Biosynthesis of Threonylcarbamoyl Adenosine (t^6A), a Universal tRNA Nucleoside*

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Christopher Deutsch†, Basma El Yacoubi‡, Valérie de Crécy-Lagard†1, and Dirk Iwata-Reuyl†2

From the †Department of Chemistry, Portland State University, Portland, Oregon 97207 and the ‡Department of Microbiology and Cell Science, University of Florida, Gainesville, Florida 32611-0700

Background: The modified nucleoside t^6A is important for tRNA function.

Results: The proteins YrdC/YgjD/YeaZ/YjeE are necessary and sufficient for the biosynthesis of t^6A in bacteria.

Conclusion: Only the universal protein families YrdC and YgjD are conserved in the biosynthesis of t^6A among all organisms.

Significance: Elucidating the enzymes responsible for t^6A biosynthesis, a universal modification of tRNA, is central to understanding its physiological role.

The anticodon stem-loop (ASL) of transfer RNAs (tRNAs) drives decoding by interacting directly with the mRNA through codon/anticodon pairing. Chemically complex nucleoside modifications found in the ASL at positions 34 or 37 are known to be required for accurate decoding. Although over 100 distinct modifications have been structurally characterized in tRNAs, only a few are universally conserved, among them threonylcarbamoyl adenosine (t^6A), found at position 37 in the anticodon loop of a subset of tRNA. Structural studies predict an important role for t^6A in translational fidelity, and in vitro work supports this prediction. Although pioneering work in the 1970s identified the fundamental substrates for t^6A biosynthesis, the enzymes responsible for its biosynthesis have remained an enigma. We report here the discovery that in bacteria four proteins (YgjD, YrdC, YjeE, and YeaZ) are both necessary and sufficient for t^6A biosynthesis in vitro. Notably, YrdC and YgjD are members of universally conserved families that were ranked among the top 10 proteins of unknown function in need of functional analysis. We present here two lines of evidence supporting the involvement of YrdC and YgjD in t^6A biosynthesis. First, both families are involved in t^6A biosynthesis in vivo. Recent genome and genetic studies linked the YrdC/Sua5 (18) and YgjD/Kae1/Qri7 (10, 19) families to t^6A biosynthesis. Notably, both families are universally conserved and were ranked among the top 10 proteins of unknown function in need of functional characterization (20). However, attempts to reconstitute the pathway in vitro with purified recombinant proteins were unsuccessful (10, 19), suggesting that other proteins might be involved.

Nucleoside modification is an intrinsic feature in the maturation of many RNA species (1), and is especially prevalent in transfer RNA (tRNA), where over 100 modified nucleosides have been structurally characterized (2). Particularly striking is the presence of structurally complex modified nucleosides in tRNA, where over 100 modified nucleosides are known to be required for accurate decoding. Although over 100 distinct modifications have been structurally characterized in tRNAs, only a few are universally conserved, among them threonylcarbamoyl adenosine (t^6A) (4), a modified nucleoside located adjacent to the anticodon at position 37 in tRNAs responsible for decoding ANN codons (Fig. 1), is an example of this phenomenon. Structural studies predict an important role for t^6A in translational fidelity by preventing the formation of a U33-A37 across-the-loop base pairing interaction, as well as allowing cross-strand stacking of A_{38} and t^6A_{37}, with the first position of the codon (5–8). In vivo work is consistent with this prediction as yeast mutants devoid of t^6A exhibit translation related phenotypes such as initiation and frame maintenance defects and increased nonsense suppression (9–11).

Elucidation of the biochemical pathways responsible for RNA modifications has been an especially challenging problem (12) perhaps nowhere as striking as for t^6A. Despite the presence of t^6A in all domains of life, and the discovery over 30 years ago that t^6A biosynthesis required ATP, threonine, and bicarbonate (13–17), the biosynthetic enzyme or enzymes have remained undiscovered. Recently, comparative genomic and genetic studies linked the YrdC/Sua5 (18) and YgjD/Kae1/Qri7 (10, 19) families to t^6A biosynthesis. Notably, both families are universally conserved and were ranked among the top 10 proteins of unknown function in need of functional characterization (20). However, attempts to reconstitute the pathway in vitro with purified recombinant proteins were unsuccessful (10, 19), suggesting that other proteins might be involved.

Clues to the identity of the remaining t^6A biosynthetic enzymes came from two observations: first, YgjD was shown to form an essential interaction network with the YeaZ (a paralog of YgjD) and YjeE proteins (21). Indeed, the essentiality phenotype of all three corresponding deletion mutants is suppressed by overexpressing the response regulator RstA, and YeaZ was shown to bind both YgjD and YjeE (21). Second, complementation of the essentiality phenotype of the Escherichia coli ygjD deletion mutant by the Bacillus subtilis ygjD gene required coexpression with the B. subtilis yeaZ gene (10), suggesting that the YgjD/YeaZ physical interaction is specific and relevant to t^6A biosynthesis in vivo. These observations together with the strong physical clustering of ygjD, yeaZ, and yjeE genes (10, 21) led us to investigate whether YeaZ and YjeE might also be involved in t^6A biosynthesis in bacteria.

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† To whom correspondence may be addressed: Dept. of Microbiology and Cell Science, University of Florida, P.O. Box 110700, Gainesville, FL 32611. Tel.: 352-392-9416; Fax: 352-392-5922; E-mail: vcrecy@ufl.edu.
‡ To whom correspondence may be addressed: Dept. of Chemistry, Portland State University, PO Box 751, Portland, OR 97207. Tel.: 503-725-5737; Fax: 503-725-9525; or E-mail: iwatareuyld@pdx.edu.
§ The abbreviations used are: t^6A, threonylcarbamoyl adenosine; AMPPNP, adenosine 5′-(β,γ-imino)triphosphate.

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EXPERIMENTAL PROCEDURES

General—Oligonucleotides were purchased from Integrated DNA Technologies, Inc. Protein concentrations were based on the Bradford dye-binding procedure (22). DNA sequencing was carried out by the OHSU core sequencing laboratory. We thank Christopher Lima (Sloan-Kettering Institute) for the generous gift of the plasmid pSMT3.

Strains and Growth Conditions—Cells were grown in LB at 37 °C unless otherwise noted. When necessary, LB was supplemented with kanamycin (50 μg/ml), ampicillin (100 μg/ml) isopropyl-β-d-thiogalactopyranoside (IPTG, 1 mM), and ZnCl₂ (0.5 M). Plasmids were transformed into E. coli NovaBlue cells for isolation of plasmid DNA, or E. coli BL21(DE3) for production and isolation of recombinant proteins.

Construction of Expression Constructs for YeaZ (pCDII29) and YjeE (pCDII30)—PCR-based cloning of the E. coli yeaZ and yjeE genes was carried out using genomic DNA from E. coli K12 and the following primers: YjeE Forward: 5'-GGTATTGAGG-GTCGCATGATGAATCGAGTAATTCCG-3'; YjeE Reverse: 5'-AGAGGAGAGTTAGAGCCTTAACCGGCTAAACGCG-CCAG-3'; YeaZ Forward: 5'-GGTATTGAGG-GTCGCATGATGAATCGAGTAATTCCG-3'; YeaZ Reverse: 5'-AGAGGAGAGTTAGAGCCTTAACCGGCTAAACGCG-CCAG-3'. PCR reactions contained 500 ng of genomic DNA (E. coli K12), 200 μM dNTPs, 50 pmol primers, 1X Pfu Ultra buffer (supplied by the manufacturer), and 0.25 mg/ml lysozyme. The resulting solutions were then applied to a pre-equilibrated Ni-NTA column. After eluting with 100 mM Tris-HCl (pH 8.0), the protein pools were concentrated (Millipore Centrifugal Filter units), combined with an equal volume of glycerol, and stored at −80 °C for future use.

FIGURE 1. The secondary structure of E. coli tRNA^{15}U(UUU) (a) and the chemical structure of t^6A (b).

Overproduction and Purification of Recombinant, Affinity-tagged YrdC, YeaZ, YjeE, and YgjD—Cultures of transformed cell lines containing pBY215.1 (YrdC), pCDII29 (YeaZ), pCDII30 (YjeE), or pCD174 (YgjD) were grown overnight at room temperature until an A₆₀₀ of 2–3 was obtained. Cells were pelleted (2,500 × g, 15 min, 4 °C) and resuspended to 250 mg/ml in lysis buffer comprised of 100 mM Tris-HCl (pH 8.0), 2.0 mM 2-mercaptoethanol (BME), 1.0 mM phenylmethylsulfonyl fluoride (PMSF), 10% glycerol, and 300 mM KCl or 300 mM KCl (YgjD). Lysozyme (0.25 mg/ml) was then added, and the resulting solutions incubated for 30 min, followed by 3 cycles of freeze thaw and the addition of DNase (10 μg/ml). The solutions were centrifuged (15,000 × g, 20 min, 4 °C), and the supernatants applied to pre-equilibrated Ni-NTA (Qiagen) columns. The columns were washed sequentially with 7 volumes of lysis buffer, 3 volumes of lysis buffer + 20 mM imidazole, 4 volumes of lysis buffer without PMSF + 30 mM imidazole, followed by elution with lysis buffer (without PMSF) + 200 mM imidazole. Fractions containing purified protein were concentrated using Millipore Centrifugal Filter units and subjected to dialysis (Slyde-a-lyzer, Pierce) against 100 mM Tris-HCl (pH 8.0) and 100 mM KCl or 300 mM KCl (YgjD) at 4 °C. The fraction proteins were cleaved overnight at room temperature using either Factor Xa (1 μg/mg, YrdC/YeaZ/YjeE) (New England Biolabs) or ULP1 (10 μg/mg, YgjD) according to the manufacturer’s (Factor Xa) or published (ULP1) protocols (23), and the solutions then applied to a pre-equilibrated Ni-NTA column. After eluting with 100 mM Tris-HCl (pH 8.0), 300 mM KCl, and 2 mM BME, the protein pools were concentrated (Millipore Centrifugal Filter units), combined with an equal volume of glycerol, and stored at −80 °C for future use.
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Construction of tRNA Templates for in Vitro Transcription—
The templates for producing tRNA\textsuperscript{Lys} and tRNA\textsuperscript{Gln} transcripts were produced via a Klonek extension reaction as described (24) with the following primers: Forward tRNA\textsuperscript{Lys}: 5′-AACCATGATACGACTCCTGAG-TTCGTTAGCTAGTGG-ACTG-3′; Reverse tRNA\textsuperscript{Lys}: 5′-TGGG-TTGCAGTAATACGACTCACTATAAGTCCCGTGGGGTAGTGG-TGCCAGTGTAATACGACTCACTATAGGGGTCGTTAGCT-3′; Forward tRNA\textsuperscript{Gln}: 5′-TTGCA-TTAATACGACTCACTATAATCAACTGCTCTACC-3′; Reverse tRNA\textsuperscript{Gln}: 5′-TGGTGGTGTCGTGCAGGATTCGAACCTGCGACCAATTGATTAA-3′.

RNA Transcription and Purification—Transcription reactions to produce a 17-nucleotide RNA corresponding to the anticodon stem-loop of tRNA\textsuperscript{Lys} (17-mer RNA) or the full-length E. coli tRNAs were run overnight at 37 °C in 80 mM HEPES (pH 7.5), 2 mM spermidine, 24 mM MgCl\textsubscript{2}, 2 mM NTPs, 300 pmol template, and 2.5 μg of T\textsubscript{7} polymerase. The template for tRNA\textsuperscript{Lys} production was generated by MvaI digestion of pCDI147 (10). Prior to reactions the templates were heated to 95 °C for 5 min and allowed to anneal slowly by cooling to room temperature over 1 h. The RNA products generated in the transcription reactions were precipitated by the addition of 0.1 volume 8.0 mM ammonium acetate, 3 volumes of 100% ethanol, and cooling at -80 °C for 30 min, then pelleted by centrifugation at 15,000 × g for 30 min at 4 °C, and resuspended in 50 mM HEPES (pH 7.5), 2 mM EDTA. The RNA products were then mixed 1:1 with formamide, boiled for 5 min, and snap cooled on ice before being purified via urea-PAGE electrophoresis (20% 17-mer RNA, 10% tRNA). The RNA was extracted from the gel by crush and soak, then precipitated as above, and resuspended in 50 mM HEPES (pH 7.5) 2 mM EDTA.

Radiochemical Incorporation Assays—Reaction assays measuring the incorporation of \textsuperscript{14}C-threonine into RNA were carried out in a volume of 50 μl containing 5 μM each YrdC, SUMO-YgiD fusion, YeaZ, and YjeE, 100 mM Tris-HCl (pH 8), 300 mM KCl, 5.0 mM DTT, 10 mM ATP, 20 mM MgCl\textsubscript{2}, 1 μM ZnCl\textsubscript{2}, 1 μM MnCl\textsubscript{2}, 1 μM CaCl\textsubscript{2}, and 50 μM 17-mer RNA or full-length tRNA transcript, 50 mM NaHCO\textsubscript{3}, and 50 μM L-\textsuperscript{14}C-threonine (100,000 DPM/assay). When \textsuperscript{14}C-bicarbonate was investigated 100 μM \textsuperscript{14}C-NaHCO\textsubscript{3} (200,000 DPM/assay) and 10 mM L-threonine were present. Assays were run for 20 min at 37 °C, and the RNA precipitated with the addition of 0.1 volume 8.0 mM ammonium acetate and 3 volumes of ethanol, followed by cooling at -80 °C for 30 min. The RNA was then collected by filtration through Whatman GF/B filters, and the filters washed with 70% cold ethanol. The dried filters were combined with RPI\textsuperscript{TM} Econo-Safe counting mixture and counted on a Beckman 6500 liquid scintillation counter.

Enzymatic ATPase Assays—Reaction assays were carried out in a volume of 10 μl containing 5 μM each YrdC, SUMO-YgiD fusion, YeaZ, and YjeE, 100 mM Tris-HCl (pH 8.0), 300 mM KCl, 5.0 mM DTT, 100 μM \textsuperscript{32}P-ATP (10,000 DPM), 20 mM MgCl\textsubscript{2}, 1 μM ZnCl\textsubscript{2}, 1 μM MnCl\textsubscript{2}, 1 μM CaCl\textsubscript{2}, and 40 μM full-length tRNA transcript tRNA\textsuperscript{Lys}, 50 mM NaHCO\textsubscript{3}, and 50 μM L-threonine. The reactions were run for 20 min, then 1 μl aliquots of each assay spotted on PEI-cellulose TLC plates (Silicyle) that were prerun with water and allowed to dry prior to being used for assays. The TLC plates were developed in 0.5 M KH\textsubscript{2}PO\textsubscript{4} (pH 3.5), and the radioactivity quantified by phosphorimaging using a GE Typhoon Trio +.

Mass Spectrometric Analysis—tRNA\textsuperscript{Lys} was placed in reaction buffer containing the same components as the radiochemical incorporation assays, without \textsuperscript{14}C-labeled substrates, at a concentration of 1.8 μg/ml. This reaction mixture was incubated at 37 °C for 30 min before phenol/chloroform extraction and ethanol precipitation. This precipitate was subjected to subsequent treatment with nuclease P1, snake venom phosphodiesterase, and alkaline phosphatase as previously described (25). This treated reaction mixture was analyzed by LC/MS on a Supelco Discovery HS C18 (250 × 2.1 mm, 5 μm) with a mobile phase of 10 mM ammonium acetate (pH 6.0):acetonitrile (linear gradient of 95:5 to 60:40 over 20 min). Mass analysis utilized an LTQ Orbitrap operating in the positive mode; spray voltage 4.00 kV; capillary temp 300 °C; capillary voltage 49; tube lens voltage 100.

Protein Binding Experiments—Proteins were tested pairwise for binding interactions with one protein His\textsubscript{6}-tagged and the other untagged. Proteins were present at 20 μM each in a solution of 100 mM Tris-HCl (pH 8.0), 300 mM KCl, and 2 mM BME (Buffer A), in a final volume of 100 μl. After incubating for 20 min at room temperature the solution was added to 1 ml of Ni-NTA (pre-equilibrated with Buffer A) and exhaustively washed with Buffer A (at least 10 volumes). The bound proteins were then eluted from the column with a solution of Buffer A + 200 mM imidazole. Fractions were pooled and concentrated back to 100 μl using 10 kDa centrifugal filter units (Amicon). Samples were then analyzed by SDS-PAGE.

RESULTS

To test the hypothesis that t\textsuperscript{6}A biosynthesis in E. coli requires YrdC, YgiD, YeaZ, and YjeE, the yjeE and yeaZ genes from E. coli were expressed as N-terminal His\textsubscript{6}-fusion proteins similar to our construct for yrdC expression (10). While recombinant YrdC, YeaZ, and YjeE were all purified in their native form after removal of the fusion tag, recombinant YgiD proved recalcitrant to soluble expression and required expression as an N-terminal His\textsubscript{6}-SUMO fusion (10), which was retained to avoid subsequent precipitation.

The in vitro formation of t\textsuperscript{6}A was first probed using radiochemical based assays in which the incorporation of \textsuperscript{14}C-threonine or \textsuperscript{14}C-bicarbonate into an RNA substrate was measured by liquid scintillation counting after ethanol precipitation of the RNA and collection on glass fiber filters (10). Several RNAs were employed as potential substrates, including unfractionated yeast tRNA from a Δsua5 mutant (18), an unmodified E. coli tRNA\textsuperscript{Lys} and tRNA\textsuperscript{Gln} transcripts produced through in vitro transcription (both naturally t\textsuperscript{6}A containing tRNA), and an unmodified stem-loop RNA corresponding to the ASL of E. coli tRNA\textsuperscript{Lys}, which has recently been shown to bind YrdC (26). We also tested unfractionated wild-type yeast tRNA which is fully modified with t\textsuperscript{6}A, and an in vitro transcribed tRNA\textsuperscript{Gln} from Methanohemobacter thermautotrophicus, a tRNA that does not naturally contain t\textsuperscript{6}A.

Robust incorporation of radioactivity was observed in assays containing \textsuperscript{14}C-threonine or \textsuperscript{14}C-bicarbonate and all four pro-
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FIGURE 2. Enzyme assays of $t^6A$ formation. Assays were carried out as described under “Experimental Procedures” with the components indicated. Proteins were present at 5 μM each, with either [14C]threonine (dark bars, left axis) or [14C]bicarbonate (light bars, right axis) as the labeled substrate. a, assays with various RNA substrates. tRNA$_{Thr}$, tRNA$_{Lys}$, tRNA$_{Gln}$, and 17-mer indicate in vitro transcribed RNA, ΔsuA5 refers to unfraccionated tRNA isolated from a ΔsuA5 yeast strain (17), and WT indicates unfraccionated tRNA isolated from wild-type yeast. b, assays with the indicated proteins or ATP absent from the reaction. c, HPLC chromatogram of nucleosides generated from the digestion and dephosphorylation of tRNA$_{Lys}$ before (bottom trace) and after (top trace) a reaction assay containing YrdC, YgjD, YeaZ, and YjeE. d, partial mass spectrum of compound eluting at ~9.9 min in the chromatogram.

Proteins when the tRNA$_{Thr}$ and tRNA$_{Lys}$ transcripts were used, as well as with unfraccionated yeast tRNA from the suA5 Δ deletion mutant (Fig. 2a), but not when any of the other RNAs were employed. In assays in which one of the proteins or ATP was omitted, no incorporation of radioactivity was observed (Fig. 2b). The observation of specific radiochemical incorporation when either 14C-labeled threonine or bicarbonate is used, and the failure to observe the incorporation of radioactivity when unfraccionated wild-type yeast tRNA or tRNA$_{Gln}$ were used in the assays, is consistent with the formation of $t^6A$, not the non-specific precipitation of the labeled substrate. Furthermore, the failure to observe incorporation of radioactivity when any one of the four proteins is omitted clearly demonstrates that all four are necessary for $t^6A$ biosynthesis. The higher activity observed with the purified tRNA$_{Thr}$ and tRNA$_{Lys}$ transcripts likely reflect the higher concentration of substrate tRNA in these samples compared with unfraccionated tRNA from the ΔsuA5 mutant. While the failure of the ASL of tRNA$_{Lys}$ to serve as a substrate is surprising in the context of its established binding to YrdC (26), it is consistent with prior in vivo studies using Xenopus oocytes in which full-length tRNA in its native structure was shown to be necessary for $t^6A$ formation (27).

As a complement to the radiochemical data, and to unambiguously confirm the formation of $t^6A$, we carried out in vitro reactions with the tRNA$_{Lys}$ transcript and unlabeled substrates, digested the modified tRNA to the free mononucleosides (28), and subjected the reaction to analysis by LC-MS (Fig. 2c). A new peak with the retention time of authentic $t^6A$ is present in the chromatogram of digested tRNA from the reaction assay that is not present in the control sample of unreacted tRNA, and MS analysis of this peak shows it to possess a parent ion with an m/z of 413.14, consistent with protonated authentic $t^6A$, as well as a prominent daughter ion at an m/z of 281.05 corresponding to the protonated base.

Having confirmed the in vitro biosynthesis of $t^6A$, we investigated the specific role of the four proteins in this process. Conversion of bicarbonate to the urea moiety of $t^6A$ requires two activation steps with ATP to satisfy the energetic requirements of the acyl-transfer chemistry (29). The first is needed to form an activated bicarbonate, presumably carboxyl phosphate or carboxy-AMP (Fig. 3, I), which can then condense with either the α-amine of threonine or N$^\beta$ of A$_{37}$, to form an N-carboxyl product (Fig. 3, II). Activation of this product by a second ATP, again via transfer of either phosphate or AMP to generate a second acyl-phospho species (Fig. 3, III), leads to the second condensation reaction with the remaining component to give $t^6A$. Thus, AMP and/or ADP should be a co-product of the reaction.

To elucidate which product of ATP activation is generated in the reaction we employed assays containing [α-32P]ATP so that the product or products formed could be directly observed by TLC analysis. Remarkably, both AMP and ADP are produced (Fig. 4a, lane 2), suggesting activation by two distinct mechanisms. In assays in which one of the proteins is absent, AMP production is seen only when YrdC is present in the assay (Fig. 4a, lanes 3, 5, 6), while the formation of ADP is unaffected by the absence of YrdC (Fig. 4a, lanes 7–8), while AMP formation is eliminated completely in the absence of threonine (Fig. 4a, lanes 7–8). With assays containing all substrates and a single protein (Fig. 4b, lanes 3–6) or pairs of proteins (Fig. 4b, lanes 7–12), AMP production occurs only in the presence of YrdC, where it is comparable to production in a complete assay, while ADP production is significantly decreased in all assays, and eliminated entirely in the assay where YrdC was used alone (Fig. 4b, lane 6). The observation of both AMP and ADP in assays can suggest the presence of contaminating adenylate kinase, however the absence of AMP when threonine is absent but all of the proteins
are present (Fig. 4a, lane 8) indicates that AMP/ADP are legitimate co-products of t^6A biosynthesis.

The above observations clearly show that ATP consumption can be uncoupled from RNA modification, and thus might also occur in the absence of tRNA. Indeed this is the case, when assays lacking tRNA are carried out with varying combinations of proteins and remaining substrates, formation of both AMP and ADP is observed (Fig. 4c). Because uncoupled ATP consumption is common in ATP-dependent systems (30–32) and t^6A formation requires the hydrolysis of two phosphoanhydride bonds, the simplest explanation of the data is that ATP is utilized to generate two types of activated intermediates, an acyl-phosphate and an acyl-adenylate. It is not yet known whether the observed uncoupling is due to partial reactions or hydrolysis, but this issue is currently being investigated.

The observation that AMP is generated only in the presence of YrdC, and is generated with YrdC alone, clearly establishes that YrdC is responsible for AMP formation. ADP production is clearly more complex, as it drops in the absence of any of the remaining three proteins. This is not entirely surprising, as YjeE and an archaeal YgjD homolog (Kae1) have been previously shown to bind ATP and/or possess weak ATPase activity (33, 34), consistent with our data, and YeaZ is a homolog of YgjD, although it lacks the characteristic ATP binding site of other ATPases of the ASKHA superfamily (35).

Three of the proteins that comprise the t^6A biosynthetic machinery have been shown to interact physically with each other (YeaZ binds both YjeE and YgjD) (21, 36) raising the possibility that t^6A formation might occur in a complex involving some or all four proteins. To address this possibility we investigated binding interactions in pull-down experiments utilizing the N-terminal His<sub>6</sub> fusion construct in pairwise experiments in which one protein retained the His<sub>6</sub> fusion and the other had been removed. We confirmed the binding of YeaZ to YjeE and YgjD and the lack of binding between YjeE and YgjD (21, 36) (data not shown). Notably, although we observed that YrdC and YjeE do not interact (Fig. 5), YrdC does bind to both YeaZ and YgjD (Fig. 5), consistent with the formation of a complex or complexes to carry out t^6A biosynthesis.

**DISCUSSION**

While the present data do not allow us to conclude unequivocally which proteins are responsible for the specific catalytic activities required for t^6A formation, our data clearly demonstrate a threonine-dependent ATPase activity for YrdC that generates AMP. These observations are consistent with recent x-ray crystal structures of the *Sulfolobus* YrdC homolog Sua5 containing bound threonine and AMPPNP (37), and HypF, a hydrogenase maturation protein that possesses both YrdC- and YgjD-like domains (38). In the Sua5 structure the α-N of bound threonine points toward the α-phosphate of AMPPNP, with an intervening space capable of accommodating the carboxyl group of an N-carboxy-threonine substrate. Indeed, N-carboxy-threonine has been successfully docked (10) to an earlier structure of Sua5 containing bound AMP (39). In one of the HypF crystals soaked with ATP and carbamoylphosphate, carbamoyl-AMP is bound to the YrdC-like domain, demonstrating that the YrdC-like domain in HypF is capable of supporting the formation of acyl-adenylates. Taken together, our observations, and the structural data above, are consistent with YrdC catalyzing the adenylation of N-carboxy-threonine (Fig. 3, rxn 3). Given the observation that *E. coli* YrdC binds selectively to unmodified tRNA (18) and ASL (26) sequences that correspond to t^6A containing tRNA, YrdC may also catalyze the condensation of N-carboxyAMP-threonine with A<sub>37</sub> (Fig. 3, rxn 4). Elucidating the mechanistic details of these steps is currently underway, as is clarifying the roles of the remaining proteins in the formation of carboxyphosphate and its subsequent condensation with threonine.

Although all four proteins (YrdC, YgjD, YeaZ, and YjeE) are strictly required for t^6A formation in *E. coli*, this may not be the case in all bacteria. Indeed, unlike YrdC, YgjD, and YeaZ, which are found in all sequenced bacteria to date (10), YjeE is absent in many intracellular and symbiotic bacteria (such as *Mycoplasma, Ureaplasma, Wolbachia, Buchnera*, and *Wigglesworthia* species) as well as *Cellulomonas flavi- gena*, suggesting that some bacteria can carry out t^6A synthesis in the absence of YjeE. The absence of both YjeE and

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![Diagram of t^6A biosynthesis]

**FIGURE 3. Illustration of the potential chemical paths and intermediates in t^6A biosynthesis.**
YeaZ in Eukarya and Archaea demonstrates that the functions or functions of YjeE and YeaZ are carried out by other enzymes in these organisms. Since Kae1 (the YjgD homolog) was first identified as a component of the EKC/KEOPS complex involved in telomere maintenance (40) and transcription of galactose- and pheromone-responsive genes (41), natural candidates for these missing partners are the other members of the KEOPS complex: Bud32, Pcc1, and Cgi121. Indeed, yeast bud32 and pcc1 mutants displayed altered t6A contents (9, 19). Conservation of KEOPS complex proteins in Archaea is consistent with a primary role in t6A biosynthesis (a universal modification), but further work is required to biochemically characterize the role of KEOPS complex subunits in t6A synthesis.

While finally resolving the long-standing problem of elucidating the biosynthetic pathway to t6A, the discovery of the enzymes necessary for t6A biosynthesis also clarifies the function of four conserved and essential enzyme families that have been the focus of extensive investigation, and functional speculation, for over a decade (reviewed in Refs. 10 and 9, 21, 42–45). Indeed, now that a specific role in the biosynthesis of t6A has been established for these proteins, the diversity of pleiotropic phenotypes observed in the absence of these proteins or of their homologs in various organisms can be reexamined in light of this defined functionality. Furthermore, the fact that YeaZ and YjeE are specific to bacteria, and that all four proteins are essential in bacteria, establishes t6A biosynthesis as a novel target for antimicrobial development. Given their newly established enzymatic role in t6A biosynthesis, and the previous choice of TsaA (i.e. threonylcarbamoyl-6-adenosine, or t6A) (46) for the methyltransferase involved in the formation of

![TLC analysis of assays with various components of the t6A synthesis system using [α-32P]ATP at 100 μM and the E. coli tRNALys transcript.](image-url)
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FIGURE 5. Protein binding studies. SDS-PAGE analysis of protein pairs, one without the His6 fusion and the other retaining the His6 fusion. Proteins were incubated in buffer for 5 min then applied to a small column of Ni$^{2+}$-affinity resin. The column was washed with buffer to completely remove nonspecifically bound protein (verified by SDS-PAGE), then the bound proteins eluted with buffer containing 200 mM imidazole. Odd-numbered lanes contain protein that did not bind to the column (FT, flow-through), even numbered lanes contain protein that was retained on the column (BD, bound). Far-left lane contains molecular weight markers.

m$^6$A, we propose to rename the YeaZ, YrdC, YgiD, and YjeE enzymes TsAB, TsaC, TsaD, and TseA, respectively.

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