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Methods to analyze post-transcriptional modifications applied to stable RNAs in *Staphylococcus aureus*

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Abstract

RNA modifications contribute to the various functions of RNAs in all living organisms. Some of these modifications are dynamic and contribute to the regulation of gene expression. In bacteria, their roles in stress, environmental adaptation, and in infections caused by pathogens have been recently fully recognized. In this review, we describe several methodologies including mass spectrometry, next generation RNA sequencing methods, biochemical approaches, and cryo-EM structural analysis that are used to detect and localize the modifications in tRNAs and rRNAs. We illustrate how the combination of methods was necessary to avoid technical biases for a successful mapping of the modifications in tRNAs and rRNAs in *Staphylococcus aureus*.

Keywords: RNA modifications; *Staphylococcus aureus* ; tRNA; rRNA; Mass Spectrometry; RNA-seq; cryo-EM structure

1 Introduction

RNA molecules are chemically modified at different positions of the nitrogenous bases or the ribose cycle. These modifications are common to all domains of life and are present in all RNA classes, including stable non-coding RNAs (tRNAs, rRNAs, some regulatory RNAs) and generally less stable mRNAs. They involve covalent alterations (i.e. methylations, acetylation and deamination) or isomerization (i.e. pseudouridines) of nucleotides (**Figure 1A**). To date, over 170 modifications have been found in RNAs from different organisms, with the most common modification being methylation (see MODOMICS database) (Boccaletto et al. 2022). Overall, they influence chemical and physical properties of RNAs with consequences on their stability, structure, recognition, transport, and cellular localization (Frye et al. 2016). Specific enzymes are responsible for their deposition and removal, providing a large degree of variability (de Crécy-Lagard and Jaroch 2020; Ishitani et al. 2008). These modulations add a sophisticated regulatory layer in gene expression and often connect external stimuli to various cellular processes. This is particularly the case for bacteria, which need to rapidly adapt their growth to environmental stresses, to the host responses, to nutrient availability, and to antimicrobials or dangerous chemical reactive species.

Epitranscriptomic regulation often targets the most modified RNA species, tRNAs and rRNAs, influencing the translation process. Both codon decoding and peptide bond formation are affected by specific modifications on tRNAs and rRNAs, which influence translation rates and accuracy. Consequently, they directly impact protein yields, their proper folding, and activity (Samatova et al. 2020; Antoine et al. 2021). Translation regulation fine tunes protein synthesis to balance the relative abundance of proteins in the same pathways or to provide the correct stoichiometry of complexes (Li et al. 2014). In addition, translational control is well appropriate when fast adaptation is required (Tollerson and Ibba 2020; Duval et al. 2015). In different stress conditions, modulation of RNA modifications can be rapidly achieved by changing the level of the modification enzymes or by affecting their activity.

Stable non-coding RNAs, rRNAs and tRNAs, which represent in bacteria >95% of total RNA fraction are extensively modified during their maturation. Despite decades of studies, the comprehensive and reliable profiling of tRNA and rRNA modifications was accomplished only for a few model species. For bacterial species, such comprehensive profiling was performed for

Escherichia coli as a prominent representative of Gram-negative bacteria (see MODOMICS) and rather recently, for *Bacillus subtilis* (de Crecy-Lagard et al. 2020) and *Staphylococcus aureus* (Antoine et al. 2019) as representative Gram-positive species. Despite the fact that MODOMICS and tRNADB (Jühling et al. 2009) databases show that all known bacterial tRNAs have numerous common modifications, they also revealed specificity among bacterial species. Dihydrouridine (D), pseudouridine (ψ), 5-methyluridine (m^5U/T) and 7-methylguanosine (m^7G) are highly common, while 2'-O-methylations (Nm), 4-thiouridine (s^4U), and anticodon loop modifications are rarer, and others are unique for specific tRNA species (**Figure 1B**). Although Gram-negative and Gram-positive bacterial species share a number of common tRNA modifications, characteristics were identified in the D-arm, e.g. Gm18 is only found in Gram-negative bacteria, while m^1A22 is primarily observed in several Gram-positive bacteria. In contrast to tRNAs, rRNA modification profiles are less diverse among bacteria. However, the exact modification position is not necessarily conserved between different bacterial species but a similar modification is often present in the same rRNA structural domain (Piekna-Przybylska et al. 2008). Hence, it is of importance to gain more knowledge on the evolution of RNA modifications across bacterial phylogeny, and analyze the structural environment of chemical modifications to gain insights into their role and mode of interaction with neighboring residues.

Accurate detection of RNA modifications is still an extremely difficult task, as today there is still not a unique method that provides the whole pattern of RNA modifications. These approaches include various chromatographies (i.e. TLC, HPLC), reverse transcriptase analyses, affinity gel electrophoresis, and more sophisticated methods including liquid chromatography-tandem mass spectrometry analyses of nucleosides or RNA fragments (LC-MS/MS), and deep sequencing approaches (Schaefer et al. 2017; Wetzel and Limbach 2016; Motorin and Helm 2022; Yoluç et al. 2021). Recent nanopore technologies can potentially provide access to multiple modifications in native RNA molecules (Garalde et al. 2018; Begik et al. 2021). Even if these methods offer an extraordinary amount of information and depth, they nevertheless suffer from limitations and biases.

Here we describe the methodologies that were used to determine the modification patterns of stable RNAs (tRNAs and rRNAs) from *Staphylococcus aureus*, which is an opportunistic

human bacterial pathogen causing a large variety of infections. We will especially underline the power of combining complementary methodologies.

2 RNA purification

2.1 Isolation of total RNA from S. aureus

Sample requirements differ depending on the type of technique used for detecting RNA modifications. High amounts of homogenous RNAs are often required for successful analyses and appropriate methods for RNA purification should be optimized. A minimal number of purification steps following efficient cell lysis and RNA stabilization is crucial for correct RNA yield, purity, and integrity. The main challenge in RNA extraction from *S. aureus* is the disruption of the thick peptidoglycan cell wall. Successful approaches use enzymatic treatment with lysostaphin (Bastos et al. 2010), mechanical bead-beating or a combination of both methods in the presence of protein denaturants to inhibit RNase activities and abolish RNA-protein interactions (i.e., phenol, guanidine isothiocyanate) (Atshan et al. 2012; Beltrame et al. 2015; França et al. 2011). *S. aureus* RNA samples are routinely prepared with the FastRNA® Pro blue kit (MP Biomedical), which uses mechanical bead-beating of bacteria suspended in the commercial solution designed to prevent RNA degradation. For large scale preparations (up to 500 mL of culture in logarithmic phase), bead-beating lysis of *S. aureus*, suspended in a 1:1 mixture of aqueous buffer and acidic phenol/chloroform/isoamyl alcohol, is performed. RNA is further purified by chloroform extraction and ethanol precipitation.

2.2 Purification of rRNAs and tRNAs

As total RNA samples contain a mixture of different species (rRNAs, tRNAs, mRNAs, sRNAs, etc.), enrichment of the RNA molecule of interest is often beneficial for the analysis of modifications. Several approaches are based on size selection of RNAs (Poulson 1973). A rapid strategy is the use of specific filters (cutoff 50 kDa) to enrich for rRNAs (retentate) or tRNAs (filtrate) after ultrafiltration. Depending on the specific analysis to be performed on the RNAs, more laborious purification methods might be required. To purify small amount of individual 16S and 23S rRNAs as well as tRNAs, *S. aureus* RNA species can be separated by polyacrylamide gel electrophoresis (PAGE) and subsequently eluted from the excised bands (electroelution or “crush and soak”) (Meyer and Masquida 2016; Petrov et al. 2013). To get larger amounts of RNAs and

to avoid gel impurities, chromatographies performed under native conditions are preferred (Kanwal and Lu 2019). For instance, size-exclusion chromatography can be used to purify the different rRNAs (23S, 16S and 5S) and the tRNA fractions (Chionh et al. 2013; McKenna et al. 2007). Alternatively, weak anion exchange chromatography using DEAE (diethylaminoethyl) matrix is widely used to separate tRNAs and rRNAs, which elute at low and high salt concentration, respectively (Easton et al. 2010). Finally, to analyze modification profiles of rRNA incorporated in fully matured and actively translating ribosomes, rRNAs (23S, 16S and 5S) can be recovered by phenol-chloroform extraction of purified *S. aureus* total ribosomes (Khusainov et al. 2016) or polysomes samples, obtained from sucrose gradients fractionation (Brielle et al. 2017).

2.3 Purification of individual tRNAs

Individual tRNAs can be purified from total tRNA using biotinylated oligo DNA probes which specifically hybridize to the target tRNA molecule. Yokogawa and co-workers (Yokogawa et al. 2010) optimized this method for thermostable tRNAs and we adapted it for the purification of *S. aureus* tRNAs. The procedure consists of four steps: (i) design and preparation of the DNA probe, (ii) probe-resin immobilization, (iii) tRNA-probe hybridization, and (iv) tRNA isolation. The DNA probe is composed of 35 to 40 nucleotides and contains a biotin at the 5' or 3' end. Its sequence is usually complementary to the region between the D-arm and the anticodon loop. The biotinylated probe is immobilized on a streptavidin Sepharose resin using standard procedures. Hybridization of target tRNA and DNA probe requires denaturation of tRNA under conditions which maintain tRNA:DNA interaction. The buffer containing tetra-ethylammonium chloride has been reported as the best to destabilize the tRNA tertiary structure and to enhance the interaction between tRNA and DNA probes (Yokogawa et al. 2010). After hybridization, the remaining non-target tRNAs are removed by washing the resin with a non-denaturing buffer. Then, the desired tRNA is eluted with the non-denaturing buffer pre-heated at 60°C. The quality of the purification is assessed by denaturing PAGE and the identity of the tRNA is confirmed by mass spectrometry (MS). Depending on the yield of the purification, eventual contaminations can be removed by gel purification or ion-exchange chromatography. The purification method described above is efficient for most tRNAs, but further optimization is often required for tRNA isoacceptors, poorly transcribed tRNAs, and tRNAs with high GC content. Methods such as reciprocal circulating chromatography (RCC) (Miyachi et al. 2007) and chaplet column chromatography (CCC)

(Suzuki and Suzuki 2007) have been optimized to increase the yield of GC-rich tRNAs (Ohira et al. 2022).

3 Mass spectrometry to identify and localize modifications in RNAs

Liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) is the most trusted method for direct and unambiguous detection of RNA modifications, as it provides precise measurements of molecular masses (accuracy < 1 Da) even from very low amounts of material (Giessing and Kirpekar 2012; Jora et al. 2019). In this set up, the RNA sample is separated by reverse-phase high-performance liquid chromatography (RP-HPLC) and the eluting species are directly injected into a mass spectrometer equipped with two analyzers (Thomas and Akoulitchev 2006). An electrospray ionization (ESI) source (Kearle and Tang 1993) evaporates and ionizes the analyte, and the formed (parent) ions are separated in the first analyzer according to their mass-to-charge (m/z) ratio. Ions with a defined m/z are selected for collision-induced dissociation (CID) (Huang et al. 2008) and the resulting fragments (daughter ions) are resolved in the second mass analyzer. The recorded MS and MS/MS spectra (plot of m/z values and their relative abundance) are used to depict mass shifts and fragmentation profiles that evidence the presence of additional chemical groups attached to the canonical nucleotides (Thakur et al. 2021).

Two types of complementary LC-MS/MS analyses are performed for complete mapping of modifications in *S. aureus* RNAs. In the first analysis, the RNA of interest is hydrolyzed to its nucleoside (sugar + nucleobase) building blocks to identify all resident modifications (Pomerantz and McCloskey 1990). In the second, the RNA is digested with a nucleobase-specific RNase, and the sequences of the resulting oligonucleotides including modifications are determined and placed within the full-length RNA sequence (available from genomic data) (Kowalak et al. 1993). Both analyses require previous purification of the target RNA to obtain reliable data, limiting this “bottom up” mapping strategy to the analysis of one RNA at a time.

3.1 Analysis of nucleosides

Samples for nucleoside analysis are prepared through enzymatic treatment of the RNA with nuclease P1, snake venom phosphodiesterase I, and bacterial alkaline phosphatase (Crain 1990). However, the reaction is performed under sub-optimal mildly acidic conditions (pH 5) (Wolff et al. 2020) to avoid alteration of labile modifications at higher pH (Jora et al. 2021; Miyauchi et al.

2013). Removal of phosphate groups is critical to make the nucleosides sufficiently hydrophobic to be separated on a reverse-phase column. Canonical nucleosides are the most abundant species and readily resolve into well-defined peaks, eluting according to their hydrophobicity ($C < U < G < A$) (Gehrke and Kuo 1990). Modified nucleosides are less abundant and they often appear earlier (e.g. D, ψ) or later (e.g. m^2G , t^6A) than their unmodified counterparts depending on the chemical nature of the modification (Su et al. 2014). Positional isomers (e.g. m^1A , m^6A , m^2A) have the same mass and cannot be distinguished by tandem MS analysis; however, they exhibit different retention times that facilitate their identification when standards are available (Jora et al. 2018).

RP-HPLC of nucleosides is performed with mobile phases (A: water, B: methanol) containing 0.1% formic acid to facilitate protonation of nucleobases during ESI (Cai et al. 2015). MS analysis is performed in positive mode using a triple quadrupole (QQQ) instrument consisting of two quadrupole mass analyzers separated by a collision cell. In most cases, CID of the nucleoside ion occurs at the N-glycosidic bond, resulting in a nucleobase ion and an uncharged ribose moiety (Ross et al. 2016). The recorded m/z values of parent (MS) and daughter ions (MS/MS) as well as the associated neutral loss of unmodified (132 Da) or 2'-O-methylated (146 Da) ribose, enable identification of the modified nucleoside. Pseudouridine does not contain an N-glycosidic bond and shows a different but unique fragmentation profile allowing its identification (Dudley et al. 2005). Two strategies of data acquisition can be employed depending on the aim of the experiment. Dynamic multiple reaction monitoring (DMRM) allows identification and quantification of a defined set of modifications by restraining detection to only those specific m/z values (Thuring et al. 2016). In contrast, neutral loss scan (NLS) is used to identify the full range of modified nucleosides in the sample including novel modifications, and consists in detecting all precursors ions that lose a mass equivalent to unmodified or methylated ribose (Kellner et al. 2014).

3.2 Analysis of oligonucleotides

As RNase digestion of tRNA mixtures or long rRNAs often produce fragments with the same sequence, purification of the target molecule is critical to unambiguously map modifications by oligonucleotide analysis. Most of all individual tRNAs of *S. aureus* can be isolated using two-dimensional polyacrylamide gel electrophoresis (2D-PAGE), in which tRNAs are first separated by their length on a denaturing gel, and then by their conformation on a semi-denaturing gel (**Figure 2A**) (Antoine and Wolff 2020; Antoine et al. 2019). For rRNAs, specific sequences can

be obtained by RNase H cleavage using DNA probes flanking the region of interest followed by PAGE purification (Hansen et al. 2002; Nakai et al. 1994). In both cases, digestion is performed directly “in-gel” using RNase T1 (cleaving after G, m²G and I leaving a 3' phosphate end) and the resulting oligonucleotides elute passively from the gel (Taoka et al. 2010). Other RNases, such as RNase A (cleaving after pyrimidines and ψ and leaving a 3' phosphate end) and RNase U2 (cleaving after purines, preferably A, leaving a 3' phosphate end), are frequently used in separated analyses to increase the sequence coverage, particularly in G-rich regions (Houser et al. 2015).

The negatively charged backbone of oligonucleotides raises some challenges for LC-MS/MS analysis. A concern in sample preparation is the presence of alkali cations (Na⁺, K⁺), which must be replaced by “volatile” NH₄⁺ cations (Stults et al. 1991). Otherwise, multiple salt adducts are formed upon ESI, leading to peak broadening and interferences that complicate interpretation of spectra (Bleicher and Bayer 1994). In-gel digests are routinely desalted using “ZipTip” C18 reverse phase pipette tips (Millipore), but other methods such as RNA precipitation with ethanol and ammonium acetate, cation exchange chromatography, and micro-dialysis can also be used (Castleberry et al. 2008). Due to the high hydrophilicity of phosphate groups, RP-HPLC of oligonucleotides requires a mobile phase containing ion-pairing agents such as triethylamine (TEA) and triethylammonium acetate (TEAA) (Apffel et al. 1997). The ammonium moiety of these molecules masks the charges in the oligonucleotide backbone, while the alkyl group provides hydrophobic surfaces for its interaction with the reverse phase column. Hexafluoroisopropanol (HFIP) is also included in the mobile phase to improve the ionization of oligonucleotides (McGinnis et al. 2013), which occurs in negative mode (deprotonation). Because TEA/TEAA/HFIP contaminations are difficult to eliminate and interfere with proteomic analyses performed in positive mode (Wetzel and Limbach 2016), it is better to use a dedicated LC-MS/MS system for oligonucleotides analysis.

As ESI of oligonucleotides produces several multicharged anions (Potier et al. 1994), data-dependent acquisition (DDA) is used to select the most abundant *m/z* species for CID, which are typically [M-2H⁺]⁻² and [M-3H⁺]⁻³ ions. On high-resolution instruments, as the quadrupole time-of-flight (Q-TOF, accuracy < 0.05 Da), the charge (and thus the mass) of the parent ion can be inferred by inspecting the spacing of isotope peaks in the MS/MS spectra (Polo and Limbach 2000). If the spacing between the selected peak (usually the monoisotopic ion) and its consecutive

isotope peak is $1/x$, the charge of the parent ion will be $-x$ (**Figure 2B**). CID of oligonucleotides occurs along the phosphodiester backbone and generates a ladder of fragments that allow sequence determination (Nakayama et al. 2011; Ni et al. 1996). Among the four possible cleavage sites (McLuckey et al. 1992), fragmentation of the 5' P-O bond is the most frequent and produces a series of complementary *c*- and *y*-type fragment ions (**Figure 2C**). Deconvolution of the multi charge MS/MS spectrum (m/z) to mono charge spectrum (mass) simplifies the sequencing process, which consists in finding the known terminus (*e.g.* 3' Gp end for RNase T1 digests). Then the masses of individual nucleotides are added to match the different peaks until the mass of the parent ion is reached (**Figure 2C**). The presence of a modified nucleotide is evidenced by a unique mass shift resulting from the attached chemical group. Only pseudouridine (same mass as U) cannot be mapped with this method. A specific chemical derivatization on pseudouridine with either acrylonitrile (Mengel-Jørgensen and Kirpekar 2002) or *N*-cyclohexyl-*N'*-(2-morpholinoethyl)carbodiimide metho-*p*-toluene-sulfonate (CMCT) (Patteson et al. 2001) produces a detectable mass shift. Although oligonucleotide analysis readily locates single-methylated nucleotides within the RNA sequence, the position of the methyl group on the ribose or the nucleobase cannot be determined, and further analyses are required (Wolff et al. 2020).

4 Biochemical analyses to easily map specific RNA modifications

4.1 Primer extension

RNA modifications induce changes in the physicochemical properties of the nucleobases and ribose rings, including changes in their polarity (Charette and Gray 2000), base stacking (Roost et al. 2015), and coordination with water and ions (Agris 1996). All these events have a direct impact on the activity of RNA-directed DNA polymerases, which are viral enzymes commonly known as reverse transcriptases (RTs) (Potapov et al. 2018). These effects can be exploited to assign modification sites using simple laboratory settings. RNA modifications at the Watson-Crick edge, (*e.g.* m^1A , m^1G , m^2G) readily interfere with cDNA synthesis, while other modifications (*e.g.* 2'-O-methylation) are less problematic for primer extension, but can be detected with specific protocols. For example, under sub-optimal polymerization conditions, the presence of a modification at the nucleotide base or its ribose in the RNA template induces reverse transcription stop at the +1 position to the modification site (or, rarely, at the neighboring nucleotide) (**Figure 3A, left**) (Rebane et al. 2002; Maden et al. 1995). Commonly used conditions include low deoxynucleotides

(dNTPs) concentration (Motorin et al. 2007). This stop is readily detected after separation of the cDNA products by PAGE, along with RNA sequencing profile. Most commonly, primer extension is performed with a radiolabeled DNA oligonucleotide and the radioactive products are detected by autoradiography (**Figure 3A, right**). RTs from avian myeloblastosis virus (AMV) and Moloney murine leukaemia virus (MMLV) are extensively used. Noteworthy, RT-signatures appear typical for a given modification, but they also depend on the reverse transcriptase used. A recent comparative study, performed on a similar RNA template using several RTs, revealed that RNA modifications can be distinguished by their RT-signature (Werner et al. 2020).

Although this technique is simple and inexpensive, it is sometimes difficult to distinguish between modifications and other possible causes of RT stops, such as RNA accidental breaks or presence of stable structures (Stern et al. 1988). To overcome misinterpretations, RT reactions are often conducted in parallel on *in vitro* transcribed RNAs, devoid of modifications, or on RNA purified from organisms in which specific RNA modification enzymes have been deleted or inactivated (Deryusheva et al. 2012).

Finally, it should be mentioned that many modifications cannot be directly detected by primer extension analysis, like pseudouridines, dihydrouridines or 5-methylcytosines. For these modifications a specific pre-treatment of the sample prior to the primer extension is necessary (**Figure 3B**, see below).

4.2 Nucleotide derivatization coupled to primer extension analysis

Given the specific chemical properties that modification brings to nucleotides, diverse options of derivatization inducing RT pauses/stops or nucleotide misincorporation have been developed for RT-silent modified nucleotides (**Figure 3B**) (Behm-Ansmant et al. 2011). To map pseudouridines, chemical derivatization with CMCT is commonly used (Bakin and Ofengand 1993). CMCT modifies the N1 and N3 positions in guanosine and uridine respectively, while pseudouridines are modified both at N1 and N3 positions. Removal of the derivatization from guanosine, uridine and N1 of ψ is achieved by alkali treatment using Na_2CO_3 at pH 10 to 11. Only the N3 modification in pseudouridine is maintained due to its specific chemical environment (Ho and Gilham 1971) and can be easily detected by primer extension. Special attention is required at all steps of the labeling procedure to avoid false positive stops. Therefore, it is important to run, in

parallel, samples without treatment, or treated only with CMCT and Na₂CO₃ separately, to accurately discriminate stops induced by CMCT on pseudouridines from accidental cleavages of the RNA chain during the treatment (Adachi et al. 2019).

The main characteristic of dihydrouridine (D) is the absence of double bond between carbon 5 and 6 and the non-planar and non-aromatic nucleobase ring (Dalluge et al. 1996). The treatment under mild alkaline condition induces the disruption of the saturated nucleobase to produce beta-ureidopropionic acid (Xing et al. 2004) (**Figure 3B**). This opened form of the nucleobase induces a specific RT stop during primer extension. Alternatively, the reduction with sodium borohydride (Cerutti and Miller 1967) causes also specific RT stops. Again, to avoid misinterpretation, it is always important to add controls with untreated samples (Igo-Kemenes and Zachau 1969; Xing et al. 2004).

4.3 Affinity gels

Affinity electrophoretic methods rely on the strong interaction of specific functional groups present on proteins or nucleic acids with a specific reactant contained in the gel matrix (Nakamura and Takeo 1998). The displacement of the sample after gel migration provides signals about the presence or the absence of the RNA modification. For instance, boronate and organomercurial affinity gel electrophoresis have been widely used to identify specific tRNA modifications in various organism (Tuorto et al. 2018).

Boronate affinity electrophoresis, is based on the specific interaction between N-acryloyl-3-aminophenylboronic acid (APB) and free *cis*-hydroxyls of RNA. This is particularly the case of the queuosine (Q) modification, which possesses a *cis* diol group that delays the migration of the tRNAs during electrophoresis (Igloi and Kössel 1985). On the same principle, an interaction between the sulfur-modified (S) RNA with organomercurials leads to derivatized Hg-S adducts (Igloi 1988). The modified RNA with *cis* diol or mercury-sulfur can be visualized by different methods. For instance, ethidium bromide staining of the gel is often used for purified tRNA species while Northern blot analyses using 5' end labeled oligonucleotide allows the detection of one tRNA species among total tRNAs samples. Although boronate affinity gels are efficient to separate Q-containing RNA, it is noteworthy that the slower migration can also be observed for RNA species co-transcriptionally modified by NAD (Nübel et al. 2017). Nevertheless, this modification has not

yet observed in tRNAs. Finally, for organomercurial gels, different sensitivity has been reported for specific U modified RNA, like s⁴U, and s²U (Zheng et al. 2017).

5 RNAseq to map modifications on single RNA and complexed RNA samples

Historically, profiling of *E. coli* tRNA modifications was first performed using classical methods of RNA modification analysis (i.e., semi-denaturing 2D RNA gels and RNA fingerprinting), and later combined with LC-MS analysis (Chakraburttu 1980; Kiesewetter et al. 1987; Maden et al. 1995; Mims et al. 1985). However, these analytical approaches are extremely laborious and require highly purified tRNAs, limiting their applicability to low-abundant tRNA species. Similarly, exhaustive profiling of rRNA modifications was hindered by the necessity of targeted rRNA fragmentation into short oligonucleotides of 50-100 nt long for further analysis (Yang et al. 2016). Although CMCT-RT mapping for pseudouridine (ψ) detection in the middle of 1980s (see paragraph 4.3) allowed precise profiling of this abundant modification in tRNAs and rRNAs, other RNA modifications remained understudied.

The development and implementation of deep sequencing mapping approaches as routine analytical protocols for RNA modification mapping was one of the major advances in the field. Major breakthroughs were made for comprehensive mRNA analysis, but also for rRNA and tRNA modification profiling (Motorin and Helm 2019; Motorin and Marchand 2021). Some adaptations had to be introduced for tRNAs characterized by stable tertiary structure and small size. These efforts allowed rapid and simultaneous profiling of different RNAs present in the same biological sample, but most of these methods are still able to detect only one given modification at a time. Analysis of low-abundant RNA species frequently requires enrichment of the RNA of interest, but this limitation does not hinder rRNA and tRNA analysis, since these species are very well represented in total RNA. In practice, fractionation of total RNA to rRNA and tRNA fractions may be beneficial to limit the required sequencing depth, but this step is optional and generally may be omitted. As already mentioned above, the presence of tRNA isodecoders and isoacceptors, which frequently differ only by one of few nucleotides, render the mapping difficult. Several approaches were suggested to alleviate this difficulty. For instance, only unambiguously mapped reads or “modification-aware” alignment algorithms can be retained (Arimbasseri et al. 2015; Behrens et al. 2021). Another possibility is to allow ambiguous or even multiple mapping for the same tRNA sequencing read. Finally, identical and highly similar tRNA sequences can be collapsed into one

generic sequence used for reads' alignment (Pichot et al. 2021). This approach was proven to be efficient to reduce ambiguous mapping, but showed modification biases in the case of rare tRNA species.

In addition to single-nucleotide resolution mapping, a comprehensive analysis of RNA modification profiles requires appreciation and/or quantification of the modification stoichiometry site-by-site. This goal can be achieved by LC-MS analysis (with careful calibration of appropriate standards) and by deep sequencing-based methods. Quantification with Ab-based enrichment protocols is relatively imprecise, but many chemicals-based NGS approaches determine modification stoichiometry with ~5-10% error, as it was demonstrated for the RNA Bisulfite sequencing (RBS), RiboMethSeq and HydraPsiSeq protocols. Hence, these methods are extensively applied to monitor RNA modification dynamics.

Considering the tRNA and rRNA modifications commonly found in bacterial species, the most relevant deep sequencing methods for analysis of these RNAs are AlkAnilineSeq to detect m⁷G, D and ho⁵C (Marchand et al. 2021a; Marchand et al. 2018), HydraPsiSeq for pseudouridine (ψ), some 5-substituted U residues (namely m⁵U and wobble base modifications), and lysidine (k²C) (Marchand et al. 2022; Marchand et al. 2020) and RiboMethSeq for 2'-O-methylated (Nm) nucleotides (Marchand et al. 2016; Marchand et al. 2017; Motorin and Marchand 2018). Other approaches include RNA bisulfite sequencing to identify m⁵C (and also m⁴C) and analysis of RT misincorporation signatures for Watson-Crick edge-modified nucleobases (m¹A, m¹G, etc). These methods and their applications are discussed below.

5.1 AlkAnilineSeq

AlkAnilineSeq protocol (Marchand et al. 2018) takes advantage of RNA scission at the abasic site created by specific cleavage of the fragile N-glycosidic bond formed by certain RNA modifications. Exposure of RNA containing D, m⁷G, m³C and ho⁵C to elevated pH at high temperature leads to opening and subsequent loss of the modified nucleobase exposing RNA abasic site. Such chemical structures are known to be readily cleaved upon aniline treatment, leading to phosphodiester bond scission and liberating 5'-phosphate group at the N+1 nucleotide relative to modification. This free 5'-phosphate is used for specific adapter ligation, providing unprecedented specificity of the detection. AlkAnilineSeq was successfully used for mapping all D and m⁷G residues in tRNAs, and ho⁵C which is a rather rare rRNA modification found in *E. coli*

and closely related bacterial species. Specificity and sensitivity of AlkAnilineSeq are very high, low background allows detection of even substoichiometric modifications (minimum ~ 0.05 mole of modification/site) (Marchand et al. 2021a; Marchand et al. 2018; Marchand et al. 2021b).

5.2 *HydraPsiSeq*

Detection of pseudouridines (ψ) and 5-substituted U residues is based on their resistance to hydrazine cleavage, while unmodified uridines (U) are efficiently cleaved under these conditions (Marchand et al. 2022; Peattie 1979). Thus, protected U residues in RNA may correspond to modified bases and the level of this protection is related to modification stoichiometry. Other bases in RNA (A, C and G) are insensitive to hydrazine and cleaved only at the background level allowing normalization of U signals. In addition, some other non U-derived modified residues show a high sensitivity towards hydrazine cleavage, as shown for lysidine (k^2C) present in bacterial tRNA^{Ile} (CAU). A similar observation was also made for m^7G , but this methylation can be also detected by AlkAnilineSeq. Since protection of the modified nucleotide may also be related to its accessibility/reactivity within the RNA structure, the proportion of false-positive hits is substantially higher for HydraPsiSeq compared to AlkAnilineSeq. Hence, it is highly recommended that pseudouridine (ψ) mapping should be further validated either by conventional CMCT-RT primer extension (§ 4.3) or by its high-throughput variant approach PseudoU-Seq (Schwartz et al. 2014). Recently, direct RNA sequencing by Oxford Nanopore Technology (ONT) have been proposed as a new means to detect different modifications including pseudouridine (ψ), which produce characteristic base-calling “error” signatures (Begik et al. 2021; Huang et al. 2021).

5.3 *RiboMethSeq*

RiboMethSeq protocol is extensively used for mapping and quantification of Nm residues in rRNAs, tRNAs and mRNAs (Birkedal et al. 2015; Marchand et al. 2016). Detection of Nm residues is based on the resistance of the RNA phosphodiester bond following Nm residue, to cleavage induced by alkaline pH and high temperature. These resistant phosphodiester bonds create ‘gaps’ in random RNA cleavage profile. The depth of the ‘gap’ corresponds to protection, which is related to the level of the 2'-O-methylation, allowing precise quantification.

As HydraPsiSeq, RiboMethSeq is measuring protection against RNA cleavage, the number of false-positive signals is relatively high. Additional validation of the modified candidates can be done using quantitative or semi-quantitative RTL-P or primer extension at low [dNTP] in standard or high-throughput version (2OMe-Seq) (Incarnato et al. 2017). When applied to *S. aureus* RNA analysis we have observed high rate of false positive assignation for pseudouridines (ψ) by HydraPsiSeq, while Nm residues have been detected with high confidence.

5.4 RNA BisulfiteSeq

RNA bisulfite sequencing is derived from well-established DNA bisulfite sequencing and insures detection of m^5C and m^4C (m^4Cm in rRNA) in RNAs, together with precise quantification of the modification stoichiometry (Khoddami et al. 2019; Schaefer et al. 2009). Bisulfite treatment at moderate alkaline pH (to reduce RNA degradation) leads to chemical deamination of all C to U, while m^5C and m^4C are resistant to bisulfite, and thus can be detected as non-deaminated C residues. Application of the method is relatively straightforward for bacterial rRNAs, while m^5C was only rarely reported in bacterial tRNAs. Two major limitations have to be considered. First, only A, G, and U residues persist in RNAs after chemical deamination creating highly ambiguous alignment. Indeed, over 50% of RNA-derived reads may show multiple alignments to the reference, and thus should be excluded. Second, deamination level of C residues in highly structured RNA regions may be incomplete and these non-deaminated Cs show up as false-positive hits (Squires et al. 2012).

5.5 Analysis of RT signatures (RT-Seq)

Analysis of RT signatures, *e.g.* misincorporation/deletion and abortive cDNA synthesis was proposed for tentative mapping of RNA modifications having altered base-pairing properties, like I, m^1G , m^1A , m^3U , $m^1acp^3\psi$, and other modifications altering Watson-Crick edge. Since RNA structure or even specific RNA sequences perturb cDNA synthesis by RT, these data can be used only as tentative assignment of potential modifications. More reliable mapping data can be obtained using combinations of several RT enzymes with different properties (Werner et al. 2020), or using specific RT conditions, *e.g.* in the presence of Mn^{2+} ions (Kristen et al. 2020). Detection of misincorporation and RT-arrests require adapted protocols, but simple misincorporation/deletion can be directly extracted from RiboMethSeq, HydraPsiSeq, and AlkAnilineSeq raw sequencing data sets. Misincorporation and deletion profiles need to be

analyzed in parallel since the signal will appear in one or another scoring system depending on the nature of RNA modification and of the RT enzyme used. For bacterial tRNAs, these protocols allow reliable detection of s⁴U8, m¹A22, I34, m¹G37, and of many D and m⁷G residues.

Combination of the deep sequencing protocols cited above allows, in principle, almost exhaustive mapping of bacterial rRNA and tRNA modifications. Few RNA modifications (*i.e.*, m²G, Q, m⁶A, i⁶A and its derivatives), which are unreactive towards chemical reagents and/or do not lead to particular RT signature, can be detected by LC-MSMS (§ 3) or by specific protocols adapted for them (*i.e.*, NO-Seq (Werner et al. 2021) or specific RT for m⁶A (Aschenbrenner et al. 2018)).

6 Analysis of rRNA modifications using cryo-EM structural studies

In the past decade, cryo electron microscopy (cryo-EM) has emerged as an extraordinary tool to resolve ribosomal structures at high resolution. The tremendous advancements in the development of direct electron detectors and other technical improvements including advanced image processing and structure sorting (Kühlbrandt 2014; Klaholz 2019) have allowed cryo-EM reconstructions to reach resolution levels around 3 Å and better, which permits to visualize post-transcriptional modifications on bacterial and eukaryotic - including human – ribosomes (Cottilli et al. 2022; Natchiar et al. 2017; Stojković et al. 2020; Watson et al. 2020). Nevertheless, the presence of some rRNA elements that, more dynamic than others, limits the assignment to the most stable ribosomal regions. While structures cannot always identify the chemical nature of a modification, they provide key insights into the structural environment of a given chemical modification. For example, 2'-O-methylation can create a more hydrophobic environment that favors van der Waals contacts with hydrophobic side chains of amino acids, pseudouridines provide an additional possibility for hydrogen bonds through their N1 position, that classical uridines do not have, and some base modifications can increase π -stacking between neighboring nucleotide bases (Natchiar et al. 2018) (**Figure 4**). The structural analysis, combined with the various methods for chemical identification discussed above, thus provides important information on the structural role and function of chemical modifications.

Structures of several bacterial ribosomes in complex with mRNA and tRNAs have shown the importance of conserved tRNA and rRNA modifications in functional sites of the small (30S) and

large ribosomal (50S) subunits. On the 30S ribosomal subunit, where the tRNA anticodon interacts with the mRNA codon in the decoding center, different rRNA modifications have been shown to kinetically regulate the initiation process and to be involved in proofreading (Demirci et al. 2010). For instance, m^2G966 and $m^4Cm1402$ (*E. coli* numbering) both contribute to stabilize the interactions taking place between the tRNA, mRNA and rRNA in the P-site (Burakovsky et al. 2012; Jenner et al. 2010; Polikanov et al. 2015). On the 50S subunit, several conserved modifications are located at the peptide exit tunnel or are clustered at the Peptidyl Transferase Center (PTC), which may indicate that they assist peptide bond formation. Interestingly, Gm2251 in the P-loop of the 23S rRNA (*E. coli* numbering) directly interacts with the CCA sequence of the tRNA and thus is expected to contribute to the correct positioning of the tRNA acceptor end (Fischer et al. 2015; Golubev et al. 2020; Polikanov et al. 2015; Watson et al. 2020).

Various strategies were used to analyze modifications from high-resolution structures of ribosomes. With X-ray structures, difference density mapping (between real densities and those artificially obtained from modification-less models) helps to indicate the position of modified nucleotides (Polikanov et al. 2015). With cryo-EM structures, the refinement of specific regions of the ribosomal subunits (focused refinement) allowed reaching better resolution to recognize rRNA modifications in combination with previous annotation using complementary methods (Watson et al. 2020; Golubev et al. 2020). Several cryo-EM structures of *S. aureus* ribosomes are now available (Belinite et al. 2021; Belousoff et al. 2017; Cimicata et al. 2022; Golubev et al. 2020; Halfon et al. 2019; Khusainov et al. 2020; Khusainov et al. 2017; Khusainov et al. 2016; Matzov et al. 2017; Wright et al. 2020), and 10 different methylation sites have been mapped (Golubev et al. 2020) (**Figure 4A** and **4B**), but not all modifications could be assigned. Dihydrouridines (D) and pseudouridines (ψ) can be mapped with good confidence at very high resolution, for example on the 2 Å structure of the *E. coli* ribosome (Watson et al. 2020). Dihydrouridines (D) impose a characteristic bent shape to their pyrimidine ring and can be easily discriminated from uridines (**Figure 4C**). Pseudouridines (ψ) can be assigned indirectly, by analyzing in which manner they change the pattern of ions or water interactions. The isomerization of N1 and C5 in ψ maintains the same Watson-Crick base pairing property (W-C edge) but includes an additional hydrogen bond donor (N1 imino proton) on the C-H edge, which is responsible for water or ions binding (**Figure 4D**) (Watson et al. 2020). Assignations of ions, water molecules and

other interactions are still challenging but they will certainly contribute to alleviate better density interpretation (Wang et al. 2021).

To facilitate the analyses of thousands of nucleotides of the ribosome, dedicated software has been recently developed, which automatically calculates difference density maps. Positioning of the RNA modification signals, obtained at specific contour levels, offers now the possibility to restrict the analysis by other methods on only a subset of nucleotides (**Figure 4E**) (Stojković et al. 2020). However, efforts are still needed to complete the mapping of all the modifications in *S. aureus* rRNAs and to gain an evolutionary analysis of the conserved and specific sites by comparing with the extensive analysis done for *E. coli* (Watson et al. 2020) and *T. thermophilus* (Polikanov et al. 2015). Structures at higher resolution will help overcoming uncertainty in extra density coming from methylations, to discriminate pseudouridines according to their specific interaction network, and to observe conformational changes induced by modified nucleobases.

7 Discussion

Global analysis of the bacterial epitranscriptome and of its variations occurring in response to environmental changes and to various stresses, is still a major technical challenge. Combination of approaches involving high resolution structures of RNA machineries, mass spectrometry, and next-generation sequencing is certainly one way to render this objective amenable. As discussed in the review, the choice of the approaches depends on the RNA species to be studied. Recent advances in the Nanopore technology allow now the direct sequencing of full-length native RNA molecules without the need of RT and PCR amplification, and the obtained data normally contain all the information to assign the modifications (reviewed in (Begik et al. 2021)). However, Nanopore sequencing is still in its infancy and further improvement in the direct RNA sequencing are required for routine application. In most cases, Nanopore data are used only for validation, but not for *de novo* mapping of RNA modifications. Moreover, application to non-polyadenylated RNA species, like rRNA and tRNA, requires the use of polyadenylation by polyA-polymerase or specific custom primers. The complexity of Nanopore ion current profiles also requires further development of appropriate interpretation informatics pipelines, frequently based on machine learning algorithms or artificial intelligence.

Another major issue is to gain more knowledge on the protein machineries that are required for the modification deposition (writer) or which prevent the modification to take place (eraser). Even if phylogeny can be used to predict the enzymes and pathways required for RNA modifications, little is known in *S. aureus*. Many questions still remained to be addressed: are modification enzymes regulated upon stress conditions? Are there specific modification enzymes for mRNAs, regulatory RNAs, tRNAs and rRNAs? What are the enzymes required for biofilm formation and for virulence? Are the modified mRNAs conserved among bacteria? Previous sensitive LCMS approach has revealed that m⁶A/A ratio in mRNAs was higher in Gram-negative than in Gram-positive bacteria including *S. aureus* (Deng et al. 2015). Transcriptomic analysis performed in *E. coli* and *Pseudomonas aeruginosa* revealed that m⁶A was enriched in mRNAs involved in energy metabolism, stress responses, and in several sRNAs (Deng et al. 2015). Due to the rather small genome size, the high diversity of species, and the existing tools to delete genes, the bacteria represent ideal model organisms to decipher the extent of the functions of RNA modifications and of the associated enzyme machineries. No doubt the development of innovative sequencing approaches will be essential to gain full understanding of the bacterial epitranscriptome and to monitor its impact in gene regulation.

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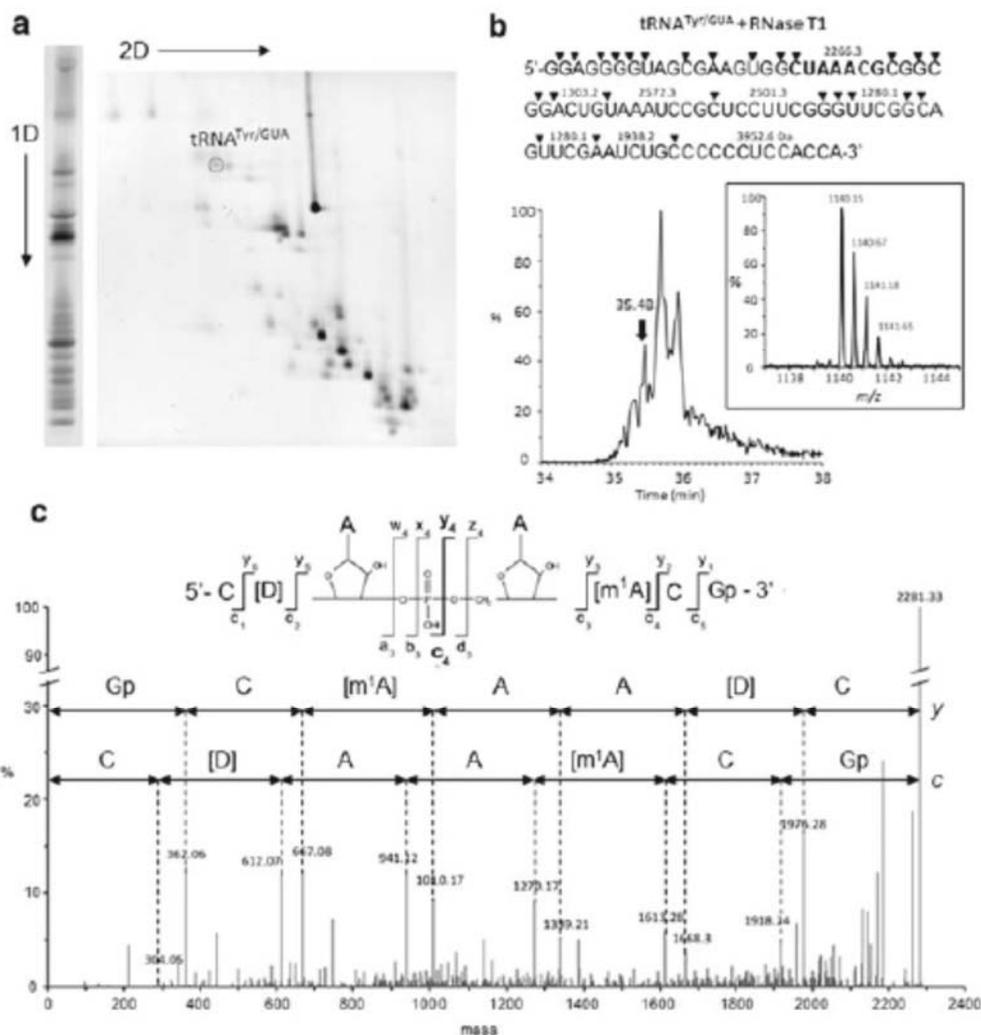


Fig. 2 Mapping of tRNA modifications by LC-MS/MS analysis of RNase digests. **a** Isolation of individual tRNAs by 2D-PAGE. Total tRNAs (10 μ g) are first separated by size on a denaturing polyacrylamide gel (left). Then, the whole lane is excised and embedded on the top of a semi-denaturing gel. After electrophoretic separation, the different bands migrate into multiple spots, most of which contain individual tRNA species (right). Arrows indicate the direction of migration on the first and second dimensions. Both gels are stained with ethidium bromide. The position of tRNA^{Tyr/GUA} in the 2D gel is indicated. **b** LC-MS analysis of in-gel T1 RNase digests of tRNA^{Tyr/GUA}. The unmodified sequence of the tRNA is shown and the cleavage sites of RNase T1 are labeled with arrowheads. Theoretical masses calculated with MongoOligo (<http://rna.rega.kuleuven.be/masspec/mongo.htm>) are displayed above the fragments of ≥ 4 nucleotides. The peak pointed on the elution profile corresponds likely to the oligonucleotide CUAACG ($m = 2267.3$ Da) in bold. Indeed, the spacing of the isotopic peaks in its MS spectrum (inset) is 1/2, indicating thus a double-charged anion $[M-2H^+]^{2-}$ with $m = 2281.3$ Da. The discrepancy between the expected and measured mass already evidences the presence of modifications. **c** Deconvoluted CID spectrum of the parent ion with $m/z = 1140.15$. Manual interpretation of the MS/MS data yielded the sequence C[D]AA[m¹A]CGp containing two modified nucleotides: dihydrouridine and a N¹-methyladenosine. Although the position of the methyl group on the adenosine cannot be determined from this analysis, it is known that Gram-positive bacteria display m¹A at position 22 of tRNA. The phosphodiester bond between the two unmodified adenosines is shown explicitly to indicate the four possible cleavages during CID. Most abundant breakages occur at the 5' P-O bond, generating c- and y- ion series (bold) that were used for sequencing

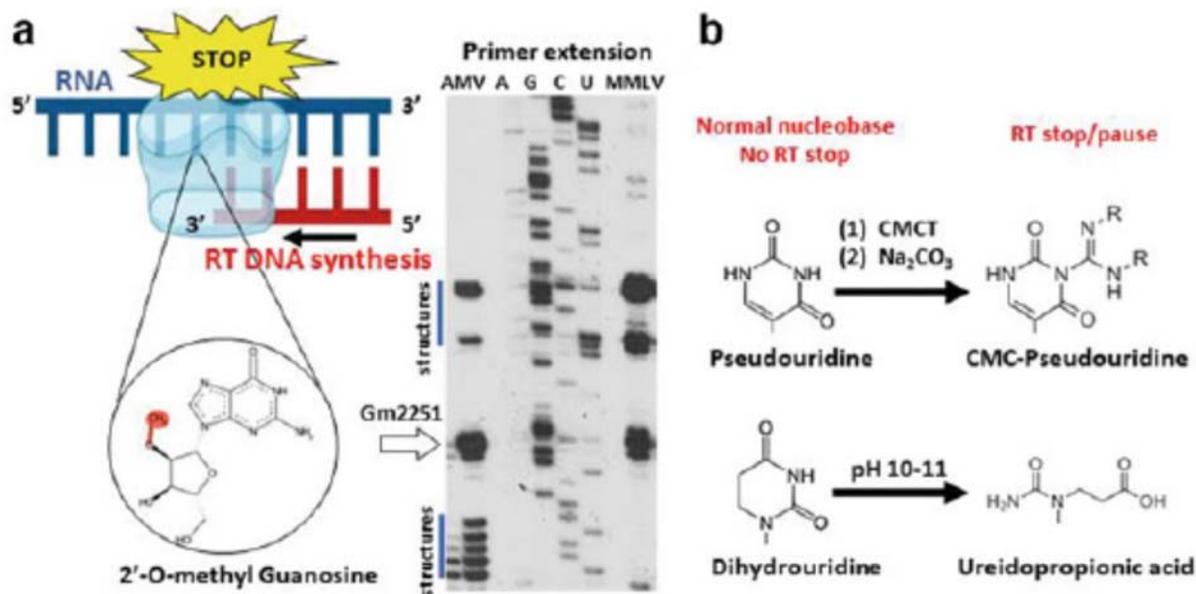


Fig. 3 Primer extension analysis to detect several RNA modifications. **a** On the right, a graphical representation explaining the principles of primer extension. Reverse transcriptase (RT, transparent light blue) stops DNA synthesis (red) at RNA modification sites, in this case at a 2'-O methyl guanosine in RNA (dark blue). On the left, an autoradiography film showing RT stop patterns induced by modifications or stable RNA structures, or unspecific cleavages. Avian myeloblastosis virus (AMV) and Moloney murine leukemia virus (MMLV) RT reactions were used at low nucleotides concentration (0.02 nM), to extend a radiolabeled DNA oligonucleotide complementary to *S. aureus* 23S rRNA. Using this procedure, the modification Gm 2251 (*E. coli* numbering) could be confirmed. **b** Chemical nucleobase derivatizations induced by the presence of pseudouridines and dihydrouridines to be detected by RT stops. Pseudouridines are modified by 1-cyclohexyl-(2-morpholinoethyl)carbodiimide metho-p-toluene sulfonate (CMCT) followed by alkali treatment with Na₂CO₃, which leaves only the CMC adduct attached to the N3 position. Dihydrouridines subjected to alkaline hydrolysis (pH 10–11) produce ureidopropionic acid after ring opening

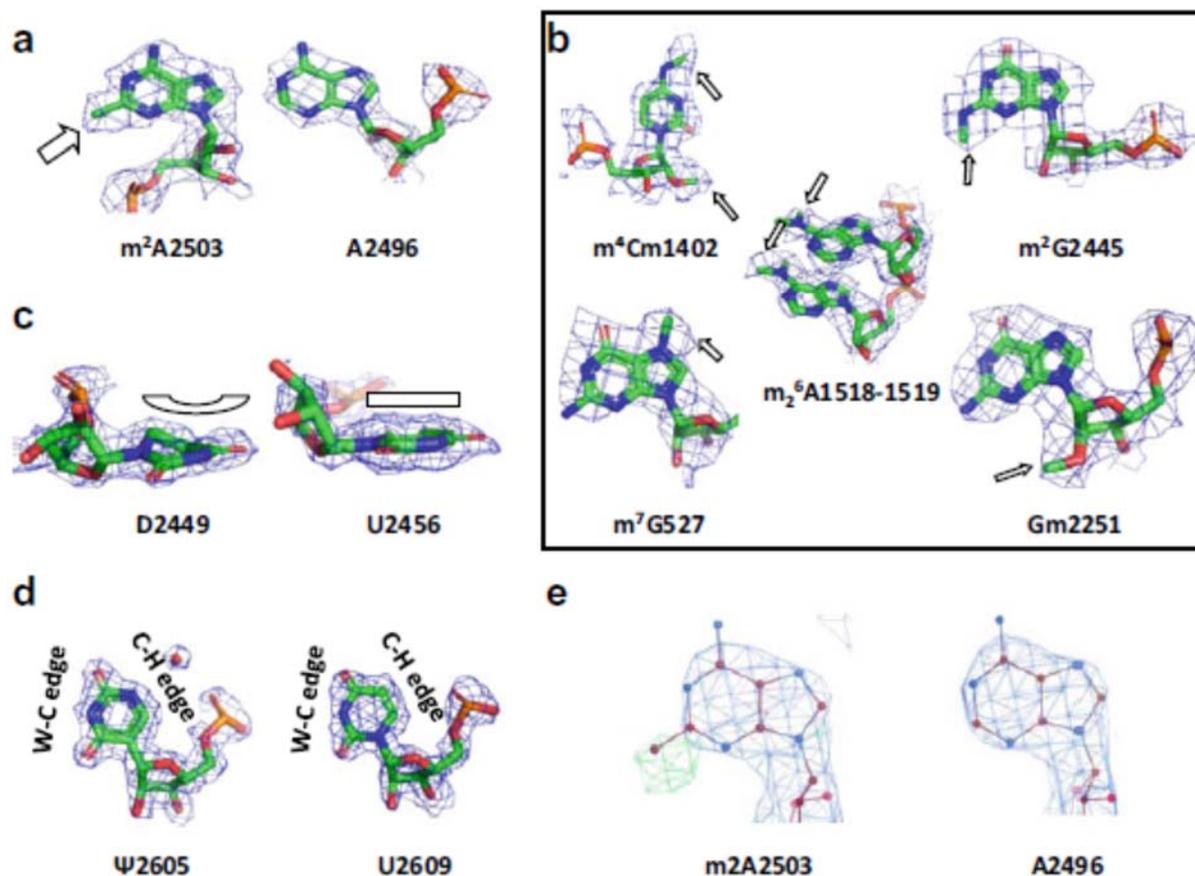


Fig. 4 rRNA modifications assigned by cryo-EM structure analyses. Nucleotides are numbered according to *E. coli* rRNA sequences. **a** *S. aureus* rRNA methylations can be assigned by cryo-EM looking at extra densities present at specific positions on the nucleotides. For example, an electron density shape difference between the two adenine residues A2496 and A2503 in 23S rRNA has assigned at position 2 a methyl group at the adenine nucleobase A2503 (model: PDB 6YEF; map: EMD-10791 at 3.2 Å, Wang et al. 2021). In this cryo-EM structure, several other *S. aureus* rRNA methylations have reported (**b**): m⁴Cm: 4-methyl 2-O' methyl cytidine; m²G: 2-methyl guanosine; m³U: 3-methyl uridine; m⁷G: 7-methyl guanosine; m₂⁶A: 6, 6-dimethyl guanosine, Gm: 2-O' methyl guanosine. **c** Characteristic bent nucleobase density shape of Dihydrouridine (D) observed on *E. coli* cryo-EM structure (model: PDB 7K00; map: EMD- 22,586 at 1.98 Å, Suzuki and Suzuki 2007). **d** Interaction between *E. coli* pseudouridine Ψ2605 with a possible water molecule and comparison with unmodified U2596 from the same 23S rRNA structure (model: PDB 7K00; map: EMD-22,586 at 1.98 Å). W-C edge: Watson-Crick edge; CH edge. **e** Example of the results obtained by using qptm software (Taoka et al. 2010). Calculated (blue) against difference (green) map from m²A2503 (modified) against A2496 (non-modified)

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