Review

Biosynthesis of Sulfur-Containing tRNA Modifications: A Comparison of Bacterial, Archaeal, and Eukaryotic Pathways

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Abstract: Post-translational tRNA modifications have very broad diversity and are present in all domains of life. They are important for proper tRNA functions. In this review, we emphasize the recent advances on the biosynthesis of sulfur-containing tRNA nucleosides including the 2-thiouridine (s^2U) derivatives, 4-thiouridine (s^4U), 2-thiocytidine (s^2C), and 2-methylthioadenosine (ms^2A). Their biosynthetic pathways have two major types depending on the requirement of iron–sulfur (Fe–S) clusters. In all cases, the first step in bacteria and eukaryotes is to activate the sulfur atom of free L-cysteine by cysteine desulfurases, generating a persulfide (R-S-SH) group. In some archaea, a cysteine desulfurase is missing. The following steps of the bacterial s^2U and s^4U formation are Fe–S cluster independent, and the activated sulfur is transferred by persulfide-carrier proteins. By contrast, the biosynthesis of bacterial s^2C and ms^2A require Fe–S cluster dependent enzymes. A recent study shows that the archaeal s^4U synthetase (ThiI) and the eukaryotic cytosolic 2-thiouridine synthetase (Ncs6) are Fe–S enzymes; this expands the role of Fe–S enzymes in tRNA thiolation to the Archaea and Eukarya domains. The detailed reaction mechanisms of Fe–S cluster depend s^2U and s^4U formation await further investigations.

Keywords: tRNA modification; sulfur; iron–sulfur cluster; translation

1. Introduction

Transfer RNAs (tRNAs) play a crucial role in protein synthesis by serving as a linkage between messenger RNAs (mRNAs) and amino acids. Amino acids are attached to tRNAs during aminoacylation catalyzed by aminoacyl-tRNA synthetases (aaRSs) [1]. The aaRSs define the genetic code by accurately matching cognate tRNAs with their corresponding amino acids. They mischarge tRNA once in ~10^4 reactions, similar to the error rate of transcription (~10^{-4}) and ribosomal decoding (~10^{-4}) [1,2]. One reason for these high accuracy levels of aminoacylation and ribosomal decoding reactions is tRNA posttranscriptional modifications that include over 100 different types [3,4]. In order to fullfil their canonical roles in protein synthesis as well as their non-canonical cellular roles, tRNAs are heavily modified to the fully functional states–including removing the 5′-end ladder, splicing introns, adding the 3′-end CCA tail, and numerous post-translational chemical modifications of specific nucleosides [5]. The functions of tRNA posttranscriptional modifications are diverse, such as stabilizing tRNA structures, enabling identification of tRNAs by aaRSs, enhancing ribosomal binding to aminoacylated tRNAs, maintaining reading frame, and ensuring proper codon-anticodon base pairing [6]. While some posttranscriptional modifications are conserved in all domains of life (for example, methylation), other types are specific to one domain (for example, archaeosine in archaea) [7]. In this review, we will focus on sulfur-containing tRNA modifications and recent advances in their biosynthetic pathways.

2. Sulfur-Containing Modifications and Their Physiological Roles

Sulfur-containing modifications are commonly found at seven different tRNA positions: 8, 9, 32, 33, 34, 37, and 54 (Figure 1). These modifications include the 2-thiouridine (s^2U) derivatives, 4-thiouridine (s^4U), 2-thiocytidine (s^2C), and 2-methylthioadenosine (ms^2A). Only thiolated guanine has not been reported to date. These thio-modifications fulfill versatile functions, and their roles differ according to their positions on tRNAs. Thio-modifications outside the anticodon loop often improve tRNA structural stability, while thio-modifications in the anticodon loop are usually important for translational fidelity and efficiency.
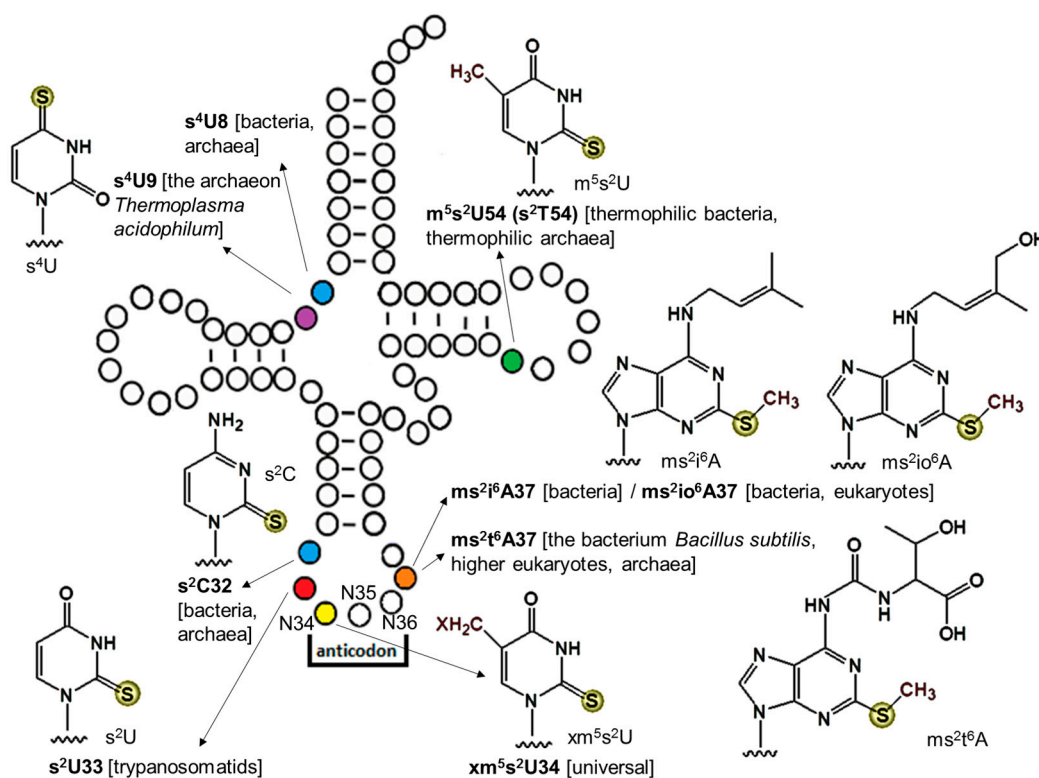

Figure 1. The location and structure of known tRNA thio-modification in three domains of life. The distribution of each modification is indicated in square brackets. Abbreviations: s^4U, 4-thiouridine; s^2C, 2-thiocytidine; s^2U, 2-thiouridine; m^5s^2U, 5-methyl-2-thiouridine; s^2T, 2-thioribothymidine; ms^2i^6A, 2-methylthio-*N^6*-isopentenyladenosine; ms^2io^6A, 2-methylthio-*N^6*-hydroxyisopentenyladenosine; ms^2t^6A, 2-methylthio-*N^6*-threonylcarbamoyladenosine. The xm^5s^2U stands for 5-methyl-2-thiouridine derivatives: 5-methylaminomethyl-2-thiouridine (mnm^5s^2U), 5-carboxymethylaminomethyl-2-thiouridine ($cmnm^5s^2U$), 5-methoxycarbonylmethyl-2-thiouridine (mcm^5s^2U), and 5-taurinomethyl-2-thiouridine (τm^5s^2U).

The 4-thiouridine modification at tRNA position 8 (s^4U8) is conserved in Bacteria and Archaea. It has not yet been reported in eukaryotes, although the gene homolog of the prokaryotic s^4U8 modification enzyme (ThiI) are present in some eukaryotic genomes [8]. The s^4U modification has also been reported at position 9 of tRNA$^{Leu}_{UAG}$ in the archaeon *Thermoplasma acidophilum* identified by liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis, along with several other novel modifications [9]. According to the online tRNA database (http://trnadb.bioinf.uni-leipzig.de), s^4U8 has been found in the virus Enterobacteria phage T4, suggesting that this modification is also present in viruses [10]. Physiologically, s^4U8 acts as a photosensor of near-ultraviolet (UV) radiation. Following irradiation, U8 cross-links with the structurally nearby cytidine at position 13. This causes

tRNA structural changes that prevent tRNA from aminoacylation, and consequently the accumulation of uncharged tRNA triggers stringent cellular responses [11].

The 2-thio (s^2) modification without any other modifications on the same nucleoside can be found at tRNA positions 32 and 33 as s^2C32 and s^2U33, respectively. The s^2C32 modification is present in *Escherichia coli* and *Salmonella enterica* tRNA$^{Arg}_{ICG}$ (decodes CGU/C/A), tRNA$^{Arg}_{CCG}$ (decodes CGG), tRNA$^{Arg}_{mnm}{}^5{}_{UCU}$ (decodes AGA/G), and tRNA$^{Ser}_{GCU}$ (decodes AGC/U) [11]; and it has also been found in Archaea [12]. Although the lack of s^2C32 did not influence the monitored bacterial growth rate, it may affect translation efficiency of rare codons that are intrinsically inefficient in decoding [11,13]. The s^2U33 modification has been reported in trypanosomatids tRNA$^{Trp}_{CCA}$ [14,15]. This modification acts as a negative regulator of the C34 → U34 editing [14]. Without this modification, almost all C34 would be converted to U34, making the UGG codon unreadable.

The s^2U derivative in the form of 5-methyl-2-thiouridine (m^5s^2U), also known as 2-thioribothymidine (s^2T), at tRNA position 54 has been reported in thermophilic organisms, such as the bacterium *Thermus thermophilus* and the archaeon *Pyrococcus furiosus* [4]. The s^2 modification of m^5s^2U54 raised at elevated temperatures, and the lack of s^2 led to a temperature sensitive phenotype [4]. Accordingly, m^5s^2U54 is proposed to enhance the thermostability of tRNA structures possibly by forming a reverse Hoogsteen base pair with m^1A58 and stacking with G53 and ψ55 [4].

The U34 located at the first position of the anticodons of tRNA$^{Gln}_{UUG}$, tRNA$^{Lys}_{UUU}$, and tRNA$^{Glu}_{UUC}$ is universally 2-thiolated in all three domains of life. Depending on the organism and the subcellular location, U34 can be hypermodified to different s^2U derivatives (xm^5s^2U) as summarized in Table 1. Several functions have been proposed for the s^2U34 modification. (i) The rigid conformation of s^2U—favorably in the C3'-*endo* form [4,16]—at the wobble position leads to a preference for codon:anticodon base paring with A-ending codons [17]. This may be explained by the greater stability of the s^2U-A vs. s^2U-G pair [18–20]; (ii) The s^2 group of xm^5s^2U acts as an identity element in aminoacylation reactions [21–24]. In vivo, the mutation of the enzyme (MTO2) responsible for s^2U34 modification in yeast mitochondria decreased tRNA aminoacylation levels [25]; (iii) The xm^5s^2U34 modifications preserve translation fidelity by preventing +1 [26] and +2 [27] ribosome frameshifting; (iv) The s^2 group of xm^5s^2U34 enhances translation efficiency on the ribosome by increasing the binding affinity of aminoacylated tRNAs to the ribosome A-site as well as the GTP hydrolysis rate [21]. Because the s^2U34 modification has important roles in translation, its absence leads to pleotropic phenotypes in yeast and various diseases in humans. In yeast, the lack of the s^2U34 modification results in defects in invasive growth [28]; hypersensitivity to high temperatures, rapamycin, caffeine, or oxidative stress [29,30]; inability to maintain normal metabolic cycles [31]; and protein misfolding and aggregation [32]. In humans, impaired s^2U34 modification of mitochondrial tRNAs has been associated with acute infantile liver failure [33,34] and myoclonic epilepsy with ragged-red fibers [35,36].

Table 1. Diversity and distribution of the s^2U derivatives (xm^5s^2U) at position 34 of tRNA$^{Gln}_{UUG}$, tRNA$^{Lys}_{UUU}$, tRNA$^{Glu}_{UUC}$.

xm^5s^2U	Name	Distribution
mnm^5s^2U	5-methylaminomethyl-2-thiouridine	bacteria, archaea
$cmnm^5s^2$U	5-carboxymethylaminomethyl-2-thiouridine	bacteria, yeast mitochondria
mcm^5s^2U	5-methoxycarbonylmethyl-2-thiouridine	eukaryotic cytosol
$τm^5s^2$U	5-taurinomethyl-2-thiouridine	mammalian mitochondria

The sulfur-containing hypermodified A37, adjacent to the anticodon, is present in tRNAs decoding the UNN codons. In bacteria, either 2-methylthio-N^6-isopentenyladenosine (ms^2i^6A) or 2-methylthio-N^6-hydroxyisopentenyladenosine (ms^2io^6A) can be found, depending on the presence of the MiaE enzyme responsible for the hydroxylation of i^6 → io^6 [11]. The ms^2i^6A37 modification is also present in eukaryotes [37,38] and viruses [10]. Additionally, the 2-methylthio-

N^6-threonylcarbamoyladenosine (ms^{2}t^{6}A37) modification has been reported in the bacterium *Bacillus subtilis*, higher eukaryotes [39], and archaea [12]. These modifications are important for the fidelity and efficiency of translation by stabilizing the A-U base paring at the first codon position and preventing +1 frameshift [13]. This is because the A37 modifications bring orders in tRNAs by (i) preventing hydrogen bonding within the anticodon loop and thus ensuring an open loop structure that is required for efficient and correct base paring [18] and (ii) structuring the loop towards the canonical U-turn structure and enhancing the 3'-stack of the codon-anticodon interaction [13]. In bacteria, a ms^{2}i^6 deficiency resulted in a decrease in the polypeptide chain elongation rate leading to a reduced growth rate and a pleiotropic phenotype [11].

3. Fe–S Cluster-Dependent and Independent tRNA Thiolation Processes

The biosyntheses of sulfur-containing tRNA modifications usually require multiple enzymes for sulfur transfer. This process generally starts with the activation of sulfur from free L-cysteine by cysteine desulfurases—e.g., IscS in bacteria [40] and Nfs1 in eukaryotes [41]—forming a persulfide (R-S-SH) enzyme adduct and free L-alanine [42]. The persulfidic sulfur, which is covalently linked to a conserved Cys residue of cysteine desulfurase, is then donated via downstream sulfur carrier proteins to the tRNA thiolation enzymes and eventually to tRNA nucleosides. Beside tRNA thiolation, the persulfide on cysteine desulfurase is also the sulfur donor for the biosyntheses of Fe–S clusters and many sulfur-containing vitamins [43]. The tRNA thiolation enzymes are either Fe–S cluster dependent or independent as summarized in Table 2. Domain structures of the s^{4}U8, s^{2}U34, m^{5}s^{2}U54, and s^{2}C32 synthetases are showed in Figure 2. The biosynthetic pathways of each tRNA thionucleoside are described below.
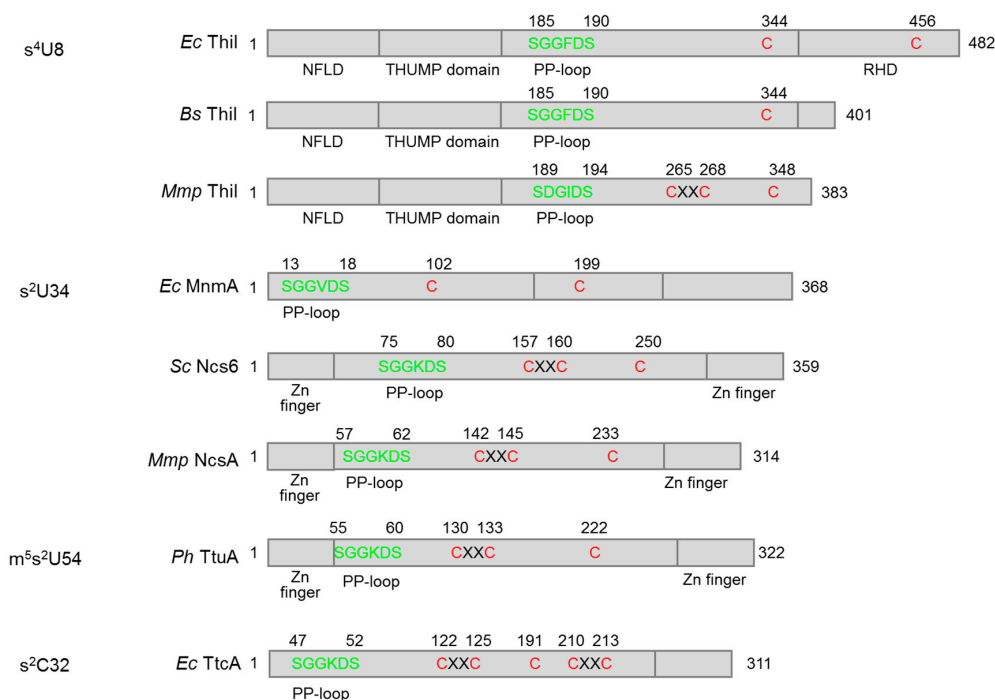

Figure 2. Domain structures of the s^{4}U8 synthetase (ThiI), s^{2}U34 synthetase (MnmA, Ncs6, or NcsA), m^{5}s^{2}U54 synthetase (TtuA), and s^{2}C32 synthetase (TtcA). The PP-loop (ATP-binding) motif and putative catalytic site Cys residues are colored in green and red, respectively. The domain structures of ThiI, MnmA, and Ncs6 are based on the solved crystal structures of *Bacillus anthracis* ThiI, *E. coli* MnmA, and *Pyrococcus horikoshii* TtuA, respectively. Abbreviations: *Ec*, *E. coli*; *Bs*, *Bacillus subtilis*; *Mmp*, *Methanococcus maripaludis*; *Sc*, *Saccharomyces cerevisiae*; *Ph*, *Pyrococcus horikoshii*; NFLD, N-terminal ferredoxin-like domain; THUMP, thiouridine synthases, methylases and pseudouridine synthases; RHD, rhodanese homology domain.

Table 2. The diversity and distribution of tRNA thionucleosides and sulfurtransferases involved in each thiolation.

Nucleoside	Distribution	Model Organisms [1]	Modification Enzymes (Sulfurtransferases) [2]	Fe–S Cluster Dependency	Modified tRNA Species	References
s^4U8	Bacteria	*E. coli*	IscS, ThiI	independent		[44,45]
	Archaea	*M. maripaludis*	S-donor?, ThiI	dependent		[46,47]
s^4U9	Archaea	*T. acidophilum*	S-donor?, ThiI	independent	$tRNA^{Leu}_{UAG}$	[9]
s^4U33	Eukaryotes	Trypanosomatids	Nfs1/Isd11, Mtu1	independent	$tRNA^{Trp}_{CCA}$	[14]
mcm^5s^2U34	Eukaryotes	*S. cerevisiae* cytosol	Nfs1, Tum1-RLD, Urm1, Uba4-RLD, Ncs2/Ncs6	dependent	$tRNA^{Gln, Lys, Glu}$	[4]
$cmnm^5s^2U34$	Eukaryotes	*S. cerevisiae* mitohondrion	Nfs1/Isd11, Mtu1	independent	$tRNA^{Gln, Lys, Glu}$	[25]
$τm^5s^2U34$	Eukaryotes	*H. sapiens* mitochondrion	hMTU1	independent	$tRNA^{Lys}$	[48,49]
$cmnm^5s^2U34/$ mnm^5s^2U34	Bacteria	*E. coli*, *S. enterica*	IscS, TusA, TusBCD, TusE, MnmA	independent	$tRNA^{Gln, Lys, Glu}$	[4]
	Bacteria	*B. subtilis*	YrvO, MnmA	independent	$tRNA^{Gln, Lys, Glu}$	[50]
mnm^5s^2U34	Archaea	*H. volcanii*, *M. maripaludis*	S-donor?, SAMP2, UbaA, NcsA	dependent	$tRNA^{Gln, Lys, Glu}$	[46,51,52]
m^5s^2U54 (s^2T54)	Bacteria	*T. thermophilus*	IscS/SufS, TtuA, TtuB, TtuC, TtuD	dependent?		[4,53–55]
	Archaea	*P. furiosus*	S-donor?, TtuA, TtuB, TtuC	dependent?		[4]
s^2C32	Bacteria	*E. coli*	IscS, TtcA	dependent	$tRNA^{Arg, Ser}$	[56,57]
	Archaea		to be determined			
$ms^2i^6A37/$ ms^2io^6A37	Bacteria	*E. coli*, *S. enterica*	IscS, MiaB	dependent	$tRNA^{Phe, Tyr,}_{Leu, Ser, Cys, Trp}$	[39]
ms^2i^6A37	Eukaryotes	*H. sapiens*	CDK5RAP1	dependent	$tRNA^{Phe, Trp, Tyr}$	[58]
$ms^2(c)t^6A37$	Bacteria	*B. subtilis*	IscS, MtaB	dependent	$tRNA^{Phe, Tyr}$	[39]
	Higher eukaryotes	*H. sapiens*	CDKAL1	dependent	$tRNA^{Ile, Met, Thr,}_{Asn, Lys, Ser, Arg}$	[59]
	Archaea		to be determined			

[1] Abbreviations: *E. coli*, *Escherichia coli*; *M. maripaludis*, *Methanococcus maripaludis*; *T. acidophilum*, *Thermoplasma acidophilum*; *S. cerevisiae*, *Saccharomyces cerevisiae*; *H. sapiens*, *Homo sapiens*; *S. enterica*, *Salmonella enterica*; *B. subtilis*, *Bacillus subtilis*; *H. volcanii*, *Haloferax volcanii*; *P. furiosus*, *Pyrococcus furiosus*. [2] The known Fe–S cluster dependent enzymes are highlighted in red. Abbreviations: Isd11, iron sulfur biogenesis desulfurase interacting protein 11; Mtu1, mitochondrial tRNA-specific 2-thiouridylase 1; Tum1, tRNA thiouridine modification protein 1; RLD, rhodanese-like domain; Urm1, ubiquitin-related modifier 1; Uba4, ubiquitin-like protein activator 4; hMTU1, human Mtu1; TusA–E, two-thiouridine synthesis protein A–E; MnmA, 5-methylaminomethyl-2-thiouridine synthetase A; SAMP2, small archaeal modifier protein 2; UbaA, archaeal Uba4 homolog; TtuA-D, two-thiouridine synthesis protein A-D; TtcA, two-thio-cytidine synthetase A; MiaB, N^6-isopentenyladenosine methylthiotransferase B; CDK5RAP1, cyclin-dependent-like kinase 5 repressor/activator site-binding protein 1; MtaB, N^6-threonylcarbamoyladenosine methylthiotransferase B; CDKAL1, cyclin-dependent-like kinase 5 repressor/activator site-binding protein 1-like 1.

3.1. Biosynthesis of s^4U8

3.1.1. Bacteria

The biosynthesis of s^4U8 in *E. coli* is Fe–S cluster independent and requires only two proteins—the cysteine desulfurase IscS and the s^4U8 formation enzyme ThiI (Figure 3A). ThiI contains a PP-loop (ATP-binding) motif and uses ATP to activate the C4 atom of tRNA U8 [60] yielding an adenylated intermediate. For sulfur transfer, IscS first forms a persulfide enzyme adduct using free L-cysteine as the sulfur donor. Then the persulfidic sulfur from IscS is transferred to the first catalytic Cys456 of

E. coli ThiI, forming a persulfide group on ThiI [44,61]. Subsequently, the second catalytic Cys344 forms a disulfide bond with Cys456 assisting the release of the sulfur from ThiI persulfide [62,63], which is then incorporated into the activated U8 forming s^4U8. The in vitro reaction requires exogenous reductant (e.g., dithiothreitol) to break the Cys344–Cys456 disulfide bond before the next catalytic round [62], but the physiological reductant is unclear. Notably, the rhodanese homology domain (RHD) that contains the catalytic Cys456, which carries the persulfide, is absent in many bacteria [8,45]; therefore, the sulfur transfer mechanism of RHD-lacking ThiI remains unanswered. The genome of *B. subtilis* encodes four functionally active cysteine desulfurases: SufS, NifZ, NifS, and YrvO [50]. Among them, the *nifZ* gene is adjacent to the *thiI* gene without RHD. Both NifZ and ThiI are essential for s^4U8 formation in *B. subtilis* [45]; this suggests that the sulfur transfer mechanism of *B. subtilis* ThiI without RHD depends on a specific interaction between NifZ and ThiI.
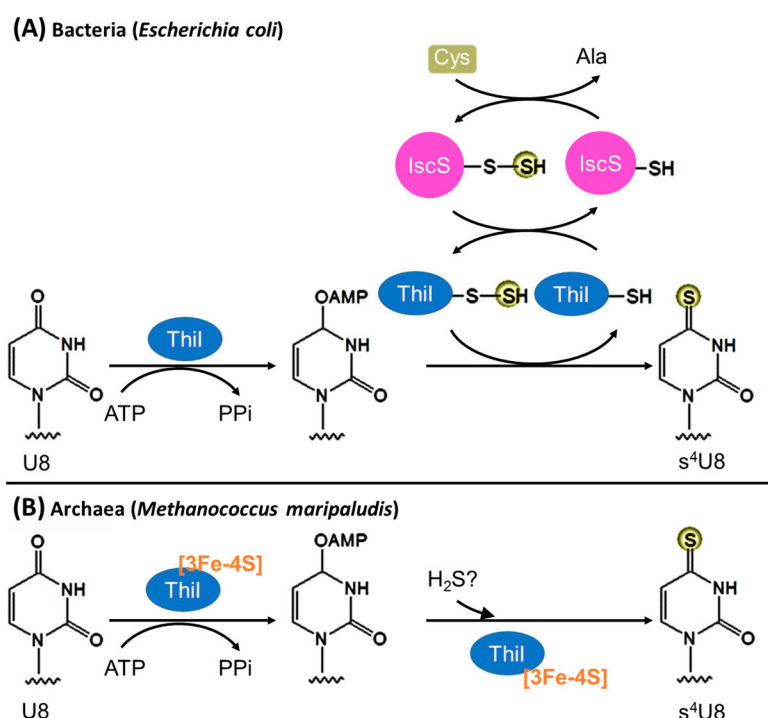

Figure 3. The biosynthetic pathways of tRNA s^4U8 in Bacteria and Archaea. (**A**) The biosynthetic pathway of s^4U8 in the bacterium *E. coli* is Fe–S cluster independent, and the sulfur transfer involves persulfide enzyme adducts; (**B**) The biosynthetic pathway of s^4U8 in the archaeon *M. maripaludis* is Fe–S cluster dependent.

3.1.2. Archaea

The archaeal s^4U8 biosynthesis presumably resembles the bacterial ThiI pathway because ThiI is widely distributed in Archaea and the deletion of the *thiI* gene in *Methanococcus maripaludis* results in the elimination of s^4U in tRNAs [47]. However, the *E. coli* s^4U biosynthetic mechanism cannot fully explain the archaeal process because the gene encoding a cysteine desulfurase is missing in many sequenced archaeal genomes [8,64] and most archaeal ThiI homologs lack the RHD essential for sulfur transfer. Although the physiological sulfur donor is not known, *M. maripaludis* ThiI can use Na_2S as an in vitro sulfur donor for tRNA thiolation [47]. The K_M of Na_2S is ~1 mM, close to the estimated intracellular concentrations of free sulfide in methanococci (~1–3 mM) [47]; this suggests that sulfide is a physiologically relevant sulfur donor. Furthermore, free L-cysteine is not a sulfur donor for the biosynthesis of Fe–S cluster [64] and tRNA thionucleosides [65] in methanogens; this suggests that a cysteine desulfurase is not required as a central sulfur donor for the biosynthesis of sulfur containing compounds. Notably, the methanogenic archaeal ThiI homologs have three conserved Cys residues

(two from a CXXC motif) in the putative catalytic domain [46,47]. A single mutation of any of the three Cys residues abolished *M. maripaludis* ThiI activity [47], implying that all three Cys residues are crucial. Recently, it has been revealed that these three Cys residues coordinate a [3Fe-4S] cluster indispensable for *M. maripaludis* ThiI activity [46]; this indicates that the s^4U8 biosynthesis in methanogenic Archaea is Fe–S cluster dependent and distinct from the persulfide-based reaction mechanism of bacterial ThiIs (Figure 3B).

3.2. Biosynthesis of s^2U34

3.2.1. Bacteria

In bacteria, the biosynthesis of s^2U34 is Fe–S cluster independent (Figure 4A). In *E. coli*, the persulfide from the cysteine desulfurase IscS is transferred via multiple intermediate sulfur carriers (Tus A, TusBCD complex, and Tus E) in a persulfide-based manner to the s^2U34 formation enzyme MnmA [66]. Similar to *E. coli* ThiI, MnmA has a PP-loop motif and two active site Cys residues. The PP-loop binds ATP that is consumed to activate the C2 atom of U34 by adenylation. The first catalytic Cys199 receives the sulfur and generates a persulfide enzyme adduct [60]. Then the second catalytic Cys102 forms a disulfide bond with Cys199 facilitating the release of the sulfur from MnmA persulfide, which is finally introduced to the activated U34 forming s^2U34. Presumably, the Cys344–Cys456 disulfide bond needs to be reduced before the next catalytic round.

Recently, a truncated pathway of s^2U34 biosynthesis has been revealed in *B. subtilis* [50]. In this bacterium, the intermediate sulfur carries (TusA/BCD/E) are missing (Figure 4B). The cysteine desulfurase YrvO and MnmA are sufficient to introduce s^2 to U34 [50]; this suggests a direct sulfur transfer from a cysteine desulfurase to the s^2U34 formation enzyme.

3.2.2. Eukaryotic Mitochondria

The tRNA modification enzyme Mtu1 (mitochondrial tRNA-specific 2-thiouridylase 1) is homologous to bacterial MnmA and responsible for the 2-thiolation reaction of mitochondrial tRNA U34 [48]. Because eukaryotes lack the gene homologs of the intermediate sulfur carriers (TusA/BCD/E) [8], the mitochondrial pathway may resemble the abbreviated *B. subtilis* pathway that requires only a cysteine desulfurase and a 2-thiolation enzyme (Figure 4C). In *Saccharomyces cerevisiae* mitochondria, the cysteine desulfurase Nfs1 forms a complex [67,68] with a small mitochondrial protein Isd11, which is not conserved in prokaryotes. Isd11 is proposed to stabilize Nfs1 in mitochondria.

3.2.3. Eukaryotic Cytosol

The mechanism by which sulfur is incorporated into tRNA s^2U34 in eukaryotic cytosol differs greatly from that in bacteria (Figure 4D). This process requires the Fe–S cluster assembly machinery [69]. A small amount of the cysteine desulfurase Nfs1 is present in yeast cytosol and participates in tRNA thiolation [70]. The sulfur relay from Nfs1 to the s^2U34 formation enzyme complex Ncs6/Ncs2 involves several RHD containing proteins and a ubiquitin-like protein. Specifically, from Nfs1, sulfur is transferred to the RHD of Tum1 and then to the RHD of Uba4 as a persulfide group. Uba4 is an E1-like protein that activates Urm1 (ubiquitin-related modifier 1) by adenylation. Then Urm1 receives the sulfur from Uba4, forming a C-terminal thiocarboxylate on Urm1 that may be the proximal sulfur donor for tRNA thiolation [4]. Similar to methanogenic archaeal ThiI, Ncs6 has a PP-loop motif and three conserved Cys residues (two from a CXXC motif) in its putative catalytic domain. The PP-loop binds ATP that is used to adenylate U34, resembling the reaction schemes of ThiI and MnmA. The three Cys residues coordinate a [3Fe-4S] cluster [46], which is probably involved in sulfur transfer. The function of Ncs2 in the Ncs6/Ncs2 complex is still unclear.
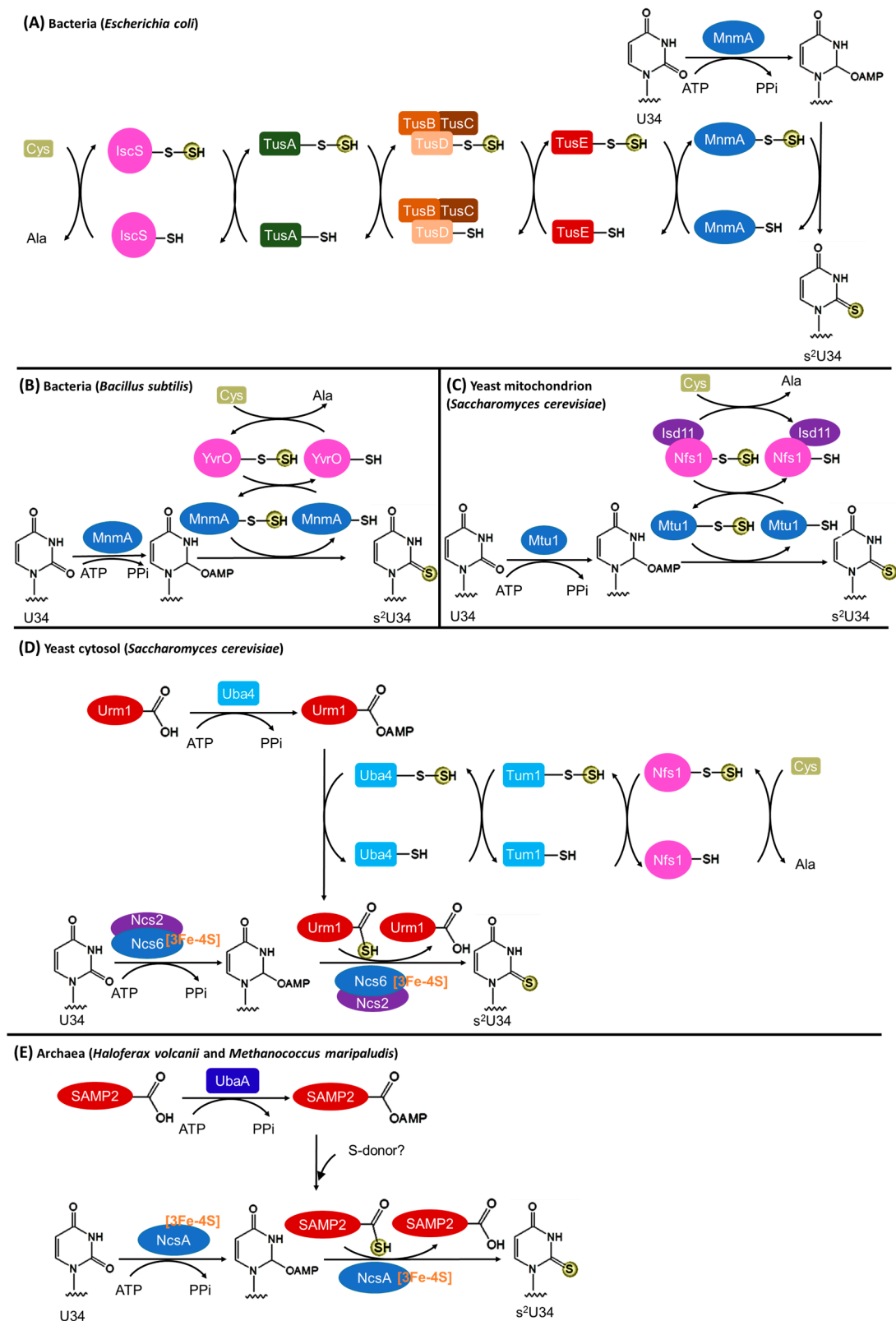
Figure 4. The biosynthetic pathways of tRNA s^{2}U34 in all domains of life. (**A**) In *E. coli*, the pathway is Fe–S cluster independent, and the sulfur transfer involves persulfide enzyme adducts; (**B**) *B. subtilis* uses a truncated pathway that is Fe–S cluster independent; (**C**) The yeast mitochondrial pathway may resemble the *B. subtilis* pathway; (**D**) The yeast cytosolic pathway is Fe–S cluster dependent, and the sulfur transfer involves persulfide and thiocarboxylate enzyme adducts; (**E**) The archaeal pathway may resemble the yeast cytosolic pathway.

3.2.4. Archaea

The archaeal s^2U34 biosynthetic pathway is proposed to resemble the eukaryotic cytosolic Ncs6 pathway (Figure 4E). This is based on the observations that (i) the *ncs6* gene homologs are widespread in archaeal genomes [8]; (ii) the deletion of the *ncs6* homolog (*ncsA*) in *Haloferax volcanii* resulted in only non-thiolated $tRNA^{Lys}_{UUU}$ [71]; (iii) Ncs6 homologs form complexes with the ubiquitin-like small archaeal modifier protein (SAMP), which has high structural homology to Urm1, in *H. volcanii* [72] and in *M. maripaludis* [51]; (iv) the *H. volcanii* E1-like protein UbaA activates SAMP in formation of a thioester intermediate [73]; (v) the deletion of either *samp2* or *ubaA* in *H. volcanii* eliminated thiolated $tRNA_{Lys}^{UUU}$ [52]; and (vi) the *M. maripaludis* Ncs6 homolog has a [3Fe-4S] cluster [46]. These findings suggest that both an Fe–S cluster containing Ncs6 homolog and an activated ubiquitin-like protein are required for s^2U34 formation in Archaea.

3.3. Biosynthesis of m^5s^2U54

The 2-thiolation process of m^5s^2U54 is similar to the Ncs6 pathway in eukaryotic cytosol that requires a RHD containing protein(s), an E1-like enzyme, and an ubiquitin-like protein for sulfur transfer (Figure 5) [4]. The sulfur from free L-cysteine is activated by a cysteine desulfurase (IscS or SufS in *T. thermophilus*) [55,74], which is then transferred as a persulfide group to a recently identified RHD containing protein TtuD [53]. The ubiquitin-like protein TtuB is activated by adenylation catalyzed by an E1-like enzyme TtuC and then receives the activated sulfur, forming a C-terminal thiocarboxylate [54]. TtuA, a homolog of Ncs6, presumably activates m^5U54 by adenylation and then introduces the sulfur from TtuB thiocarboxylate to tRNA. Similar to Ncs6 and methanogenic archaeal ThiI, three Cys residues (two from a CXXC motif) in the putative catalytic domain of TtuA are important for the thiolation activity [75]. Although a TtuA crystal structure did not reveal the presence of an Fe–S cluster [75], the homology between TtuA and Ncs6 suggests that TtuA may be an Fe–S cluster dependent enzyme.
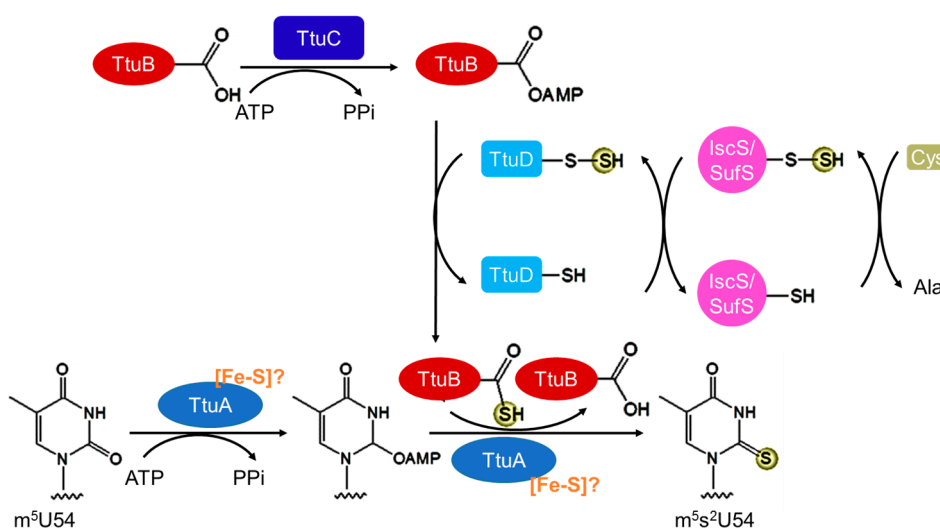

Figure 5. The 2-thiolation process of tRNA U54 in the thermophilic bacterium *T. thermophilus*.

3.4. Biosynthesis of s^2C32

In bacteria, the biosynthesis of s^2C32 is dependent on Fe–S cluster formation [76]. For Fe–S cluster assembly, the sulfur from free L-cysteine is transferred via the cysteine desulfurase IscS to IscU, an Fe–S cluster assembly scaffold protein [77]. Fe–S clusters are assembled on IscU and then incorporated into various Fe–S cluster enzymes. The s^2C formation enzyme TtcA (two-thio-cytidine A), which introduces sulfur to tRNA C32, belongs to the TtcA/TtuA protein family [57]. TtcA has two

CXXC motifs, within which three Cys residues coordinate a [4Fe-4S] cluster essential for the thiolation activity [56]. Although the reaction mechanism remains unclear, one Fe atom in the [4Fe-4S] cluster is proposed to transiently ligate a sulfide (–SH) group that is the proximal sulfur donor to generate s^2C [56].

3.5. Biosynthesis of ms^2A37

The tRNA A37 can be methylthiolated to various ms^2A37 derivatives. In bacteria, the N^6-isopentenyladenosine (i^6A37) → 2-methylthio-N^6- isopentenyladenosine (ms^2i^6A37) transformation is catalyzed by MiaB, and the N^6-threonylcarbamoyladenosine (t^6A37) → 2-methylthio-N^6-threonylcarbamoyladenosine (ms^2t^6A37) conversion is catalyzed by MtaB, a homolog of MiaB [39]. The human homologs of MiaB and MtaB are CDK5RAP1 (cyclin-dependent-like kinase 5 repressor/activator site-binding protein 1) [58,78] and CDKAL1 (cyclin-dependent-like kinase 5 repressor/activator site-binding protein 1-like 1) [59,79], respectively. In organisms with TcdA, an enzyme that converts t^6A to a cyclic form ct^6A, ms^2ct^6A37 is formed by MtaB [80]. MiaB/MtaB homologs are also present in archaeal genomes [79], but their functions in methylthiolation have not yet been examined.

Both MiaB and MtaB are methylthiotransferases (MTTases), belonging to the radical *S*-adenosylmethionine (SAM or AdoMet) superfamily of enzymes that catalyze the attachment of a methylthio (-SCH_3) moiety on unactivated carbon centers. MiaB has two [4Fe-4S] clusters [81,82]. One cluster is coordinated by the characteristic radical SAM motif (CX3CX2C) [81] and is essential for the reductive cleavage of SAM, generating a 5′-deoxyadenosyl radical ($Ado^•$) and methionine [83,84]. The second cluster is coordinated by three N-terminal Cys residues and plays a central role in constructing a -SCH_3 group and attaching it to tRNA [84,85]. The two Fe–S clusters remain intact during catalysis; this indicates that an exogenous sulfur donor possibly attached to the second cluster, instead of the sulfur in Fe–S clusters, is required for this reaction [84].

4. Conclusions

The biosynthetic pathways of sulfur-containing tRNA nucleosides are very complex because (i) they usually involve a cascade of sulfur carrier proteins rather than a direct transfer from the ultimate sulfur donor to the substrate; and (ii) the sulfur flows vary significantly between different organisms. Many details (especially in Archaea and eukaryotes) are still waiting to be answered. Just a few examples: (i) because many Archaea lack cysteine desulfurases, the initial sulfur donor for the biosynthesis of thio-modifications in Archaea remains to be identified; (ii) it is unclear whether any unidentified intermediate sulfur carriers are involved in the sulfur transfer between the initial and terminal sulfur transferases for the biosyntheses of s^4U in Archaea and s^2U in Archaea and eukaryotes; (iii) the reaction mechanisms of the [4Fe-4S] cluster containing TtcA, the [3Fe-4S] cluster containing methanogenic archaeal ThiI, and the [3Fe-4S] cluster containing archaeal and eukaryotic Ncs6 homologs are still not known; (iv) it remains to be clarified whether TtuA, which is involved in m^5s^2U54 biosynthesis in thermophilic prokaryotes, is an Fe–S protein.

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