catalyzed G_{-1} addition to C_{73}-$tRNA^{His}$ is also shown (9).

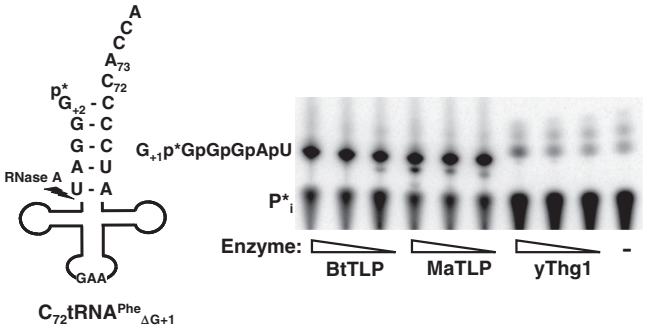


Figure 7. BtTLP catalyzes robust repair of 5′-truncated $tRNA^{Phe}$ substrates. The phosphatase protection assay for G_{+1} addition was conducted using 5′-^{32}P-labeled C_{72}-$tRNA^{Phe}_{\Delta G+1}$ (see tRNA diagram) with serial dilutions of BtTLP, MaTLP or yeast Thg1 (yThg1). All reactions contained 1.0 mM GTP and 0.1 mM ATP for activation. The migration of the phosphatase-protected species is consistent with the predicted 6-nt reaction product (see diagram), also confirmed by the addition of a single nucleotide to the 5′-truncated $tRNA^{Phe}_{\Delta G+1}$ substrate observed using primer extension (see Supplementary Figure S4).

generated a 5′-truncated variant of yeast $tRNA^{Phe}$ lacking G_{+1}, and tested G_{+1} addition using a 5′-^{32}P monophosphorylated substrate. Both BtTLP and MaTLP produce a prominent phosphatase resistant product indicative of addition of the missing G_{+1} to this substrate, whereas yeast Thg1 exhibits little or no detectable formation of this product (Figure 7). In the absence of a bona fide hexanucleotide standard for G_{+1} addition to this substrate, we used a primer extension assay (13) to confirm the addition of missing nucleotides to the 5′-end of $tRNA^{Phe}_{\Delta G+1}$, and to a second $tRNA^{Phe}$ substrate missing both G_{+1} and G_{+2} residues ($tRNA^{Phe}_{\Delta G+2}$) (Supplementary Figure S4). Reactions with either of the 5′-truncated $tRNA^{Phe}$ substrates yielded longer primer extension products than for control untreated tRNAs by 1 or 2 nt, indicating that missing 5′-nt were added to restore a fully base paired aminoacyl acceptor stem (Supplementary Figure S4). A similar kinetic preference was observed for the 5′-end repair reaction over the analogous G_{-1} addition reaction to full-length C_{73}-$tRNA^{Phe}$ (Supplementary Table S2). Notably, in contrast to assays with 5′-truncated $tRNA^{His}$ (Figure 5A), we did not observe evidence for further activation/addition reactions beyond the +1 position of full-length $tRNA^{Phe}$.

BtTLP weakly complements wild type Yeast Thg1 function *in vivo*

In yeast, *THG1* is essential for optimal growth and the requirement for *THG1* can only be bypassed by providing additional copies of both $tRNA^{His}$ and HisRS to the cells (14). Therefore, the ability of Thg1 homologs to add G_{-1} to $tRNA^{His}$ *in vivo* in yeast can be assessed using a plasmid shuffle assay (9). A yeast *thg1Δ* strain, made viable by the presence of a wild-type yeast *THG1 URA3* plasmid, is transformed with a *CEN LEU2* plasmid containing any Thg1/TLP gene of interest, expressed under the control of a galactose inducible promoter. If the Thg1/TLP complements the essential function of yeast *THG1 in vivo*, the resulting strains are able to grow on media containing 5-fluoroorotic acid (FOA), which causes loss of the *URA3 THG1* covering plasmid. Using this assay, we previously showed that four different archaeal TLPs individually supported growth of the yeast *thg1Δ* strain, but did so only in the presence C_{73}-$tRNA^{His}$ (9), mirroring the ability of these archaeal Thg1/TLP family members to add only templated, but not non-templated, G_{-1} to $tRNA^{His}$.

However, BtTLP supports growth of the yeast *thg1Δ* strain even in the presence of only A_{73}-$tRNA^{His}$ and addition of a plasmid expressing C_{73}-$tRNA^{His}$ confers no additional growth advantage to the BtTLP-complemented strain (Figure 8). This result was surprising, given the relatively weak levels of G_{-1} addition activity exhibited by BtTLP in the *in vitro* assays with A_{73}-$tRNA^{His}$ (Table 1). A primer extension assay was used to assess the 5′-end status of tRNA isolated from the complemented strains, confirming the presence of a −1 nt on $tRNA^{His}$ (Supplementary Figure S5).

The relatively similar k_{cat}/K_M values observed for G_{-1} and U_{-1} addition to wild-type (A_{73}) yeast $tRNA^{His}$ catalyzed by BtTLP (Table 1) suggest that either of these nucleotides may be present at the −1 position of the mature tRNA. The effect of U_{-1} on histidylation by HisRS in yeast has not been specifically investigated, but A_{-1}- or C_{-1}-containing $tRNA^{His}$ variants are substrates for HisRS, albeit with decreased catalytic efficiencies, consistent with a predominant role for the 5′-terminal monophosphate in recognition by HisRS (3,26).

DISCUSSION

We have revealed distinct biochemical features of bacterial and archaeal TLPs consistent with a novel physiological function for these enzymes in tRNA 5′-end repair. Initial

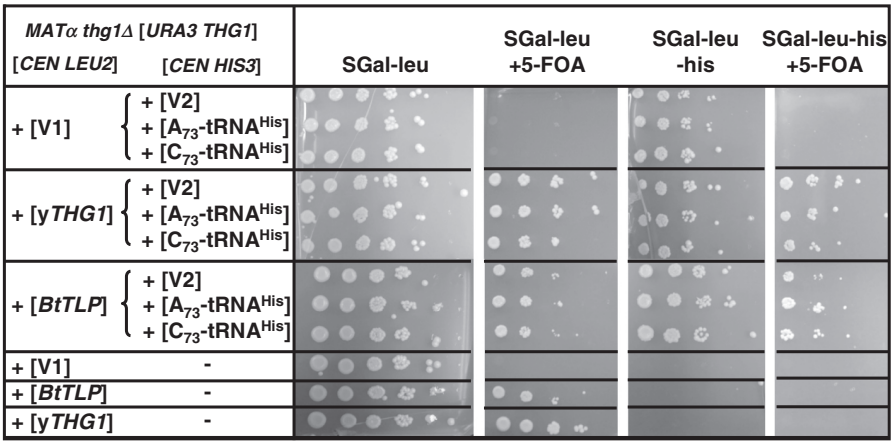


Figure 8. Expression of BtTLP in yeast complements the growth defect of the yeast *thg1Δ* strain. Plasmid shuffle assays were performed with a yeast *thg1*Δ strain (9) transformed with *CEN LEU2* plasmids containing either BtTLP [*BtTLP*] or yeast Thg1 [*yTHG1*], or no Thg1 [*V1*]. The top three panels also contained a second *CEN HIS3* plasmid encoding [A_{73}- $tRNA^{His}$], [C_{73}-$tRNA^{His}$] or no tRNA [*V2*], as indicated. Positive transformants were grown overnight in selective media, diluted to OD_{600} = 1 and used to make 10-fold serial dilutions; 2 µl of each dilution was spotted to media (as indicated) and images were taken after 3–4 days of growth at 30°C.

characterization of the TLP from the bacterium *B. thuringiensis* (BtTLP) demonstrated a biochemical preference for Watson–Crick template-dependent 3′–5′ nt addition (Figures 1 and 2, Table 1), similar to that observed previously with TLPs from several archaea (9). Upon further investigation, we identified four distinct features of bacterial TLP activity that could be exploited for an alternative function. First, unlike for yeast Thg1, GTP does not effectively compete with other Watson–Crick base pair-forming NTPs for addition by BtTLP (Figure 3 and Supplementary Figure S1). Second, BtTLP adds any of the 4 nts to 5′-truncated $tRNA^{His}$ substrates with significantly enhanced catalytic efficiency over that observed for nucleotide addition to full-length $tRNA^{His}$ (Figures 4–6, Supplementary Table S1). Third, while BtTLP adds N_{-1} nucleotides to $tRNA^{His}$ with widely varied catalytic efficiencies, with 5′-truncated $tRNA^{His}$ all four +1 nts are added with similarly high k_{cat}/K_M values (Table 1 and Supplementary Table S1, Figure 6). Fourth, BtTLP adds missing nucleotides to a tRNA species other than $tRNA^{His}$ (Figure 7 and Supplementary Figure S4, Supplementary Table S2). We propose that these distinct biochemical features are well-suited for a physiological role for BtTLP in tRNA 5′-end repair. Similar properties of the archaeal TLP from *M. acetivorans* (Figures 4 and 7, Supplementary Figures S2 and S3, Supplementary Table S1) suggest a parallel biological function in Archaea, thus greatly expanding the potential scope of 3′–5′ nt addition reactions beyond a simple role for Thg1/TLP family members in $tRNA^{His}$ maturation.

Identification of *bona fide* physiological substrates for the 5′-end repair activity is an important future goal that can not be addressed by *in vitro* characterization alone. In recent years, an increasing number of tRNA quality control mechanisms have been identified, allowing cells to maintain a high-quality cellular pool of tRNAs and thus ensuring optimal fidelity and efficiency of translation (27–34). The TLP-catalyzed tRNA 5′-end repair activity we have identified is well-suited to participating in tRNA quality control. tRNA 5′-end repair mechanisms have not yet been demonstrated in any organism, but several mechanisms for production of 5′-truncated tRNA species provide potential substrates for the 5′-end repair activity. 5′-processing of tRNAs typically generates mature tRNAs initiating at the +1 position [with the notable exception of $tRNA^{His}$ from certain bacteria and organelles (6,7,11,35)], since removal of the precursor tRNA 5′-leader sequence catalyzed by RNase P occurs for the most part with high fidelity. Nonetheless, miscleavage events occur with significant frequency in bacteria, generating aberrent tRNA 5′-ends, including those that lack one or more nucleotides from the 5′-end (36,37). TLP-catalyzed 5′-end repair of such mis-processed tRNA species would rescue a pool of tRNAs that would otherwise be unusable for translation. In this respect, the 5′-end repair function we propose may be similar to the well-known mechanisms for repair of tRNA 3′-ends catalyzed by the CCA-adding enzyme, which functions to add the 3′-CCA to tRNAs for which this sequence is not genomically encoded, but also functions to repair 3′-ends of tRNA species damaged by cellular nucleases (38,39).

5′-truncated tRNA species could also be generated by the action of 5′–3′ exonucleases that act on tRNA; 5′–3′ exonucleolytic degradation of tRNA has been recently identified in yeast, where the *XRN1*/*RAT1* enzymes act to degrade several hypomodified tRNA species via the rapid tRNA decay pathway (27,40). *XRN1*/*RAT1* family members with unknown functions are widely distributed throughout the bacterial and archaeal domains, including organisms that contain TLPs, and moreover a role for some of these family enzymes in tRNA or rRNA processing or degradation has been proposed (41). Finally, in Archaea, a growing number of alternative tRNA processing/generation pathways have been identified, including production of at least some tRNA species as leaderless

transcripts, where it remains unclear how uniformity of 5′-ends is accomplished (42). It is an important future direction to determine the essentiality of TLPs in archaea and bacteria. However, such tests might face the same caveats encountered with tRNA 3′-end repair pathways, which are not inherently essential for viability, but may be particularly required under conditions of stress (39).

Interestingly, the tRNA 5′-end repair reaction identified here is not the first biochemical process proposed to use 3′–5′ nt addition to restore a fully base-paired aminoacyl acceptor stem in tRNA. Previously, a 5′-tRNA editing activity was identified that occurs in the mitochondria of lower eukaryotes, including organisms such as *S. punctatus*, *A. castellani* and *P. polycephalum* (17,19,21,25), and which requires as one of its components an analogous tRNA 5′-end repair activity to the activity described here. 5′-tRNA editing exists to correct genomically encoded mismatches present at the 5′-end of certain mitochondrial tRNAs by first excising the incorrect nucleotides, and then using a 3′–5′ nt addition activity to add the correct nucleotides to the 5′-truncated tRNA, thus creating a fully base paired aminoacyl acceptor stem (17,25). The identity of the protein(s) that catalyze either the nuclease or 5′-end repair components of this activity are not known. The archaeal/bacterial TLP 5′-end repair activity is not likely to function in 5′-tRNA editing *in vivo*, since sequenced archaeal/bacterial tRNA genes do not contain 5′-mismatched nucleotides that would require editing to generate a functional tRNA. Nonetheless, the existence of the protozoan 5′-tRNA editing activity reinforces the idea that pathways exist for generation of the type of 5′-truncated tRNA substrates that we have associated with bacterial and archaeal TLP function.

The ability of BtTLP to complement the growth defect of the yeast *thg1Δ* strain was somewhat surprising, given the lack of complementation observed with archaeal TLPs tested previously (9), all of which exhibit similar biochemical activities to BtTLP, including the kinetic preference for 5′-end repair activities over N_{-1} addition reactions. Interestingly, the reproducibly weaker growth observed in the BtTLP-complemented strain compared to the yeast *THG1* control strain (Figure 8) is unlikely to be directly limited by the slower kinetics of G_{-1} addition to A_{73}-$tRNA^{His}$ catalyzed by BtTLP, since providing the C_{73}-$tRNA^{His}$ that is the kinetically preferred substrate for BtTLP activity (Table 1) did not enhance growth (Figure 8). This suggests the interesting possibility that the weaker growth of the BtTLP-complemented strain may reflect alternative activities catalyzed by BtTLP when it is expressed in yeast, perhaps related to the ability of the enzyme to use other substrates for 3′–5′ nt addition (Figure 7).

Alternative 5′-end repair activities of bacterial and archaeal TLPs would resolve the mystery surrounding the presence of TLPs in many organisms that do not inherently require post-transcriptional addition of G_{-1} to $tRNA^{His}$. Nonetheless, these data do not preclude additional roles for bacterial or archaeal TLPs in addition of G_{-1} to $tRNA^{His}$, even in organisms that already contain a genomically encoded G_{-1}. This activity would be required if the encoded G_{-1} is removed by RNase P-catalyzed processing (11), or by 5′-end degradation pathways such as those described above. A recent independent report of G_{-1}-addition activity catalyzed by two bacterial TLPs (including BtTLP) (16) is consistent with this possibility, and with the various N_{-1} addition activities demonstrated with $tRNA^{His}$ substrates in this work (Figures 1 and 2). Moreover, TLPs derived from Archaea that lack a genomically encoded G_{-1} and thus predictably function in $tRNA^{His}$ maturation (9), such as *M. thermoautotrophicus*, also catalyze 5′-end repair with the tRNA substrates tested here (data not shown). Thus prokaryotic TLPs may catalyze both $tRNA^{His}$-specific G_{-1} addition and tRNA 5′-end repair reactions, and further study of these enzymes may yield important insights into the evolution of 3′–5′ addition activities and their varied uses in biology.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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