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Identification and quantification of (t)RNA modifications in *Pseudomonas aeruginosa* by liquid chromatography-tandem mass spectrometry

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Abstract: Transfer RNA (tRNA) modifications impact the structure and function of tRNAs thus affecting the efficiency and fidelity of translation. In the opportunistic pathogen *Pseudomonas aeruginosa* translational regulation plays an important but less defined role in the adaptation to changing environments. In this study, we explored tRNA modifications in *P. aeruginosa* using LC-MS/MS based approaches. Neutral Loss Scan (NLS) demonstrated the potential to identify previously unknown modifications, while Multiple Reaction Monitoring (MRM) can detect modifications with high specificity and sensitivity. In this study, the MRM-based external calibration method allowed for quantification of the 4 canonical and 32 modified ribonucleosides, of which 21 tRNA modifications were quantified in the total tRNA pool of *P. aeruginosa* PA14. We also purified the single tRNA isoacceptors tRNA-ArgUCU, tRNA-LeuCAA and tRNA-TrpCCA and determined, both qualitatively and quantitatively, their specific modification pattern. Deeper insights into the nature and dynamics of tRNA modifications in *P. aeruginosa* will pave the way for further studies on posttranscriptional gene regulation as a relatively unexplored molecular mechanism of controlling bacterial pathogenicity and life style.

Introduction

Transfer RNA (tRNA) is the most post-transcriptionally modified type of RNA and is also target of the highest chemical diversity of modifications.^[1,2] Chemical modifications can modulate the structure and the function of tRNAs and thus ensure accuracy and efficiency of protein biosynthesis.^[3–6] The modifications located in the tRNA core are mainly ribonucleotide methylations or hydrogenations found in a broad range of different tRNA isoacceptors. They seem to contribute to the stability and structural integrity of the tRNA molecule.^[7,8] Complex chemical ribonucleotide modifications can often be found in the anticodon region of only a subset of tRNAs. They have been demonstrated to be involved in the decoding process and to be important for anticodon-codon binding, codon recognition, as well as reading frame and structure maintenance.^[9–16] There is growing evidence that tRNA modifications play an important role in translational regulation. Through selective, codon-biased translation of specific mRNAs, the cell can control protein biosynthesis.^[6,17–19] Loss of individual tRNA modifications, e.g. due to the inactivation of the tRNA modifying enzymatic activity, result in impaired phenotypes that affect global behaviors such as growth, virulence, pathogenicity and overall fitness.^[20–23] Additionally, a ribonucleotide at a certain tRNA position can be modified in all or only in a fraction of the tRNA molecules within the cell.^[24,25] Thus, changing the modification state of selected tRNAs can result in the modulation of gene expression profiles in a codon-biased genomic background. Indeed the modulation of the level of tRNA modifications has been described as a mechanism to change the proteome profile in response to different environmental stimuli and stresses.^[26–29]

A comprehensive characterization of tRNA modifying enzymes and the landscape of modifications in tRNAs has been achieved only for a few organisms such as *Escherichia coli*.^[4] Knowledge of the nature and dynamics of tRNA modifications is, an important prerequisite for studies aiming at the elucidation of the contribution of tRNA modification on the establishment of cellular phenotypes.

Pseudomonas aeruginosa is an opportunistic, Gram-negative pathogen causing acute and chronic infections, such as in the lung of cystic fibrosis patients.^[30–32] *P. aeruginosa* is able to adapt to many different environments. This is mainly due to a large repertoire of complex adaptation strategies controlled at the well-studied transcriptional level.^[33–35] However, beside transcriptional control also post-transcriptional regulatory processes are important for changes in the proteome profile and the bacterial phenotype.^[36,37] A recent study showed that modification of *P. aeruginosa* tRNA is involved in the regulation of the RhlR-receptor dependent quorum sensing phenotypes and affects *P. aeruginosa* virulence.^[38] Furthermore, tRNA modifications have been implicated to play a role in the oxidative stress response indicating an important, yet not well studied role of tRNA modifications in the translational control of bacterial adaptation.^[39,40]

For the analysis of RNA modifications, liquid chromatography–tandem mass spectrometry (LC-MS/MS) has emerged as a powerful tool.^[41–46] Here, we established different LC-MS/MS methods to analyze tRNA

modifications in *P. aeruginosa*. We quantified tRNA-modifications in the total *P. aeruginosa* PA14 tRNA pool as well as in single purified tRNA isoacceptors. New knowledge on the identity of chemical tRNA modification as well as on the modification level within the cellular tRNA pool provides an important foundation for further studies on the role of modifications on the structure and function of tRNAs. Especially reversible modifications may play active roles in regulating diverse biological processes including bacterial pathogenic states and life-style switches.

Results

LC-MS/MS methods to identify RNA modifications

To identify RNA modifications in *P. aeruginosa* PA14 two different LC-MS/MS methods were established. In a targeted approach we used a triple quadrupole MS in the Multiple Reaction Monitoring (MRM) mode with Q1 and Q3 set to selected masses in order to detect specific modifications with known fragmentation patterns (Figure 1A). To also identify unknown modifications in PA14 RNA, a Neutral Loss Scan (NLS) was applied. In this mode, the RNA sample is scanned in Q1 for masses in a defined range. After fragmentation, the product ions are scanned in Q3 for those that have lost a ribose (- 132 Da) or a 2'-O-methylribose (- 146 Da) during fragmentation (Figure 1A).

To compare both approaches, total purified PA14 RNA was hydrolyzed into ribonucleosides and analyzed by MRM and NLS. In the MRM method 27 modifications were included for target compound analysis and out of those, 20 modifications could be detected and identified as PA14 RNA modifications (Figure 1B, Table S1). The levels of the remaining 7 modifications (ac⁴C, m¹A, m⁴C, m⁸A, mo⁵U, nm⁵U and nm⁵s²U) were below the detection limit (Table S1). By using NLS, the RNA was scanned for modifications that lost the ribose during fragmentation. This resulted in the detection of 27 modifications (Figure 1B, Table S1). For most modifications more than one peak for a particular neutral loss was detected. This is exemplary shown for the modified ribonucleosides cm⁵U and ct⁶A (Figure 1A). Additionally, four peaks were detected by NLS that were isotopomers and could not be discriminated by mass. Examples are the modifications m⁴Cm and m⁵Cm, which both have a mass of 272.1 Da (Table S1). The identity and retention time was confirmed by comparison with synthetic standards, yielding 15 unambiguously identified PA14 RNA modifications by NLS. Overall we found an overlap of 15 modifications, which were identified by both, the NLS and the MRM method. By applying NLS, 12 peaks were exclusively detected but not identified due to non-availability of synthetic standards. MRM revealed five modifications that were exclusively identified in the total *P. aeruginosa* RNA. These were modifications, which either exhibited a modification pattern that was not associated with loss of ribose or the modifications were present in only low amounts (Figure 1C). Our results demonstrate on the one hand the potential of NLS to identify previously unknown modifications and on the other hand the high specificity and sensitivity of MRM.

Figure 1: Identification of PA14 RNA modifications by using Multiple Reaction Monitoring (MRM) and Neutral Loss Scan (NLS). **A)** In MRM the mass selection in Q1 and Q3 was fixed to one precursor-fragment pair that is specifically detected as exemplarily shown for $\text{mnm}^5\text{s}^2\text{U}$. In NLS the RNA was scanned in Q1 and Q3 for modifications that lost a ribose or a 2'-O-methylribose during fragmentation leading to complex chromatograms as e.g. for cm^5U and ct^6A . **B)** Number of modifications in PA14 RNA that were included, detected and identified by NLS and MRM, respectively. **C)** Comparison of the detected modifications by both, NLS and MRM.

Quantification of RNA modifications by MRM-based external standard calibration

For further analysis of RNA modifications in *P. aeruginosa* we quantified the modifications by using external standard calibration. The MRM was extended to the 4 canonical ribonucleosides and to 32 RNA modifications including simple modifications such as methylations but also modifications with more complex structures synthesized in multiple enzymatic steps such as $\text{ms}^2\text{i}^6\text{A}$ (Figure S1). Modifications that were not available as synthetic standards (such as $\text{mnm}^5\text{s}^2\text{U}$) were synthesized (Experimental Section S1). For quantification of these RNA modifications, an external calibration curve containing the synthetic standard of all modifications was prepared. This covered a concentration range from 13.4 pM up to 5 μM . To correct for interfering influences (biological and technical variances), the internal standard (IS) Tenofovir was added in fixed concentrations to the calibrators and the calibrator concentration was correlated to the peak area ratio between analyte and IS. To detect the linear fit of the calibration curve, 15 sets of calibrators were measured as exemplarily shown for the calibration curve of the modified ribonucleoside $\text{mnm}^5\text{s}^2\text{U}$ (Figure S2). The lower and the upper limits of quantification (LLOQ and ULOQ) were determined for all target modified and canonical ribonucleosides included in the MRM method (Table S2). The LLOQ represents the lowest and the ULOQ the highest calibrator point included with an

accuracy deviation not higher than 20 %. In addition, the signal of the LLOQ should have a signal-to-noise ratio larger than 10 and the signal of the ULOQ should not exceed detector saturation. For mnm⁵s²U, the LLOQ was 0.61 nM and the ULOQ was 800.3 nM (Figure S2). This corresponds to 6.1 fmol and 8.0 pmol mnm⁵s²U injected onto the column (10 µl injection volume). Among all 32 modifications, Ψ had the highest LLOQ with 87.3 fmol. The modifications with the lowest LLOQ were io⁶A and Am with 0.8 fmol (Table S2). These values demonstrate that the MRM method is sensitive and allows the quantification of modifications also in low abundant tRNA isoacceptors.

Quantities of global tRNA modifications in *P. aeruginosa*

We next used the established LC-MS/MS MRM-based external calibration method to quantify global tRNA modifications in *P. aeruginosa*. For this, RNA was further purified to enrich total tRNA (Quality control, Figure S3) that was mixed with the IS Tenofovir and subjected to the analysis (Figure 2A). The ratio from the peak area between the modified ribonucleoside and the IS allowed to quantify the respective tRNA modification. We focused our MRM search on overall 32 target modifications. 21 modifications were quantified in PA14 total tRNA (Figure 2B), whereas the lower limit for quantification (LLOQ) was not reached for the modifications: ac⁴C, ac⁴Cm, m¹A, m⁴C, m⁵C, m⁸A, mo⁵U, ms²i⁶A, nm⁵s²U, nm⁵U and s²U (Figure 2C). The extent of modification was expressed as the ratio of target modification to total canonical ribonucleosides (mole/mole) and depicted as modification per 1000 ribonucleosides. In order to determine the total amount, the canonical ribonucleosides were quantified following the same procedure as for the modified ribonucleosides. Since the amount of canonical ribonucleosides was higher than that of modified ribonucleosides, the sample was diluted to not exceed the ULOQ (Table S3). The modification D, Ψ or the single methylated ribonucleosides m⁵U exhibited a higher modification extent (27.8, 38.3 and 8.8 modifications per 1000 ribonucleosides, respectively), whereas ribonucleoside modifications, which exhibit a more complex chemical structure were present at lower quantities in the global tRNA profile. Among the latter we identified the modified ribonucleosides mnm⁵U (0.003 modifications per 1000 ribonucleosides) and i⁶A (0.005 modifications per 1000 ribonucleosides) (Figure 2B).

Figure 2: The quantities of modified ribonucleosides in total tRNA (ttRNA) of PA14. **A)** The workflow of tRNA purification, hydrolysis and quantification by MRM-based external calibration is shown. **B)** The quantities of the modifications were determined by using the established MRM-based external calibration method and depicted as modifications per 1000 nucleosides (modification (fmol) / sum of canonical ribonucleosides (fmol)). The data represent means $\pm$ SD for three biological replicates. **C)** The lowest limit of quantification (LLOQ) was not reached for the listed modifications in PA14 tRNA.

Modification pattern and level of single *P. aeruginosa* tRNA isoacceptors

We also exemplarily purified the tRNA for arginine with the anticodon UCU (tRNA-ArgUCU), the tRNA for leucine with the anticodon CAA (tRNA-LeuCAA) and the tRNA for tryptophan with the anticodon CCA (tRNA-TrpCCA) (Quality control, Figure S4) and analyzed their modifications using the established MRM method. The amount of modified and canonical ribonucleosides in each analyzed tRNA isoacceptor was determined by MRM-based external calibration (Table S3). The amount of the target modification per amount tRNA is depicted in Figure 3. Our quantitative analysis revealed 8 modifications in tRNA-ArgUCU, 6 modifications in tRNA-LeuCAA and 6 modifications in tRNA-TrpCCA above the background level of 0.1 (Figure 3). The common modifications Cm, m²A and m⁷G were present in all three analyzed tRNA isoacceptors. However, the level varied among the different tRNA isoacceptors and was lower than 1, indicating a non-complete modification at the respective positions. The modifications mnm⁵s²U with a modification level of 0.1 and s²C with a level of 0.2 were exclusively identified in tRNA-ArgUCU (Figure 3).

We quantified m⁵U modifications with a mean modification level of 0.9, 1.2 and 1.0 in the three tRNAs, respectively, indicating that all three tRNAs harbor one m⁵U modification. Furthermore, in all three tRNAs ~2 (mean 2.4, 2.1 and 2.3, respectively) uridines were modified to D. Furthermore, we found >2 Ψ modifications in tRNA-ArgUCU (mean 2.6) and tRNA-TrpCCA (mean 2.6), and 3 Ψ modifications (mean 2.98) in tRNA-LeuCAA (Figure 3).

Figure 3: The quantities of modifications in *P. aeruginosa* tRNA-ArgUCU, tRNA-LeuCAA and tRNA-TrpCCA. The workflow to purify and hydrolyze single tRNA isoacceptors followed by quantification of the modifications is schematically depicted. The quantities of modifications in PA14 tRNA-ArgUCU, tRNA-LeuCAA and tRNA-TrpCCA were determined by MRM-based external calibration. The modification level (modification (fmol) x number of parent ribonucleosides in tRNA / parent ribonucleoside (fmol)) was calculated. A modification level lower than 0.1 (black dashed line) was considered background noise. Data represent means $\pm$ SD for three biological replicates.

Figure 4: Assignment of the measured data to experimentally verified positions within the three *E. coli* tRNA isoacceptors. The genomic tRNA sequence of each tRNA isoacceptor from *E. coli* as well as from *P. aeruginosa* PA14 is depicted inside the circles. The nucleotides are marked in green if present in *E. coli* and *P. aeruginosa*. At positions with a deviation in the tRNA sequence the nucleotides marked in black indicate the *E. coli* sequence and those marked in red the PA14 sequence. The genomic PA14 tRNA sequences were previously published.^[47] The modifications marked in black and green are experimentally verified positions of modifications in *E. coli*.^[1] The green modifications have been quantified also in the corresponding PA14 tRNAs in this study. There are modifications that were exclusively detected in *P. aeruginosa* but not in *E. coli*. Those are indicated in red and listed in the black box, except $\text{mnm}^5\text{s}^2\text{U}$ that is placed at the position of the described *E. coli* derivative.

We did not experimentally map the quantified modifications to specific positions of the PA14 tRNA sequence; however, a comparison with the map of modifications in *E. coli* is presented (Figure 4). Most of the modifications identified in PA14 have been mapped previously to distinct positions of *E. coli* tRNA isoacceptors. The modifications t^6A at position 37 (t^6A_{37}) in tRNA-ArgUCU, s^4U_8 , Gm_{18} and $\text{ms}^2\text{i}^6\text{A}_{37}$ in tRNA-LeuCAA and s^4U_8 and $\text{ms}^2\text{i}^6\text{A}_{37}$ in tRNA-TrpCCA were not quantified in *P. aeruginosa*. Vice versa, all three *P. aeruginosa* tRNA isoacceptors displayed modifications not found in the corresponding *E. coli* tRNAs, such as m^2A (in all three tRNAs), m^7G (in tRNA-ArgUCU and tRNA-LeuCAA) and Cm (in tRNA-ArgUCU). In addition, *P. aeruginosa* tRNA-ArgUCU exhibited $\text{mnm}^5\text{s}^2\text{U}_{34}$, a derivative of *E. coli* $\text{mnm}^5\text{U}_{34}$ (Figure 4).

Discussion

P. aeruginosa is an opportunistic pathogen and able to adapt to large-scale environmental changes.^[33,35] There is growing evidence that modifications in tRNA contribute to fidelity and efficiency of translation and can selectively control biosynthesis of specific proteins and impact *P. aeruginosa* virulence.^[6,19,38] Thus, detailed knowledge on the nature and dynamics of chemical tRNA modifications promises to pave the way for further studies on posttranscriptional gene regulation as a fundamental yet relatively unexplored molecular mechanism of shaping bacterial behavior.

In this study, we describe on the application of LC-MS/MS methods to identify RNA- and quantify tRNA-modifications in *P. aeruginosa*. Two LC-MS/MS techniques, MRM and NLS, were established. The use of NLS has been previously demonstrated to allow identification of known but also of previously unknown ribonucleoside modifications such as ms²A in *E. coli*.^[45] Here, we detected 27 RNA modifications in the NLS, of which 15 were unambiguously identified in *P. aeruginosa*. 12 peaks could not be attributed to any modification (Figure 1, Table S1). Further comparative analysis and the use of synthetic standards will be needed to unambiguously assign them to a previously unknown chemical ribonucleoside modification in *P. aeruginosa*.

In the MRM method we included the four canonical and 32 modified ribonucleosides, which cover diverse chemical structures and reflect most of the tRNA modifications found in eubacteria (Figure S1).^[1,16] In a similar MRM study, a maximum of 28 modified ribonucleosides has been analyzed simultaneously.^[43] The repertoire of tRNA modifications has been extensively studied in *E. coli* as a model organism.^[4] In a recent study also the global profile of tRNA modifications in *P. aeruginosa* was characterized.^[39] The study uncovered 25 *P. aeruginosa* PA14 tRNA modifications, while we were able to quantify 21 modifications in *P. aeruginosa* PA14 using the MRM method (Figure 2). Despite differences in growth conditions and methods, remarkable 16 modifications were found in our and the previous study, with I and t⁶A showing different signal intensities in the two studies (Table S4). Interestingly, four tRNA modifications (namely ac⁴C, ms²t⁶A, nm⁵s²U and nm⁵U (Table S4)), which have been identified before in *E. coli*^[1,4], were found in neither study in *P. aeruginosa*. Vice versa, the modification Am (previously identified in *Saccharomyces cerevisiae* tRNA^[1]) was found in both studies in *P. aeruginosa* tRNA but not in *E. coli*. Jaroensuk *et al.* exclusively identified cmnm⁵s²U, cmo⁵U, m¹A, mo⁵U and s²U all of which have been described in *E. coli* tRNA.^[39] Additionally, they found four modifications that have previously been identified in *S. cerevisiae* tRNA and/or bacterial rRNA^[1,2], namely m^{2,2}G, m³C, m⁵C and m^{6,2}A. In this study we exclusively quantified D, io⁶A, m²G, mnm⁵s²U and s²C in *P. aeruginosa* total tRNA (Table S4). All of these tRNA modifications have been previously found in *E. coli* or other Gram-negative bacteria (*Salmonella typhimurium* and *Thermus thermophilus*).^[1]

In addition to the identification of the chemical nature, MRM-based external calibration allows for the quantification of RNA modifications. The sensitivity of the established method was comparable to that of other described methods with a quantitation range from low fmol to double-digit pmol (Table S2).^[42,46] Previous studies observed that between 4% and 16.88% of the ribonucleotides per tRNA molecule were modified in Gram-negative bacteria.^[16] In agreement, in this study the overall extent of tRNA modifications in the three single *P. aeruginosa* tRNA isoacceptors was between 9% and 11% (Figure 3). The vast majority

of the modifications quantified in the three *P. aeruginosa* tRNA isoacceptors have previously been identified in the corresponding *E. coli* tRNA isoacceptors (Figure 4). Quantification of tRNA modifications revealed that D, Ψ and m⁵U modifications were most abundant. Those uridine modifications have been shown to be located at the tRNA-core of almost every tRNA isoacceptor and to contribute to the tRNA structural integrity.^[48–50] We found one methylated uridine (m⁵U) in each of the analyzed *P. aeruginosa* tRNA isoacceptors (Figure 3). The modification m⁵U has been shown to be located at position 54 within tRNA sequences.^[16] Thereby position 54 is usually completely methylated to ribothymidin (m⁵U or T) in all tRNAs.^[25] We also found 2 uridines modified to D in the three analyzed tRNA-ArgUCU, tRNA-LeuCAA and tRNA-TrpCCA (Figure 3). The modification D has been previously identified at position 16, 17 and 20 in tRNA isoacceptors.^[16] However, all three *P. aeruginosa* tRNAs harbor an uridine only at two of these three sites (see Figure 4). Furthermore, 2–3 uridines were modified to Ψ in the three *P. aeruginosa* tRNAs (Figure 3). The modification Ψ has been described to be commonly found at the conserved position 55 in many tRNA isoacceptors.^[16] In addition, in *E. coli* Ψ was also identified at other positions: at position 40 in tRNA-ArgUCU (position 40 is however a cytosine in *P. aeruginosa* (see Figure 4)) and at positions 32 and 39 in tRNA-LeuCAA.^[16] Indeed, in *P. aeruginosa*, the positions 32 and 39 are potential Ψ sites in tRNA-LeuCAA (Figure 4). Although an overestimation of the presence of Ψ modifications cannot be ruled out in our analysis and, it remains to be determined whether there are additional yet unidentified Ψ modifications in the *P. aeruginosa* tRNAs.

In conclusion, by applying sensitive LC-MS/MS methods we explored tRNA modifications in the opportunistic pathogen *P. aeruginosa*. We characterized the global profile of tRNA modifications as well as the modification patterns of three single tRNA isoacceptors. Quantification of tRNA modifications revealed information about the amount of tRNA modifications and the level of specific modifications present in single tRNA isoacceptors. The established methods will allow for a thorough investigation of the dynamic changes and the regulatory functions of tRNA modifications on *P. aeruginosa* pathogenicity and life style. New information on how bacterial behavior is controlled might ultimately serve the development of novel strategies to inhibit bacterial adaptation to the human host and thus may result in the design of new ways to interfere with bacterial virulence.

Experimental Section

Bacterial strains, media, and growth conditions

The *Pseudomonas aeruginosa* UCBPP-PA14 reference strain (PA14)^[51] and the *P. aeruginosa* PA14 *ladS* transposon mutant (NR PA14 transposon mutant library ID 38371 Gm')^[51] containing the pUCP20 plasmid (*Escherichia-Pseudomonas* empty shuttle vector with beta-lactamase (*bla*) and LacZ alpha peptide (*lacZ* alpha) genes; Ap^r/Cb^r)^[52] were used in this study. Bacterial cells were cultivated in Luria broth (LB) media at 37 °C and harvested at an OD₆₀₀ of 2 by centrifugation for 15 min at 4700 rpm and 4°C. Carbenicillin (400 µg/ml) was added to the media for pUCP20 plasmid maintenance.

Chemicals and reagents

All chemicals were purchased from Sigma Aldrich (Munich, Germany) or Carl Roth (Karlsruhe, Germany) unless specified otherwise. Ribonucleosides used as analytical standards were purchased from different vendors (Table S5) or synthesized as described in Experimental Section S1 in the Supporting Information.

Isolation of RNA from *P. aeruginosa*

RNA isolation was performed according to^[53]. All centrifugation steps were performed at 4 °C. Briefly, bacterial culture (50 ml) was centrifuged at 3800x g for 15 min and the pellet was dissolved in resuspension buffer (300 mM sodium acetate (pH 4.5), 10 mM Na₂EDTA). RNA was extracted two times with equal volumes of acidic phenol by vortexing for 30 s, 60 s and 60 s with 60 s intervals on ice between each step followed by centrifugation for 15 min at 15,000x g. The aqueous phase was mixed with 2.5x volumes of ethanol and incubated for 2 h. RNA was recovered by centrifugation for 15 min at 15,000x g and the pellet was dissolved in sodium acetate (0.3 M, pH 4.5) followed by RNA precipitation with 2.5x volumes of ethanol for at least 2 h. The RNA was recovered by centrifugation for 15 min at 15,000x g and washed three times with ethanol (70% v/v). After drying the pellet, the RNA was resuspended in RNase-free water. The concentration was determined using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA USA) and the integrity of the RNA was checked by agarose gel electrophoresis.

Purification of total tRNA from *P. aeruginosa* RNA by solid phase extraction

The total tRNA in the RNA fraction was purified by solid phase extraction using an anion exchange column (CHROMABOND SB 1 ml; Macherey-Nagel, Düren, Germany) placed on a vacuum chamber (VacMaster; Biotage, Uppsala, Sweden). After column conditioning with sample buffer (20 mM Tris-HCl pH 7.5, 200 mM NaCl, 10 mM MgCl₂), RNA (1 µg) mixed with sample buffer was applied to the column. The column was washed with sample buffer and then the total tRNA was eluted in three steps by increasing the salt concentration (500 mM NaCl to 1 M and to 1.3 M NaCl). The total tRNA was precipitated by adjusting the NaCl concentration (2.5 M) and incubation with 2.5x volumes of ethanol at -20°C overnight. After recovering by centrifugation at 3800x g for 45 min, washing with ethanol (70% v/v) and drying the pellet, the elution fractions containing the total tRNA were combined after resuspension in water. The

concentration was determined using the NanoDrop and the quality was characterized using a Bioanalyzer (RNA 6000 Nano Kit; Agilent, Waldbronn, Germany) (Figure S3).

Purification of specific tRNA from total tRNA

A specific tRNA molecule was purified following a previously described protocol with the following slight modifications.^[54] We used Dynabeads-M280 Streptavidin (Thermo Fisher Scientific, Waltham, MA USA). After washing the beads the DNA oligonucleotide complementary to the target tRNA was attached following the manufacturer's instructions. The following DNA-oligonucleotides were used: tRNA-ArgUCU 5'-ACCATCCGCTTAGAAGGCGGATGCTCTATCT-3'biotin, tRNA-LeuCAA 5'-CGCCAGAAACGGATTTTGAATCCGCCGCG-3'biotin and tRNA-TrpCCA 5'-CTGCGGTTTTGGAGACCGCCGCTTGCCA-3'biotin. The total tRNA (100 µg) was mixed with an equal volume of 2x hybridization buffer (20 mM Tris-HCl pH 7.5, 1.8 M TMACl and 0.2 mM EDTA) and incubated at 65°C for 10 min with gentle mixing. The beads were washed (10 mM Tris-HCl, pH 7.5) and the buffer was completely removed before mixing them with the total tRNA. The samples were first incubated at 65°C for 10 min and then at room temperature for 3 h with gentle mixing. The unbound tRNAs were removed and the hybridized resin was washed three times (10 mM Tris-HCl, pH 7.5). The target tRNA was eluted by suspending the hybridized resin in buffer (10 mM Tris-HCl, pH 7.5) and incubation at 75°C for 5 min. The supernatant was transferred to a new reaction tube and desalted by adding 1/10 volume of sodium acetate and 2.5x volumes of ethanol incubating at -20°C overnight. The tRNA was recovered by centrifugation at 15,000x g for 30 min at 4°C, washing the pellet twice with ethanol (70% v/v) and drying it followed by resuspension in water. The tRNA concentration was determined by using the NanoDrop and the quality was checked using a Bioanalyzer (Small RNA Kit; Agilent, Waldbronn, Germany) (Figure S4).

Enzymatic hydrolysis into ribonucleosides

The enzymatic hydrolysis into ribonucleosides was adapted from^[41]. Total RNA (10 µg) and specific tRNA (1 µg) was incubated in RNA hydrolysis solution for 3 h at 37°C with slight agitation. The hydrolysis solution per RNA sample consisted of Tris-HCl (0.5 M, pH 8.0), MgCl₂ (50 mM), BSA (1 mg/ml), benzonase nuclease (15 U/µl), (Sigma Aldrich, Munich, Germany), PDE-I (0.1 U/µl), (Worthington Biochemical Corporation, Lakewood, NJ, USA) and alkaline phosphatase (30 U/µl) (Thermo Scientific, Bremen, Germany). After incubation, the hydrolyzed RNA sample was mixed 1:1 with the internal standard (IS) Tenofovir (100 ng/ml). The enzymes were removed by centrifugation of the enzymatic hydrolysate in an equilibrated 5 kDa centrifugal filter device (Vivaspin500; Sartorius, Göttingen, Germany) for 30 min at 4°C and 15,000x g. The RNA hydrolysis solution treated as described served as a matrix sample.

Separation of ribonucleosides using High Performance Liquid Chromatography (HPLC)

Prior to MS analysis, the ribonucleosides were separated on a Shimadzu Nexera HPLC system (Shimadzu, Duisburg, Germany) using a Zorbax Eclipse XDB-C18 RP-HPLC column (50 x 4.6 mm, 1.8 µm particle size) (Agilent, Santa Clara, CA, USA) equipped with a column saver (2 µm) (Supelco, Bellefonte, PA, USA) and a

C18 RP security guard (Phenomenex, Aschaffenburg, Germany). The column oven was set to 30°C, the autosampler to 4°C and the injection volume was 10 µl. The mobile phase A consisted of 0.1% formic acid (Merck, Darmstadt, Germany) in HPLC-grade water (J.T. Baker, Deventer, The Netherlands) and the mobile phase B of 0.1% formic acid in HPLC-grade methanol. The flow rate was 0.4 ml/min. For the MS/MS method comparison, a gradient duration of 12.1 min was used with 5% mobile phase B for 0.3 min as starting condition and a gradient as follows: 20% at 6 min, 75% at 8 min and 9 min, 5% at 9.1 min and reequilibration for 3 min. For the analysis of tRNA modifications, the gradient was prolonged to 21.1 min. In this case, the gradient started with 5% mobile phase B for 0.3 min followed by 95% at 13.9 min and 14.9 min, 5% at 15 min until 21 min.

General Mass Spectrometry setup

The HPLC system was directly coupled to a Linear Ion Trap Quadrupole QTRAP5500 mass spectrometer equipped with an electrospray ion (ESI) source (DuoSpray™) (Sciex, Framingham, MA, USA). All samples were analyzed in positive ionization mode. The MS parameters were as follows: source temperature, 400°C; ion spray and detector voltage, 5.5 kV and 2.3 kV; curtain gas, 30 psi; nitrogen collision gas level, 9; nebulizer gas and interface heater gas, 60 psi and 75 psi.

Analytical parameters and data processing for ribonucleoside analysis using HPLC-MS/MS in neutral loss scanning (NLS) mode

The mass spectrometer was working in neutral loss scanning mode with a fixed mass shift offset of -132.06 Da between the first (Q1) and third (Q3) quadrupole in case of a ribose loss or -146.04 Da for any 2'-O-methylribose loss. The declustering potential (DP), entrance potential (EP) and collisional cell exit potential (CXP) were set to 60 V, 10 V and 20 V, respectively. For the collisional energy (CE) an energy spread ranging from 20-60 V was applied for precursor ion fragmentation. The overall scan rate with unit resolution was set to 200 Da/s and the scan range was 200-500 Da with a cycle time of 1.5 s per cycle.

The raw data were processed using the MarkerView 1.2.1.1 software (Sciex). The recorded peaks were aligned and assigned with the following data extraction parameters: subtraction offset, 10 scans; subtraction multiplication factor, 1.3; noise threshold, 1000 counts; spectral peak width, 0.7 Da; minimum retention time peak width, 3 scans; mass tolerance, 0.2 Da; retention time tolerance, 15 s; and peak area integration, from original raw data. The processed data were exported into Microsoft Excel and filtered for a signal to noise ratio higher than 10. For further peak identification the processed data were matched against a created database containing all known modified ribonucleosides allowing a m/z difference of ± 0.3 Da. ^[1]

Analytical parameters, data processing and quantification of ribonucleoside analysis using HPLC-MS/MS in multiple reaction monitoring (MRM) mode

The analytical parameters for acquiring the IS and ribonucleosides are shown in Table S6.

The data were processed using Analyst 1.6 software (Sciex) with the following parameters: smoothing width, 3 points; noise percent, 90% and peak-splitting factor, 2. In order to quantify the ribonucleosides an external calibration curve was generated by mixing all ribonucleoside standards with the IS Tenofovir in the RNA hydrolysis matrix in a concentration ranging from 13.4 pM up to 5 μ M with 2.5-fold serial dilutions in between. For the calibration curve quadratic regression with 1/x weighting and accuracy with $100 \pm 20\%$ was applied to detect the linear fit. The modification amount was calculated by using the ratio of the peak area from the modified ribonucleoside and the IS for the calibration curve. The lowest limit of quantification (LLOQ) was defined as the lowest concentration of the calibration curve, which was used for the regression with an accuracy deviation $\pm 20\%$ and a signal-to-noise ratio larger 10. The upper limit of quantification (ULOQ) is the highest calibrator point included for regression with an accuracy deviation $\pm 20\%$ and that does not reach detector saturation. For any ribonucleoside the most intense fragment ion was selected for quantification and at least one other fragment ion was used as identifier with an intensity above the limit of detection (LOD) that is defined as 3-fold higher than the background noise. In addition to the mass transition, the retention time of the ribonucleoside should match the respective standard substance.

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Table of Contents

In this study, we explored, both qualitatively and quantitatively, (t)RNA modifications in *P. aeruginosa* PA14 by LC-MS/MS methods. The analytical biological approach sheds light on the dynamic landscape of tRNA modifications. Enzymes that modulate the modification status at selected tRNAs could represent dynamic regulators of gene expression with an impact on protein levels.

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Identification and quantification of (t)RNA modifications in *Pseudomonas aeruginosa* PA14 by liquid chromatography-tandem mass spectrometry