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1 Mapping post-transcriptional modifications in *Staphylococcus aureus*

2 tRNAs by nanoLC/MSMS

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23 13 **Running title:** Identification of tRNA modifications using mass spectrometry
24

25 14 **Keywords:** post-transcriptional tRNA modifications; nanoLC/MSMS; *Staphylococcus*
26
27 15 *aureus*; 2D gel isolation

28
29 16
30 17 **Abbreviations:**

31
32 18 LC: Liquid Chromatography

33
34 19 MS: Mass Spectrometry

35
36 20 BHI: Brain Heart Infusion

37
38 21 EtBr: Ethidium Bromide

39
40 22 TBE: Tris Borate EDTA

41
42 23 HFIP: Hexafluoro Isopropanol

43
44 24 TEA: Triethylamine

45
46 25 TEAA: Triethylamine Acetate

47
48
49 26

50 27 **Highlights**

- 51
52 28
53 29 • Protocols for nanoLC/MSMS to identify and localize tRNA modifications in
54 30 *Staphylococcus aureus*.
55 31 • Methodology of MS approaches to study tRNA modifications.
56 32 • Map of *Staphylococcus aureus* tRNA modifications in at least one tRNA isoacceptor
57 33 for each amino acid.
58
59 34
60 35

36 **Abstract (171 of max 250 words)**

37 RNA modifications are involved in numerous biological processes. These modifications
38 are constitutive or modulated in response to adaptive processes and can impact RNA
39 base pairing formation, protein recognition, RNA structure and stability. tRNAs are the
40 most abundantly modified RNA molecules. Analysis of the roles of their modifications in
41 response to stress, environmental changes, and infections caused by pathogens, has
42 fueled new research areas. Nevertheless, the detection of modified nucleotides in RNAs
43 is still a challenging task. We present here a reliable method to identify and localize
44 tRNA modifications, which was applied to the human pathogenic bacteria,
45 *Staphylococcus aureus*. The method is based on a separation of tRNA species on a two-
46 dimensional polyacrylamide gel electrophoresis followed by nano liquid
47 chromatography-mass spectrometry. We provided a list of modifications mapped on 25
48 out of the 40 tRNA species (one isoacceptor for each amino acid). This method can be
49 easily used to monitor the dynamics of tRNA modifications in *S. aureus* in response to
50 stress adaptation and during infection of the host, a relatively unexplored field.

51

1. Introduction

1.1 A brief tour on the roles of tRNA modifications in bacteria

RNA modifications alter the chemical and physical properties of nucleotides and the biological functions of RNA. They are involved in numerous biological processes because they can impact RNA base-pairing formation, the structure of specific RNA motifs, protein recognition, translation and decoding properties, and RNA stability (for a review see [1]). RNA modifications can be present in many RNA species, but abundant noncoding RNAs such as tRNAs, rRNAs, and spliceosomal RNAs are the most heavily modified ones and depend on the modifications for their biogenesis and function. Today more than 170 modifications have been identified, primarily in tRNAs and rRNAs (<http://modomics.genesilico.pl/modifications/>) [2], and numerous RNA-modifying enzymes have been characterized [3-8]. The expression and activity of these enzymes can be modulated in response to environmental stresses, adding another layer of complexity in gene regulation. For example, in bacteria, deregulations of modifications in ribosomal RNAs (rRNA) can promote antibiotic resistance [9-11]. In tRNAs, modifications are central for folding, quality control and efficient and accurate decoding. Also, they control gene expression during stress adaptation [12] and modulate interaction with human host [13].

tRNAs contain the largest number of modifications and the widest chemical diversity [2]. The complexity of tRNA modifications ranges from simple methylations on the base or the ribose to rather complex and large base hypermodifications, the synthesis of which generally depend on a cascade of enzymatic reactions [14]. Modifications have been shown to contribute to the highly conserved L-shaped structure, which is crucial for the interaction with a variety of proteins, the ribosome, and mRNAs. Interestingly, the distribution of the modified nucleotides along the tRNA molecule is conserved across evolution and reflects their two major functions in maintaining the tRNA structure and, ultimately, in decoding. Modified nucleotides concentrate in two hotspots - the anticodon loop and the tRNA core region, where the D- and TΨ-loops interact with each other (reviewed in [15, 16]). tRNA modifications of the core are important for the stability of the tRNA structure. In this context, it was shown that they affect temperature adaptation in thermophilic as well as in psychrophilic organisms [17]. The modifications of the core region are also thought to be important for EF-Tu binding (U to T change at position 54 in the TΨ loop)[18] and since they are involved in the

85 tertiary interactions maintaining the L-shape they are expected to influence the binding
86 of several proteins to the tRNA (i.e. aminoacyl tRNA synthetases and anticodon
87 modification enzymes) [19, 20]. tRNA modifications also facilitate tRNA-mediated
88 transcriptional control through T-box riboswitches to sense intracellular availability of
89 amino acids [21] and participate in quality control of tRNA integrity [22, 23]. More
90 recently, it was demonstrated that hypomodification of tRNAs in *Vibrio cholerae* induces
91 their rapid decay mediated by the degradosome, demonstrating a new quality control
92 mechanism [24].

93 Their major role in translation is due to their direct or indirect influence on the
94 decoding process and fidelity of translation (i.e., [25]). Because the genetic code is
95 degenerated, the 20 natural amino acids are encoded by 61 triplets that are recognized
96 by 22-46 distinct tRNAs depending on the organism. Therefore, several tRNAs recognize
97 more than one synonymous codon (i.e., the tRNA isoacceptors carry the same amino
98 acid). **The first nucleotide at position 34 of the anticodon (wobble base) can potentially
99 form pairs non-complementary in the Watson-Crick sense (for a review see [26, 27]).**

100 Detailed structural, physicochemical and kinetic studies of bacterial ribosomes
101 associated with mRNA and aminoacyl-tRNAs have shown that the decoding center of the
102 ribosome imposes specific constraints for the selection of a given codon by its cognate
103 aminoacyl-tRNA, and that base-pairs with non-Watson-Crick geometries are not allowed
104 [25, 28-32]. In miscoding induced by GU pairs, these pairs are in a Watson-Crick-like
105 state promoted by tautomerization [28, 29]. Interestingly, post-transcriptional
106 modifications of nucleotide 34 can change the physicochemical behavior of the base
107 (frequency of tautomerism), the spatial preference of the nucleotide (*syn/anti*,
108 puckering of the ribose), the stability of codon-anticodon base-pairing and the structure
109 of the anticodon loop itself, all contributing to the fidelity of the decoding process [33-
110 38]. A typical example is given by tRNA^{Ty}_{GUA}, tRNA^{Asn}_{GUU}, tRNA^{Asp}_{GUC} and tRNA^{His}_{GUG},
111 which can recognize both NAU and NAC codons, allowing G-C Watson-Crick base-pair
112 with NAC codons and wobble G·U pair with NAU codons. These tRNAs (collectively
113 harboring GUN anticodons), in eukaryotes and bacteria, present the Queuosine (Q)
114 hypermodification on G34 [39]. Recently, Tuorto and colleagues [40] have demonstrated
115 that, in mammals, the presence of Q affected translation speed. In the absence of Q
116 modification, ribosomes pause on NAU codons, while NAC codons are much less affected
117 [40]. Protein aggregation was observed for those genes enriched in NAU codons.

118 Although not yet investigated, such deregulation based on mRNA codon content in
119 response to the tRNA anticodon modification states, might take place in bacteria which
120 display distinct codon usage bias (GtRNAdb [41]). Because tRNA modifications including
121 the anticodon loop modifications can be modulated by various environmental changes,
122 the proteome is expected to vary during adaptation [12]. To date, perturbations of tRNA
123 modifications following physiological stress such as oxidation, temperature, pH, salinity,
124 and nutrient starvation are well established in eukaryotes, but less well studied in
125 bacteria [42, 43].

1.2 Roles of tRNA modifications in pathogenic bacteria

127 During infection, pathogenic bacteria must adapt to the host environment, which is
128 often scarce in nutrients and oxygen, and fastened with toxic metabolites and enzymes.
129 They express virulence genes to interact with host tissues, to compete with the
130 commensal flora, to avoid detection of the bacteria by the host immune system or to
131 fight against it. Thus, pathogenic bacteria rapidly adapt to continuously changing
132 environmental conditions and often regulate virulence gene expression at both the
133 transcriptional and post-transcriptional levels. The analysis of the impact of RNA
134 modifications in pathogenic bacteria during growth adaptation and infection has just
135 started to be appreciated [44]. Recent studies in Gram-negative bacteria, have
136 demonstrated that deletion of genes encoding RNA modification enzymes led to
137 infection attenuation in mice, reduced colonization, decreased levels of the pro-
138 inflammatory cytokines, reduced bacterial motility, replication in macrophages, and
139 invasion of epithelial cells ([45] and references therein). For example, the deletion of the
140 tRNA guanine transglycosylase gene (*tgt*) responsible for Q modifications reduces the
141 translation of VirF, the transcription factor orchestrating *Shigella flexneri* virulence [46].
142 Moreover, tRNA modifications in pathogenic bacteria have been shown to modulate
143 interaction with human host [13], or to **regulate the expression of genes involved in the**
144 **protection from oxidative stress, one of the main challenges that pathogenic bacteria**
145 **must cope with during infection [47].** In mycobacteria, hypoxia modulates tRNA
146 modification state to selectively translate codon-biased mRNAs for persistence genes
147 [48]. In numerous Gram-negative (*Escherichia coli*, *Salmonella enterica*, *Aeromonas*
148 *hydrophila*, *Pseudomonas fluorescens*, *P. aeruginosa* and *P. syringae*) and some Gram-
149 positive pathogens (*Streptococcus pyogenes* and *S. suis*), the GidA/MnmE tRNA

151 modification pathway, which leads to methylation of U34 in different tRNAs, has been
152 implicated in the regulation of bacterial virulence [49]. Even if the precise mechanism
153 remains unclear, an increased frequency of two-base frameshift has been reported [50].
154 Although these examples illustrate how the dynamic nature of tRNA modifications
155 regulates virulence in various pathogenic bacteria, the roles of RNA modifications in
Staphylococcus aureus have not been yet studied.

S. aureus is a Gram-positive opportunistic pathogen that is present in a third of the
population on skin and in nostrils [51] as commensal bacteria, but is also responsible for
a wide range of infections from minor skin abscesses to life-threatening diseases such as
septicemia or toxic shock syndrome [52]. *S. aureus* represents a major health problem
due to the insurgence of antibiotic multi-resistant strains in the hospital and community
settings. Given the importance of tRNA modifications in several aspects of bacterial life,
we decided to implement a rapid and easy method for their detection in *S. aureus* tRNAs
for which little knowledge existed.

1.3 Analysis of tRNA modifications by mass spectrometry approaches

Mapping of post-transcriptional RNA modifications using mass spectrometry (MS)
has existed for over 30 years [53]. The ability to get precise measure of molecular
masses (less than 1 Da) provided the possibility to characterize modified residues in
tRNAs using liquid chromatography coupled to mass spectrometry (LC/MSMS). Two
complementary MS approaches have been developed. The first one implies total digest
of tRNAs into nucleosides in order to identify the whole repertoire of modified
nucleosides in one experiment [54]. The resulting nucleosides are thereby separated by
reversed-phase high-performance liquid chromatography (HPLC) [55] and
modifications detected using spectroscopy (e.g., UV) or by mass spectrometry [56]. The
comparison of the HPLC chromatogram with known standard profiles and the
characteristic measured mass-to-charge ratio (m/z) of the ionized nucleosides are
contributing to the precise nucleoside identification. Atomic composition is finally
obtained *via* collision induced dissociation (CID) tandem mass spectrometry (MSMS),
providing a sensitive method for obtaining a survey of modified nucleosides in the tRNA
sample. The second method is based on the generation of tRNA fragments produced
from specific endoribonuclease digestion (e.g., RNase T1 cleaves every guanosine at
their 3' side). The generated oligonucleotides with known 5' and 3'-nucleotide identity

184 [57] are then separated by HPLC and detected by MS. Fragments along the
185 phosphodiester backbone by CID MSMS can be manually interpreted to localize the
186 modification to a specific nucleotide. Thus, the mass and the sequence of each
187 oligonucleotide is precisely identified by LC/MSMS allowing determination of the nature
188 of each modification and their location in the tRNA sequence [58].

189 These two complementary methods have provided the first insights into tRNA
190 modification dynamics following various stresses [53, 59, 60]. For nucleoside analysis,
191 an ensemble of tRNAs can be studied to follow easily the changes of their modification
192 patterns upon growth conditions while the LC/MSMS analysis of oligonucleotides
193 requires purification of specific tRNA prior to digestion. This is usually done by affinity
194 chromatography with a complementary DNA oligonucleotide linked to a solid resin or
195 bead [60]. The recent development of Ultra-Performance LC (nanoLC) permits the
196 careful separation of RNA fragments prior to the mass spectrometry analysis at very
197 slow flow rates (hundreds of nL/min) in a column with a reduced resin diameter (<
198 2µm). This method requires smaller sample amounts, generates less chromatographic
199 dilution and higher sensitivity. Because it is possible to detect very small amounts of
200 tRNA-derived oligonucleotides, a two-dimensional gel electrophoresis (2D-PAGE) can be
201 used to fractionate each tRNA species from bulk RNA extracts.

202 Here, we describe the use of this method and its application to the analysis of post-
203 transcriptional modifications in *S. aureus* tRNAs. We also provide here for the first time a
204 map of *S. aureus* tRNA modifications present in 26 out of the 40 tRNAs with different
205 sequences, including at least one isoacceptor tRNA for each amino acid. We will also
206 discuss applications to better address the roles of tRNA modifications in the
207 physiopathology of *S. aureus*.

2. Material and methods

2.1 Bacterial growth

208
209
210
211 *S. aureus* HG001 strain, a derivative of RN1 (NCT8325) strain with restored *rbsU* (a
212 positive activator of SigB), was used for tRNA isolation. The strain still contains a
213 mutation at *tcaR*, a transcriptional regulator involved in teicoplanin susceptibility and
214 biofilm [61]. *S. aureus* strain was manipulated under a specific hood in a dedicated room
215 for manipulating class II pathogens. Stock culture in glycerol 25%, conserved at -80°C
216 were plated on a blood-agar petri dish and grown at 37°C overnight in Memmert model

100 incubator. One single clone was picked and grown in 2 mL Brain Heart Infusion (BHI from Sigma-Aldrich), incubated at 37°C with a 180 rpm agitation overnight in a Stuart® orbital incubator S1600C. A preculture (100 µL) was added in 10 mL BHI medium (OD_{600nm}=0.05), the bacterial growth was regularly checked until it reached an OD_{600nm}=3 with a Thermospectronic Genesys 20 spectrophotometer (~4 h of culture at 37 °C stirring at 180 rpm).

2.2 Cell lysis and total RNA extraction

Cells were pelleted by centrifugation at 3750 rpm (3000 g) for 15 min at 4 °C in a Beckman Coulter Allegra X12-R centrifuge with a SX4750A rotor. Supernatant was carefully removed and the pellet (200 mg) was resuspended in 1 mL of RNAPro® Solution (MP Biomedicals, CA). Cells lysis was performed using a FastPrep®-24 with the 24x2 mL holder at 6 m/s speed in 2 mL FastPrep® tubes containing 0.1 mm zirconium silicate beads from FastRNA® pro blue kit (MP biomedical, CA).

Total RNA purification followed the procedure described for the FastRNA Pro Blue Kit (MP Biomedicals, CA). RNA was further purified by adding 1/2 volume of chloroform:isoamyl alcohol (19:1), vortexed 10 seconds before precipitation of the RNA in the aqueous phase obtained by adding 3 volumes of cold absolute ethanol (1.2 mL) in 2 mL Eppendorf tubes at -20°C overnight. RNA was precipitated by centrifugation at 13000 rpm (18000 g) for 15 min at 4°C using an Eppendorf 5417R centrifuge with a F45-30-11 rotor. The RNA pellet was washed with 500 µL of 80% cold ethanol and dried 20 min under vacuum in a Speed Vac® from Savant, before being resuspended in 200 µL of milliQ water. RNA concentration was determined by measuring absorbance at 260 nm with a Nanodrop ND-1000 spectrophotometer. Total RNA concentration was adjusted to 2 µg/µL. For phenol or chloroform extraction, work has to be done under a fume hood. Eppendorf tubes, tips, and buffers were RNase-free.

2.3 tRNA purification, 1st dimension PAGE

The first dimension gel electrophoresis was performed under denaturing conditions using 15% polyacrylamide gel (15% acrylamide:bisacrylamide 19:1 solution from Roth, 8 M urea, 1×TBE buffer from Euromedex®) (**Figure 1A**). Before loading the RNA sample, the gel was pre-run at 900 V for 20 min. One volume of urea-blue solution (urea 8 M, bromophenol blue and xylen cyanol 0.025%) was added to 5 µL containing

10µg of total RNA. The sample was heated at 90°C during 2 min before loading on the gel. After 15 h migration at 500 V at 20°C, the gel was stained with 0.5 µg/mL EtBr solution (from Sigma-Aldrich) by soaking the gel during 5 min, then washed with milliQ water and visualized on a High performance 2UV™ transilluminator from UVP. The gel slice containing 5S rRNA and tRNAs was cut to perform the second dimension gel.

2.4 Individual tRNA purification, 2nd dimension PAGE

The second dimension was performed under semi-denaturing conditions. The first dimensional gel slice (see § 2.3) was first placed horizontally at the top and between two glass plates (20x20 cm). Then a solution containing 20% polyacrylamide (polyacrylamide-bisacrylamide 19:1), 4 M urea, 1X TBE and 0.06% of ammonium persulfate and TEMED was poured between the plates. Once polymerized, the gel was run at 6 W (200 V) for 24h at 20°C using BioRad Protean II xi Cell. The gel was stained with EtBr as described in paragraph § 2.3 before UV visualization (Figure 1B). Gel pictures were taken with a Gel Doc™ EZ imager with its corresponding 16x12 cm UV tray.

2.5 In gel RNase digestion

Gel spots containing tRNAs were excised with a blade scalpel and dried under vacuum for 10 min (Speed Vac®). Digestion of the tRNAs with specific RNase was carried out directly on the gel fragments. The gel spots were incubated with 20 µL of 1U/µL of RNase T1 from Thermo Fisher Scientific (cleavage at G residues) in ammonium acetate 100 mM (pH 6.8) and incubated at 50°C for 3h followed by a 37°C overnight incubation under 300 rpm constant agitation. Alternatively, 20 µL of 0.01 U/µL of RNase A from Thermo Fisher Scientific (cleavages at C and U residues) in ammonium acetate 100 mM were added to the gel spots and the samples were incubated at 60°C during 3h, followed by a 37°C overnight incubation under 300 rpm constant agitation. The samples were stored at -20°C or immediately desalted, after removal of the gels by centrifugation (10 min, at 18000 g, at 4°C), using ZipTIP C18 (Millipore) prewet with acetonitrile 50% in milliQ water (3x10 µL) and 200 mM ammonium acetate (3x10 µL). The supernatant containing the digested tRNA fragments passively eluted from the gel, was loaded on the column, which was then washed with 200 mM ammonium acetate (7x10 µL) followed by milliQ water (5x10 µL). Finally, the fragments were eluted with 10 µL of 50%

283 acetonitrile in milliQ water and dried for 25 min under vacuum (Speed Vac®) (**Figure**
1 284 **1C**).

2.6 nanoLC/MSMS of RNA oligonucleotides

285
286
287 The pellet containing the oligonucleotides is resuspended in 3 μ L of milliQ water.
288 The oligonucleotides were then separated on nanoLC Acquity UPLC peptide BEH C18
289 column (130 Å, 1.7 μ m, 75 μ m x 200 mm) using a nanoAcquity UPLC system (Waters).
290 The column was pre-equilibrated in Buffer A (200 mM HFIP (Hexafluoroisopropanol),
291 7mM TEA (Triethylammonium), 7.5 mM TEAA (Triethylammonium acetate, pH 7.5) at a
292 flow rate of 300 nL/min. After loading on the column, the RNase digestion products
293 were eluted using a step gradient with two segments. The first consists of a gradient 15
294 to 35% of solution B (100 % methanol (MeOH)) for 2 min, followed by the second step
295 where the solution B reaches 50% (during 20 min at the same flow rate). Finally, the
296 MeOH solution is decreased to 15% during 25 min. The column is then re-equilibrated in
297 Buffer A during 20 min (**Figure 1D**).

298
299 MS and MSMS analysis were performed using a SYNAPT G2-S HDMS from Waters
300 equipped with a NanoLockSpray-ESI source in negative sensitivity mode with a capillary
301 voltage set to 2.6 kV and sample cone to 30 V. Source was heated to 130°C. For MS
302 analysis, a mass range from 550 to 1500 (m/z) was used followed by Collision Induced
303 Decay (CID) fragmentation of most intense signals with a m/z detection range of
304 fragments from 50 to 2000 with 1s scans (**Figure 1E**).

2.7 Data analysis

305
306 Chromatograms and spectra were processed using the Masslynx software from
307 Waters (V4.1). CID fragment spectra were deconvoluted by MaxEnt3 tool to convert the
308 m/z measured from multi-charged ions into singly charged ones. Spectra were then
309 manually sequenced using oligonucleotide fragmentation ion nomenclature [62].
310 Sequences are deduced by calculating mass difference between consecutive fragment
311 ions singly charged. Typically, the most intense ion series (y and c) were used. Correct
312 manual annotation was checked using MongoOligo online calculator
313 (<http://mods.rna.albany.edu/masspec/Mongo-Oligo>), with a maximum tolerance of 0.05
314 Da between the calculated mass and the measured mass for parent and fragment ions.
315 The tRNA genomic sequences were obtained from the HG001 genome [63].

3. Results and discussion

3.1A global view of *S. aureus* tRNA modifications

The above protocol is fast and easily implementable. Briefly, the pool of tRNAs extracted from *S. aureus* cultures was isolated by collecting RNA species of sizes smaller than the 5S rRNA (118 nucleotides), which are clearly visible on a denaturing (8 M Urea) 1D gel (**Figure 1A**). The tRNA samples were loaded on two different lanes on 1D gel. One lane was used for direct nanoLC/MSMS analysis and the other one was deposited on top of semi-denaturing gel for the second-dimension. The bands from the 1D gel were cut and analyzed by nanoLC/MSMS method to assign each tRNA (**Figure 1A**). Several bands contained different RNA species (i.e., band *b* contains both a 5S fragment and tRNA^{Ser}_{GCU}; and band *j* contains both tRNA^{Arg}_{ACG1-2} and tRNA^{Met}_{CAU1}). To assign unambiguously fragments to the different tRNAs, we then proceeded to a further purification step using the second-dimension gel electrophoresis under semi-denaturing conditions (4 M urea) (**Figure 1B**). The 1D bands gave rise to several spots where tRNAs are migrating individually (i.e. band *b* was resolved into spots 2 and 3 containing 5S rRNA and tRNA^{Ser}_{GCU}, respectively; and band *j* was resolved into spots 10 and 12 corresponding to tRNA^{Arg}_{ACG1-2} and tRNA^{Met}_{CAU1}, respectively). Each spot was subjected to the in-gel RNase T1 digestion (**Figure 1C**) followed by nanoLC/MSMS analysis (**Figure 1D-E**), which provided information on the sequences and on the presence of modifications.

Sixty-one tRNA genes are present in *S. aureus* HG001 [63]. They are transcribed from eleven *loci* as single genes (tRNA^{Ser}_{UGA1}, tRNA^{Arg}_{CCG}, tRNA^{Arg}_{UCU} and tRNA^{Gln}_{UUG3}) or organized into seven operons (I to VII, **Figure 2**) sometimes together with rRNAs. The number of isoacceptor tRNAs varies between one (Cys, Trp and elongator tRNA^{Mets}) to seven (tRNA^{Gly}) (**Figure 2**). With the exclusion of the non-proteinogenic tRNA^{Gly} and the tRNA^{Cys}, we could determine fragments belonging to 25 different tRNAs (**Table 1**) representing 46 tRNA genes, at least one isoacceptor for each tRNA. Some isoacceptors have identical sequences and cannot be distinguished in our analysis (the three tRNA^{Ala}_{UGC}, the two tRNA^{Arg}_{ACG}, the three tRNA^{Asn}_{GUU}, the four tRNA^{Asp}_{GUC}, two of the three tRNA^{Gln}_{UUG}, the two tRNA^{Gly}_{GCC}, the two proteinogenic tRNA^{Gly}_{UCC}, two of the three non-proteinogenic tRNA^{Gly}_{UCC}, the two tRNA^{Ala}_{GAU}, the two tRNA^{Leu}_{UAA}, the two tRNA^{Pro}_{UGG}, the three tRNA^{Thr}_{UGU} and the three tRNA^{Val}_{UAC}). For the other tRNAs, we could determine exactly from which gene they were transcribed.

349 Only fourteen tRNAs could not be detected (indicated with blue dots in **Figure 2**;
1 350 tRNA^{Glu}_{UUC2} and tRNA^{Glu}_{UUC3} are identical and therefore counted as single species).
2
3 351 Three of them are transcribed from a single gene while the others belong to operons
4
5 352 from which other tRNAs were isolated in our analysis. Since the most intense spots on
6
7 353 the 2D-gel were all analyzed, it is possible that they are less abundant (less transcribed
8
9 354 or more rapidly degraded) and more difficult to be detected. To complete the *S. aureus*
10
11 355 tRNA analysis, it would be necessary to purify them individually using biotinylated
12
13 356 oligonucleotides, which could specifically fish them from total RNA extracts [60].

14
15 357 Several modifications were detected on the tRNA fragments (**Figure 3**) deriving
16
17 358 from the core region (D and TΨ stem-loops), the variable region, and the anticodon loop.
18
19 359 Modifications at nucleotides in the core region and variable loop include dihydrouridine
20
21 360 (D), 1-methyladenosine (m¹A), 7-methylguanosine (m⁷G), and thymidine (T). A high
22
23 361 diversity of modifications was found in the anticodon loop and ten of them concentrate
24
25 362 at positions 34 and 37 (**Table 1, Figure 3**). Using the present strategy, pseudouridine
26
27 363 (Ψ) cannot be detected since its mass is indistinguishable from that of uridine. In order
28
29 364 to trace them, we use N-cyclohexyl-N'-β-(4-methylmorpholinium) ethylcarbodiimide *p*-
30
31 365 tosylate (CMC) derivatization which modified only Ψ under alkaline conditions (at pH
32
33 366 above 10) [64]. Although such modification can be visualized by mass spectrometry [65,
34
35 367 66], we unfortunately lost it during the MSMS fragmentation step preventing the
36
37 368 detection of any Ψ. This technical issue could be solved by the use of acrylonitrile, which
38
39 369 is more resistant to the fragmentation [67].

40
41 370 **Table 1** shows the regions that were probed for each analyzed tRNA. Four tRNAs are
42
43 371 almost complete (coverage > 70% for the tRNA^{Ser}_{UGA3}, the tRNA^{Pro}_{UGG1-2}, the
44
45 372 tRNA^{Leu}_{SUUU2-3} and the tRNA^{Glu}_{UUC1}). On average almost 60% of the tRNA sequences
46
47 373 were analyzed and, for most of the tRNAs, the core region and the anticodon loop were
48
49 374 covered with the exception of tRNA^{Leu}_{UAA1-2} and tRNA^{Trp}_{CCA}, for which we could not
50
51 375 cover any nucleotide of the anticodon loop, and tRNA^{Arg}_{ACG1-2} and tRNA^{Gln}_{UUG1-2}, for
52
53 376 which we could not detect fragments including their anticodons (nucleotides 34-36). In
54
55 377 this study, we used RNase T1 and RNase A since they are highly active enzymes able to
56
57 378 hydrolyze RNA in the gel. RNase T1 cleaves specifically at guanines while RNase A
58
59 379 cleaves at pyrimidine residues. To enhance the coverage, we tested other RNases. We
60
61 380 first used RNase MC1, which cleaves at the 5' of uridine and pseudouridine [68] but no
62
63 381 significant gel digestion of the tRNAs could be obtained (data not shown). We finally

382 adapted RNase U2, which cleaves at purines with a slight specificity to adenosine [69],
383 and preliminary data revealed that RNase U2 is able to digest RNAs contained in gel. By
384 using multiple RNases with different base specificity, oligonucleotide fingerprints can be
385 overlapped to provide full sequence coverage and to complete the mapping of tRNA
386 modifications.

387 Overall, *S. aureus* tRNA modification types and their localization are similarly found
388 in other Gram-positive bacteria such as *Bacillus subtilis* [2] and *Lactococcus lactis* [70]
389 (Figure S1). *S. aureus* specific features are discussed thereafter.

3.2 Individual tRNA modification maps

391 All modifications that were detected in the analyzed *S. aureus* tRNAs are detailed in
392 Table 1. As expected, most of them were localized in the core regions and in the
393 anticodon loops. In general, the modifications were also conserved in many other
394 bacteria.

395 Our work highlights the possible roles of the modifications at position 34 and 37 in
396 tRNA anticodons for fidelity maintenance, codon selection and discrimination in *S.*
397 *aureus*. For instance, Q at position 34 of tRNA^{Tyr}_{GUA} was proposed to efficiently
398 discriminate tyrosine codons (UAU and UAC) from stop codon during translation in
399 eukaryotes (review in [14]) and to increase affinity for UAU codons [40] in bacteria. As
400 expected, we could detect Q34 in tRNA^{Tyr}_{GUA2}, but also in tRNA^{Asn}_{GUU1-3}, tRNA^{Asp}_{GUC1-4}
401 and tRNA^{His}_{GUG1-2}.

402 Modification at U34 is usually found in tRNAs that read split codon-box families
403 where the synonymous A- and G-ending codons encode a different amino acid than the
404 U- and C-ending codons. We detected three major modifications at U34 (Table 1). The
405 mo⁵U (5-methoxyuridine (5)) [71] was present in eight *S. aureus* tRNAs. This
406 modification is only found in Gram-positive bacteria and is believed to enhance
407 translational fidelity by the ribosome [72]. We found cmnm⁵s²U (5-
408 carboxymethylaminomethyl-2-thiouridine (\$)) in the *S. aureus* tRNA^{Lys}_{UUU2-3}. This
409 hypermodification is ubiquitous in all bacteria and is usually detected in several tRNAs
410 (Lys_{UUU}, Gln_{UUG}, Glu_{UUC}, Trp_{UCA} or Leu_{UAA}). Finally, mnm⁵s²U (5-methylaminomethyl-2-
411 thiouridine (S)) was observed in tRNA^{Glu}_{UUC} and tRNA^{Lys}_{UUU} as shown in several Gram-
412 negative bacteria like *Escherichia coli*. The hypermodifications (S and \$) at U34 are
413 primarily observed on tRNAs carrying U34 and U35 (Table 1). In fact, these two
414

415 uridines are essential recognition determinants for the enzyme MnmA, which catalyzes
416 the thiolation at position 2 of U34 [73]. Intriguingly, *S. aureus* tRNALys^{UUU2-3} presented
417 both “\$” and “S” modifications at U34 (Figure S2). Such heterogeneity was also observed
418 at position 37 where t⁶A (N6-threonylcarbamoyladenine (6)) was present on the same
419 fragment presenting S at U34, while an unknown adenosine hypermodification (for a
420 total mass of 530 Da for the nucleotide) was observed for the fragment containing \$34.
421 To estimate the relative abundance of the two different modification patterns of
422 tRNALys^{UUU2-3}, the area of the peaks of the two distinct oligonucleotides in the mass
423 chromatogram was determined (Figure S2E-F). The comparison reveals that the
424 oligonucleotide containing the anticodon with unknown modified adenosine (H)
425 represents 34.6% of the total tRNALys^{UUU2-3}. Further analysis will be required to identify
426 this novel modification, to carefully quantify its abundance and to understand the
427 functional consequences of such heterogeneity in tRNALys.

428 The modification contents were also analyzed on the unique elongator tRNAMet^{CAU}
429 and one initiator tRNAMet^{iCAU} species. The important determinants of the initiator tRNA
430 in all bacteria are the absence of a Watson–Crick base pair between positions 1 and 72
431 in the acceptor stem and the presence of three conserved consecutive G:C base pairs in
432 the anticodon stem [74]. The peculiarity of the acceptor stem accounts for the high
433 specificity of the methionyl-tRNA^{Met} transformylase (FMT) for methionylated tRNAs
434 over other aminoacylated species [75, 76], while the anticodon stem-loop is important
435 for IF3 discrimination of non-initiator tRNAs in the 30S translation initiation complex
436 [77-80]. In addition, formylation of the methionine prevents misrecognition of the
437 initiator tRNA by EF-Tu [81, 82]. No modification could be detected in the anticodon of
438 the initiator tRNAMet (Table 1). A search in the Modomics database
439 (<http://modomics.genesilico.pl> [2, 83]) shows that the absence of modification in the
440 anticodon is a trait common to other bacterial initiator tRNAs (e.g., *Lactococcus lactis*
441 and *Streptomyces griseus*), except that a methylation on the ribose at C32 was observed
442 in *Thermus thermophilus* and in *E. coli*. The absence of modification for the initiator tRNA
443 anticodon could be another peculiarity of the translation initiation complex in which
444 decoding is monitored at level of the 30S subunit bound to the initiation factors IF1, IF2
445 and IF3 [84].

446 For the elongator tRNAMet^{CAU}, we identified a N4-acetylcytidine (M) at position 34
447 and a N6-methyladenosine (=) at position 37. Modifications at C34 and A37 are present

448 in most of the bacterial elongator tRNAMet and believed to be important to maintain
1 449 fidelity. For instance, the M34 modification in *E. coli* elongator tRNAMet was shown to
2 450 prevent misreading of the AUA isoleucine codon [85]. Indeed, the NAU anticodons are
3 451 particular because besides the tRNAMet, one tRNAIle isoacceptor also carries the
4 452 anticodon CAU (**Figure 2**). In addition, *S. aureus* encode two other tRNAIle isoacceptors
5 453 with the GAU anticodons. In various organisms, there are some variations in the tRNAIle
6 454 content. Indeed, only in some bacteria, some tRNAIle have the UAU anticodons, common
7 455 also in eukaryotes. Most eukaryotes have two isoacceptor tRNAIle, one with the
8 456 anticodon IAU (I =inosine), which reads all three isoleucine codons (AUU, AUC and AUA),
9 457 [86] while the other, with the anticodon ΨAΨ, is thought to read only AUA [87]. In
10 458 bacteria, tRNAIleGAU reads two of the isoleucine codons (AUU and AUC) and carries a t⁶A
11 459 modification at position 37, as it is also in *S. aureus* (**Table 1**). The other tRNAIleCAU
12 460 reads the third isoleucine codon AUA and usually carries a lysidine residue (Lysine
13 461 modified C at position 34). The enzyme TilS, responsible for this modification, is not only
14 462 essential for the correct recognition of the AUA codon but also for the amino-acid
15 463 specificity from methionine to isoleucine [88, 89]. This modification enzyme is also
16 464 present in the *S. aureus* genome suggesting that the lysidine modification is most likely
17 465 present in tRNAIleCAU to maintain fidelity. Purification of this isoacceptor tRNAIle will be
18 466 necessary to assert this hypothesis.
19 467

3.3 tRNA modification dynamics under different stress conditions

468 The protocol presented here can be easily applied to analyze specific stress
469 conditions that might alter the modification state or the nature of modifications in
470 tRNAs. The possibility to probe several tRNAs in one single experiment using 2D-gel
471 isolation procedure, without the need of biotinylated probes to capture (single) tRNA,
472 has removed one of the major bottlenecks for the LC/MSMS analyses. The recent
473 advances in mass spectrometry technologies and software [90, 91] now enable the
474 routine sequence mapping of modifications from much less material than in the past.
475 Prior to modification mapping on individual tRNAs, a complete nucleoside profile of
476 total tRNAs would be complementary to screen for modification changes taking place
477 under specific conditions. The analysis of the dynamics of tRNA modifications upon
478 stress encountered during infection in *S. aureus* might reveal unexpected strategies
479 developed by the pathogen to survive, persist, and fight against host immune defenses.
480

481 The increase in the sensitivity of nanoLC/MSMS methodologies and the new
482 development of high throughput RNA sequencing analysis [92] are promising
483 developments amenable to gain a full picture of RNA modifications, opening new areas
484 in the field of RNA regulatory functions in bacteria.

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Author contributions

LA conducted the experiments, analyzed the data and wrote the paper; PW developed the MS analysis approach, conducted the preliminary experiments, analyzed the data and wrote the paper; EW designed the research and wrote the paper; PR and SM designed the research, analyzed the data and wrote the paper; All authors have read and approved the manuscript.

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740 **Figure legends.**

741 **Figure 1. Complete protocol for nanoLC/MSMS analysis of *S. aureus* tRNAs from**

742 **isolation to modification mapping.** The five main steps of the protocol are as follow. A.

743 *First-dimension PAGE.* Total RNA was migrated on the first-dimension gel

744 electrophoresis (denaturing 15 % PAGE with 8 M Urea). The gel slice containing “tRNA-

745 size” (25000 to 150000 Da) RNA species migrating close to 5S rRNA, was cut. Arrow 1

746 indicates the direction of gel migration. The gel slice containing the tRNAs is deposited

747 on the top of the second-dimension gel. In parallel, the 21 visible bands (colored by

748 Ethidium Bromide and named from *a* to *t*) were cut individually, digested with RNase T1

749 and analyzed by nanoLC/MSMS to determine which tRNAs are present in each band (see

750 at the end of the legend). B. *Second-dimension PAGE.* Separation of the tRNAs from the

751 first-dimension gel slice in the second-dimension gel electrophoresis (semi-denaturing

752 20 % PAGE with 4 M Urea). Arrow 2 indicates the direction of migration. After staining,

753 the gel was divided into three large areas to be analyzed by the Gel Doc™ EZ imager.

754 Here only the middle (left) and bottom (right) parts are reported but the two pieces are

755 coming from the same experiment. No spots were visible on the upper part of the gel

756 (not shown). Each of the 23 spots (numbered from 1 to 23) was cut and used for the

757 following steps. The identity of these spots, as obtained from the complete analysis is

758 given at the end of the legend. C. *RNases in gel digestion.* The spots were subjected to

759 RNase T1 or RNase A digestion, desalted, and used for the liquid chromatography

760 separation of the fragments. D. *nanoLC (Ultra-Performant Liquid Chromatography) and*

761 *MS analysis.* The RNase derived fragments from each 2D spot are separated on the BEH

762 C18 column with a 300 nL/min flow rate. An example from spot 17 (tRNAVal_{UAC1-2-3})

763 digested with RNase T1 is given. Each peak is subjected to MS (insert) and MSMS (E) to

764 determine its sequence and the presence of modifications. E. *MSMS analysis.*

765 Electrospray-Ionization Collision-Induced Dissociation (ESI CID) spectrum obtained for

766 the ⁵CCU5UAC=AGp³ oligonucleotide (m/z = 972.87, triple charged (-3H⁺)) from the *S.*

767 *aureus* tRNAVal_{UAC1-2-3}. The profile obtained from the MSMS (fragmentation) analysis is

768 shown with its sequence given from its 5' nucleotide (sequence in green, c ion series) or

769 from its phosphorylated 3' nucleotide (sequence in red, y ions series). The localization of

770 a N6-methyladenosine (m⁶A) is showed with its symbol “=”. **1D bands:** *a:* tRNASer_{UGA3};

771 *b:* 5S rRNA fragment + tRNASer_{GCU}; *c:* 5S rRNA fragment + tRNASer_{UGA1} ; *d:* tRNATyr_{GUA2};

773 *e*: tRNA^{Leu}_{UAG}; *f*: tRNA^{Leu}_{UAA1-2}; *g*: tRNA^{Leu}_{CAA}; *h*: tRNA^{His}_{GUG1-2}; *i*: tRNA^{Thr}_{UGU1-2-3} +
 1 774 tRNA^{His}_{GUG1-2}; *j*: tRNA^{Arg}_{ACG1-2} + tRNA^{Met}_{CAU1}; *k*: tRNA^{Gly}_{GCC1-2p} (proteinogenic); *l*:
 2 775 tRNA^{Asp}_{GUC1-2-3-4} + tRNA^{Ala}_{GAA1-2}; *m*: tRNA^{Met}_{CAU1}; *n*: tRNA^{Val}_{UAC1-2-3}; *o*: tRNA^{Val}_{UAC1-2-3}
 3 776 + tRNA^{Glu}_{UUC1}; *p*: tRNA^{Pro}_{UGG1-2} + tRNA^{Ala}_{UGC1-2-3}; *q*: tRNA^{Ala}_{UGC1-2-3} + tRNA^{Phe}_{GAA}; *r*:
 4 777 tRNA^{Gly}_{UCC1-2p} + tRNA^{Asn}_{GUU1-2-3} + tRNA^{Lys}_{UUU2-3}; *s*: tRNA^{Gln}_{UUG1-2} + tRNA^{Trp}_{CCA}; *t*:
 5 778 tRNA^{Gly}_{UCC1-3np} (non-proteinogenic). **2D spots**: 1: tRNA^{Ser}_{UGA3}; 2: 5S rRNA fragment; 3:
 6 779 tRNA^{Ser}_{GCU}; 4: tRNA^{Ser}_{UGA1}; 5: tRNA^{Tyr}_{GUA2}; 6: tRNA^{Leu}_{UAG}; 7: tRNA^{Leu}_{UAA1-2}; 8:
 7 780 tRNA^{Leu}_{CAA}; 9: tRNA^{His}_{GUG1-2}; 10: tRNA^{Arg}_{ACG1-2}; 11: tRNA^{Thr}_{UGU1-2-3}; 12: tRNA^{Met}_{CAU1};
 8 781 13: tRNA^{Gly}_{GCC1-2}; 14: tRNA^{Asp}_{GUC1-2-3-4}; 15: tRNA^{Ala}_{GAA1-2}; 16: tRNA^{Met}_{CAU1}; 17:
 9 782 tRNA^{Val}_{UAC1-2-3}; 18: tRNA^{Glu}_{UUC1}; 19: tRNA^{Pro}_{UGG1-2}; 20: tRNA^{Ala}_{UGC1-2-3}; 21:
 10 783 tRNA^{Phe}_{GAA1}; 22: tRNA^{Gly}_{UCC1-2p} (proteinogenic); 23: tRNA^{Asn}_{GUU1-2-3}; 24: tRNA^{Lys}_{UUU2-3};
 11 784 25: tRNA^{Trp}_{CCA}; 26: tRNA^{Gln}_{UUG1-2}; 27: tRNA^{Gly}_{UCC1-3np} (non-proteinogenic). 1D bands
 12 785 and 2D spots are also summarized in Table 1.
 13 786

787 **Figure 2. *Staphylococcus aureus* HG001 tRNA operons organization.** The sixty-one
 788 genes present in *S. aureus* HG001 [63] were placed on a circular genome representation
 789 using DNAPlotter [93]. The tRNA genes are expressed from eleven loci sometimes as
 790 single gene, and more often as operons, which also contain rRNA genes. tRNAs which
 791 were not detected in the present study are marked with a blue dot. tRNA genes on the
 792 positive and negative strands are represented as long blue and green tracks,
 793 respectively. rRNA genes on the positive and negative strands are represented as yellow
 794 and pink tracks, respectively. Coding sequences on the positive and negative strands are
 795 represented as red and cyan tracks, respectively. Mean centered GC% plot is
 796 represented in mustard (above-mean) and violet (below mean).

797 **Figure 3. Atlas of modifications characterized on *S. aureus* tRNAs.** A. The different
 798 modifications observed on *S. aureus* tRNAs are reported on the typical cloverleaf two-
 799 dimensional structure of a tRNA. The acceptor stem, the variable region, the D, TΨ and
 800 anticodon loops are indicated. The nucleotide positions where the modifications were
 801 found are highlighted by light blue filled circles. B. Table summarizing the modifications
 802 with their abbreviations, the tRNAs for which each modification was found and its
 803 nucleotide position.

806 **Table 1. Characterization of RNA modifications in 26 *Staphylococcus aureus* tRNAs.**

1
2 807 Sequences of characterized tRNAs are reported with their respective coverage
3
4 808 percentages. In the sequences the color code is as follow: **red** nucleotides indicate
5 809 oligonucleotides fragments obtained by RNase T1 digestion, **green** nucleotides indicate
6
7 810 oligonucleotides fragments obtained by RNase A digestion (overlapping sequences are
8
9 811 in red) while **black** nucleotides represent regions that could not be analyzed. The
10
11 812 various regions of the tRNAs are visualized by colors according to the tRNA database
12
13 813 <http://trna.bioinf.uni-leipzig.de/> as follow: sequences highlighted in orange are for the
14
15 814 two complementary strands of the anticodon arm; sequences highlighted in green are
16
17 815 for the two complementary strands of the D-stem; sequences highlighted in blue are for
18
19 816 the two complementary strands of the acceptor-stem; sequences highlighted in pink are
20
21 817 for the two complementary strands of the T-stem; sequences highlighted in light blue
22 818 are for the CCA at the 3'. Modifications are indicated with symbols described in Figure
23
24 819 3B.

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Table1
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ID	2D	A.A	Anticod.	Acc-stem	D-stem	D-loop	D-stem	Ac-stem	Ac-loop	Ac-stem	V-region	T-stem	T-loop	T-stem	Acc-stem	CCA	Cover. (%)	HQ001	Open
20.	Ala	UGC 1-2-3	GGGGGU	GCUC	AGUAGDA	GAGC	CCUGC	UUGGAC	GCAGG	AGUUC		AGGG	UUCGAC	CCGU	AGUCCU	CCA	38	00443	II
10.	Arg	ACG 1-2	GCGCCG	UA GCUC	AAUDGGALIA	GAGC	UUUSA	CUACGA	UCAG	AGUUU		AUGGG	UUCGACU	CCUAG	CGGGCC	G CCA	38	00441	II
23.	Asn	GUU 1-2-3	UCCACAG	UA GCUC	AGUGGUA	GAGC	UUCGG	CUQUA	CCGAU	CGGUC		GUAGG	UUCGAGU	CCUAC	CUUUGGA	G CCA	49	01758	IV
14.	Asp	GUC 1-2-3-4	GGUCCG	UA GUGU	AGCGDDA	ACAC	CCUGC	CUQUAC	GCAG	AGAUC		GCGG	TUCGALU	CCGU	CGAGAC	G CCA	63	00021	I
26.	Gln	UUG 1-2	UUGGCUA	UA GCCA	AGCGGUA	AGGC	ACGGA	CUUUGAC	UCCGU	CACUC		GUUGG	UUCGAAU	CCAGC	UAGCCCA	G CCA	27	01799	V
18.	Glu	UUC 1	GCCUCCU	UG GUCA	AGCGDDA	*GAC	ACGCC	CUQUAC	GGCGG	UAAC		ACGG	TUCGAGU	CCCGU	AGGAGUC	A CCA	85	00020	I
13.	Gly	GCC 1-2	GCAGAG	UA GUUC	AGCGGUA	GAUU	ACGGA	CUUAAA	GUUG	GGGUC		GCGG	UUCGAAU	CCCGU	CUUCCG	U CCA	59	00439	II
22.	Gly	UCC 1-2 p	GCGGUG	UA GUUU	AAUGGA	AAAC	CCGAC	CUCCAA	CCUA	UGUU		GUGGG	UUCGAAU	CCCAU	CACCCG	U CCA	61	01759	IV
9.	His	GUG 1-2	GCGGUU	UG GUGA	AGUGDDA	*CAC	ACGGA	UUQUK/GU	UCCGA	CAUUC		GAGGG	TUCGALC	CCCUU	CAGCCG	C CCA	68	01760	IV
15.	Ile	GAU 1-2	GGGCCUA	UA GCUC	AGCUGDDA	GAGC	CAAGC	CUAUBA	GUG	AGGUC		GGUGG	UUCGAGU	CCACU	UAGGCC	A CCA	53	01804	V
8.	Leu	CAA	GCCGGUG	UG GCGG	AAUDGGCAG	ACGC	CGGGA	CUBSA+A	UCCGU	UUCACAUUGGAGU		GUCCG	UUCGACC	CCGAC	CACCCGU	A CCA	52	01794	V
7.	Leu	UAA 1-2	GCCGGGG	UG GCGG	AACDGGCAG	ACGC	ACGGA	CUUAAA	UCCUG	CGGUGAGAUACCCGU		ACCG	TUCGALU	CCGGU	CCUCCG	A CCA	57	01855	V
6.	Leu	UAG	GCGGGUG	UG GCGG	AAUDGGCAG	ACGC	ACGGA	CUAAGA	UCCAG	GCUUACGGGUGU		GGGG	TUCGACU	CCCUU	CACCCG	A CCA	66	01817	V
24.	Lys	UUU 2-3	GAGCCAU	UA GCUC	AGUUGGUA	GAGC	ACGGA	CUSSUUA/HA	UCAGA	GGGUC		AGAG	TUCGAAU	CCUJU	AUGGUC	A CCA	74	01818	V
16.	Met	CAU 1	GCGGGA	UG GAGC	AGUUGGUA	GUC	UCCGG	CUAUA	CCCGA	AGGUC		GGUGG	TUCAAU	CCGCC	UCCCGCA	A CCA	44	01763	IV
12.	Met	CAU	GCGGGUG	UA GCUC	AGCUGGCA	GAGC	UACGG	UUUAU+C	CCGU	AGGUC		GGGG	TUCGALC	CCUJC	CACCCG	A CCA	65	01811	V
21.	Phe	GAA 1	GGUCCAG	UA GCUC	AGUUGGUA	GAGC	ACGGA	UUGAAC	UCCAU	GUGUC		GGCAG	UUCGACU	CUGUC	CUAACC	A CCA	45	01761	IV
19.	Pro	UGG 1-2	CGGGAAG	UA GCUC	AGCUGGUA	GAGC	CUUGC	UUGGCA	CCAG	GGmUC		GCAG	TUCGAAU	CCUJU	CUUCCG	A CCA	72	00442	II
3.	Ser	GCU	GGAAAG	UA CUCA	AGUUGGUA	*GAG	GCCG	CUUUBA	GUUG	UAGUCGCAAGCGGC		GAGG	TUCGAAU	CCUJC	GGUUC	G CCA	66	00873	III
4.	Ser	UGA 1	GGAGGA	UA CCA	AGUCCGGCUGA	AGGG	UCCGG	CUSSA+A	ACCGA	CAGGGCCUUAACGGGCGC		GGGG	UUCGAAU	CCUJC	UCCUCC	G CCA	31	00383	-
1.	Ser	UGA 3	GGAGGA	UA CCA	AGUCCGGCUGA	*GGG	UCCGG	CUSSA+A	ACCGA	CAGGGCCUUAACGGGCGC		GGGG	TUCGAAU	CCUJC	UCCUCC	G CCA	70	01807	V
11.	Thr	UGU 1-2-3	GCCGCC	UA GCUC	AAUDGGUA	GAGC	ACUGA	CUUUBA	UCAU	AGGUU		GGGG	TUCAAGU	CCUJU	GGCCCG	A CCA	34	00437	II
25.	Trp	CCA	AGGGCA	UA GUUC	AACGGUA	GAUU	GAGGU	CUCCAA	ACCGU	UGGU		GUGGG	UUCGAAU	CCUAC	UCCCGU	G CCA	45	01801	V
5.	Tyr	GUA 2	GGAGGG	UA GCSA	AGUUGGUA	*CCG	GCGG	CUUUA+A	UCCG	UCCUCCGGUUC		GGCAG	UUCGAAU	CCGCC	CCCCUCC	A CCA	64	01395	VII
17.	Val	UAC 1-2-3	GGAGAU	UA GCUC	AGCUGGUA	GAGC	ACUGC	CUSSA	GCAGA	GGGUC		GGGG	UUCGAAC	CCUJC	AUUCUCC	A CCA	54	01820	V

Figure1
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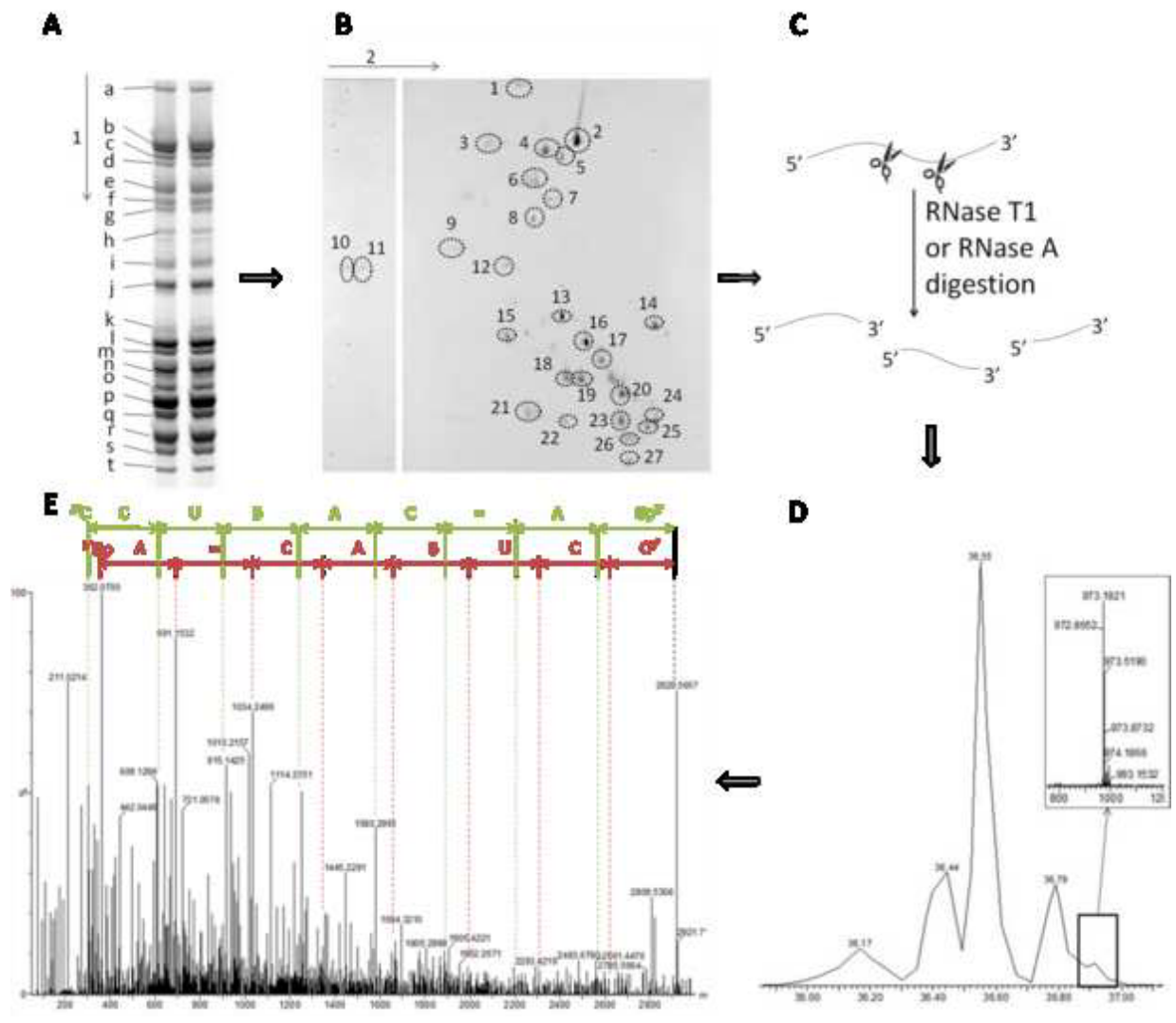


Figure2
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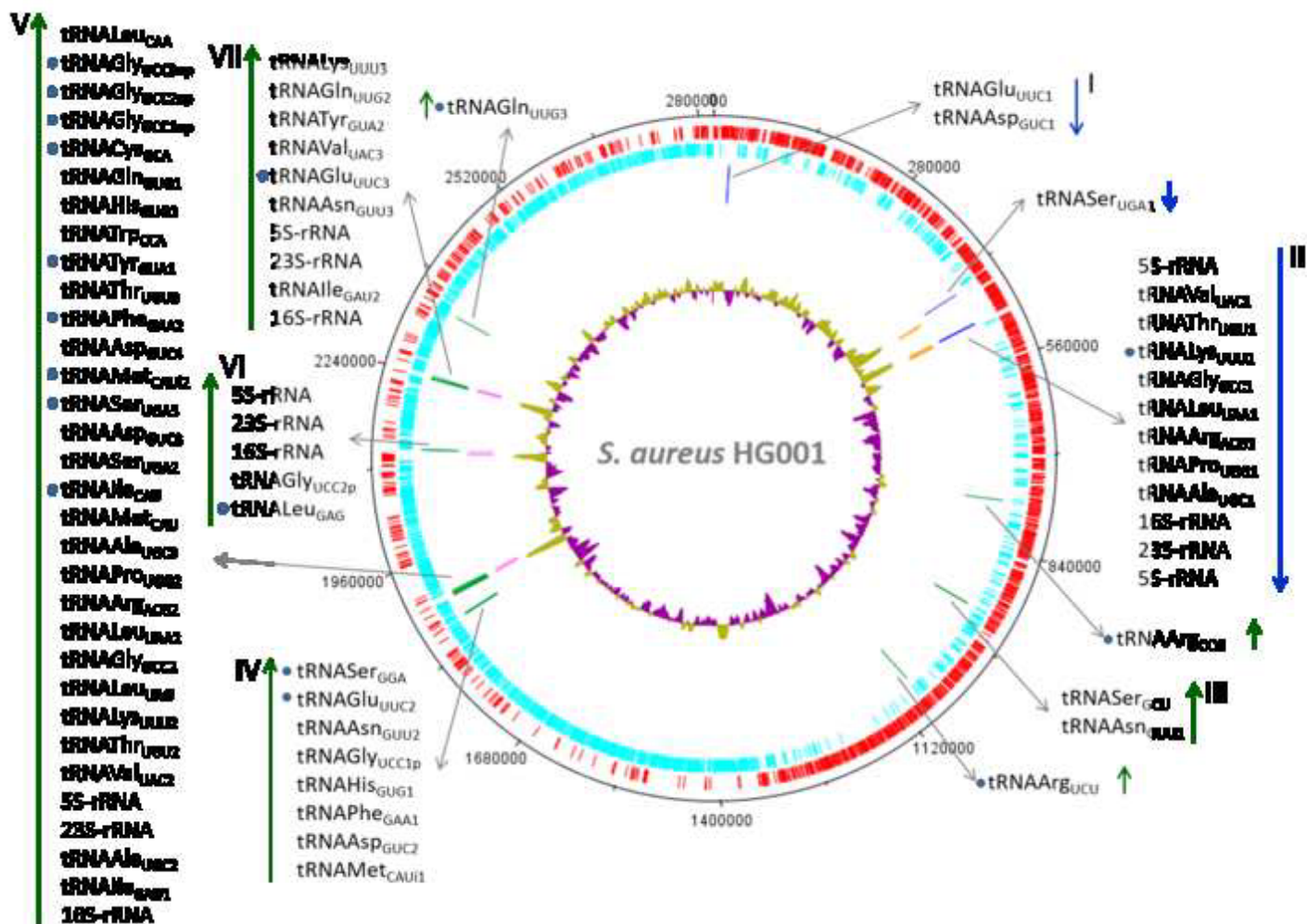
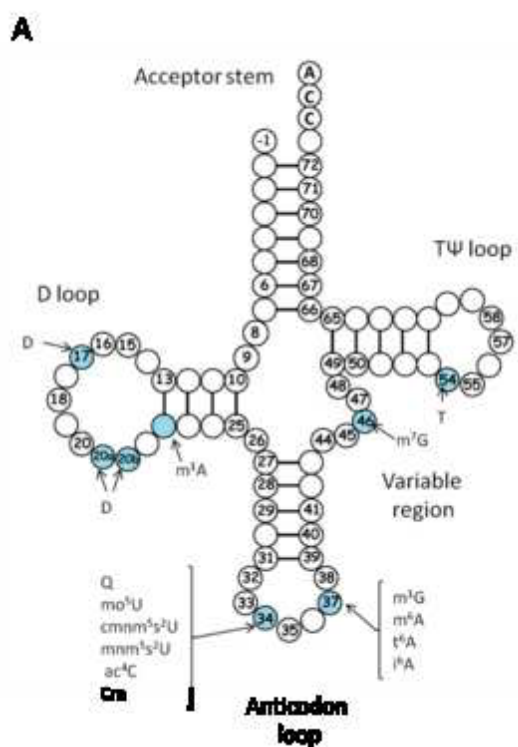


Figure3
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B

Modification	Symbol	tRNA	position	Modification	Symbol	tRNA	position
Dihydrouridine	D	Leu _{UAA}	U 18 U 20a, b	5-carboxymethylaminomethyl-2-thiouridine	S (cmnm ⁵ s ² U)	Lys _{UAG}	U 34
		Leu _{UAG}		5-methylaminomethyl-2-thiouridine	S (minm ⁵ s ² U)	Glu _{UUC} Lys _{UAA}	U 34
		Leu _{GAA}		1-methylguanosine	K (m ¹ G)	His _{UAG} Pro _{UAG} Arg _{AGC} Leu _{UAG}	G 37
		Thr _{UGU}		N ⁶ -methyladenosine	= (m ⁶ A)	Met _{CAU} Val _{UAC}	A 37
1-methyladenosine	= (m ¹ A)	His _{UAG}	A 22	N ⁶ -threonylcarbamoyladenosine	6 (t ⁶ A)	Ile _{GAU} Asn _{GUU} Thr _{GUU} Ser _{GCU}	A 37
		Asp _{GUU}		N ⁶ -isopentenyladenosine	+ (i ⁶ A)	Tyr _{UUA} Ser _{UUA} Leu _{CAA}	A 37
		His _{GUU}		Unknown modified adenosine	H (x ⁶ A)	Lys _{UAA}	A 37
2'-O-methylcytidine	B (Cm)	Leu _{CAA}	C 34	7-methylguanosine	7 (m ⁷ G)	Arg _{AGC}	G 46
N ⁴ -acetylcytidine	M (ac ⁴ C)	Met _{CAU}	C 34	5-methyluridine	T (m ⁵ U)	Met _{CAU} Asp _{GAU} Pro _{UGG} Leu _{UAG} Thr _{UAA} Glu _{UUC} Leu _{UAA} Ser _{GCU} Ser _{UGA}	U 53
Queuosine	Q	His _{UUG} Tyr _{GUU} Asn _{GUU} Asp _{GUU}	G 34				
5-methoxyuridine	5 (mo ⁵ U)	Gly _{UCC}	U 34				
		Val _{UAC}					
		Pro _{UGG}					
		Ala _{UUC} Leu _{UAG} Thr _{UGU} Ser _{UGA}					