tRNA 5′-end repair activities of tRNAHis guanylyltransferase (Thg1)-like proteins from Bacteria and Archaea

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ABSTRACT
The tRNAHis 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 tRNAHis maturation. However, in most Bacteria and Archaea, G–1 is genomically encoded; thus post-transcriptional addition of G–1 to tRNAHis 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 Bacteriodes 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 N–1 nucleotides to 5′-truncated tRNAs over analogous additions to full-length tRNA (kcat/KM 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 tRNAHis. Based on these distinct biochemical properties, we propose that rather than functioning solely in tRNAHis 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 tRNAHis guanylyltransferase (Thg1), originally identified in yeast, adds a single essential G residue (G–1) to the 5′-end of tRNAHis in eukaryotes (1). The presence of G–1 is a nearly universal feature of tRNAHis in all three domains of life, since G–1 is an important recognition element for aminoacylation of tRNAHis by its cognate histidyl-tRNA synthetase (HisRS) (2–5). In Escherichia coli and chloroplast, G–1 is incorporated into tRNAHis by an alternative pathway; the G–1 residue is genomically encoded, incorporated into the precursor tRNA during transcription, and retained in the mature tRNAHis 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 tRNAHis (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 tRNAHis 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 tRNAHis may be used, since RNase P-catalyzed removal of a genomically encoded G–1 from tRNAHis in plant mitochondria has been reported (11).

Yeast Thg1 adds G–1 to tRNAHis using an unusual 3′–5′ nucleotide (nt) addition reaction, employing a three-step chemical mechanism for nucleotidylytransfer (1) that proceeds via formation of a 5′-adenylated 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 tRNAHis occurs opposite a universally conserved A73 residue, however yeast Thg1 also catalyzes Watson–Crick template-dependent 3′–5′
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 A73-containing tRNAHis), but preferentially add Watson–Crick base paired nucleotides to tRNAHis 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 tRNAHis genes, and thus a role for these proteins in tRNAHis 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 tRNAHis 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 tRNAHis substrates. Surprisingly, we also find that BtTLP exhibits substantial activity with truncated tRNA substrates lacking their mature 5′-end. In each case, the kcat/Km for templated N−1 addition is dramatically greater than for the analogous addition at the −1 position of tRNAHis. 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 tRNAHis. 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 His6-tagged protein in E. coli. tRNA constructs were derived from previously described yeast tRNAHis and yeast tRNA^phe plasmids for T7 RNA polymerase-dependent in vitro transcription (24); alterations to N73 or N77 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 \(-20^\circ C\). Purified protein concentrations were determined by BioRad protein assay.

3'-5' nt addition assays

Nucleotide addition assays were performed using tRNA substrates prepared by \textit{in vitro} transcription followed by 5'-end labeling with \(32P\) using T4 polynucleotide kinase and \([\gamma-32P]-ATP\) (24). Activity assays contained \(\sim 10-30\) nM 5'-32P-tRNA (specific activity 6000 Ci/mmol) in Thg1 assay buffer [25 mM HEPES pH 7.5, 10 mM MgCl2, 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 \(\mu\)l each) were initiated using 1 \(\mu\)l enzyme (undiluted or serial dilutions, \(\sim 0.01-15\) ng of each purified protein) and were incubated at room temperature for 2-3h. 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 \(\mu\)g 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:NH4OH:H2O (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\(^{\text{Phe}}\) substrates lacking G+1 only, or lacking both G+1 and G+2, were generated by \textit{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 \(\mu\)M unlabeled tRNA, 0.1 mM ATP, 1 mM GTP and 48 \(\mu\)M BtTLP or 25 \(\mu\)M MaTLP in Thg1 assay buffer, and were carried out at room temperature for 2-3h. The resulting tRNAs (3-4 pmol) were purified by phenol extraction followed by ethanol precipitation and used as the template for primer extension with \(\sim 1\) pmol 5'-32P-labeled tRNA\(^{\text{Phe}}\)-specific DNA primer (5'-GCTCT CCCAACCTGAGCTAAA-3').

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

\textbf{In vivo genetic complementation of Thg1 function by BtTLP}

\textit{In vivo} complementation was tested using the previously described yeast strain (JJY20: relevant genotype, \textit{Mato thg1A::kanMX his3-1 leu2A met15A ura3 [CEN URA3 \textit{PTHG1-THG1}]}) (9). Drop tests were performed with strains transformed with plasmids for galactose-inducible expression of yeast \textit{THG1} or BtTLP [\textit{CEN LEU2 pgal\textunderscore 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 [\textit{CEN HIS3}] expressing either yeast wild-type A\(\_73\)-tRNA\(^{\text{His}}\), C\(\_73\)-tRNA\(^{\text{His}}\), or empty vector (14).

\textbf{RESULTS}

BtTLP catalyzes template dependent N-1 addition to tRNA\(^{\text{His}}\)

The recombinantly expressed and purified TLP from the bacterium \textit{B. thuringiensis} serovar \textit{israelensis} (BtTLP) was tested for its ability to catalyze the prototypical Thg1 reaction, G+1 addition to yeast tRNA\(^{\text{His}}\) (24). Addition to the 5'-end of 5'-32P labeled monophosphorylated yeast tRNA\(^{\text{His}}\) (p\(\_\_73\)-tRNA\(^{\text{His}}\)) results in protection of the labeled phosphate from removal by phosphatase, and reaction products, such as G-1p*GpC (Figure 1A), can be resolved from \(32\)P generated from unreacted substrate using TLC. BtTLP only weakly catalyzes addition of a non-templated G-1 to A\(\_73\)-tRNA\(^{\text{His}}\) as evidenced by the relatively small amount of G-1p*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 (Figure 1B). The preferential addition of the Watson–Crick paired G+1 over non-templated G-1 to yeast tRNA\(^{\text{His}}\) is the same pattern of reactivity previously observed with archael TLPs (9).

In assays with A\(\_73\)-tRNA\(^{\text{His}}\) substrate in the presence of ATP and GTP, BtTLP accumulates two different lower migrating products, both of which correspond to activated tRNA\(^{\text{His}}\) intermediates (Figure 1). The first of these two products (App*GpC) migrates slightly below the G+1 addition product and corresponds to 5'-adenylated tRNA\(^{\text{His}}\), which is also produced by yeast Thg1 when GTP is omitted from the reaction (9). The second, more slowly migrating product corresponds to 5'-guanylated tRNA\(^{\text{His}}\) (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\textsuperscript{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\textsuperscript{His} variant substrates with each of the four possible nucleotides at position 73 (N\textsubscript{73}-tRNA\textsuperscript{His}). Using 5'\textsuperscript{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\textsubscript{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 Y\textsubscript{-1}p\textsuperscript{*}GpC products (where R = A or G) and to detect pyrimidine addition, RNase T1 was used to generate Y\textsubscript{-1}p\textsuperscript{*}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\textsubscript{-1}p\textsuperscript{*} nt (data not shown).

BtTLP, like yeast Thg1, can add any of the 4 nts at the –1 position of tRNA\textsuperscript{His} (Figure 2). However, BtTLP is distinct from yeast Thg1 in its selective preference for Watson–Crick templated N\textsubscript{-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\textsubscript{-1} addition products (RNase A) versus U\textsubscript{-1} or C\textsubscript{-1} addition products (RNase T1) from the same assay (Figure 3). While yeast Thg1 added ~5-fold higher amounts of G\textsubscript{-1} than U\textsubscript{-1} or C\textsubscript{-1} to A\textsubscript{73}-tRNA\textsuperscript{His} in the presence of equimolar GTP and UTP, the nucleotide preference for BtTLP was reversed, with ~40-fold higher amounts of U\textsubscript{-1} added over G\textsubscript{-1}. A similarly enhanced preference of BtTLP for templated C\textsubscript{-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\textsubscript{-1} addition to C\textsubscript{73}-tRNA\textsuperscript{His} was ~50-fold greater than for addition of G\textsubscript{-1} to the A\textsubscript{73}-tRNA\textsuperscript{His} substrate, whereas the $k_{cat}/K_M$ values exhibited by yeast Thg1 for G\textsubscript{-1} addition these two
substances are nearly identical (Table 1). While \( k_{\text{cat}}/K_M \) 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 Thgl/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\({}_{\text{His}}\)**

Although BtTLP adds N\(_{1}\) nucleotides to tRNA\({}_{\text{His}}\), albeit with varying catalytic efficiencies (Table 1), a role for the enzyme in tRNA\({}_{\text{His}}\) 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\({}_{\text{His}}\) 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\({}_{\text{His}}\)\(_{\Delta G+1}\), Figure 4). Yeast Thgl 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‘,32P labeled monophosphorylated C\(_{72}\)tRNA\({}_{\text{His}}\)\(_{\Delta G+1}\), BtTLP, unlike yeast Thgl, 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\({}_{\text{His}}\), which is essentially the same molecule as the A\(_{73}\)tRNA\({}_{\text{His}}\) tested previously (Figure 1), we observed addition of nucleotides to truncated tRNA substrates.

| Enzyme | tRNA\({}_{\text{His}}\) | N\(_{1}\) | \( k_{\text{cat}} \) (h\(^{-1}\)) | \( K_M \) (\( \mu \text{M} \)) | \( k_{\text{cat}}/K_M \) (M\(^{-1}\)s\(^{-1}\)) |
|--------|-----------------|---------|----------------|----------------|-----------------|
| yThgl A\(_{73}\) | G | 8.4 ± 0.9\(^a\) | 0.42 ± 0.13\(^a\) | 5500 ± 1200\(^a\) | |
| yThgl 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 A\(_{73}\) | A | 4.2 ± 0.7 | 12 ± 4 | 94 ± 13 | |
| BtTLP C\(_{73}\) | U | 1–2\(^c\) | ~1\(^c\) | 230\(^c\) | |

\(^{a}\)Values reproduced from ref. (9).

\(^{b}\)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 \( \mu \text{M} \)). The lower limit for \( k_{\text{cat}} \) and \( K_M \) were extrapolated from this value.

\(^{c}\)Due to slow rates of U\(_{1}\) addition observed in the assays, estimates for \( k_{\text{cat}} \) and \( K_M \) were made based on the apparent saturation of the initial rate of the reaction at >1 \( \mu \text{M} \) A\(_{73}\)tRNA\({}_{\text{His}}\) and average observed rates of reactions performed at 2, 5 and 10 \( \mu \text{M} \) tRNA (ranging from 1 to 2 h\(^{-1}\)). The estimate for \( k_{\text{cat}}/K_M \) 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\({}_{\text{His}}\)\(_{\Delta N+1}\) substrates. These assays revealed significant (from 5- to 160- fold) enhancements of \( k_{\text{cat}}/K_M \) for addition of each missing N\(_{1}\) nucleotide over the analogous N\(_{1}\) addition reactions measured with full-length tRNA\({}_{\text{His}}\) (Supplementary Table S1, Figure 6). Moreover, \( k_{\text{cat}}/K_M \) 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 \( k_{\text{cat}}/K_M \) 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.
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/{C}0_{1} to tRNA_{His}. We tested the TLP from Methanosarcina acetivorans, a methanogenic archaeon in which G/{C}0_{1} is genomically encoded, for its ability to add nucleotides to truncated tRNA_{His}/C_{1}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}/C_{1}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}/C_{1}G+1 occurs more efficiently than the corresponding G/C_{0}_{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/C_{0}_{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
generated a 5'-truncated variant of yeast tRNA\textsubscript{Phe} lacking G\textsubscript{+1}, and tested G\textsubscript{+1} addition using a 5',3'-P monophosphorylated substrate. Both BtTLP and MaTLP produce a prominent phosphatase resistant product indicative of addition of the missing G\textsubscript{+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\textsubscript{+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\textsubscript{Phe}_{\textsubscript{AG+1}}, and to a second tRNA\textsubscript{Phe} substrate missing both G\textsubscript{+1} and G\textsubscript{-2} residues (tRNA\textsubscript{Phe}_{\textsubscript{AG+2}}) (Supplementary Figure S4). Reactions with either of the 5'-truncated tRNA\textsubscript{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 aminocycl acceptor stem (Supplementary Figure S4). A similar kinetic preference was observed for the 5'-end repair reaction over the analogous G\textsubscript{-1} addition reaction to full-length C\textsubscript{73}-tRNA\textsubscript{Phe} (Supplementary Table S2). Notably, in contrast to assays with 5'-truncated tRNA\textsubscript{His} (Figure 5A), we did not observe evidence for further activation/addition reactions beyond the +1 position of full-length tRNA\textsubscript{His}.

**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\textsubscript{His} and HisRS to the cells (14). Therefore, the ability of Thg1 homologs to add G\textsubscript{-1} to tRNA\textsubscript{His} \textit{in vivo} in yeast can be assessed using a plasmid shuffle assay (9). A yeast thg1A strain, made viable by the presence of a wild-type yeast THG1 URA3 plasmid, is transformed with a \textit{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 \textit{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 \textit{thg1A} strain, but did so only in the presence C\textsubscript{73}-tRNA\textsubscript{His} (9), mirroring the ability of these archaeal Thg1/TLP family members to add only templated, but not non-templated, G\textsubscript{-1} to tRNA\textsubscript{His}.

However, BtTLP supports growth of the yeast \textit{thg1A} strain even in the presence of only A\textsubscript{73}-tRNA\textsubscript{His} and addition of a plasmid expressing C\textsubscript{73}-tRNA\textsubscript{His} confers no additional growth advantage to the BtTLP-complemented strain (Figure 8). This result was surprising, given the relatively weak levels of G\textsubscript{-1} addition activity exhibited by BtTLP in the \textit{in vitro} assays with A\textsubscript{73}-tRNA\textsubscript{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 \textendash{}1 nt on tRNA\textsubscript{His} (Supplementary Figure S5).

The relatively similar \(k_{\text{cat}}/K_{M}\) values observed for G\textsubscript{-1} and U\textsubscript{-1} addition to wild-type (A\textsubscript{73}) yeast tRNA\textsubscript{His} catalyzed by BtTLP (Table 1) suggest that either of these nucleotides may be present at the \textendash{}1 position of the mature tRNA. The effect of U\textsubscript{-1} on histidinylation by HisRS in yeast has not been specifically investigated, but A\textsubscript{-1} or C\textsubscript{-1}-containing tRNA\textsubscript{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
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 tRNAHis substrates with significantly enhanced catalytic efficacy over that observed for nucleotide addition to full-length tRNAHis (Figures 4–6, Supplementary Table S1). Third, while BtTLP adds N-1 nucleotides to tRNAHis with widely varied catalytic efficiencies, with 5′-truncated tRNAHis 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 tRNAHis (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 tRNAHis 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 tRNAHis 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 aberrant 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 tRNA 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 mitochondrial 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 archael/bacterial TLP 5'-end repair activity is not likely to function in 5'-tRNA editing in vivo, since sequenced archael/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 archael TLP function.

The ability of BtTLP to complement the growth defect of the yeast thyg1Δ strain was somewhat surprising, given the lack of complementation observed with archael TLPs tested previously (9), all of which exhibit similar biochemical activities to BiTLP, 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 A73-tRNAHis catalyzed by BiTLP, since providing the C73-tRNAHis that is the kinetically preferred substrate for BiTLP 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 BiTLP 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 archael 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 tRNAHis. Nonetheless, these data do not preclude additional roles for bacterial or archael TLPs in addition of G-1 to tRNAHis, 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 tRNAHis 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 tRNAHis maturation (9), such as M. thermoautothrophus, also catalyze 5'-end repair with the tRNA substrates tested here (data not shown). Thus prokaryotic TLPs may catalyze both tRNAHis-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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