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Discovery and Characterization of the Proteins Involved in the Synthesis of N⁶-
Threonylcarbamoyl Adenosine, a Nucleoside Modification of tRNA

by

Christopher Wayne Deutsch

A dissertation submitted in partial fulfillment of the
requirements for the degree of

Doctor of Philosophy
in
Chemistry

Dissertation Committee:
Dirk Iwata-Reuyl, Chair
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Portland State University
2016

Abstract

N⁶-threonylcarbamoyl adenosine (t⁶A) is a universally conserved tRNA modification found at position 37 of tRNAs which decode ANN codons. Structural studies have implicated its presence as a requirement for the disruption of a U-turn motif in certain tRNAs, leading to the formation of properly structured anticodon stem loop. This structure is proposed to enhance the base pairing between U₃₆ of tRNA and A₁ of the codon which aids in translational frame maintenance.

Despite significant effort since its discovery in the 1970s the enzymes involved in its biosynthesis remained undiscovered. Bioinformatic analysis identified two proteins as likely candidates for t⁶A synthesis, YrdC and YgjD. Subsequent gene knockout experiments in yeast were consistent with their involvement in t⁶A biosynthesis *in vivo*. Furthermore, clustering between the bacterial genes *ygjD*, *yeaZ* and *yjeE* as well as the identification of a protein interaction network between YgjD, YeaZ, and YjeE suggested that YeaZ and YjeE might be involved in t⁶A biosynthesis.

The genes encoding *ygjD*, *yeaZ*, *yrdC* and *yjeE* were cloned from *E. coli* and the recombinant protein was purified. Experiments analyzing the incorporation of [U-¹⁴C]-L-threonine and [¹⁴C]-bicarbonate (substrates previously indicated in its biosynthesis) into tRNA in the presence of these four proteins demonstrated the first reconstitution of the t⁶A pathway *in vitro*. LC-MS analysis verified the formation of t⁶A, and these proteins were renamed TsaD (YgjD), TsaB

(YeaZ), TsaC (YrdC), and TsaE (YjeE).

Biochemical characterization of this pathway suggested that the formation of t⁶A proceeds through an unstable threonylcarbamoyl adenosine monophosphate (TC-AMP) intermediate, which is produced by TsaC from its substrates CO₂, L-threonine and ATP. To investigate this reaction in more detail a coupled assay was developed, enabling sensitive detection of turn over. TsaC is a promiscuous enzyme which readily accepts several amino acids as substrates. The formation of t⁶A from TC-AMP is catalyzed by TsaD, TsaB, and TsaE. Of these three proteins only TsaD is universally conserved suggesting it is the protein catalyzing the transfer of the threonylcarbamoyl moiety to A₃₇ of tRNA. This transfer is not promiscuous as only TC-AMP serves as an efficient substrate for t⁶A formation. Structural investigation of these proteins are consistent with the formation of a single protein complex potentially alleviating issues with the reactivity and instability of TC-AMP.

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Glossary

Δ sua5 tRNA: Total transfer ribonucleic acid from yeast cells lacking the *tsaC* gene

ψ (nucleoside): Pseudouridine

μ g: Microgram

μ L: Microliter

μ M: Micromolar

6 (nucleoside): N⁶-threonylcarbamoyl adenosine

6x Histidine: Hexahistidine

7 (nucleoside): 7-methylguanosine

A (amino acid): Alanine

A (nucleotide): Adenosine-5'-diphosphate

A (ribosome): Aminoacyl site of ribosome

A₃₄₀: Absorbance at 340 nanometers

A₆₀₀: Optical density at 600 nanometers

AA: Amino acid

aaRS: Aminoacyl transfer ribonucleic acid synthetase

Acceptor stem: Transfer ribonucleic acid, site of aminoacylation

ADP: Adenosine-5'-diphosphate

Ala: Alanine

ALS: Anticodon stem and loop

AMP: Adenosine-5'-monophosphate

Arg: Arginine

ArgS: Arginyltransfer ribonucleic acid synthase

Asp: Aspartic acid

Asn: Asparagine

ATP: Adenosine-5'-triphosphate

BME: 2-mercaptoethanol

BP: Nucleotide base pair

BPB: Bromophenol blue

B. subtilis: *Bacillus subtilis*

C (nucleotide): Cytidine-5'-triphosphate

CAMP: Cationic antimicrobial peptides

CCA: Cytosine-cytosine-adenine

cm: Centimeter

CTP: Cytidine-5'-triphosphate

CV: Column volume

Cys: Cysteine

Cys-tRNA^{Cys}: Cysteine specific transfer ribonucleic acid, aminoacylated with cysteine

D (nucleotide): Dihydrouridine

D stem and loop: Transfer ribonucleic acid stem loop containing dihydrouridine modification

DEPC: Diethylpyrocarbonate

Dicer: Endoribonuclease which cleaves double stranded ribonucleic acids

DNA: Deoxyribonucleic acid

DNase: Deoxyribonuclease

dNTPs: Deoxyribonucleotide triphosphates

DPM: Disintegration per minute, units to express radioactivity

DTT: Dithiothreitol

E (ribosome): Exit site of ribosome

E. coli: *Escherichia coli*

EDTA: Ethylenediaminetetraacetate

eEF-1: Eukaryotic elongation factor 1

EF-TU: Elongation factor thermo unstable

eIF2: Elongation factor 2

eIF4: Elongation factor 4

eIF4A: Elongation factor 4A

FPLC: Fast protein liquid chromatography

G (nucleotide): Guanosine-5'-triphosphate

g⁶A: N⁶-glycinylicarbamoyl adenosine

GC-AMP: Glycinylicarbamoyl adenosine monophosphate

Gcn2P: Eukaryotic elongation factor 2 protein kinase

GDP: Guanosine-5'-diphosphate

GlcNAc: N-acetylglucosamine

Gln: Glutamine

Glu: Glutamic acid

Gly: Glycine

GTP: Guanosine-5'-triphosphate

HEPES: 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid

His: Histidine

HPLC: High pressure liquid chromatography

hn⁶A: N⁶-hydroxynorvalylcarbamoyl adenosine

HNC-AMP: Hydroxynorvalylcarbamoyl adenosine monophosphate

H. sapiens: *Homo sapiens*

I (nucleotide): Inosine

IEX: Ion exchange chromatography

Ile: Isoleucine

IPTG: Isopropyl-β-D-thiogalactopyranoside

K: Lysine

kV: Kilovolt

L (volume): Liter

LB: Luria Broth

LC: Liquid chromatography

LC-MS: Liquid chromatography mass spectrometry

Leu: Leucine

Lys: Lysine

M: Molar

m⁶A: N⁶-methyladenosine

m¹G: 1-methylguanosine

m⁵U: 5-methyl uridine

MES: 2-(N-Morpholino)ethanesulfonic acid

Met: Methionine

metRS: Methionyl-tRNA synthetase

Met-tRNA_i^{Met}: Methionine aminoacylated, methionine specific transfer ribonucleic acid

min: Minute

mg: Milligram

mm: Millimeter

mM: Millimolar

MprF: transfer ribonucleic acid dependent aminoacyl transferase

mRNA: Messenger ribonucleic acid

MS: Mass spectrometry

ms²t⁶A: 2-methylthio-N⁶-threonylcarbamoyl adenosine

M. thermoautotrophicus: *Methanothermobacter thermoautotrophicus*

MurNAc: N-acetylmuramic acid

N (amino acid): Asparagine

NADH: Nicotinamide adenine dinucleotide, reduced form

NAD⁺: Nicotinamide adenine dinucleotide, oxidized form

Ni-NTA: Nickel nitrilotriacetic acid

nm: Nanometer

NMR: Nuclear magnetic resonance spectroscopy

NT: Nucleotide

N-terminus: The amino terminus of a peptide

NTPs: Ribonucleotide triphosphates

P (ribosome): Peptidyl site of ribosome

PAGE: Polyacrylamide gel electrophoresis

PCR: Polymerase chain reaction

PEI-Cellulose: Polyethyleneimine cellulose

Phe: Phenylalanine

Phosphoseryl-tRNA^{Cys}: Phosphoserine aminoacylated, cysteine specific transfer ribonucleic acid

pmol: Picomol

PMSF: Phenylmethylsulfonyl fluoride

ppGpp: 5'-diphosphate 3'-diphosphate guanosine

Pi: Phosphate

PIPES: 1,4-Piperazinediethanesulfonic acid

PPi: Pyrophosphate

pppGpp: 5'-triphosphate 3'-diphosphate guanosine

Pro: Proline

RCF: Relative centrifugal force

RelA: Ribosome-associated ppGpp synthase

S (nucleoside): 5-methylaminomethyl-2-thiouridine

SEC: Size exclusion chromatography

SepCysS: O-phospho-L-seryl-tRNA:Cys-tRNA synthase

SpoT: 5'-diphosphate 3'-diphosphate guanosine hydrolase and synthase

SUMO: Small Ubiquitin-like Modifier Protein

R (amino acid): Arginine

RelA: Ribosome-associated ppGpp synthase

RNA: Ribonucleic acid

RNase: Ribonuclease

RNase P: Ribonuclease which cleaves the 5' end of transfer ribonucleic acid

RNase Z: Ribonuclease which cleaves the 3' end of transfer ribonucleic acid

s⁶A: N⁶-serinylcarbamoyl adenosine

S nucleoside: 5-methylaminomethyl-2-thiouridine

SC-AMP: Serinylcarbamoyl adenosine monophosphate

S. cerevisiae: *Saccharomyces cerevisiae*

SDS-PAGE: Sodium dodecyl sulfate polyacrylamide gel electrophoresis

Ser: Serine

T (nucleoside): Thymine

t⁶A: N⁶-threonylcarbamoyl adenosine

TC-AMP: Threonylcarbamoyl adenosine monophosphate

Thr: Threonine

TLC: Thin layer chromatography

TobZ: O-carbamoyltransferase

Tris-HCl: Tris(hydroxymethyl)aminomethane hydrochloride

tRNA: Transfer ribonucleic acid

tRNA^{Ala}: Alanine specific transfer ribonucleic acid

tRNA^{Asp}: Aspartic acid specific transfer ribonucleic acid

tRNA^{Lys}(UUU): Lysine specific transfer ribonucleic acid, codon UUU

tRNA^{Phe}: Phenylalanine specific transfer ribonucleic acid

tRNA^{Met}: Methionine specific transfer ribonucleic acid used for initiation

Trp: Tryptophan

t_{sa}B: N⁶-threonylcarbamoyl adenosine gene B

T_{sa}B: N⁶-threonylcarbamoyl adenosine protein B

t_{sa}C: N⁶-threonylcarbamoyl adenosine gene C

T_{sa}C: N⁶-threonylcarbamoyl adenosine protein C

t_{sa}D: N⁶-threonylcarbamoyl adenosine gene D

T_{sa}D: N⁶-threonylcarbamoyl adenosine protein D

t_{sa}E: N⁶-threonylcarbamoyl adenosine gene E

T_{sa}E: N⁶-threonylcarbamoyl adenosine protein E

T stem and loop: tRNA stem loop containing thymine-pseudouridine-cytosine (TΨC) modification

Tyr: Tyrosine

U (Nucleotide): Uridine-5'-triphosphate

UNN: Uridine, any nucleotide, any nucleotide

Urea-PAGE: Urea polyacrylamide gel electrophoresis

UTP: Uridine-5'-triphosphate

UTR: Untranslated region

Val: Valine

wY: Wybutosine

X (nucleoside): 3-(3-amino-3-carboxypropyl)uridine

XC: Xylene cyanol

XC-AMP: Derivative of N⁶-threonylcarbamoyl adenosine where X = any amino acid

yeaZ: N⁶-threonylcarbamoyl adenosine gene B

YeaZ: N⁶-threonylcarbamoyl adenosine protein B

ydiE: *Bacillus subtilis* N⁶-threonylcarbamoyl adenosine gene D

ydiC: *Bacillus subtilis* N⁶-threonylcarbamoyl adenosine gene B

ygjD: N⁶-threonylcarbamoyl adenosine gene D

YgjD: N⁶-threonylcarbamoyl adenosine protein D

yjeE: N⁶-threonylcarbamoyl adenosine gene E

YjeE: N⁶-threonylcarbamoyl adenosine protein E

yrdC: N⁶-threonylcarbamoyl adenosine gene C

YrdC: N⁶-threonylcarbamoyl adenosine protein C

1.0 Introduction

1.1 Transfer Ribonucleic Acids

tRNA Structure

Transfer ribonucleic acids (tRNAs) are small single stranded ribonucleotides which are essential to protein biosynthesis. Through interactions between both nucleic acids and proteins they serve as adapter molecules in translation.

Canonical tRNAs consist of 73-94 ribonucleotides. Their secondary structure is often represented by a clover-leaf comprised of four stems and 3 loops (D, T, anticodon and acceptor stems and D, T, anticodon loops) (Figure 1). The acceptor stem contains 7-9 base pairs which include the phosphorylated 5' terminal nucleotide, the 3' terminal nucleotide, and a cytosine-cytosine-adenine (CCA) terminus (appended to the 3' terminus of the tRNA). The D stem is composed of 4-6 base pairs ending in a 4-12 nucleotide loop containing dihydrouridine (D). The anticodon stem is composed of 6 base pairs ending in a 7 nucleotide loop which contains the anticodon. The T stem is composed of 4-5 base pairs ending in a 7 nucleotide loop which contains the sequence thymine-pseudouridine-cytosine (TΨC) (1). In some tRNAs there is an additional variable loop between the T and anticodon stems which is typically 4-23 nucleotides in length (2).

The universally conserved tertiary structure of tRNAs is the result of coaxial stacking of RNA helices (3, 4) (Figure 2). This structure is formed as a

result of the acceptor stem stacking with the T stem and the D stem stacking with the anticodon stem. All tRNAs share this common tertiary structure which allows for their transfer between the peptidyl and aminoacyl sites of the ribosome.

Canonical Function

Transfer ribonucleic acids are adapter molecules which bridge the gap between the genetic information storage of nucleic acids and the amino acid sequence of proteins. They accomplish this role through the addition of an amino acid (AA) to their 3' terminus (aminoacylation) by aminoacyl tRNA synthetases (aaRS). Specific interactions between tRNAs and aminoacyl tRNA synthetases confer specificity of tRNA aminoacylation by amino acids. These aminoacylated-tRNAs subsequently serve as activated intermediates in protein translation, allowing for the transfer of an amino acid to a growing polypeptide chain. After aminoacylation, a tRNA is delivered to the ribosome with the aid of elongation factors (EF-TU in bacteria, eEF-1 in eukaryotes) (5). At the ribosome the aminoacyl-tRNA interacts with messenger RNA (mRNA) via a three base pair interaction between the codon of mRNA and the anticodon of tRNA. Once the appropriate tRNA is bound, the ribosome catalyzes the transfer an amino acid from its 3' terminus to the growing polypeptide chain (Figure 3).

tRNA Maturation

tRNA maturation is a complex and necessary sequence of events which

convert the pre-tRNA (the initial transcription product of a tRNA gene by an RNA polymerase) to a fully functional mature tRNA. Initially, RNA polymerase transcribes a pre-tRNA consisting of the canonical nucleobases cytosine (C), adenine (A), uracil (U), and guanine (G). This pre-tRNA may contain introns which are spliced out by tRNA endonucleases in archaea and eukaryotes (6-8). In bacteria, introns contained in the tRNA gene are self-splicing through the formation of a catalytically active RNA structure (a ribozyme) (9-11). Additionally, the 5' and 3' termini of the pre-tRNA are cleaved by RNaseP and RNaseZ respectively (8, 12, 13). Subsequently, this cleaved transcript is further modified by the addition of a CCA terminus to the 3' end by a nucleotidyl transferase. Before the tRNA can become fully mature it is exported to the cytoplasm (in eukaryotes) where it undergoes extensive nucleoside modification (14-16). The order of maturation, and the steps required for maturation, are not universally conserved.

Nucleoside Modification

Nucleoside modification is prevalent in tRNAs, with over 100 modifications being characterized to date (17, 18). Typically 10-25% of nucleosides are modified in tRNA (19). These are broadly grouped into two categories, simple or hyper-modifications (Figure 4) (20), and all are derived from the canonical nucleobases A, U, C, and G (21). Simple modifications typically involve relatively minor chemical modifications such as methylations, reductions, and

isomerizations, and are generally formed in a single enzymatic step. Hyper-modification typically imparts substantial changes in the nucleotide structure, and involves multiple enzymatic steps to complete. The importance of modified nucleosides can be highlighted by the abundance of genes involved in modifications of nucleotides (~1% of an organisms genome) (22).

Function of Modified Nucleobases

The function of many modifications are yet to be elucidated but are proposed to play roles in translation, tRNA identity and/or stability/folding. Modifications occurring in the main body of tRNAs often influence stability or folding properties. For example, the loss of T₅₄ leads to a reduction in thermostability of tRNA^{Phe} and tRNA^{fMet}, with the T_m decreasing by 2-6 °C (23, 24). The loss of m¹A₉ in tRNA^{Lys} leads to an alternative structure (25) and the lack of Ψ can destabilize helices (26). The biological consequences of these structural changes are not entirely understood, but emerging evidence suggests the lack of modifications leads to degradation of the tRNA (27). Modifications at various positions influence the identity of tRNAs. For example, lysidine at position 34 of tRNA^{Ile} prevents misacylation by methionyl-tRNA synthetase (metRS) (28), and m¹G₃₇ of tRNA^{Asp} prevents misacylation by arginyltransfer ribonucleic acid synthase (ArgS) (29).

An abundance of these modifications occur in or around the anticodon stem loop (ASL), typically improving translational fidelity and efficiency.

Modifications found in the ASL frequently effect tRNA function by regulating codon-anticodon interactions. Positions 34 and 37 are the most frequently modified tRNA positions, often serving to regulate wobble-base pair interactions. These interactions are discussed in detail in the following section.

Degeneracy of the Genetic Code and the Importance of the ASL

Degeneracy of genetic code refers to the fact that each codon is specific for an amino acid (or encodes a stop) but some amino acids are coded for by more than one codon (Table 1). The limited number of tRNAs available for translation (typically about 40 per organism vs 64 codons) leads to the requirement that some tRNA recognize more than one codon (21, 30).

These observations led Francis Crick to propose the wobble hypothesis in 1966 (31). The wobble hypothesis suggested that the first two base pairings of the codon to the anticodon would be canonical Watson-Crick base pairs and would provide coding specificity, while the third nucleotide of the codon (wobble position) would determine the number of codons that a tRNA would recognize. Specifically, if the wobble position was a C or A only the Watson-Crick base pair would be possible. Conversely, if the wobble position was a U or G more than one base pairing would be possible (G with C or U, U with A or G). These properties would allow for some tRNAs to recognize more than one codon, solving the problems associated with the limited number of tRNAs available for decoding.

The first tRNA sequenced (*Saccharomyces cerevisiae* tRNA^{Ala}) was found to contain an inosine at the wobble position. This was important as the replacement of an adenosine with inosine substantially increases the number of codons which can be recognized (A with U vs I with A , U, or C) consistent with the wobble hypothesis, and encouraging the investigation of the importance of nucleoside modification in the ASL (21, 27).

In the 50 years since Crick's proposal many exciting discoveries on the importance of modification of the ASL have been observed, leading to an "expanded wobble hypothesis" (Table 2) (21). This hypothesis includes modifications occurring at position 34 (wobble) and 37 (conserved purine adjacent to the anticodon) of the anticodon stem loop.

The wobble position is commonly modified in order to expand recognition and selectivity while maintaining the required ASL architecture for translation. Inosine, lysidine, 5-methylene uridine derivatives, 2-thiouridine derivatives, and the 5-oxyuridine derivatives have all been shown to play critical roles in wobble base pairing (Figure 5). It is now understood that approximately 30-40% of all codon recognition occurs through wobble base pairing, underlying its importance in translation (21).

The conserved purine 37 adjacent to the anticodon has also been shown to be critical to translation. Its modification has been proposed to be linked to base pairing between the first base of the codon and the third of the anticodon. Specifically, N⁶-isopentyladenosine (or derivatives) are found in tRNA which

decode UNN codons while t⁶A (or derivatives) are found in tRNA which decode ANN codons (where N = any nucleobase). It is proposed that these modified bases are necessary to increase the stability of U₃₆-A₁ or A₃₆-U₁ base pairings while codon-anticodon pairings involving a C₃₆-G₁ or G₃₆-C₁ would be stable enough to not require modification of the 3' adjacent purine (21, 30).

Regulation a Non-canonical Function of tRNA

Recently, tRNAs have been shown to perform numerous additional functions in both prokaryotes and eukaryotes (Figure 6) (27, 32, 33). Non-aminoacylated (uncharged) tRNAs have important roles in signaling and regulation of metabolic and cellular processes. Numerous examples of this have been reported and include the stringent response, the T box control system, activation of the protein kinase Gcn2p, accumulation of tRNA in the P site of the ribosome, and the formation of tRNA fragments.

The stringent response is a global regulatory system which is mediated by the production of alarmone, 5'-diphosphate 3'-diphosphate guanosine (ppGpp), and 5'-triphosphate 3'-diphosphate guanosine (pppGpp). In *E. coli*, the production of ppGpp is mediated by two enzymes, RelA and SpoT. RelA is a ribosome-associated ppGpp synthase which uses uncharged tRNAs as effector molecules to activate its synthesis of pppGpp and ppGpp from guanosine-5'-triphosphate (GTP) or guanosine-5'-diphosphate (GDP) and adenosine 5'-triphosphate (ATP). The enzyme SpoT is a dual ppGpp hydrolase/synthase

which is currently poorly understood. ppGpp then serves as an effector molecule binding between the ω and β' subunits of RNA polymerase to downregulate global transcription levels (34).

The T box control system is found in many gram negative bacteria such as *Bacillus subtilis* (*B. subtilis*). The T box regulatory system is controlled by the binding of tRNA to specific, conserved regions in the 5' untranslated region (UTR) of mRNA known as T box leader sequences. The T box leader sequence acts as a riboswitch which prevents translation in the presence of an aminoacyl-tRNA while allowing translation in the presence of an uncharged tRNA through the formation of terminator or anti-terminator structures, respectively. Numerous amino acid related genes are controlled by this mechanism including the isoleucyl-tRNA synthetase in *Actinobacteria* (33).

In eukaryotes, uncharged tRNAs serve as effector molecules to activate the protein kinase Gcn2p. Once activated this kinase phosphorylates serine 51 of the α subunit of eIF2. eIF2 is a protein involved in translation initiation (specifically the binding of GTP and Met-tRNA^{Met}) which upon phosphorylation is downregulated, lowering the overall level of gene translation (35).

In both eukaryotes and bacteria uncharged tRNAs can accumulate in the P site of the ribosome. This accumulation inhibits protein synthesis.

tRNAs regulate gene expression through the production of tRNA fragments. These fragments can function as other small non-coding RNA (small interfering RNA, micro RNA, etc.) which bind mRNA for degradation. tRNA

fragments have also been suggested to interfere with translational initiation through displacement of eIF4/eIF4A from capped and uncapped mRNA (36, 37). Recent studies have also indicated that tRNAs and their fragments can play a critical role in regulating apoptosis in the cell (38). Cytochrome c induced caspase-9 activation leads to apoptosis of the cell through a process known as the intrinsic pathway. Microinjections of tRNA have been shown to reduce this activation while injection of tRNA specific RNase (leading to the formation of tRNA fragments) enhances it. Taken together it is likely that tRNA binds cytochrome c, preventing caspase-9 activation while tRNA fragments enhance caspase-9 activation by cytochrome c.

Aminoacyl-tRNA as substrates in cellular processes

Aminoacyl-tRNAs serve as substrates for a variety of cellular processes beyond their canonical role in translation. An example of this is the non-ribosomal addition of amino acids to proteins targeting them for degradation. The transfer of an amino acid to the N-terminus of proteins by an aminoacyl-tRNA transferase can drastically influence the rate of degradation of the protein. In β -galactosidase the addition of amino acids can influence the stability of the protein significantly (half lives of 3 minutes to over 20 hours). This trend of protein instability due to an N-terminal amino acid is called the N-end rule and has been observed in bacteria, plants, fungi and mammals (39).

Additionally, aminoacyl-tRNAs are used in the non-ribosomal formation of

peptides bonds in peptidoglycan, a critical structural element of bacterial cell walls. Peptidoglycan is a polymer of N-acetylglucosamine (GlcNAc) and N-acetylmuramic acid (MurNAc). The lactyl groups of MurNAc are further modified with the substitution of small penta stem peptides. These penta stem peptides are then either directly cross-linked or cross-linked with an additional amino acid. The crosslinking of penta stem peptides with additional amino acids is dependent upon aminoacylated-tRNAs as a substrate for aminoacyl-tRNA ligases (40).

Aminoacyl-tRNAs serve as substrates in the modification of phospholipids in the cell membrane. One example of this is a response to cationic antimicrobial peptides (CAMPs) which have strong affinities for negatively charged bacterial lipids. Specifically, the MprF enzyme aminoacylates anionic phospholipids with L-alanine or L-lysine using aminoacylated tRNAs as substrates. This aminoacylation imparts positive charges preventing the association between bacterial lipids and CAMPs (41). Furthermore aminoacyl-tRNA are substrates for the production of antibiotics (valanimycin, pacidamycin, cyclopeptide synthesis, etc.), precursors for other aminoacyl-tRNA (phosphoseryl-tRNA^{Cys} conversion to Cys-tRNA^{Cys} by SepCysS), and the formation of tetrapyrrole (42, 43).

1.2 N⁶-threonylcarbamoyl adenosine

N⁶-threonylcarbamoyl adenosine (t⁶A) is a nucleoside modification found in the ASL at position 37 of tRNAs which decode ANN codons, and is one of the 15

known universally conserved modifications. It is essential in most bacterial systems while being dispensable in some eukaryotic systems (44). Despite not being essential in model eukaryotic systems its absence leads to severe growth defects.

Radioactive substrate incorporation examined in the 1970s showed its biosynthesis was dependent upon threonine, bicarbonate and ATP (45-50) (Figure 7). Numerous structural and biochemical studies (51-58) have provided a great deal of insight into the function of t⁶A. These works were consistent with t⁶A improving translational fidelity by promoting cross-strand stacking of t⁶A₃₇ and A₃₈ with the first position of the codon and preventing across-the-loop base pairing of U₃₃-A₃₇, allowing for a properly structured ASL (Figure 8) (53-56). This hypothesis is supported by *in vivo* work, as yeast mutants deficient in t⁶A have increased rates of initiation and frame maintenance defects (51, 52). Additionally, t⁶A modification appears to be required for tRNA^{Lys} translocation (59, 60).

Elucidation of RNA modification pathways is a particularly challenging problem. This is primarily due to the lack of obvious phenotypes of modification deficient strains and the difficulty of biochemical assays used to detect these modifications. Despite t⁶A being found in all domains of life, the discovery of its biochemical precursors over 30 years ago, and the presence of obvious phenotypes in deficient strains, the proteins involved in the biosynthesis pathway had remained undiscovered.

Bioinformatic analysis identified two protein families which were potential candidates for t⁶A biosynthesis, YgjD (TsaD) and YrdC (TsaC). These proteins were of unknown function and universally conserved. Significantly, the YgjD and YrdC protein families were found fused in proteins catalyzing chemistry similar to what is expected in t⁶A biosynthesis and were listed by Galperin *et al.* (61) as among the top ten proteins of unknown function in need of functional characterization. An example of YgjD and YrdC domain fusion is the O-carbamoyltransferase TobZ (Figure 9). TobZ carbamoylates the antibiotic tobramycin to form nebramycin. This is accomplished by the formation of a carbamoyladenylate by a YrdC (TsaC) like domain. This intermediate is subsequently channeled to a Kae1 (TsaD) like domain, where carbamoyl transfer occurs (Figure 10) (62). Yeast knock out strains of *sua5* (*tsaC*) or *kae1* (*tsaD*) genes confirmed the involvement of these proteins in t⁶A biosynthesis as the loss of these genes lead to the absence of t⁶A *in vivo* (Figure 11) (51, 52).

In addition to t⁶A, numerous related nucleoside modifications have been identified (Table 3, Figure 12). These modifications differ from t⁶A by either the substitution of threonine with another amino acid (N⁶-glycinylocarbamoyl adenosine, N⁶-hydroxynorvalylocarbamoyl adenosine) or by additional chemical appendages in addition to t⁶A (2-methylthio-N⁶-threonylocarbamoyl adenosine, N⁶-methyl-N⁶-threonylocarbamoyl adenosine). The similarity of these modifications with t⁶A suggested that some common biochemical precursors and/or enzymes may be involved in their synthesis. These modifications have only been

identified in tRNA with little to no characterization on their effects and biosynthesis occurring to date.

Specific Aims

The overall objectives of this work were to identify the proteins involved in t⁶A biosynthesis, elucidate the chemical mechanism of its formation, and determine the function of the proteins involved in its synthesis.

1. Identification of Proteins Involved in the Biosynthesis of t⁶A

The goal of these investigations was to determine both the proteins involved in t⁶A biosynthesis as well as the minimum protein requirements for its formation.

2. Elucidation of Structural Interactions of t⁶A Proteins

The goal of these experiments was to elucidate the structural interactions of the proteins involved in t⁶A biosynthesis.

3. Characterization of the t⁶A Biosynthetic Pathway, Mechanism and Substrate Specificity

The goal of these experiments was to determine the minimum substrate requirements for t⁶A formation, the chemical route its formation, and the substrate specificity of the enzymes involved in its biosynthesis.

2.0 Materials and Methods

2.1 General

Instrumentation

Ultraviolet-visible (UV-VIS) spectrophotometry was carried out using a Varian Cary 100 or Beckman DU-520 spectrophotometer. High pressure liquid chromatography (HPLC) was accomplished using an Agilent 1100 HPLC. Phosphorimaging experiments were conducted using a GE Typhoon Trio⁺. Scintillation counting was completed using either a Beckman LS6500 Liquid Scintillation Counter or a Hidex 300 SL. Analytical SDS-PAGE and Urea-PAGE analysis was performed using the Mini Protean III apparatus from BioRad. Nitrocellulose transfer was performed using a BioRad Trans-Blot Semi-Dry Transfer Cell. Preparative Urea-PAGE utilized a Dan-Kar Corp vertical gel apparatus. Centrifugation was performed using an Avanti J-20 XP centrifuge, Eppendorf 5430R, LabNet Spectrafuge 16M Microcentrifuge, or Sorvall RC 5C Plus. Mass spectrometric experiments were conducted using a Thermo LTQ Orbitrap. Polymerase chain reaction (PCR) utilized an Applied Biosystems 2720 Thermal Cycler. Agarose gel electrophoresis was performed using a GeneMate horizontal gel system and was visualized using a Transilluminator FBTIV-88 from Fisher Scientific. Lyophilization was performed using a Labconco Centrивap Concentrator equipped with a Labconco Centrивap Cold Trap. The vacuum pump utilized for lyophilization was an Oerlikon Leybold Vacuum (model trivac P 2,5 E). Protein ion exchange chromatography (IEX) was performed on a GE Healthcare

Lifesciences Akta Fast Protein Liquid Chromatography (FPLC) utilizing a GE Healthcare HiTrap Q 1 mL column.

Bacterial Cell Lines

E. coli NovaBlue (*endA1 hsdR17* ($r_{K12}^- m_{K12}^+$) *supE44 thi-1 recA1 gyrA96 relA1 lac* F'[*proA⁺B⁺ lacI^qZΔM15::Tn10*] (Tet^R)) cells were purchased from Novagen. *E. coli* BL21(DE3) ($F^- ompT hsdS_B$ ($r_B^- m_B^-$) *gal dcm* (DE3) cells were purchased from Novagen. Cells were grown in Luria Broth (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 µM). When plating bacteria Fisher LB Agar was used.

Enzymes

Nuclease P1 from *Penicillium citrinum* and Phosphodiesterase I from *Crotalus adamanteus* venom were purchased from Sigma Aldrich as lyophilized powders. These enzymes were dissolved into the manufacturer recommended buffer and stored at -20 °C. Proteinase K was purchased from Sigma Aldrich in buffered solution and was stored at -20 °C. Alkaline phosphatase, T4 DNA Ligase, and Pfu Ultra DNA Polymerase (Agilent) were purchased as solutions from Fisher Scientific and were stored at the manufacturer recommended temperature. Restriction enzymes were purchased from Fermentas and stored at -20 °C. Lysozyme was purchased as a lyophilized powder from Fisher and

stored at -20 °C. FactorXa was obtained from New England Biolabs and stored at -20 °C.

Chemicals and Reagents

All reagents, salts, buffers, and enzymes were obtained from Fisher Scientific unless otherwise noted. All solutions were prepared using millipore (18.2 MΩ•cm resistivity) water. Oligonucleotides were purchased from Integrated DNA Technologies. Polyethyleneimine cellulose (PEI-Cellulose) thin layer chromatography (TLC) plates were purchased from SiliCycle. [U-¹⁴C]-L-threonine and sodium [¹⁴C]-bicarbonate were purchased from Moravek. Alpha and gamma [³²P]-adenosine triphosphates were purchased from Perkin Elmer or MP Biomedical. Sodium [³²P]-pyrophosphate (PPi) was purchased from MP Biomedical. D-[U-¹³C]-Glucose and [¹⁵N]-ammonium chloride were purchased from Sigma Aldrich. Nickel nitrilotriacetic acid (Ni-NTA) affinity resin was purchased from Sigma Aldrich or Qiagen. Centrifugal filtration units were obtained from Amicon. All solutions containing RNA utilized diethyl pyrocarbonate (DEPC) treated water. DEPC was added to millipore treated water at a final concentration of 0.1% (volume/volume) and allowed to react overnight before autoclaving to produce DEPC treated water. Size exclusion chromatography (SEC) standards were purchased from Sigma Aldrich. Adenosine, guanosine, cytidine, and uridine triphosphates were purchased from Sigma Aldrich. Bradford reagent was obtained from BioRad. The QuikChange

site directed mutagenesis kit was obtained from Agilent. PCR purification kits were obtained from Qiagen. The FactorXa/LIC cloning kit was obtained from Novagen. Pyrophosphate reagent was purchased from Sigma Aldrich. EconoSafe liquid scintillation cocktail was purchased from RPI. D,L-hydroxynorvaline was obtained from Sigma Aldrich. A special thanks to Christopher Lima (Sloan-Kettering Institute) for the generous gift of the plasmid pSMT3 and pULP1 (63), Valérie de Crécy-Lagard (University of Florida) for the generous gift of plasmids containing *tsaD* (pBY216.1), *tsaC* (pBY215.1), and *tsaC* mutants K53A (pYC103.2), R110A (pYC101.1), K50A (pYC103.1) and R52A (pYC102.2), bulk tRNA isolated from wild type *S. cerevisiae*, bulk tRNA isolated from *Saccharomyces cerevisiae* (*S. cerevisiae*) with a *sua5* (*tsaC*) gene deletion, John Perona for the plasmid containing T7 Polymerase (pT7-911Q), and Paul Agris for *H. sapiens* tRNA^{Lys}-3(UUU) ASL.

2.2 Preparation of Proteins and RNA

Construction of Expression Constructs

PCR-based cloning of the *E. coli* *tsaB*, *tsaE*, and *prmC* genes was carried out using genomic DNA from the *E. coli* K12 cell line using the primers listed in Table 4. The resulting constructs were named pCDII29 (*tsaB*), pCDII30 (*tsaE*), and pCDII140 (*prmC*). Construct names were assigned as plasmid (p), Chris Deutsch (CD), lab note book (roman numeral) and page number. *E. coli* genomic DNA was isolated as described by Syn *et al.* (64). PCR reactions contained 500

ng of genomic DNA, 200 μ M deoxyribonucleotides (dNTPs), 50 picomol (pmol) primers, 1X Pfu Ultra buffer (supplied by the manufacturer), and 2.5 units of Pfu Ultra DNA polymerase in a final volume of 50 μ L. A three step PCR thermocycling protocol was utilized: 1) 94 °C for 3 minutes; 2) 30 cycles of denaturation at 94 °C for 1 minutes, annealing at 50 °C for 1 minutes, and extension at 72 °C for 2 minutes; 3) 72 °C for 3 minutes. The PCR products were then gel purified (1% agarose), the DNA isolated (Qiagen PCR purification kit) and ligated into a FactorXa/LIC vector (Novagen) as described by the manufacturer.

The *tsaC* mutants K53A, R110A, K50A and R52A were cloned into FactorXa/LIC vectors using the plasmids pYC103.2, pYC101.1, pYC103.1 and pYC102.2 respectively using the primers listed in Table 4. The PCR reactions were performed as previously described with the substitution of plasmid for genomic DNA. The resulting *tsaC* mutant constructs were renamed pCDIII24.1 (K53A), pCDIII24.2 (R110A), pCDIII24.3 (K50A), and pCDIII24.4 (R52A).

The *tsaD* gene was cloned from plasmid pBY216.1 using the primers listed in Table 4. Cloning was performed as described in (63). The resulting construct was named pCDI74 (SUMO-TsaD).

Site directed mutagenesis of plasmid pBY215.1 to obtain the *tsaC* mutant N141A was performed using the QuikChange protocol using the primers in Table 4. The resulting construct was named pCDIII24.5.

DNA Sequencing, Purification, Extraction and Restriction Digestion

DNA sequencing was carried out by the OHSU core sequencing laboratory to confirm the primary structures of all constructs. Plasmid preparations were performed using a Qiagen MiniPrep or MaxiPrep using the manufacturer recommended protocol. 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. Competent cells were produced using CaCl_2 as described by Nishimura (65). Transformations of plasmids into *E. coli* were performed by placing competent cells on ice for 5 minutes, adding 1 μL plasmid (500 ng/ μL), followed by a 30 minute incubation on ice. Cells were subsequently incubated at 42 °C for 45 seconds before being placed back on ice for an additional 5 minutes. LB (500 μL) was added to the cell/plasmid mixture and the solution was incubated at 37 °C for one hour before plating. DNA extraction from agarose was performed using a Qiagen PCR purification kit as described by the manufacturer. Restriction digestion was performed using restriction enzymes from Fermentas using the manufacturer recommended protocols. DNA ligation was performed using T4 DNA ligase using the manufacturer supplied protocols.

Overproduction and Purification of Recombinant, Affinity-tagged Proteins

Cultures of transformed BL21(DE3) cells containing plasmids to produce TsaC, TsaB, TsaE, TsaD, PrmC, ULP, T7 polymerase, TsaC K53A, TsaC R110A, TsaC K50A, TsaC R52A, and TsaC N141A were grown overnight at 25 °C until an

optical density at 600 nm (A_{600}) of 2–3 was obtained. LB was supplemented with 50 µg/L kanamycin (100 µg/L ampicillin for T7 Polymerase). Cells were pelleted (2,500 relative centrifugal force (RCF), 20 minutes, 4 °C) and resuspended in an equal volume of LB containing 50 µg/L kanamycin (100 µg/L ampicillin for T7 Polymerase). After a one hour recovery period at 20 °C (TsaD) or 37 °C (all others), IPTG was added to a final concentration of 1.0 mM, and cultures were grown for an additional 4 hours (TsaD 16 hours in the presence of 0.5 µM $ZnCl_2$). Cells were then pelleted (5000 RCF, 15 minutes, 4 °C) and resuspended to 250 mg cells/mL buffer. The resuspension buffer contained 100 mM Tris-HCl (pH 8.0), 2.0 mM 2-mercaptoethanol (BME), 1.0 mM phenylmethylsulfonyl flouride (PMSF) and 100 mM KCl (300 mM KCl, TsaD). T7 polymerase preparations also included protease inhibitor cocktail (Roche) at the manufacturer specified concentrations. Lysozyme (0.25 mg/mL) was then added, and the resulting solutions incubated for 30 minutes at 37 °C. Cells were subsequently lysed by three cycles of freezing/thawing at -80 °C/ 37 °C. In order to reduce the viscosity of the cell lysate DNase (10 µg/mL) was added and incubated at 37 °C for 30 minutes. The solutions were centrifuged (15,000 RCF, 20 minutes, 4 °C), and the supernatants applied to Ni-NTA columns which had been equilibrated with 10 column volumes (CV) equilibration buffer. Equilibration buffer was comprised of 100 mM Tris-HCl (pH 8.0), 2.0 mM BME, and 100 mM KCl (300 mM KCl, TsaD).

The columns were washed sequentially with 7 CV of equilibration buffer, 3 volumes of equilibration buffer containing 20 mM imidazole, 4 volumes of

equilibration buffer containing 30 mM imidazole, followed by elution with equilibration buffer containing 200 mM imidazole. Fractions containing purified protein were concentrated using Millipore Centrifugal Filter units to 500 μ L and subjected to dialysis (Slyde-a-lyzer, Pierce) against 4 L of 100 mM Tris-HCl (pH 8.0) and 100 mM KCl (300 mM KCl TsaD) overnight at 4 °C.

The fusion proteins were cleaved overnight at room temperature using 1 μ g FactorXa per mg of TsaC, TsaB, TsaE, or PrmC according to the manufacturer's protocol to remove the N-terminal affinity fusion peptide. SUMO-TsaD was cleaved using ULP1 (10 μ g/mg) using published protocols (63). After ULP1 cleavage TsaD became unstable, so the tag was subsequently retained for all future experiments. The cleaved proteins were then applied to a Ni-NTA column which had been equilibrated with 10 column volumes of 100 mM Tris-HCl (pH 8.0), 100 mM KCl (300 mM for TsaD), and 2 mM BME. Proteins were eluted from the column using equilibration buffer. After elution the protein pools were concentrated (Millipore Centrifugal Filter units), combined with an equal volume of glycerol, and stored at -80 °C for future use. When necessary proteins were subjected to ion exchange chromatography for a final polishing step using a HiTrap Q (GE Healthcare Life Sciences) 5x1 mm column. This was accomplished by placing up to 10 mg of protein in a 1 mL solution containing 100 mM Tris-HCl (pH 8.0), 100 mM KCl (300 mM for TsaD), and 1 mM BME. A salt gradient was subsequently run up to 1 M KCl over the course of 30 minutes using an Akta FPLC with a flow rate of 1 mL/min. Fractions were pooled and

assessed for purity by SDS-PAGE. Protein concentrations were based on the Bradford dye-binding procedure (66) or UV-VIS spectrometry. Extinction coefficients for proteins were determined using ProtParam from ExPASy (67).

Construction of tRNA Templates for *in vitro* Transcription

The templates for producing *E. coli* tRNA^{Lys}(UUU), tRNA^{Ile}(CAU), tRNA^{Ile}(GAU), and *Methanothermobacter thermoautotrophicus* (*M. thermoautotrophicus*) tRNA^{Gln}(CUG) transcripts were produced via a Klenow extension reaction as described by Sherlin (68) with the oligonucleotides listed in Table 5. *E. coli* tRNA^{Thr}(GGU) template was produced using a plasmid based method (pCDI147). Plasmid pCDI147 was produced by the ligation of six oligonucleotide pieces (Table 5) using T4 DNA ligase as described by the manufacturer. The plasmid was then transformed into *E. coli* NovaBlue cells and purified using a Qiagen Plasmid Maxi Preparation kit according to the manufacturer supplied protocols.

The ligated DNA fragment was subsequently digested with *Bam*HI/*Hind*III before ligation into similarly treated pUC18. Plasmid was subsequently linearized using *Mva*I to allow for run-off transcription. The template corresponding to the anticodon stem loop of *H. sapiens* tRNA^{Lys}-3(UUU) with a U₂₇-A₄₃ replacement of G₂₇-C₄₃ to improve stability was produced by annealing two oligonucleotides (Table 5). This was accomplished by heating equimolar sense and antisense oligonucleotides at 90 °C for 5 minutes and then allowing

them to cool at room temperature for an hour.

RNA Transcription and Purification

Transcription reactions to produce a 17-nucleotide RNA corresponding to the anticodon stem-loop of tRNA^{Lys} (17-mer RNA) or the full-length tRNAs were run for four hours at 37 °C in 80 mM HEPES (pH 7.4), 2.0 mM spermidine, 24 mM MgCl₂, 2.0 mM ribonucleotide triphosphates (NTPs), 3 µM template, and 2.5 µg/mL of T7 polymerase. The RNA products generated in the transcription reactions were precipitated by the addition of 0.1 volume 8.0 M ammonium acetate, 3 volumes of 100% ethanol, and cooling at –80 °C for 30 minutes, then pelleted by centrifugation at 15,000 RCF for 30 minutes at 4 °C, and resuspended in 50 mM HEPES (pH 7.4), 2.0 mM EDTA. The solutions were mixed 1:1 with formamide, heated at 90 °C for 5 minutes, and snap cooled on ice before being purified via Urea-PAGE electrophoresis (20% for ASL RNA, 10% for full length tRNA). The RNA was extracted by cutting the excised band from the Urea-PAGE gel, slicing it into 1 cm cubes, followed by adding 10 mL HEPES (pH 7.4) 2 mM EDTA per 1 g of gel. This suspension was then placed at 4 °C with agitation overnight. The soluble portion of the suspension was then precipitated as previously described, and resuspended in 50 mM HEPES (pH 7.4) 2 mM EDTA.

2.3 Biochemical Investigation of t⁶A Biosynthesis

tRNA Precipitation Assays

The extent of *in vitro* tRNA modification with t⁶A was monitored using a tRNA precipitation assay. Reactions contained radioactive [U-¹⁴C]-L-threonine, [U-¹⁴C]-threonylcarbamoyl adenosine monophosphate (TC-AMP) or [¹⁴C]-bicarbonate. The precipitation of tRNA was accomplished by the addition of 0.1 volumes 8 M ammonium acetate and 3 volumes 100% ethanol before incubation at -80 °C for 30 minutes, resulting in a tRNA suspension. These tRNA suspensions were then transferred to separate Whatman GF/B filters. To ensure transfer of the precipitate the microcentrifuge tubes containing the reaction were washed with an additional 1 mL of 70% cold ethanol and this fluid was placed on the same filters as the original suspension. After transfer the filters were washed with 2 L of cold 70% ethanol and dried using filtration. The dried filters were combined with 5 mL of RPI EconoSafe counting cocktail and the radioactivity quantified by liquid scintillation counting.

Protein Requirements of t⁶A Formation

Reactions contained 20 μM TsaB, 20 μM TsaC, 20 μM TsaD, 20 μM TsaE, 10 mM ATP, 100 mM Tris-HCl (pH 8), 300 mM KCl, 5.0 mM DTT, 20 mM MgCl₂, 1 μM ZnCl₂, 1 mM MnCl₂, 1 μM CaCl₂, and 50 μM tRNA. The tRNAs used were *E. coli* tRNA^{Lys}(UUU), *M. thermoautotrophicus* tRNA^{Gln}(CUG), *E. coli* tRNA^{Thr}(GGU), the ASL of *H. sapiens* tRNA^{Lys}-3(UUU), bulk tRNA isolated from a *S. cerevisiae*

$\Delta sua5$ knockout, or bulk tRNA isolated from *S. cerevisiae*. When measuring the incorporation of [U- 14 C]-L-threonine the reactions also contained 50 mM sodium bicarbonate and 50 μ M [U- 14 C]-L-threonine (100,000 DPM/assay). Reactions measuring the incorporation of [14 C]-bicarbonate contained 100 μ M added sodium [14 C]-bicarbonate (200,000 DPM/assay) and 10 mM L-threonine. Reactions were then terminated at indicated time points and the radioactive incorporation into tRNA quantified as described above.

RNA Modification of tRNA^{lle}(CAU) and tRNA^{lle}(GAU)

RNA modification of *in vitro* transcribed tRNA^{lle}(CAU) and tRNA^{lle}(GAU) was carried out in reactions containing 50 μ M tRNA^{lle}(CAU) or tRNA^{lle}(GAU), 5 μ M TsaB, 5 μ M TsaC, 5 μ M TsaD, 5 μ M TsaE, 5 mM L-threonine, 5 mM sodium bicarbonate, 10 mM ATP, 100 mM Tris-HCl (pH 8.0), 300 mM KCl, 20 mM MgCl₂, 1 mM MnCl₂, 1 μ M ZnCl₂ and 5 mM DTT at 37 °C for 20 minutes. To monitor the extent of labeling reactions were run in parallel containing 5 mM [U- 14 C]-L-threonine (250,000 DPM). After the reaction tRNA was phenol/chloroform extracted, precipitated as described above, and then resuspended in 50 mM HEPES (pH 7.4), 2 mM EDTA. This solution was snap frozen on dry ice before subsequent use. Reactions containing [U- 14 C]-L-threonine were then terminated at indicated time points and the radioactive incorporation into tRNA quantified as described above.

pH Optimization of t⁶A Enzyme Kinetics

Reactions containing 10 μ M TsaB, 10 μ M TsaC, 10 μ M TsaD, 10 μ M TsaE, 300 mM KCl, 30 μ M [U-¹⁴C]-L-threonine (100,000 DPM/assay), 10 mM sodium bicarbonate, 2 mM ATP, 50 mM MES, 50 mM MOPS, 50 mM Tris (pH 6-9 as indicated), 50 μ M bulk tRNA isolated from a *S. cerevisiae* Δ *sua5* knockout, 1 mM DTT, and 0.5 mM MgCl₂ were reacted at 37 °C for 30 minutes before termination and the radioactive incorporation into tRNA quantified as described above.

[U-¹⁴C]-Threonylcarbamoyl-AMP Incorporation Into tRNA

Reactions containing 100 mM Tris-HCl (pH 8.0), 300 mM KCl, 10 mM bicarbonate, 0.5 mM MgCl₂, 5 mM DTT, and 50 μ M Δ *sua5* tRNA were incubated for 20 minutes at 37 °C before ethanol precipitation. Reactions also contained 20 μ M TsaB, 20 μ M TsaC, 20 μ M TsaD, 20 μ M TsaE, 2 mM ATP, 30 μ M [U-¹⁴C]-L-threonine (100,000 DPM/assay), and [U-¹⁴C]-TC-AMP (100,000 DPM/assay) as indicated. Reactions were then terminated at indicated time points and the radioactive incorporation into tRNA quantified as described above.

Carbamoyl Adenosine Derivatives Incorporation Into tRNA

Reactions containing 100 mM Tris-HCl (pH 8.0), 300 mM KCl, 150 μ M added [¹⁴C]-bicarbonate (1,000,000 DPM, approximately 163 μ M due to dissolved carbonates), 10 mM ATP, 20 μ M TsaB, 20 μ M TsaC, 20 μ M TsaD, 20 μ M TsaE, 0.5 mM MgCl₂, 200 μ M Δ *sua5* tRNA, and 10 mM amino acid (either L-

threonine, L-serine, glycine, or D,L-hydroxynorvaline) were incubated for 20 minutes at 37 °C. Reactions were then terminated and the radioactive incorporation into tRNA quantified as described above.

High Pressure Liquid Chromatography of Carbamoyl Adenosine

Monophosphates Produced by TsaC

HPLC of various carbamoyl adenosine monophosphate derivatives was performed using a Supelco Discovery HS C18 (250 × 2.1 mm, 5 µM) column with a mobile phase of 10 mM ammonium acetate (pH 6.0, Buffer A) and acetonitrile (Buffer B) using a flow rate of 0.3 mL/min. After a 5 minute hold with Buffer A a linear gradient with acetonitrile (100:0 to 60:40 over 20 minutes) was applied. Reactions were subjected to centrifugal filtration (Millipore centrifugal filter units, 10 kDa cutoff) to remove TsaC and quench the reaction before HPLC analysis. The concentration of carbamoyl adenosine monophosphates was determined by the production of a standard curve using ATP. ATP areas were compared to carbamoyl adenosine monophosphates assuming identical extinction coefficients.

Steady State Kinetics of TC-AMP Production

Reactions containing 50 mM MOPS (pH 7.4), 50 mM KCl, 20 mM sodium bicarbonate, 0.5 mM MgCl₂, and 20 µM TsaC were reacted at 37 °C for 5-30 minutes then analyzed by HPLC as previously described. Reactions also

contained 50 μ M-50 mM L-threonine and 10 mM ATP when analyzing L-threonine kinetics or 10 mM L-threonine and 100 μ M-10 mM ATP when analyzing ATP kinetics.

TsaC Mutant Analysis

Relative activities of TsaC mutants were determined by using HPLC as mentioned previously. Specific conditions used for these assays were 50 mM MOPS (pH 7.4), 50 mM KCl, 10 mM L-threonine, 10 mM sodium bicarbonate, 0.5 mM MgCl_2 , 10 mM ATP, and 100 μ M TsaC mutant. The TsaC mutants tested were K53A, R110A, K50A, N141A, and R52A.

Analysis of ATP Formation by TsaC

Reactions containing 50 mM MOPS (pH 7.4), 50 mM KCl, 0.5 mM MgCl_2 , 100 μ M TC-AMP, 20 mM pyrophosphate, and 20 μ M TsaC were reacted at 37 °C for 20 minutes. When investigating the reverse reaction using phosphate MOPS was replaced with 50 mM sodium phosphate (pH 7.4) and pyrophosphate was excluded. Reactions were analyzed by HPLC as previously described.

Thin Layer Chromatography Analysis of [^{32}P]-Phosphate Derivatives

TLC was performed using PEI-Cellulose plates which had been pre-run using water and allowed to dry before use. Reactions analyzing ATP co-products contained 5 μ M TsaC, 5 μ M TsaB, 5 μ M TsaD, and 5 μ M TsaE, 40 μ M

tRNA^{Lys}(UUU), 50 mM sodium bicarbonate, and 50 μ M L-threonine as indicated. Reactions also included 100 mM Tris-HCl (pH 8.0), 300 mM KCl, 5.0 mM DTT, 100 μ M α -[³²P]-ATP (10,000 DPM), 20 mM MgCl₂, 1 μ M ZnCl₂, 1 mM MnCl₂, 1 μ M CaCl₂. The reactions were run for 20 minutes at 37 °C before a 1 μ L aliquot was spotted on a PEI-Cellulose plate. When analyzing TsaC's conversion of TC-AMP to ATP reactions contained 50 μ M TC-AMP, 10 μ M TsaC, 50 mM MOPS (pH 7.4), 50 mM KCl, 0.5 mM MgCl₂, and 50 μ M [³²P]-PPi or [³²P]-Pi (10,000 DPM). [³²P]-Pi was produced by the treatment of [³²P]-PPi with alkaline phosphatase using the manufacturer supplied protocol.

Experiments were analyzed by spotting 1 μ L of sample 1 cm from the bottom of the TLC plate. After the spots had dried developing was accomplished through the use of a 0.5 M potassium phosphate (pH 3.5) mobile phase. After the developed plates had dried the radioactive distribution of products was analyzed by phosphorimaging. This was accomplished by exposing phosphor storage screens to the TLC plate for four hours. When possible radioactive products were directly compared to standards.

[U-¹⁴C]-TC-AMP Purification

Reactions containing 50 mM MOPS (pH 7.4), 50 mM KCl, 2 mM ATP, 0.5 mM MgCl₂, 100 μ M TsaC, 20 mM sodium bicarbonate, and 100 μ M [U-¹⁴C]-L-threonine were incubated at 37 °C for 20 minutes and subsequently fractionated using HPLC. HPLC parameters for fractionation of TC-AMP were the same as

as previously described for carbamoyl adenosine derivatives. The peak corresponding to TC-AMP (retention time 20 minutes) was subsequently collected and snap frozen on dry ice. The purified peak was lyophilized by speed vacuum and stored at -80 °C.

Mass Spectrometric Analysis of t⁶A

Reactions containing 20 µM TsaB, 20 µM TsaC, 20 µM TsaD, 20 µM TsaE, 10 mM ATP, 100 mM Tris-HCl (pH 8), 300 mM KCl, 5.0 mM DTT, 20 mM MgCl₂, 1 µM ZnCl₂, 1 mM MnCl₂, 1 µM CaCl₂, 50 mM sodium bicarbonate, 10 mM L-threonine, and 50 µM *E. coli* tRNA^{Lys}(UUU) were incubated at 37 °C for 30 minutes before phenol/chloroform extraction and ethanol precipitation. After precipitation the tRNA was resuspended in water to a concentration of 1.8 µg/mL.

In order to produce individual nucleosides from the tRNA it was sequentially treated with Nuclease P1, phosphodiesterase, and alkaline phosphatase. Nuclease P1 treatment was performed by adding 0.1 volumes 0.1 M ammonium acetate (pH 5.3) and 5 µg Nuclease P1/1 mg tRNA. This reaction was incubated for four hours at 50 °C. Phosphodiesterase treatment was performed by adding 0.1 volume of 1 M ammonium bicarbonate (pH 7.8) and 0.05 units of phosphodiesterase/1 mg of tRNA. This reaction was incubated for four hours at 37 °C. Lastly, alkaline phosphatase treatment was completed by adding 20 units of alkaline phosphatase and incubating for 2 hours at 37 °C.

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 minutes) with a flow rate of 0.3 mL/min. Mass analysis utilized an LTQ Orbitrap operating in the positive mode; spray voltage 4.00 kV; capillary temperature 300 °C; capillary voltage 49; tube lens voltage 100.

Mass Spectrometric Analysis of Carbamoyl Adenosine Monophosphate Derivatives

Reactions containing 50 mM MOPS (pH 7.4), 50 mM KCl, 20 mM sodium bicarbonate, 2 mM ATP, 0.5 mM MgCl₂, 10 μM TsaC and 10 mM amino acid (L-threonine, D,L-hydroxynorvaline, L-serine or glycine) were reacted at 37 °C for 20 minutes. These reaction mixtures were then spin filtered to remove TsaC and quench the reaction (Millipore centrifugal filter units, 10 kDa cutoff). Reactions were subsequently analyzed by HPLC as previously described or LC-MS. LC-MS analysis used 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 100:0 to 60:40 over 30 minutes) with a flow rate of 0.3 mL/min. Mass analysis utilized an LTQ Orbitrap operating in the negative mode; spray voltage 2.5 kV; capillary temperature 300 °C; capillary voltage -17; tube lens voltage -107.

Phosphorylation of t⁶A Proteins

Reactions containing 625,000 DPM γ[³²P]-ATP, 100 mM Tris-HCl (pH 8.0),

300 mM KCl, 1 mM MgCl₂, 25 mM sodium bicarbonate, and 25 mM L-threonine and when indicated 50 μM *Δsua5* tRNA, 50 μM 6x histidine tagged TsaD, 50 μM 6x histidine tagged TsaC, 50 μM 6x histidine tagged TsaB and 50 μM 6x histidine tagged TsaE, were incubated at 37 °C for 20 minutes and subsequently loaded onto a Ni-NTA column which had been equilibrated with 100 mM Tris-HCl (pH 8.0) and 300 mM KCl. The reactions were subsequently washed with 20 column volumes equilibration buffer and then eluted in 4 column volumes of equilibration buffer containing 200 mM imidazole. Fractions eluted in imidazole were concentrated using a Amicon centrifugation filter unit (10 kDa cutoff) and analyzed for radioactivity using liquid scintillation counting. This was accomplished by adding approximately 100 μL of concentrated protein solution into 5 mL RPI EconoSafe counting cocktail.

Coupled Assay to Determine Pyrophosphate Production by TsaC

The TsaC catalyzed conversion of ATP to pyrophosphate was analyzed by UV-VIS spectroscopy. The coupled assay was performed as described by Sigma Aldrich with two notable exceptions. First, the total volume of the assay was reduced from 2.95 mL to 137.5 μL, without affecting the concentration of components involved in the coupled assay. Second, the temperature of the reaction was monitored at 37 °C instead of 30 °C.

Reactions contained 50 mM MOPS (pH 7.4), 50 mM KCl, 10 mM bicarbonate, 50 μM – 20 mM amino acid (L-threonine, L-serine, D,L-

hydroxynorvaline, glycine, L-alanine, L-valine), 1 mM ATP, 10 μ M TsaC, 45 mM imidazole (pH 7.4), 5 mM citrate, 0.1 mM EDTA, 2 mM MgCl_2 , 0.2 mM MnCl_2 , 0.02 mM CoCl_2 , 0.8 mM NADH, 12 mM Fructose-6-phosphate, 5 mg/mL bovine serum albumin, 5 mg/mL sugar stabilizer, 0.5 units/mL pyrophosphate dependent phosphofructokinase, 7.5 units/mL aldolase, 5 units/mL glycerophosphate dehydrogenase, and 50 units/mL triosephosphate isomerase. Reactions were analyzed at 37 °C from 0-20 minutes.

When analyzing kinetic parameters for ATP the reactions contained 50 mM MOPS (pH 7.4), 50 mM KCl, 10 mM bicarbonate, 45 mM imidazole (pH 7.4), 5 mM citrate, 0.1 mM EDTA, 2 mM MgCl_2 , 0.2 mM MnCl_2 , 0.02 mM CoCl_2 , 0.8 mM NADH, 12 mM Fructose-6-phosphate, 5 mg/mL bovine serum albumin, 5 mg/mL sugar stabilizer, 0.5 units/mL pyrophosphate dependent phosphofructokinase, 7.5 units/mL aldolase, 5 units/mL glycerophosphate dehydrogenase, 50 units/mL triosephosphate isomerase, 10 mM L-threonine, 50 μ M - 20 mM ATP, and 10 μ M TsaC. Reactions were analyzed at 37 °C from 0-20 minutes.

Reactions containing 50 mM MOPS (pH 7.4), 50 mM KCl, 10 mM bicarbonate, 45 mM imidazole (pH 7.4), 5 mM citrate, 0.1 mM EDTA, 2 mM MgCl_2 , 0.2 mM MnCl_2 , 0.02 mM CoCl_2 , 0.8 mM NADH, 12 mM Fructose-6-phosphate, 5 mg/mL bovine serum albumin, 5 mg/mL sugar stabilizer, 0.5 units/mL pyrophosphate dependent phosphofructokinase, 7.5 units/mL aldolase, 5 units/mL glycerophosphate dehydrogenase, 50 units/mL triosephosphate isomerase, and 10 μ M - 20 mM pyrophosphate were analyzed at 37 °C from 0-20

minutes to determine the rate of NADH oxidation by the coupled enzymes.

The rate of TsaC pyrophosphate formation was determined using the following equation:

$$\Delta A_{340}/\text{min Actual} = (\Delta A_{340}/\text{min Sample}) - (\Delta A_{340}/\text{min Background})$$

$$\Delta[\text{NADH}]/\text{min} = (\Delta A_{340}/\text{min Actual}) / (6220 \cdot \text{M})$$

$$\Delta \text{PPi}/\text{min} = -(\Delta[\text{NADH}]/\text{min}) \cdot (1 \text{ mol PPi} / 2 \text{ mol NADH})$$

Carboxythreonylation of Proteins

Reactions contained 50 mM MOPS (pH 7.4), 50 mM KCl, 2 mM ATP, 0.5 mM MgCl₂, and either 10 mM L-threonine, 105 μM [¹⁴C]-bicarbonate (200,000 DPM/assay) or 30 μM [U-¹⁴C]-L-threonine (200,000 DPM/assay), 25 mM sodium bicarbonate. N-terminal 6x histidine proteins were included when mentioned at the following concentrations, 100 μM TsaC, 10 μg other proteins (PrmC, TsaB, TsaE, TsaD). After a 30 minute incubation at 37 °C reactions were washed using a 10 kDa cut off spin filter before liquid scintillation counting. Washing entailed concentrating the assay components to 50 μL, discarding the flow through, adding buffer containing 50 mM MOPS (pH 7.4) and 50 mM KCl to a volume of 500 μL. This process was repeated a total of 6 times for a 1,000,000 fold effective dilution of radioisotopes.

An aliquot from each sample was run on SDS-PAGE, transferred to a nitrocellulose membrane, and developed overnight for analysis by phosphorimaging. Nitrocellulose transfer was accomplished by soaking 4 pieces

of filter paper in transfer buffer comprised of 25 mM Tris-HCl (pH 8.3), 192 mM glycine, 1.3 mM SDS, and 20% methanol for 5 minutes. Subsequently a “transfer sandwich” was created by placing 2 pieces of wet filter paper on the anode of a semi-dry transfer apparatus, followed by a nitrocellulose membrane, a SDS-PAGE gel, and 2 additional pieces of filter paper. The semi-dry transfer apparatus was operated under a constant current of 30 mA and overall transfer of protein was monitored by transfer of the ladder to the membrane and was > 90%. Additional spots were placed over the ladder and corners of nitrocellulose membrane for orientation.

Carboxythreonylation of DNA

Reactions containing 50 mM MOPS (pH 7.4), 50 mM KCl, 5 mM ATP, 0.5 mM MgCl₂, 50 μM TsaC, 5 mM sodium bicarbonate, 50 μM [U-¹⁴C]-L-threonine (200,000 DPM), and either 25.9 μM *E. coli* tRNA^{Lys}(UUU) template DNA or 10 μg *E. coli* genomic DNA were incubated at 37 °C for 30 minutes. Reactions were then either purified using gel electrophoresis (agarose 1%) or precipitated by the addition of 3 volumes 100% ethanol and 0.1 volumes 8 M ammonium acetate before a 30 minute incubation at -80 °C. Excised gel pieces were placed in 5 mL of liquid scintillation cocktail before being heated at 90 °C for 30 minutes to liberate DNA from the agarose. Precipitated DNA was washed extensively with 70% cold ethanol before resuspension in 100 μL of water. The 100 μL DNA solutions were then placed in 5 mL of liquid scintillation cocktail. Subsequently,

liquid scintillation counting was performed to determine if the DNA had been carboxythreonylated.

Protein Interactions (SDS-PAGE)

The TsaD, TsaC, TsaE, and TsaB proteins were tested pairwise for binding interactions. In each test one protein was 6x histidine tagged and the other untagged as indicated. Proteins were present at 20 μ M 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 μ L. After incubating for 20 minutes 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 μ L using 10 kDa centrifugal filter units (Amicon). Reactions were then analyzed by SDS-PAGE.

Production of [U-¹³C, U-¹⁵N] and [U-¹⁵N] TsaC

Cultures of BL21(DE3) cells containing pBY215.1 (TsaC) were grown overnight in LB at room temperature until an A_{600} of 2–3 was obtained. Cells were pelleted (2,500 RCF, 4 °C) and resuspended in an equal volume of M9 minimal media.

M9 minimal media was comprised of 47.74 mM Na_2HPO_4 , 22.06 mM KH_2PO_4 , 8.56 mM NaCl, 18.7 mM NH_4Cl , 2 mM MgSO_4 , 100 μ M CaCl_2 , and 22.2

mM D-glucose. For [U-¹⁵N]-TsaC [¹⁵N]-ammonium chloride was substituted for NH₄Cl. For [U-¹⁵N, U-¹³C]-TsaC D-[U-¹³C]-glucose and [¹⁵N]-ammonium chloride were substituted for D-glucose and NH₄Cl respectively.

After a one hour recovery period at 37 °C, IPTG was added to a final concentration of 1.0 mM, and cultures were grown for an additional four hours and harvested, purified, and FactorXa treated as previously mentioned. The proteins were then dialyzed as previously described against 4 L of 100 mM sodium phosphate (pH 6.8), 100 mM sodium chloride and snap frozen by placing them in dry ice until completely frozen.

Protein Gel Filtration

Size exclusion chromatography was performed using a Biosep SEC-S3000 column (300 x 7.8 mm, 5µM). The mobile phase was 100 mM sodium phosphate (pH 6.8), 300 mM potassium chloride and had a flow rate of 1 mL/min over 30 minutes. Proteins were suspended at a concentration of 2 mg/mL each in mobile phase before analysis. TsaD, TsaE, TsaB, and TsaC were first run individually. When more than one protein was analyzed samples were allowed to equilibrate at room temperature for 30 minutes before analysis. Samples were then compared to Sigma-Aldrich molecular weight standards to determine approximate apparent mass.

3.0 Results

3.1 Discovery of the t⁶A Biosynthetic Pathway

The proposed chemistry of t⁶A formation (Figure 13) suggested that its biosynthetic pathway would require multiple enzymatic steps. Previous work in knockout strains of yeast had shown that *kae1* (*tsaD*) and *sua5* (*tsaC*) were involved in the biosynthesis of t⁶A (Figure 11) (51, 52). To test the hypothesis that these genes were sufficient for the formation of t⁶A *in vitro* both proteins were expressed and purified.

The plasmid pBY215.1 containing the *E. coli yrdC* (*tsaC*) gene with an N-terminal 6x histidine tag was transformed into the *E. coli* cell line BL21(DE3) for overexpression. Overexpression was robust with yields of ~60 mg per L of LB or ~25 mg per L of M9 minimal media (Figure 14) after affinity purification.

The plasmid pBY216.1 containing the *E. coli yggD* (*tsaD*) gene with an N-terminal 6x histidine tag was transformed into the *E. coli* cell line BL21(DE3) for overexpression. Initial attempts of overexpression resulted in high levels of expression, however, the recombinant protein was insoluble. Altering the expression conditions, such as including ZnCl₂ and lowering the induction temperature, proved insufficient to improve the solubility of TsaD. Therefore, the *tsaD* gene was recloned into the pSUMO vector. The pSUMO vector includes an N-terminal SUMO tag which has been shown to improve the stability and solubility of numerous proteins (63). The *tsaD* gene was cloned from the pBY216.1 plasmid using PCR before ligation into the pSUMO vector. The

resulting plasmid, pCDI74, provided robust production of a soluble N-terminal SUMO-TsaD recombinant protein. Overexpression of SUMO-TsaD in *E. coli* BL21(DE3) and subsequent purification using Ni-NTA agarose typically resulted in yields of ~20 mg per L of LB.

Initial affinity purification of the SUMO-TsaD fusion construct utilizing Ni-NTA agarose and a stepwise elution using imidazole resulted in an impure protein product which was further purified to homogeneity via ion exchange chromatography (Figure 15).

Subsequent cleavage of the N-terminal SUMO tag by the protease ULP resulted in an unstable protein which precipitated from solution. Therefore, the N-terminal SUMO tag was retained for all subsequent experiments.

Cloning and Purification of TsaB and TsaE

In vitro assays using the TsaC and TsaD proteins were insufficient to reconstitute the t⁶A pathway *in vitro*, suggesting that additional proteins were required for its biosynthesis in bacteria.

Two additional candidates implicated in the involvement of bacterial t⁶A synthesis were the highly conserved bacterial proteins YeaZ (TsaB) and YjeE (TsaE). Bioinformatic analysis revealed that there was strong physical clustering of the *tsaB*, *tsaD*, and *tsaE* genes in bacteria, suggesting the potential for involvement in the t⁶A pathway (Figure 16) (52). Furthermore, a protein interaction network between the TsaD/TsaB and TsaE/TsaB proteins (69) was

discovered using a bacterial two-hybrid model. Finally, complementation of the lethal *E. coli* *ygjD* (*tsaD*) knockout with the *B. subtilis* homolog (*ydiE*) also required the *B. subtilis* *yeaZ* homolog *ydiC* gene to permit growth (52).

The *tsaB* and *tsaE* genes were cloned from *E. coli* genomic DNA using PCR and were inserted into pET32Xa/LIC vector as N-terminal 6x histidine fusion proteins. Overexpression in *E. coli* BL21(DE3) was robust for both recombinant proteins. After purification using Ni-NTA agarose both TsaB (Figure 17) and TsaE (Figure 18) were typically obtained in yields of ~60 mg per L of LB. Cleavage with FactorXa proceeded with high specificity and efficiency typically resulting in over 90% cleavage.

Production of tRNA Templates, RNA Transcriptions and Purification

Klenow reactions to extend synthesized oligonucleotides to full length tRNA substrates for *E. coli* tRNA^{Lys}(UUU), tRNA^{Ile}(CAU), tRNA^{Ile}(GAU), and *M. thermoautotrophicus* tRNA^{Gln}(CUG) typically resulted in high yields of conversion to full length template. Isolation of the plasmid containing *E. coli* tRNA^{Thr}(GGU) and subsequent *MvaI* digestion and agarose purification, as described in experimental, led to yields of approximately 45 nmol of template. T7 polymerase purification produced approximately 50 mg of T7 Polymerase per L of LB. Transcription typically yielded 150-300 µg of purified tRNA per mL (Figure 19).

***In vitro* t⁶A Formation**

In order to ascertain if TsaC, TsaD, TsaB and TsaE were sufficient and necessary for t⁶A formation *in vitro* tRNA precipitation assays were performed. These assays were designed to measure the incorporation of either [U-¹⁴C]-L-threonine or [¹⁴C]-bicarbonate into tRNA, as previous work indicated their involvement in its biosynthesis (45-50). From these assays it was determined that in the presence of TsaC, TsaD, TsaB, and TsaD, radioactive threonine or bicarbonate were incorporated into tRNA, consistent with the formation of t⁶A₃₇-tRNA. In the absence of any one of the four proteins no detectable incorporation of radioactive threonine or bicarbonate was observed (Figure 20).

To determine the tRNA requirements of t⁶A formation, a series of tRNAs were examined by precipitation assay. Specifically, *in vitro* transcribed *E. coli* tRNA^{Lys}(UUU), *E. coli* tRNA^{Thr}(GGU), *M. thermoautotrophicus* tRNA^{Gln}(CUG), the anticodon stem loop from *H. sapiens* tRNA^{Lys}-3(UUU) (with a U₂₇-A₄₃ replacement of G₂₇-C₄₃), and *in vivo* produced tRNA isolated from *S. cerevisiae* were examined as substrates. The tRNA isolated from *S. cerevisiae* was isolated from either the wild type (WT) strain or a strain deficient in t⁶A nucleoside (Δ *sua5*) (51, 52).

Assays containing full length tRNA with an ANN decoding anticodon (*in vitro* transcribed *E. coli* tRNA^{Thr}(GGU) and *E. coli* tRNA^{Lys}(UUU) or Δ *sua5* tRNA) showed robust incorporation of both [U-¹⁴C]-L-threonine or [¹⁴C]-bicarbonate into the tRNA. In contrast, assays containing the ASL from *H. sapien* tRNA^{Lys}-

3(UUU), fully modified tRNA from wild type *S. cerevisiae* (already containing the t⁶A modification), or the non ANN decoding *in vitro* transcribed *M. thermoautotrophicus* tRNA^{Gln}(CUG) did not show detectable incorporation of radioisotope (Figure 21).

To confirm the *in vitro* production of t⁶A, LC-MS was employed to analyze the reaction products. The *E. coli* tRNA^{Lys}(UUU) transcript was reacted with unlabeled substrate in the presence of the TsaD, TsaB, TsaC and TsaE proteins, and the tRNA was subsequently digested and dephosphorylated to mononucleosides for analysis by LC or LC-MS. Comparison of tRNA digestion of a control showed a new peak in the LC trace which was consistent with authentic t⁶A (Figure 22A). Subsequent LC-MS analysis (Figure 22B) of this peak showed compounds with m/z values of 413.14 (t⁶A) and 281.05 (protonated t⁶A base) thus confirming the radiochemical assays were measuring the formation of t⁶A .

3.2 Mechanistic Investigation of the t⁶A Biosynthetic Pathway

t⁶A ATP Co-product Analysis

To determine which product(s) were formed during ATP activation of intermediates (Figure 13) a TLC based assay using α -[³²P]-ATP was employed. Both AMP and ADP were found as co-products from the activation of intermediates leading to t⁶A (Figure 23). The AMP co-product was dependent upon the presence of both TsaC and threonine (Figure 23a lanes 6, 17-19). There is a significant reduction in the production of ADP in the absence of any

one of the remaining 3 proteins (Figure 23a lanes 3-16), however, ADP production is still observed. Notably, the production of AMP and ADP is independent of tRNA (Figure 23) suggesting the formation of any activated intermediates occur independently of tRNA. An unidentified ATP co-product was also found in the presence of TsaC and threonine (Figure 23b, lanes 11, 15, 17-19). LC analysis demonstrated this intermediate to be an adenosine derivative and the structure of this intermediate was reported to be threonylcarbamoyl adenosine monophosphate (TC-AMP) in *B. subtilis* by Lauhon (71).

LC-MS of Threonylcarbamoyl Adenosine Monophosphate (TC-AMP)

To confirm the production of TC-AMP by *E. coli* TsaC activity assays were carried out with analysis by HPLC. LC traces of reaction mixtures containing ATP, L-threonine and TsaC showed the formation of a new peak (Figure 24b, and LC-MS analysis confirmed that intermediate was TC-AMP (Figure 24).

Analysis of Carbonate Requirements in TsaC

TC-AMP formation catalyzed by TsaC was shown to require L-threonine and ATP. However, the identity of the carbonate source for this reaction had not been definitively shown. Only TC-AMP and AMP were found as co-products from the TsaC reaction (Figure 23) and non stoichiometric equivalents of AMP and TC-AMP are produced (Figure 25). This suggested that the AMP observed in the TsaC assays was the result of TC-AMP degradation to AMP, threonine, and

carbonate. These observations led to two plausible mechanisms of TC-AMP formation. The first (Figure 26) proceeds through a 3 step pathway beginning with ATP and bicarbonate to produce carboxyphosphate and ADP. Subsequently, carboxyphosphate reacts with L-threonine to produce N-carboxy-L-threonine and phosphate. The N-carboxy-L-threonine subsequently reacts with the ADP produced in the first step to produce TC-AMP and a second equivalent of phosphate. The second mechanism proceeds through a 2 step pathway (Figure 27), in which TsaC catalyzes the formation of a N-carboxy-L-threonine from CO₂ followed by reaction with ATP to produce TC-AMP and pyrophosphate.

To determine if TsaC used CO₂ or bicarbonate as the source of the carbamoyl moiety in t⁶A, HPLC analysis of the reverse reaction was carried out. Purified TC-AMP was reacted with either phosphate or pyrophosphate and the extent of conversion by TsaC to ATP was monitored (Figure 28). TsaC catalyzed formation of ATP in the presence of phosphate would suggest the formation of TC-AMP occurring through a 2 step activation pathway using bicarbonate (Figure 26), while TsaC catalyzed formation of ATP by pyrophosphate would indicate a 1 step activation using CO₂ (Figure 27).

ATP formation was observed under both conditions, although the reaction appears more robust in the presence of pyrophosphate. In order to exclude the possibility ATP formation was due to contaminating phosphate or pyrophosphate in one of the reactions, the reactions were investigated with [³²P]-PPi and [³²P]-Pi followed by TLC analysis (Figure 29). [³²P]-Pi was generated by the treatment of

[³²P]-PPi with alkaline phosphatase.

While the problem of contaminating species is apparent from Figure 29, the level of ATP produced in the PPi reaction is substantially higher (Figure 29, Lanes 3, 6) than that in the Pi reaction. The low level of ATP formation observed in the Pi reaction is likely due to a low level of contaminating PPi (Figure 29, Lane 7). As such, it appears the canonical substrate for TsaC is CO₂.

Analysis of ATP Co-products Derived from TsaB, TsaE, and TsaD

Analysis of the archaeal t⁶A biosynthetic pathway suggested that the protein Bud32 (a t⁶A protein found in eukaryotes and archaea) was functioning as a P-loop ATPase, and that this activity was required for the formation of t⁶A *in vitro* (72). To determine if the ATP to ADP conversion by TsaB, TsaE, and TsaD was the result of an essential ATPase activity, the formation of t⁶A from TC-AMP as catalyzed by TsaC, TsaD, TsaE and TsaB was investigated in the presence and absence of ATP.

Radiochemical tRNA precipitation assays using [U-¹⁴C]-TC-AMP indicated that the transfer of the carbamoylthreonine moiety from TC-AMP to produce t⁶A occurs in the presence of TsaD, TsaB, and TsaE and that ATP and TsaC are not required for this transfer (Figure 30).

To investigate whether the ADP formation by TsaD, TsaB, or TsaE was a result of protein phosphorylation a bioinformatic analysis of the proteins for possible phosphorylation sites was performed using NetPhosBac, which

indicated that all four proteins have potential phosphorylation sites (Table 6). To biochemically address this issue TsaD, TsaB, TsaE, and TsaC were incubated together or pairwise, in the presence or absence of tRNA, with γ -[^{32}P]-ATP. The proteins were then analyzed for ^{32}P incorporation from ATP after isolation from the reaction mixture using affinity tag binding or by phosphorimaging after SDS-PAGE, and nitrocellulose transfer. No incorporation of ^{32}P into protein was observed in any sample tested.

Enzyme Optimization and Kinetics

The pH optimum of t⁶A formation was assessed by tRNA precipitation assays. t⁶A Formation was observed throughout the tested pH range (Figure 31). The rate of t⁶A formation drops off significantly after a pH of 8. The pH optimum of the reaction was determined to be approximately pH 7.5.

Steady state kinetics of TsaC catalyzed formation of TC-AMP were initially investigated by HPLC analysis of TsaC reaction products (Figure 32, 33). Kinetic parameters were determined for ATP and L-threonine substrates. Subsequent analyses were performed through the use of a coupled enzyme assay as described below.

In order to rapidly determine the rate of the TsaC catalyzed reaction a coupled enzyme system was developed. The coupled assay proceeded through the following enzymatic conversions:

1. $\text{ATP} + \text{CO}_2(\text{aq}) + \text{amino acid}$ is converted by TsaC to a carbamoyl

adenosine monophosphate derivative and pyrophosphate.

2. Pyrophosphate + D-fructose-6-phosphate is converted by a pyrophosphate dependent phosphofructokinase to D-fructose-1,6-diphosphate + phosphate.
3. D-Fructose-1,6-diphosphate is converted to dihydroxyacetone phosphate + D-glyceraldehyde-3-phosphate by aldolase.
4. D-glyceraldehyde-3-phosphate is converted to dihydroxyacetone phosphate by triosephosphate isomerase.
5. 2 dihydroxyacetone phosphates + 2 β -nicotinamide adenine dinucleotide (reduced, NADH) are converted to 2 glycerol-3-phosphate + 2 β -nicotinamide adenine dinucleotide (oxidized, NAD^+) by glycerophosphate dehydrogenase.

The extent of the reaction was measured from the conversion of NADH to NAD^+ by monitoring UV absorbance at 340 nm. Assays were initially run to ensure that the observed rate of NADH oxidation correlated with the TsaC reaction, not the coupled assay rate. Subsequent work by others in the lab revealed an apparent V_{max}/K_m of 1.6 min^{-1} for the coupled assay, which was greater than that determined for TsaC, confirming that TsaC was not the rate limiting step. Furthermore, this work demonstrated that the coupled assay was not inhibited by ATP concentrations below 3 mM. For a UV-VIS trace showing maximum coupled assay rate, background oxidation rate of NADH, and the TsaC

reaction see Figure 34.

Kinetic parameters were elucidated for ATP (Figure 35), L-threonine (Figure 36), L-serine (Figure 37), and D,L-hydroxynorvaline (Figure 38), and are summarized in Table 7. Relative activity using glycine was determined to be 1.95% while the amino acids L-alanine and L-valine did not serve as substrates.

Analysis of TsaC Mutants

In order to determine the solution structure of TsaC, isotope labeled protein was produced ([U-¹⁵N, U-¹³C]-TsaC and [U-¹⁵N]-TsaC) and analyzed by NMR. The resulting solution structure (Figure 39) provided insights on the amino acids involved in substrate binding (73). Specifically, saturation transfer difference NMR had implicated the residues K53, R110, and N141 in ATP binding and the residues K50 and R52 in phosphate coordination (73). Additionally, gene complementation studies with TsaC mutants implicated R52 as critical for t⁶A formation *in vivo* (51). All of the residues are found in close proximity to bound TC-AMP in a crystal structure of *Sulfolobus tokadaii* Sua5 (TsaC) (Figure 40) (74).

To confirm that these residues were important to catalysis by TsaC, a preliminary analysis of the TsaC mutants K53A, R110A, K50A, N141A, and R52A was carried out by measuring their relative activities using HPLC to monitor TC-AMP formation. The results are summarized in Table 8.

3.3 Structural Investigation of the Proteins Involved in t⁶A Biosynthesis

Protein Interactions (SDS-PAGE)

To determine if the TsaD, TsaB, TsaC, and TsaE proteins were capable of binding to each other *in vitro* protein pull down experiments were carried out (Figure 41). Protein pairs (one with an affinity tag, one without) were incubated followed by Ni-NTA chromatography and SDS-PAGE analysis. These experiments indicated TsaD interacts with both TsaC and TsaB, TsaC interacts with TsaB, and TsaE interacts with TsaB, consistent with the formation of a single protein complex.

Protein Gel Filtration Chromatography

Size exclusion chromatography was performed in order to investigate the quaternary structure of the proteins involved in t⁶A biosynthesis. A Sigma Aldrich size exclusion standard was used to create a standard curve (Figure 42). Apparent molecular masses of the individual proteins are reported in Figure 43. These results are consistent with TsaB being dimeric and TsaC being monomeric. TsaE primarily runs with an apparent molecular mass between a dimer and trimer with some monomer being observed. TsaD primarily runs as a monomer with an additional peak observed corresponding to a trimer.

Definitive determination of subunit arrangement of multiple enzyme combinations was difficult to determine. This was due to the similar sizes of TsaC, TsaB and TsaE, the tendency of these proteins to form dimers with

themselves and/or each other, and TsaD having extremely broad elution peaks. The formation of a peak consistent with a TsaD + TsaB heterodimer is illustrated by an overlay of TsaD, TsaB, and TsaD + TsaB chromatograms shown in Figure 44. It is important to note that TsaD was analyzed as a fusion protein (SUMO-TsaD) which likely interfered in this analysis. A summary of determined apparent weights for all the protein complexes is reported in Table 9.

3.4 Peripheral Analysis of the t⁶A Biosynthetic Pathway

Analysis of s⁶A, hn⁶A and g⁶A

Analysis of reactions using non-threonine amino acids suggested that TsaC is promiscuous in its production of carbamoyladenosine derivatives. Specifically, LC analysis indicated TsaC is able to catalyze the formation of a product with a similar retention time to TC-AMP in the presence of hydroxynorvaline (Figure 45), serine (Figure 46), and glycine (Figure 47). In contrast, no product was observed via HPLC when L-alanine or L-valine were included in the assay. To confirm the formation of hydroxynorvalyl (HNC-AMP), serinyl (SC-AMP), and glycinyl (GC-AMP) carbamoyl adenosine monophosphates the relevant assays were analyzed by LC-MS (Figures 48-50).

In the presence of D,L-hydroxynorvaline a m/z ion of 505.108 was found. This corresponds to the predicted mass of 505.11 for HNC-AMP with an error of 3.86 ppm. In the presence of L-serine a m/z ion of 477.080 was found. This corresponds to the predicted mass of 477.08 for SC-AMP with an error of 0.16

ppm. In the presence of glycine a m/z ion of 447.067 was found. This corresponds to the predicted mass of 447.07 GC-AMP with an error of 5.79 ppm.

In order to determine if carbamoyl adenosine derivatives other than TC-AMP could be incorporated into tRNA using the t⁶A biosynthetic machinery tRNA precipitation assays were performed (Figure 51). Within the limits of detection of the assay (1.4% relative activity of L-threonine), none of the non-threonine carbamoyl adenosine derivatives are incorporated into tRNA.

Carboxythreonylation of Proteins, Cloning and Purification of PrmC

TC-AMP has a stability on the lower end of biologically relevant phosphoric anhydrides (71, 75). This instability in water suggests the potential to react with other nucleophiles, which led us to investigate if TC-AMP was capable of reacting with proteins and DNA. In particular, the methyl transferase PrmC is often found clustered with the t⁶A proteins (76) and is also involved in translation, leading to the hypothesis that carboxythreonylation of PrmC by TC-AMP might serve as a regulating mechanism.

To test this hypothesis the *prmC* gene was cloned from *E. coli* genomic DNA and was inserted into pET32Xa/LIC vector as a N-terminal 6x histidine fusion protein. Overexpression in *E. coli* BL21(DE3) was robust. After purification using Ni-NTA agarose (Figure 52) PrmC was typically obtained in yields of ~30 mg per L of LB.

Carboxythreonylation of proteins was assessed by the generation of [U-

^{14}C]-TC-AMP in the presence of various proteins. After incubation in the presence of [U- ^{14}C]-TC-AMP SDS-PAGE and nitrocellulose transfer were performed and radioactive incorporation into proteins was assessed via phosphorimaging. Carboxythreonylation of proteins was examined for the following combination of proteins: TsaC alone, TsaC with PrmC, TsaC with TsaD, TsaB and TsaE. In all tested protein combinations there were no observable incorporation of radioisotope.

Carboxythreonylation of DNA

The non-enzymatic carboxythreonylation of DNA by TC-AMP was examined to determine if its build up in the cell resulted in DNA modification. This was accomplished by the generation of [U- ^{14}C]-TC-AMP in the presence of either genomic DNA or tRNA^{Lys}(UUU) template DNA. Examination of excised agarose gel fragments by scintillation counting indicated that neither genomic DNA or small dsDNA was capable of being non-enzymatically carboxythreonylated. Subsequent DNA precipitation assays supported this result with no activity observed above background.

4.0 Discussion

4.1 Elucidation of the t⁶A Biosynthetic Pathway

The first clues to the identity of the enzyme(s) involved in t⁶A biosynthesis were revealed when comparative genomic and genetic studies (51, 52, 77) linked the Sua5 (TsaC) and Kae1 (TsaD) protein families to its synthesis. Notably, these protein families were ranked among the top ten protein families of unknown function requiring characterization (61). Specifically, the knockout of the yeast genes *sua5* and *kae1* (homologous genes to *E. coli yrdC* (*tsaC*) and *ygjD* (*tsaD*)) led to the loss of t⁶A *in vivo* (Figure 11).

Despite direct evidence of the involvement of the TsaC and TsaD proteins in t⁶A biosynthesis, they proved insufficient to reconstitute t⁶A biosynthesis *in vitro*, suggesting the involvement of additional proteins (52, 77). Three observations narrowed the search for these additional proteins; YgjD (TsaD) forms an interaction network with the YeaZ (TsaB) and YjeE (TsaE) proteins (69), complementation of the lethal *E. coli ygdD* (*tsaD*) knockout with the *B. subtilis* homolog (*ydiE*) also requires the *B. subtilis yeaZ* homolog *ydiC* gene to permit growth (52), and there is strong bacterial clustering between *ygjD* (*tsaD*), *yjeE* (*tsaE*) and *yeaZ* (*tsaB*) (Figure 16). These observations led to investigation of *ygjD* and *yeaZ* genes in bacterial t⁶A biosynthesis.

Generation of expression constructs of the *tsaB*, *tsaC*, *tsaD*, and *tsaE* genes and the subsequent purification of their gene products from *E. coli*, allowed probing of their involvement in t⁶A biosynthesis. Radiochemical

incorporation of [^{14}C]-bicarbonate and [$\text{U-}^{14}\text{C}$]-L-threonine into tRNA in the presence of TsaD, TsaB, TsaC, and TsaE (Figure 20) demonstrated the first reconstitution of its pathway *in vitro*. Significantly, these experiments showed that t⁶A formation was dependent upon all four proteins in bacteria. Notably, YeaZ and YjeE are not found in eukaryotes, suggesting that bacterial t⁶A biosynthesis is a potential target for antibiotic development.

4.2 Mechanistic Studies of the t⁶A Biosynthetic Pathway

RNA Requirements of t⁶A Formation

Analysis of the tRNA substrate requirements of t⁶A formation (Figure 21) demonstrated that modification of tRNA was not a requirement for its formation *in vitro*. The failure of an anticodon stem loop (*H. sapiens* tRNA^{Lys}-3(UUU)) to serve as a substrate, despite evidence that TsaC binds the unmodified ASL with high affinity and specificity (78), suggested that full length tRNA was required for the formation of t⁶A. This observation was consistent with previous studies in *Xenopus laevis* oocytes that suggested the L-shaped tRNA architecture was required for t⁶A modification (79). Furthermore, non ANN decoding tRNA also failed as substrates, consistent with previous studies demonstrating that t⁶A formation is specific to ANN decoding tRNA (79). Analysis of tRNA^{Phe}(GAA) has provided some insight to the specificity of this reaction (71). Specifically, native tRNA^{Phe}(GAA) does not serve as a substrate for t⁶A formation while the tRNA^{Phe}A₃₆ to U₃₆ mutant does. These results suggest that the tRNA requirements for t⁶A

formation are a U_{36} , A_{37} , and the L-shaped architecture of full length tRNA.

Identity of Activated Intermediates in t^6A Production

Canonical carbamoyl transfer reactions proceed through a two step activation pathway (Figures 10, 53) (62, 80, 81). Typically, the first step is the activation of bicarbonate to either carboxyphosphate or carboxyadenylate. Subsequently, this activated carbonate is reacted with an amine to form a carbamoyl group with the loss of the first activating group. The carbamoyl group is then activated to either a carbamoyladenylate or carbamoylphosphate before subsequent transfer. In the context of t^6A biosynthesis, one would expect 2 equivalents of ATP to satisfy its energy requirements for carbamoyl transfer. The first of these intermediates would likely be either a carboxyadenylate or carboxyphosphate which could subsequently react with either the N^6 of A_{37} or the α -amine of threonine. After the carbamoyl linkage has formed, a second activation step would be required to form an acyl-phosphate or adenylate to react with the remaining component to produce t^6A (Figure 13).

Analysis of ATP co-products produced in the t^6A reaction suggested both AMP and ADP were produced (Figure 23) consistent with the formation of at least two activated intermediates in t^6A synthesis. The ADP co-product is produced as a co-product by TsaD, TsaB, and TsaE and is enhanced by the inclusion of all three proteins (Figure 23b lanes 4, 12-14). The AMP co-product is dependent upon the presence of both TsaC and threonine (Figure 23a lanes 6, 17-19).

Notably, the production of AMP and ADP is independent of tRNA (Figure 23) suggesting that a non tRNA dependent intermediate is formed.

The first intermediate identified in the t⁶A pathway was TC-AMP (71) which is produced by TsaC utilizing ATP and L-threonine. TC-AMP is already activated for carbamoyl transfer, suggesting no additional activation is required by the other t⁶A proteins. The generation of a carboxyadenylate by TsaC was excluded by HPLC analysis as only one equivalent of ATP is used in the production of TC-AMP (Figure 25). This suggested that TsaC was either proceeding through a carboxyphosphate intermediate (Figure 26) or using CO₂ as a substrate (Figure 27).

Subsequent investigation (Figure 29) demonstrated the production of pyrophosphate in the generation of TC-AMP consistent with CO₂ as the carbonate source for t⁶A formation. This was consistent with the previously reported proposal of a N-carboxy-L-threonine intermediate being formed in the active site of TsaC (71). This intermediate would likely be formed by the localized concentration of CO₂ and L-threonine in the active site of TsaC as CO₂ is significantly more electrophilic than bicarbonate and aliphatic amines have been shown to form stable carbamoyl moieties in the presence of high concentrations of CO₂ under alkaline conditions (82). As such, the function of TsaC in TC-AMP production is likely two fold, increase the localized concentration of reactants and to catalyze acid base catalysis to facilitate the reaction of L-threonine with CO₂.

Analysis of ADP Formation by TsaD, TsaB, and TsaE

Recent investigations (72) suggested that the archaeal protein Bud32 (a protein involved in t⁶A biosynthesis in archaea and eukaryotes) was acting as a P-loop ATPase and ADP production was required for t⁶A formation. However, experiments in which [U-¹⁴C]-TC-AMP was used as a precursor to t⁶A formation (Figure 30) excluded the possibility that TsaD, TsaB or TsaE were functioning as an essential ATPase in *E. coli*, as the transfer of the carbamoylthreonine of [U-¹⁴C]-TC-AMP to tRNA to produce t⁶A occurred readily in the absence of ATP. Furthermore, analysis of TsaE mutants deficient in ATPase activity are still capable of forming t⁶A, suggesting that the formation of ADP is not required for t⁶A formation (84), consistent with our observations that TC-AMP is sufficient for t⁶A formation in the absence of ATP. As such, ADP formation linked to TsaB, TsaD, and TsaE is likely not directly related to the chemistry of t⁶A formation.

Structural investigations have implicated TsaE's ATPase activity in the formation of a stable TsaB-TsaD-TsaE heterotrimer (84). This hypothesis is supported by the increased ATP hydrolysis in the presence of these three proteins (Figure 23). As such, the ATPase activity of TsaD, TsaB, and TsaE could be responsible for the maintenance of a carbamoyl acceptor complex but this has yet to be definitively shown. The current understanding of the biochemical mechanism of t⁶A formation determined from this work is summarized in Figure 54.

4.3 Peripheral Analysis of the t⁶A Biosynthetic Pathway

Substrate Analysis of the TsaC Reaction

Previous experiments revealed that tRNAs can contain a variety of adenosine N⁶ modifications in addition to t⁶A. Specifically, N⁶-hydroxynorvalylcarbamoyl adenosine (hn⁶A) (85) and N⁶-glycinylicarbamoyl adenosine (g⁶A) (86). These modifications differ only in the identity of the amino acid appended to the nucleoside (Figure 12). Given the similarity of these modifications to t⁶A it was hypothesized that the t⁶A biosynthetic pathway may be promiscuous in its incorporation of amino acids.

Analysis of a series of amino acids similar to threonine indicated two that were efficiently converted to their XC-AMP equivalents, hydroxynorvaline (Figure 45) and serine (Figure 46). Additionally, low levels of glycine conversion were observed (Figure 47). Kinetic analysis of TsaC catalyzed XC-AMP formation demonstrated that the production of HNC-AMP and SC-AMP occurs at comparable efficiency to the formation of TC-AMP (Table 7) suggesting that the formation of these XC-AMP derivatives can occur *in vivo*.

Subsequent analysis of HNC-AMP and SC-AMP demonstrated that they were not incorporated into tRNA and that the TsaD, TsaB, and TsaE proteins provide the selectivity to ensure only t⁶A is produced through this pathway in *E. coli*. As g⁶A was found in yeast and hn⁶A in thermophilic bacteria and archaea it is important to note that the proteins involved in t⁶A formation in these organisms may be less selective in their incorporation of XC-AMP derivatives.

Carboxythreonylation of DNA and Proteins

TC-AMP is a reactive compound that rapidly hydrolyzes to AMP, L-threonine, CO₂ and can rearrange to *trans*-(4S,5R)-5-Methyl-2-oxazolidinone-4-carboxylic acid (L-Thr-ox) (71). Its stability is on the lower end of biologically relevant phosphoric anhydrides (approximately 1/3 the stability of carbamoylphosphate) due to multiple intramolecular degradation pathways and the higher free energy of hydrolysis of adenylates (71, 75, 87, 88). Given this reactivity it is possible that nucleophiles other than water might react with TC-AMP such as proteins and DNA.

A protein of particular interest for investigation of carboxythreonylation was PrmC. PrmC is a protein-(glutamine-N5) methyltransferase which has been shown to have activity for release factors RF1 and RF2, proteins involved in translation (89, 90). This protein is often clustered with the TsaC protein and is found fused to TsaC in *Mycoplasma mobile*. It was hypothesized that enzymatic or non-enzymatic carboxythreonylation of PrmC could regulate its methyltransferase activity. This would be significant as both TsaC and PrmC are involved in translation. In a situation where excess TC-AMP was being produced by TsaC, PrmC could be carboxythreonylated to regulate its activity. However, experiments demonstrated that neither DNA, PrmC, or any of the t⁶A related proteins were non-enzymatically carboxythreonylated in the presence of excess TC-AMP.

4.4 Structural Investigation of t⁶A Pathway

The instability of TC-AMP, coupled with involvement of the KEOPS complex in archaea and eukaryotes, and previous examples of the channeling of activated carbamoyls (91, 92), suggested that the proteins involved in t⁶A synthesis might utilize substrate channeling through the formation of a protein complex. Protein pulldown experiments demonstrated that the TsaD, TsaC, TsaB, and TsaE proteins interacted with one or more protein partners *in vitro*, consistent with the formation of a single protein complex (Figure 41), supporting this hypothesis. Complementary gel filtration analysis of the *E. coli* proteins demonstrated the formation of a protein complex between TsaD and TsaB (Figure 44) consistent with a TsaD-TsaB₂ complex or a TsaD-TsaB complex with a non-idealized protein shape, which have been shown to effect the apparent molecular mass of proteins in gel filtration experiments (93-96).

No direct evidence of the hypothetical TsaD, TsaB, TsaC, and TsaE protein complex has been shown but the evidence supporting such a complex continues to grow. In eukaryotes t⁶A formation is catalyzed by the proteins Kae1 (TsaD), Sua5 (TsaC), Bud32 and Cgi121 (44). Crystal structures of both the eukaryotic Kae1-Bud32-Cgi121 heterotrimer (97) and bacterial TsaD₂-TsaB₂ heterotetramer have been solved (Figures 55, 56) (84). Furthermore, gel filtration utilizing the the TsaD, TsaB, and TsaE proteins from *Thermotoga maritima* have demonstrated the formation of an $\alpha_2\beta_2\gamma_2$ complex (98). Given the unstable nature of the TC-AMP intermediate it is likely that TsaC binds the

already formed TsaE-TsaB-TsaD heterohexamer and channels TC-AMP to the active site of TsaD.

4.5 The Essentiality of t⁶A, Emerging Differences in Eukaryotic and Bacterial Systems

Bioinformatic analysis of the t⁶A biosynthetic pathway has shown that there are substantial differences in t⁶A formation between eukaryotes and prokaryotes. In both systems the TsaC and TsaD proteins are conserved. Bioinformatic and biochemical investigations have demonstrated that the TsaC protein is a threonylcarbamoyl adenosine monophosphate synthase and TsaD is a N⁶-threonylcarbamoyl adenosine synthase. In prokaryotes, two additional proteins are required for the synthesis of t⁶A, TsaB and TsaE. In archaea and eukaryotes there are several different protein complexes which can synthesize t⁶A. In most eukaryotes the proteins TsaC, TsaD, Cgi121, Pcc1, and Bud32 are required (99). In yeast, an additional protein, Gon7 is also required. The mitochondrial protein Qri7 requires only TsaC to produce t⁶A (44).

Beyond the differences in the protein architecture required for t⁶A formation there is also a difference in the essentiality of t⁶A. In most prokaryotes t⁶A is essential. In numerous eukaryotes it is dispensable but its loss leads to severe growth defects. Recently, an important difference between eukaryotic and bacterial isoleucyl-tRNA synthetases was found (44). Specifically, two isoforms of tRNA^{Ile} (CAU and GAU), with or without t⁶A, were examined as

substrates for isoleucyl-tRNA synthetases from both eukaryotes and bacteria. In bacteria, t^6A_{37} -tRNA^{Ile}(CAU) and t^6A_{37} -tRNA^{Ile}(GAU) are positive determinants for aminoacylation by isoleucyl-tRNA synthetase while in eukaryotes they are not. This result is consistent with differences in eukaryotic and prokaryotic translational machinery as a result of t^6A modification of tRNA and may explain the essentiality of t^6A in bacteria. The major architectural differences between the t^6A biosynthetic machinery in the different domains of life, and the strictly required nature of the t^6A modification in prokaryotes suggests that this system has a potential as a future antibiotic target.

4.6 Future Work

Despite significant advancements in the understanding of t^6A biogenesis numerous questions remained unresolved. One such question is if TsaC homologs in other organisms are also promiscuous and how this promiscuity is dealt with. In the case of *E. coli*, the TsaD, TsaB, and TsaE proteins have been demonstrated to have high selectivity for TC-AMP over other XC-AMP derivatives. Due to the discovery of g^6A in yeast and hn^6A in archaea and thermophilic bacteria, it is necessary to show the selectivity of their t^6A machinery for XC-AMPs. Specifically, examination of eukaryotic and archaeal t^6A machinery could lead to the formation of g^6A and hn^6A *in vitro*.

Additional investigation of the ATPase activity of TsaB and TsaE is also required. Despite the demonstration that the ATPase activity of these proteins is

not required for the formation of t^6A it is unknown if this activity effects its rate of formation. This could be addressed through monitoring the rate of t^6A formation from TC-AMP in the presence or absence of ATP. Similarly, this could be addressed through kinetic analysis of t^6A formation utilizing TsaE and/or TsaB mutants deficient in ATPase activity.

To determine the TsaC residues involved in the formation of TC-AMP additional investigation would be required. This could be accomplished through additional characterization of the mutants investigated in this work. Specifically, full kinetic characterization of these mutants could provide insight on their relative binding of TsaC substrates to confirm their suggested roles in the catalysis of TC-AMP (73). R52 of TsaC is a residue that merits particular attention. Previous investigation demonstrated the R52A mutant of TsaC was not capable of rescuing t^6A production in $\Delta sua5$ yeast (51). Furthermore, HPLC analysis indicates that the R52A mutant is incapable of producing TC-AMP *in vitro*. This would suggest that the mutation of R52A is either detrimental to the structure or stability of TsaC, or that this residue is directly involved in the catalysis of TC-AMP.

To access the structural implications of the R52A mutant circular dichroism could be employed to determine if any major architectural changes are occurring due to this mutation. Furthermore, conservative mutations such as R52K could be generated. If the R52K mutant was capable of rescuing TC-AMP formation it would provide further evidence that R52 of TsaC was directly involved in its

formation.

Table 1: Degeneracy in the genetic code

Second Base									
First Base	U		C		A		G		Third Base
U	UUU	Phe	UCU	Ser	UAU	Tyr	UGU	Cys	U
	UUC		UCC		UAC		UGC		C
	UUA		UCA		UAA	Stop	UGA	Stop	A
	UUG		UCG		UAG	Stop	UGG	Trp	G
C	CUU	Leu	CCU	Pro	CAU	His	CGU	Arg	U
	CUC		CCC		CAC		CGC		C
	CUA		CCA		CAA	CGA	A		
	CUG		CCG		CAG	Gln	CGG		G
A	AUU		ACU	Thr	AAU	Asn	AGU	Ser	U
	AUC		ACC		AAC		AGC		C
	AUA	Ile	ACA		AAA	Lys	AGA	Arg	A
	AUG	Met	ACG		AAG		AGG		G
G	GUU	Val	GCU	Ala	GAU	Asp	GGU	Gly	U
	GUC		GCC		GAC		GGC		C
	GUA		GCA		GAA	Glu	GGA		A
	GUG		GCG		GAG		GGG		G

The genetic code showing degeneracy. Amino acids which have six codons are colored pink, four yellow, three blue, two orange, and one green. Stop codons are colored red.

Table 2: Expanded wobble hypothesis

Wobble Hypothesis		Expanded Wobble Hypothesis		
tRNA 34	Codon 3	tRNA 34	Codon 3	tRNA 37
G	C, U	G	C, U	t ⁶ A
C	G	C	G	m ⁶ A
I	U, C, A	I	U, C, A	m ¹ G
U	A, G	5-methylene uridine	G	wY
		2-thiouridine	A, G	
		5-oxyuridine	A, G, C, U	

Initial wobble hypothesis vs expanded wobble hypothesis. 5-methylene uridine constitutes all derivatives of m⁵U excluding 2-thiouridine. 2-thiouridine and 5-oxyuridine include all derivatives. tRNA 37 corresponds to the conserved 3' adjacent purine. Abbreviations of nucleosides: t⁶A (N⁶-threonylcarbamoyl adenosine), m⁶A (N⁶-methyladenosine), m¹G (1-methylguanosine), and wY (wybutosine). Table reproduced from (21).

Table 3: N⁶-carbamoyladenine modifications

Modification	Similarity to t ⁶ A
t ⁶ A	-NA-
ms ² t ⁶ A	Methylthio derivative
m ⁶ t ⁶ A	Methyl derivative
hn ⁶ A	Threonine is replaced by hydroxynorvaline
ms ² hn ⁶ A	Threonine is replaced by hydroxynorvaline and methylthiolated
g ⁶ A	Threonine is replaced by glycine

Examples of modifications in tRNA that differ only slightly in composition from t⁶A. Abbreviations used N⁶-glycylcarbamoyladenine (g⁶A), N⁶-threonylcarbamoyladenine (t⁶A), 2-methylthio-N⁶-threonylcarbamoyladenine (ms²t⁶A), N⁶-methyl-N⁶-threonylcarbamoyladenine (m⁶t⁶A), N⁶-hydroxynorvalylcarbamoyladenine (hn⁶A), 2-methylthio-N⁶-hydroxynorvalylcarbamoyladenine (ms²hn⁶A).

Table 4: Oligonucleotides used for the cloning of *tsaD*, *tsaB*, *tsaE*, *tsaC* mutants and *prmC* genes

Target	Primer	Primer Sequence
<i>tsaE</i>	Sense	5'-GGTATTGAGGGTCGCATGATGAATCGAGTAATTCCG-3'
<i>tsaE</i>	Antisense	5'-AGAGGAGAGTTAGAGCCTTAACCGGCTAAACGCGCCAG-3'
<i>tsaB</i>	Sense	5'-GGTATTGAGGGTCGCATGCGAATTCTGGCTATCG-3'
<i>tsaB</i>	Antisense	5'-AGAGGAGAGTTAGAGCCTCATTCTTTGCCCGGAAG-3'
<i>tsaD</i>	Sense	5'-CCGTATATATATTGGATCCCGTGACTTGATTGA-3'
<i>tsaD</i>	Antisense	5'-CTAGAAGTACAGTAAGCTTACGCAGCCGGTA-3'
<i>prmC</i>	Sense	5'-GGTATTGAGGGTCGCATGGAATATCAACACTGG-3'
<i>prmC</i>	Antisense	5'-AGAGGAGAGTTAGAGCCTCATTGATAATAGCGGCCG-3'
<i>tsaC</i>	Sense	5'-GGTATTGAGGGTCGCAATAATAACCTGCAAAG-3'
<i>tsaC</i>	Antisense	5'-AGAGGAGAGTTAGAGCCTTACCCCTGTGCGAAAC-3'
<i>tsaC</i> N141A	Sense	5'-GGTTTCTACCAAGTCCCGCCTTGAGTGGATTGCC-3'
<i>tsaC</i> N141A	Antisense	5'-GGCAATCCACTCAAGGCGGCACTGGTAGAAAC-3'

tsaE, *tsaB*, *tsaC*, and *prmC* denote oligonucleotides that used standard FactorXa/LIC cloning. A single set of oligonucleotides were used to clone the *tsaC* K53A, R110A, K50A, and R52A genes into a FactorXa/LIC vector. *tsaD* cloning was accomplished as described in (63). N141A denotes oligonucleotides used for QuikChange mutagenesis to generate the *tsaC* N141A mutant.

Table 5: Oligonucleotides used for tRNA templates

#	Oligonucleotide Sequence
1	AATTCCTGCAGTAATACGACTCACTATAGGGGTCGTTAGCTCAGTTGGTAGAGCAG
2	TGGTGGGTCGTGCAGGATTCGAACCTGCGACCAATTGATTAAAAGTCAACTGCTCTACC
3	AATTCCTGCAGTAATACGACTCACTATAGGGCCCCTTAGCTCAGTGGTTAGAGCAGGCG
4	TGGTGGCCCCTGCTGGACTTGAACCAGCGACCAAGCGATTATGAGTCGCCTGCTCT
5	AATTCCTGCAGTAATACGACTCACTATAAGGCTTGTAGCTCAGGTGGTTAGAGCGC
6	TGGTAGGCCTGAGTGGACTTGAACCACCGACCTCACCTTATCAGGGGTGCGCTCTAAC
7	TGCAGTAATACGACTCACTATAAGTCCCGTGGGGTAGTGGTAATCCTGC
8	TGGTAGTCCCGAGCGGAGTCGAACCGCTGTGCGCCGGGTCCAAAGCCCAGCAGGATTAC
9	TAATACGACTCACTATAGGCAGACTTTTAATCTGC
10	GCAGATTAAAAGTCTGCCTATAGTGAGTCGTATTA
11	AGCTTTAATACGACTCACTATAGGGGCTGATATGGCTCAG
12	TTGGTAGAGCGCACCTTGGTAGGGGTGGGGTCCCCAGTTCGACTCTGGG
13	TATCAGCACCATATGCTAGTTATTGCTCAGG
14	GATCCCTGAGCAATAACTAGC
15	ATATGGTGCTGATACCCAGAGTCGACTGGGGACCCCACCCCTACCAAGGG
16	TGCCTCTACCAACTGAGCCATATCAGCCCCTATAGTGAGTCGTATTAA

All oligonucleotides are oriented 5'-3'. Oligonucleotide numbers correspond as follows: *E. coli* tRNA^{Lys}(UUU) sense (1) and antisense (2). *E. coli* tRNA^{Ile}(CAU) sense (3) and antisense (4). *E. coli* tRNA^{Ile}(GAU) sense (5) and antisense (6). *M. thermoautotrophicus* tRNA^{Gln}(CUG) sense (7) and antisense (8). Human tRNA^{Lys}-3(UUU) sense (9) antisense (10). *E. coli* tRNA^{Thr}(GGU) sense (11-13) and antisense (14-16). tRNA^{Thr} was produced by ligating six oligonucleotide pieces together before ligation into pUC18.

Table 6: Phosphorylation sites of proteins involved in t⁶A formation

Protein	Sequence ¹	Residue	Residue #	Score ²
TsaD	SDGSSEIFF	S	35	0.767
TsaD	KIKKTTPLR	T	44	0.648
TsaD	IKKTTPLRR	T	45	0.527
TsaD	QTGGSRVLG	S	125	0.618
TsaD	TVGRSLAFA	S	221	0.501
TsaD	AGGVSANRT	S	394	0.605
TsaD	SANRTLRAK	T	398	0.567
TsaD	DLGVSVRPR	S	448	0.804
TsaB	RGPGSFTGV	S	67	0.649
TsaB	KNGATRVLA	T	109	0.63
TsaB	KPLVSTSAN	S	263	0.584
TsaB	LVSTSANLS	S	265	0.632
TsaB	RLNPSEIRD	S	302	0.547
TsaC	ETIFSRWPG	S	98	0.501
TsaC	KPLVSTSAN	S	148	0.584
TsaC	LVSTSANLS	S	150	0.632
TsaC	RLNPSEIRD	S	187	0.547
TsaE	SAVSSAGEL	S	142	0.663

1) Predicted phosphorylation sites of t⁶A proteins. **2)** Scores above 0.5 indicate a high potential for phosphorylation. Scores were calculated using the NetPhosBac algorithm.

Table 7: Summary of TsaC kinetic parameters

Substrate	k _{cat} (min)	K _m (mM)	Method ¹
ATP	0.039 +/- 0.003	0.11 +/- 0.04	HPLC
ATP	0.30 +/- 0.01	0.30 +/- 0.04	Coupled Assay
L-threonine	0.063 +/- 0.005	1.4 +/- 0.5	HPLC
L-threonine	0.25 +/- 0.009	0.33 +/- 0.05	Coupled Assay
D,L-hydroxynorvaline	0.11 +/- 0.05	0.46 +/- 0.09	Coupled Assay
L-serine	0.31 +/- 0.02	0.29 +/- 0.05	Coupled Assay

1) Method indicates whether the kinetic parameters were determined from HPLC or coupled assay.

Table 8: Relative activity of TsaC mutants

TsaC	Relative Activity¹	Predicted Significance
Wild Type	100.0%	Standard
K53A	20.0%	ATP Binding
R110A	57.9%	ATP Binding
K50A	17.2%	Phosphate coordination
N141A	14.5%	ATP Binding
R52A	-ND- ²	Phosphate coordination

1) Relative activities were determined by comparing mutant activities to the wild type protein. **2)** -ND- represents no activity detected, indicating that no TC-AMP was formed by the R52A mutant.

Table 9: Size exclusion chromatography of t⁶A proteins

Proteins¹	Expected Mass (kDa)²	Apparent MM (kDa) of Peaks³
TsaDBCE	107.8	144.9, 97.4, 48.5, 22.1
TsaDBC	91.0	57.2, 23.3
TsaDBE	87.2	126.5, 45.6, 21.2
TsaDCE	82.6	156.2, 95.4, 47.9
TsaBCE	62.7	53.5, 20.9
TsaDB	70.3	151.4, 99.6, 50.6
TsaDC	65.8	243.8, 127.1, 44.2, 21.8
TsaDE	62.0	127.6, 46.1, 24.1
TsaBC	45.8	61.1, 23.5
TsaBE	42.0	53.9, 22.6
TsaCE	37.5	47.2, 22.3
TsaE homodimer	33.7	45.6, 21.5
TsaB homodimer	50.4	56.9

1) Proteins indicates the proteins present in the size exclusion experiment. **2)** Expected mass indicates the mass of a 1:1 presence of all proteins in a heteromeric structure in kilodaltons. **3)** Apparent MM indicates the determined apparent molecular masses from size exclusion experiments. Weights separated by commas indicate more than one retention peak was found.

Figure 1: Secondary structure of tRNA

Secondary structure of *E. coli* tRNA^{Lys}(UUU) represented by a clover leaf. The anticodon stem loop is colored in green, the D stem and loop is orange, the T stem and loop is gray, the aminoacyl stem is blue, and the variable loop is pink. Modified nucleoside abbreviations: D (dihydrouridine), mnm⁵s²U (5-methylaminomethyl-2-thiouridine), t⁶A (N⁶-threonylcarbamoyl adenosine), T (thymine), acp³U (3-(3-amino-3-carboxypropyl)uridine), m⁷G (7-methylguanosine), and ψ (pseudouridine).

Figure 2: Tertiary structure of tRNA

Crystal structure of yeast tRNA^{Phe}. Location of aminoacylation and the anticodon are indicated. The anticodon stem and loop is colored yellow. The acceptor stem is colored blue. The D stem and loop is colored orange. The T stem and loop is colored gray. The variable loop is colored pink. Image produced from PDB entry 4TNA (4).

Figure 3: Canonical role of tRNA in translation

Amino acids are depicted as green circles. The ribosome is shown in blue.

- 1)** An aminoacylated tRNA binds to the aminoacyl (A) site of the ribosome in a sequence specific manner based on codon-anticodon interactions.
- 2)** The growing peptide chain found bound to tRNA in the peptidyl (P) site is transferred to the amino acid of the aminoacyl-tRNA prompting transfer from the A to P sites.
- 3)** With every sequential addition of an amino acid the tRNA found in the P site is transferred to the Exit (E Site) before leaving the ribosome as a uncharged tRNA.
- 4)** The direction of ribosome movement along the mRNA.

Figure 4: Simple and hyper-modifications of nucleosides

Examples of canonical bases, simple modifications, and hyper-modifications. **A)** Adenosine serves as the starting nucleotide for both **B)** inosine and **C)** 2-methylthio- N^6 -threonylcarbamoyl adenosine (ms^2t^6A). Inosine is produced by the deamination of the exocyclic amine. ms^2t^6A is produced by the addition of a carbamoylthreonyl group to the exocyclic amine and a methylthio moiety to the 2' ring carbon. **D)** Guanosine is the starting material for both **E)** 1-methyl guanosine and **F)** Wybutosine. 1-methyl guanosine is produced by a methyl addition. Wybutosine is produced by methylation, tricyclization, and addition reactions.

Figure 5: Wobble base pairing

Examples of canonical (A:U and G:C) and some wobble base pairings. Bases are denoted by their one letter abbreviations (bold). Abbreviations: A=adenosine, G=guanosine, C=cytidine, U=uridine, I=inosine.

Figure 6: Canonical and non-canonical roles of tRNA

Uncharged tRNA play critical roles in gene expression and regulation of cellular death. Gene expression is primarily regulated by uncharged-tRNA binding either nucleic acids or activating/deactivating enzymes. The generation of tRNA fragments occurs through a variety of endonucleases (Dicer, RNaseP/Z, etc) and are postulated to inhibit translation and act as small non-coding RNAs. Regulation of cell death is primarily mediated through cytochrome interactions. Aminoacylated tRNA serve their canonical role in protein synthesis as well as a variety of amino acid addition reactions (cell walls/membranes, peptide labeling, antibiotics etc) and aminoacylated tRNA dependent pathways (tetrapyrrole, tRNA mischarging etc). Image reproduced from (32). Image of tRNA produced from PDB entry 4TNA (4).

Figure 7: Structure of t⁶A and substrates implicated in its biosynthesis
 Pioneering work completed in the 1970s identified the formation of t⁶A to be dependent upon L-threonine, bicarbonate, and ATP.

Figure 8: Structure of an ASL containing t^6A

Crystal structure of human tRNA^{Lys}(UUU) ASL (blue) bound to the 16S ribosomal subunit with mRNA (purple). The t^6A_{37} base is colored green. Image reproduced from PDB entry 1MXQ (54).

Figure 9: TobZ is comprised of both TsaC and TsaD like domains

The TobZ protein contains both YrdC (TsaC) and Kae1 (TsaD) like domains and performs chemistry similar to what is expected for the t^6A biosynthetic pathway. Image was taken from (62).

Figure 10: Chemistry catalyzed by TobZ

In the first step the TsaC like domain catalyzes the transfer of an activated carbamoyl (carbamoyl phosphate) to adenosine monophosphate. Subsequently, adenosine monophosphate is used as an activating group to transfer the carbamoyl group to a sugar by the TsaD like domain.

Figure 11: Analysis of yeast tRNA in *sua5* and *kae1* knockout strains
 Analysis of yeast *sua5* (A-D) and *kae1* (E-F) knockout strains. **A)** Wild type trace, t^6A present. **B)** *sua5* (*tsaC*) knockout strain t^6A deficient. **C)** *sua5* knockout with *sua5* expressed in trans, t^6A production is rescued. **D)** *sua5* knockout with *E. coli tsaC* expressed in trans t^6A production is rescued. **E)** *kae1* (*tsaD*) knockout, t^6A absent. **F)** *kae1* knockout with *kae1* expressed in trans, t^6A production is rescued. Figure was reproduced from (51, 52).

Figure 12: Structure of N⁶-carbamoyladenine modifications t⁶A, s⁶A, g⁶A, hn⁶A

N⁶-threonylcarbamoyl adenosine (t⁶A), N⁶-glycylcarbamoyl adenosine (g⁶A), and N⁶-hydroxynorvalylcarbamoyl adenosine (hn⁶A) have been previously reported in yeast tRNA. N⁶-serinylcarbamoyl adenosine (s⁶A) is a potential N⁶-carbamoyladenine nucleoside modification.

Figure 13: Proposed chemistry of t⁶A formation

Activated indicates either adenosine monophosphate or phosphate. The expected chemistry of t⁶A formation would initially occur through the activation of bicarbonate. Subsequently, this activated bicarbonate could condense with either threonine or the exocyclic amine of adenosine. If a N-carboxy-L-threonine adduct was formed a subsequent round of activation would be required before reacting with the exocyclic amine of adenosine. If a carboxyadenosine adduct was formed activation would also be required before reaction with threonine.

Figure 14: TsaC purification

SDS-PAGE analysis of TsaC purification

Lane 1, molecular weight marker.

Lane 2, column flow through.

Lane 3, column wash.

Lane 4, 20 mM imidazole wash.

Lane 5, 30 mM imidazole wash.

Lane 6, 200 mM imidazole elution.

Figure 15: SUMO-TsaD purification

A) SDS-PAGE analysis of SUMO-TsaD purification.

Lane 1, flowthrough.

Lane 2, 20 mM imidazole wash.

Lane 3, 200 mM imidazole elution.

Lane 4, molecular weight marker.

B) Elution profile obtained from IEX of the SUMO-TsaD protein. Fractions (0.5 mL) were pooled and protein content was assessed by monitoring the absorbance at A_{260} . SUMO-TsaD was found in the fractions corresponding to the first major peak (6-9)

C) SDS-PAGE showing purified SUMO-TsaD.

Lane 1 molecular weight marker,

Lane 2-5, purified SUMO-TsaD corresponding to fractions 6-9 from IEX.

Figure 16: Clustering of bacterial *yeaZ* and *yjeE* with *ygjD*

Select clustering of *yeaZ* (*tsaB*), *yjeE* (*tsaE*) and *ygjD* (*tsaD*) in bacterial genomes. Image taken from (52).

Figure 17: TsaB purification

SDS-PAGE analysis of expression and purification of TsaB.

Lane 1, column flow through.

Lane 2, column wash.

Lane 3, 20 mM imidazole wash.

Lane 4, 30 mM imidazole wash.

Lane 5, 200 mM imidazole elution.

Lane 6, molecular weight marker.

Figure 18: TsaE purification

SDS-PAGE analysis of expression and purification of TsaE.

Lane 1, column flow through.

Lane 2, column wash.

Lane 3, 20 mM imidazole wash.

Lane 4, 30 mM imidazole wash.

Lane 5, 200 mM imidazole elution.

Lane 6, molecular weight marker.

Figure 19: RNA transcription and purification

Urea-PAGE (10%) gel. RNA is visualized by placing the gel on a fluorescent TLC plate and exposing to a UV-light.

Lane 1, tRNA^{Ile}(GAU) transcription.

Lane 2, tRNA^{Ile}(CAU) transcription.

Lane 3, tRNA^{Lys}(UUU) transcription.

The dye bromophenol blue (BPB) runs comparable to a 12 nucleotide RNA.

Figure 20: Proteins requirements for t⁶A formation *in vitro*

In vitro tRNA precipitation assays were performed in duplicate with [U-¹⁴C]-L-threonine (left axis, black bars) or [¹⁴C]-bicarbonate (right axis, gray bars).

Assays contain either all components or with indicated protein or ATP absent. Figure taken from (70).

Figure 21: tRNA requirements of t⁶A formation

In vitro tRNA precipitation assays with [U-¹⁴C]-L-threonine (left axis, black bars) or [¹⁴C]-bicarbonate (right axis, gray bars). *M. thermoautotrophicus* tRNA^{Gln}(CUG), *E. coli* tRNA^{Lys}(UUU), *E. coli* tRNA^{Thr}(GGU), and *H. sapiens* tRNA^{Lys}-3(UUU) (ASL) were *in vitro* transcribed. Δsua5 is unfractionated tRNA from Δsua5 yeast, and wt is unfractionated tRNA from wild-type yeast extract. Figure taken from (70).

Figure 22: LC-MS of nucleosides derived from the t⁶A reaction

A) HPLC trace of digested *in vitro* transcribed *E. coli* tRNA^{Lys}(UUU) before (bottom trace) and after (top trace) reaction with TsaC, TsaD, TsaB, and TsaE.

B) Partial mass spectrum of compound eluting at ~ 9.9 minutes.

Figure taken from (70).

A

Figure 23: α -[^{32}P]-ATP co-product analysis

A) tRNA absent. **B)** tRNA present. Assay solutions were prepared as described in experimental with (+) inducing the presence and (-) the absence of each of the specific components. Lane 1 contains only α -[^{32}P]-ATP as a standard. Figures taken from (70).

B

Figure 24: LC and LC-MS analysis of TC-AMP

A partial ion chromatogram showing the expected m/z TC-AMP, 491.10. The structure of TC-AMP and an LC trace showing the formation of a new peak when ATP, L-threonine and bicarbonate are reacted in the presence of TsaC are inserted into partial ion chromatogram.

Figure 25: Non stoichiometric formation of AMP and TC-AMP

LC trace of the TsaC catalyzed conversion of ATP to TC-AMP. The formation of TC-AMP occurs in significant excess to AMP, eliminating the potential of 2 ATPs being consumed to produce one TC-AMP.

Figure 26: Proposed two step activation of TC-AMP

1) ATP and bicarbonate are converted to carboxyphosphate and ADP. **2)** carboxyphosphate and L-threonine condense to form N-carboxy-L-threonine and phosphate. **3)** N-carboxy-L-threonine and ADP condense to produce TC-AMP and phosphate. The net equation of this reaction would be: ATP + bicarbonate + L-threonine $\rightarrow$ TC-AMP + 2 phosphate.

Figure 27: Proposed formation of TC-AMP using CO₂

TsaC catalyzes the addition of CO₂ to the amine of L-threonine to form carboxy-L-threonine. ATP activates carboxy-L-threonine to form TC-AMP and PPi.

Figure 28: HPLC analysis of the formation of ATP from TC-AMP
Purified TC-AMP (blue trace), TsaC catalyzed ATP formation using pyrophosphate (red trace), TsaC catalyzed ATP formation using phosphate (yellow trace).

Figure 29: Formation of ATP from TC-AMP catalyzed by TsaC

Lane 1, α - ^{32}P -ATP standard.

Lane 2, ^{32}P -PPi standard.

Lane 3, reverse reaction using ^{32}P -PPi.

Lane 4, α - ^{32}P -ATP standard.

Lane 5, ^{32}P -PPi standard.

Lane 6, reverse reaction using ^{32}P -Pi

Lane 7, overloaded Pi standard.

Figure 30: Formation of t⁶A from TC-AMP

tRNA precipitation assays were performed in duplicate with the following components: [U-¹⁴C]-L-threonine, TsaD, TsaB, TsaC, TsaE, ATP (A), [U-¹⁴C]-TC-AMP, TsaD, TsaB, TsaC, TsaE, ATP (B), [U-¹⁴C]-TC-AMP, TsaD, TsaB, TsaE (C), [U-¹⁴C]-TC-AMP, TsaD, TsaB, TsaC, TsaE (D), [U-¹⁴C]-TC-AMP, TsaB, TsaC, TsaE (E). The reported DPM is an average of 2 experiments.

Figure 31: pH profile of t⁶A formation

tRNA precipitation assays were performed in duplicate. Velocity is reported in μM/min. Error bars indicate the standard deviation of the assays.

Figure 32: Steady state kinetics of the TsaC reaction (HPLC, L-threonine)

Assays were performed in duplicate. Velocity is reported in $\mu\text{M}/\text{min}$. k_{cat} was determined to be $0.063 \pm 0.005 \text{ min}^{-1}$. K_m was determined to be $1.4 \pm 0.5 \text{ mM}$.

Figure 33: Steady state kinetics of the TsaC reaction (HPLC, ATP)

Assays were performed in duplicate. Velocity is reported in $\mu\text{M}/\text{min}$. k_{cat} was determined to be $0.039 \pm 0.003 \text{ min}^{-1}$. K_m was determined to be $0.11 \pm 0.04 \text{ mM}$.

Figure 34: UV trace of coupled assay

UV-VIS monitoring of the coupled assay. Red trace, L-threonine was excluded from the reaction mixture, providing a background oxidation rate of NADH. Black trace, 100 μ M pyrophosphate was added to determine the maximum rate of enzymatic conversion by the coupled enzymes. Blue trace, TsaC reaction as described in experimental using 10 mM L-threonine.

Figure 35: Steady state kinetics of the TsaC reaction (coupled assay, ATP)
Assays were performed in at least duplicate. Velocity is reported in $\mu\text{M}/\text{min}$. k_{cat} was determined to be $0.30 \pm 0.01 \text{ min}^{-1}$. K_m was determined to be $0.30 \pm 0.04 \text{ mM}$.

Figure 36: Steady state kinetics of the TsaC reaction (coupled assay, L-threonine)

Assays were performed in duplicate. Velocity is reported in $\mu\text{M}/\text{min}$. k_{cat} was determined to be $0.25 \pm 0.009 \text{ min}^{-1}$. K_m was determined to be $0.33 \pm 0.05 \text{ mM}$.

Figure 37: Steady state kinetics of the TsaC reaction (coupled assay, L-serine)

Assays were performed in duplicate. Velocity is reported in $\mu\text{M}/\text{min}$. k_{cat} was determined to be $0.31 \pm 0.02 \text{ min}^{-1}$. K_m was determined to be $0.29 \pm 0.05 \text{ mM}$.

Figure 38: Steady state kinetics of the TsaC reaction (coupled assay, D,L-hydroxynorvaline)

Assays were performed in duplicate. Velocity is reported in $\mu\text{M}/\text{min}$. k_{cat} was determined to be $0.11 \pm 0.05 \text{ min}^{-1}$. K_m was determined to be $0.46 \pm 0.09 \text{ mM}$.

Figure 39: Solution structure of TsaC

Solution structure of TsaC derived from [U-¹⁵N, U-¹³C]-TsaC. PDB entry 2MX1 (73).

Figure 40: Crystal structure of *S. tokodaii* with TC-AMP bound

Crystal structure of *S. tokodaii* showing its surface. TC-AMP and the residues which were mutated in *E. coli* are drawn as sticks. The image was produced using PDB entry 4E1B (62, 74).

The *S. tokodaii* residues are indicated on the crystal structure. These residues correspond with *E. coli* as follows K57 (K50), R59 (R52), N63 (K53), R121 (R110), and N145 (N141) as determined from an alignment between *S. tokodaii* Sua5 and *E. coli* YrdC (51).

Figure 41: Analysis of protein interactions

SDS-PAGE analysis of protein interactions. Proteins labeled in white contain a 6x histidine tag. Proteins labeled in black contain no histidine tag. Lanes 1 and 8 correspond to the protein molecular weight standards.

Lane 2, non column bound fraction of the TsaC+TsaD protein incubation.

Lane 3, column bound fraction of the TsaC+TsaD protein incubation.

Lane 4, non column bound fraction of the TsaB+TsaD protein incubation.

Lane 5, column bound fraction of the TsaB+TsaD protein incubation.

Lane 6, non column bound fraction of the TsaB+TsaC protein incubation.

Lane 7, column bound fraction of the TsaB+TsaC protein incubation.

Lane 9, non column bound fraction of the TsaB+TsaE protein incubation.

Lane 10, column bound fraction of the TsaB+TsaE protein incubation.

Figure 42: Standard curve for determination of protein apparent mass
The Y-axis represents the log molecular weight. The X-axis represents the eluted volume of standard divided by the excluded volume.

E

Protein	Monomeric MM (kDa)	Apparent MM (kDa)
TsaD	45.2	127.6, 43.0
TsaC	20.6	21.3
TsaB	25.2	56.9
TsaE	16.9	45.6, 21.5

Figure 43: Gel filtration of the proteins involved in t^6A biosynthesis

Size exclusion chromatography (SEC) of individual proteins involved in t^6A biosynthesis. The Y-axis represents mAu while the X-axis represents time in minutes. **A)** SEC of the TsaE protein. **B)** SEC of the TsaC protein. **C)** SEC of the TsaB protein. **D)** SEC of the TsaD protein. **E)** Table summarizing the monomeric weight of proteins, and apparent molecular masses determined from SEC.

Figure 44: Overlay of the TsaD, TsaB, and TsaD/TsaB gel filtration chromatograms

Red trace, TsaB. Black trace, TsaD and TsaB. Blue trace, TsaD. A new peak is found when TsaB and TsaD are incubated together. This peak corresponds to an apparent molecular weight of 99.6 kDa. The expected mass of a TsaD-TsaB heterodimer is 70.3 kDa while the expected mass of a TsaD-TsaBx2 trimer is 95.6 kDa.

Figure 45: LC analysis of TsaC Reaction with D,L-hydroxynorvaline
 LC Trace of the TsaC reaction in the presence of D,L-hydroxynorvaline and the structure of the putative product HNC-AMP.

Figure 46: LC analysis of TsaC reaction with L-serine

LC Trace of the TsaC reaction in the presence of L-serine and the structure of the putative product SC-AMP.

Figure 47: LC analysis of TsaC reaction with glycine

LC Trace of the TsaC reaction in the presence of glycine and the structure of the putative product GC-AMP.

Figure 48: Extracted ion chromatogram of peak eluting at 25.38 minutes
An ion with m/z of 447.067 was observed, consistent with the predicted m/z of 447.07 (error of 5.79 ppm). The ions corresponding to GC-AMP and AMP (346.05) are highlighted in yellow.

Figure 49: Extracted ion chromatogram of peak eluting at 17.81 minutes
An ion with m/z of 477.08 was observed, consistent with the predicted m/z of 477.08 (error of 0.16 ppm). The ion corresponding to SC-AMP is highlighted in yellow.

Figure 50: Extracted ion chromatogram of peak eluting at 18.2 minutes

An ion with m/z at 505.11 was observed, consistent with the predicted m/z of 505.11 (error of 3.86 ppm for HNC-AMP). The masses corresponding to HNC-AMP and AMP (346.05) are highlighted in yellow.

Figure 51: Incorporation of carbamoyladenine derivatives into tRNA

Average background subtracted DPM of incorporation of two reactions are reported. D,L-hydroxynorvaline, glycine and serine have incorporations slightly above background, but substantially less than 1.4% of the canonical substrate, L-threonine.

Figure 52: PrmC purification

SDS-PAGE analysis of purification of PrmC.

Lane 1, column wash.

Lane 2, 20 mM imidazole wash.

Lane 3, 30 mM imidazole wash.

Lane 4, 200 mM imidazole elution.

Lane 5, molecular weight marker.

Figure 53: Biogenesis of N-carbamoyl-L-aspartate

The biochemical route to N-carbamoyl-L-aspartate. **1)** ATP and bicarbonate are converted to carboxyphosphate and ADP. **2)** The lone pair of ammonia attacks the carbonyl of carboxy phosphate producing carbamate and phosphate. **3)** Carbamate is subsequently activated by ATP producing carbamoyl-phosphate and ADP. **4)** The carbamoyl group of carbamoyl-phosphate is transferred to aspartate with the loss of phosphate producing N-carbamoyl aspartate (83).

Figure 54: Biosynthetic pathway of t⁶A formation

TsaC catalyzes the formation of TC-AMP and PPi using L-threonine, CO₂, and ATP. The carbamoylthreonine moiety is transferred from AMP to the exocyclic amine of A₃₇. Bonds to the backbone of tRNA are indicated as R groups.

Figure 55: Crystal structure of the TsaD-TsaB heterodimer

Crystal structure of the TsaD-TsaB heterodimer. The solved structure was a tetramer comprised of two TsaD-TsaB heterodimers. Red indicates the TsaD protein. Blue indicates the TsaB protein. Taken produced from PDB entry 4YDU (84).

Figure 56: Crystal structure of the Kae1-Bud32-Cgi121 protein complex
Red Kae1-Bud32 fusion protein. Blue Cgi121. Image produced from the PDB entry 3ENH (97).

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