Characterizing the Amino Acid Activation Center of the Naturally Editing-Deficient Aminoacyl-tRNA Synthetase PheRS in Mycoplasma mobile

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Characterizing the amino acid activation center of the naturally editing-deficient aminoacyl-tRNA synthetase PheRS in *Mycoplasma mobile*

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Key words: Mycoplasma mobile, aminoacyl-tRNA synthetase, PheRS, m-Tyr and translation quality control

Abbreviations: AA-AMP - aminoacyl-adenylate
AA-tRNA - aminoacyl-tRNA
aaRS – aminoacyl-tRNA synthetase

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Abstract
To ensure correct amino acids are incorporated during protein synthesis, aminoacyl-tRNA synthetases (aaRSs) employ proofreading mechanisms collectively referred to as editing. Although editing is important for viability, editing-deficient aaRSs have been identified in host-dependent organisms. In *Mycoplasma mobile*, editing-deficient PheRS and LeuRS have been identified. We characterized the amino acid activation site of MmPheRS and identified a previously unknown hyperaccurate mutation, L287F. Additionally, we report that m-Tyr, an oxidation byproduct of Phe which is toxic to editing-deficient cells, is poorly discriminated by MmPheRS activation and is not subjected to editing. Furthermore, expressing MmPheRS and the hyperaccurate variants renders *Escherichia coli* susceptible to m-Tyr stress, indicating that active site discrimination is insufficient in tolerating excess m-Tyr.

Introduction
Aminoacyl-tRNA synthetases (aaRSs) are enzymes catalyzing the reaction of attaching cognate amino acids onto their corresponding tRNAs. The aminoacylation reaction consists of two steps: first amino acids are activated in the presence of ATP to form aminoacyl-adenylates (AA-AMP), which are then transferred onto tRNAs forming aminoacyl-tRNAs (AA-tRNA) [1]. Due to the structural and chemical similarities shared by certain near and non-cognate amino acids, incorrect amino acids can
Sometimes be misactivated, leading to mistranslation. In order to maintain the fidelity of protein synthesis, some aaRSs have developed proofreading mechanisms, where misactivated or mischarged tRNAs are hydrolyzed [2]. These proofreading mechanisms are collectively referred to as "editing". The capacity to edit has been shown in both eukaryotic and prokaryotic systems to be crucial for optimal cellular viability [3–5].

*Mycoplasma* are a group of cell wall-less, phylogenetically Gram-positive bacteria belonging to the class *Mollicutes*. They are known for having the smallest genomes of any known self-replicable organisms [6]. As a consequence of their massive genome reduction, *Mycoplasma* have lost many genes associated with metabolite biosynthetic pathways, resulting in their dependence on vertebrate hosts for survival [7]. In addition to metabolic genes, *Mycoplasma* aaRSs have previously been identified with sequence degeneration in the editing domain, where crucial residues or motifs are mutated [8,9]. This degeneration in aaRS editing site can diminish the editing capacities, which leads to error-prone protein synthesis [8–10]. Among all *Mycoplasma* species, *Mycoplasma mobile* is the most studied organism regarding aaRS editing degeneration. *M. mobile* encodes naturally editing-deficient PheRS and LeuRS, and proteomics data showed it to have higher than normal Phe and Leu codon specific misincorporation frequencies, indicating that the loss of PheRS and LeuRS editing results in higher rates of mistranslation in *M. mobile* [8].

PheRS is a heterotetramer composed of two α subunits and two β subunits which are encoded by *pheS* and *pheT*, respectively. The α subunit consists of the amino acid activation center, while the β subunit contains the editing domain [11,12]. Although the β subunit has been selected evolutionarily by organisms in different domains of life, editing is not essential for cellular growth in normal conditions [13]. In some cases, the loss of editing can be compensated by stringent amino acid activation, as shown in the monomeric mitochondrial PheRS [14]. Although the editing function of MmPheRS has been studied, no research has been performed to investigate the amino acid activation center of MmPheRS. Hence it was of inherent interest to characterize the active site of MmPheRS, and to understand its evolutionary traits through studying *M. mobile* specific amino acid replacements. One way to elucidate that is by determining the amino acid activation efficiency and discrimination toward non-cognate amino acids using MmPheRS active site variants.
Several non-cognate substrates have been reported for PheRS, including ortho-Tyr, meta-Tyr, para-Tyr and L-Dopa [15]. *E. coli* editing-deficient mutant has reported to be very susceptible to *m*-Tyr compared to *p*-Tyr [16]. Unlike *p*-Tyr, *m*-Tyr is a non-canonical amino acid that is not naturally encoded in the genome. It is a byproduct of Phe oxidation, hence would naturally accumulate in cells under oxidative stress and can be used as an oxidative stress indicator [17]. As *m*-Tyr activation and editing has never been explored before using MmPheRS, we sought to elucidate by both enzymatic techniques and *in vivo* phenotypic characterization if *m*-Tyr could be excluded from the *M. mobile* translational machinery.

**Material and Methods**

*Strains, culture conditions and genetic techniques*

*Mycoplasma mobile* 163K (ATCC43663) was purchased from the American Type Culture Collection (ATCC) and revitalized according to ATCC instruction. 16s rRNA sequencing was used to confirm the genotype of the *M. mobile* strain. *M. mobile* was cultured at 25 °C without shaking in Aluotto medium, which consists of 2.1% heart infusion broth, 0.56% yeast extract and 10% heat inactivated horse serum, as previously described [18,19]. 50 μg/mL ampicillin and 25 μg/mL thallium acetate were supplemented to Aluotto media as selections for *Mycoplasma sp.*. For solid Aluotto media, 0.7% agar was used. For fusion protein expression and plasmid construction, *E. coli* strain BL21 (DE3) pLysS and XL1-Blue were used respectively and were grown in Lysogeny Broth (LB) in the presence of respective selections. Site-directed mutagenesis was carried out to generate MmPheRS PheS variants (pQE31) as previously described [20]. For the minimal media growth assays, Phe-auxotrophic (ΔpheA::kan) *E. coli pheT* (G318W) editing-deficient strain [21] was used to express various pQE31-encoded PheRS.

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**Protein and tRNA preparation**

The MmPheRS encoding genes (PheS: MMOB3170 ; PheT: MMOB5170 ) were codon optimized for optimal gene expression in *E. coli* by Genescript as previously described [10]. *E. coli* PheRS intergenic sequence 5′ ggcaggaatagatt 3′ was incorporated between *M. mobile* pheS and pheT genes to allow for optimal expression. The synthetic operon was inserted into pQE31 and transformed into BL21 (DE3) pLysS for expression. EcPheRS expression construct (XL1-Blue/pQE31) was gifted from D. A. Tirrell (California Institute of Technology). All fusion protein expression constructs were incubated overnight in LB with corresponding selections before back-diluting at 1:100 the next day. For MmPheRS expression, the back-diluted culture was induced at OD 0.2 using 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) and incubated at 16 °C for 14 hours for slow expression before harvesting. The expression of EcPheRS was induced at OD 0.4 using 0.5 mM IPTG, and incubated at 37°C for three hours before spinning down. Spun-down biomass was resuspended in lysis buffer containing 10 mM Tris- HCl (pH 8.0), 200 mM NaCl, 5 mM NaH₂PO₄, 10% glycerol and half a tablet of cOmplete™ EDTA-free protease inhibitor mixture (Roche) before being subjected to sonication. The homogenized lysate was clarified by centrifugation then subjected to affinity resin column purification as previously described with modification of the wash and elution buffer [22]. In this study, the columns were washed in 200 mM NaCl, 5 mM NaH₂PO₄, and 10% glycerol, and eluted in the same buffer containing 250 mM imidazole. Collected fractions were concentrated and dialyzed as described previously [10]. Both *M. mobile* and *E. coli* tRNAₚₚ were *in vitro* transcribed by T7 RNA polymerase then purified as previously reported [22]. For *E. coli* tRNAₚₚ, the DNA templates were prepared by PCR amplification from pUC19 EctRNAₚₚ expression construct [23]; For *M. mobile* tRNAₚₚ, the DNA templates were generated through synthetic oligo assembly, where equal volumes of top and bottom oligos were combined and heated at 95°C for two minutes (Table S1), before subjecting for PCR amplification. For both Ec and Mm tRNAₚₚ, aminoacylation assays were performed as previously instructed [12] to determine their respective active concentrations.
Pyrophosphate exchange

Pyrophosphate exchange assays were performed at 37 °C in HKM buffer (100 mM Na-Hepes, pH 7.2, 30 mM KCl, 10 mM MgCl₂, 2 mM NaF) with 2 mM ATP, 2 mM [³²P]PPi (2–4cpm/pmol), 100 nM aaRS and various concentrations of amino acids as previously described with slight modification [23]. Briefly, the reactions were quenched with charcoal solution (1% charcoal, 5.6% HClO₄, and 75 mM PPI), which was then deposited on 3 MM Whatman filter discs (GE) for radioactivity measurements. The collected results were analyzed and plotted using KaleidaGraph by fitting to a Michaelis-Menten curve to determine the kinetics parameters. Standard deviation was used for error bar calculation.

ATP consumption assay

ATP consumption assays were carried out as described previously with slight modification as shown below [12]. In this study, the reactions were performed at 37 °C in aminoacylation buffer (100 mM Na-HEPES, 30 mM KCl, 10 mM MgCl₂) including 1 or 2 mM amino acids, 2 mM [γ-³²P] ATP, 2 U/ml of yeast pyrophosphatase (Roche) and 1µM PheRS with or without 2 µM tRNA_Phe. Quenched reactions were spotted on a PEI cellulose plate and developed in 0.7 M potassium phosphate (pH 3.5) for [γ-³²P] ATP and [γ-³²P] Pi separation. The concentration of Pi formation is used as a representation for ATP consumption and was determined by multiplying the Pi/ATP intensity ratio with the initial ATP concentration (2 mM). All assays were performed with at least three replicates, and the errors were determined using standard deviation.

Growth assays

For spot assays, M. mobile cells were grown to OD 0.15 at 600 nm before being used for serial dilution. 5 fold serial dilutions were made using Aluotto medium without experimental amino acid additives, then 5 µl of each dilution was spotted on Aluotto plates (0.7% agar) with 1mM Phe, 1mM m-Tyr, 1mM p-Tyr, and one without external amino acid additive respectively. Cells were grown at
25°C for 12 days and colonies were examined using a gel-imager (Biorad). For liquid culture growth, *M. mobile* was inoculated in 96 well plates at OD 0.01 in Aluotto media with designated external amino acids as described. Growth was recorded every 24-hour using a multi-well plate spectrometer (Biorad) for around 14 days. Both assays were performed with at least three biological replicates to ensure correct interpretation of the phenotypes. Errors were determined through standard deviation.

Phe-auxotrophic PheRS-editing-deficient *E. coli* strain (*pheT* G318W, ΔpheA::kan) was used to express various plasmid-encoded PheRS including WT EcPheRS, WT MmPheRS, MmPheRS βQ306A, αL287F, αW289F, αL287F/W289F, empty vector and EcPheRS (βG318W). Cells were picked as single colonies, resuspended in water and inoculated at an initial OD<sub>600</sub> of 0.004 in M9 including 0.003 mM Phe as previously described with slight modification[16]. Designated concentration of *m*-Tyr was supplemented, and the growth was monitored every 30 minutes at 37°C by a multi-well plate spectrometer (Biorad). Three biological replicates were conducted, and every time point has at least two individual data sets. Error bars were calculated using standard deviation.

Results

**Identifying amino acid functional divergence in the active site of MmPheRS**

To begin characterizing the amino acid activation center of MmPheRS, a multiple sequence alignment (MSA) was performed using the PheS sequence from 42 bacterial species including *M. mobile* to select for *M. mobile* specific amino acid replacements (Fig.1A and S1). In addition to active site variation at conserved residues, we further selected the residues that have previously been suggested to influence the amino acid activation function in existing PheRS crystal structures, such as residues that are predicted to interact with the amino acid substrate [24,25]. By overlapping these two steps of selection, 4 residues were chosen: T166, D207, S267, and W289. Since A288 (A294 in *E.coli*) is known to affect PheRS substrate specificity from previous studies [26,27], we further included the residue upstream to A288, L287, to understand if this replacement would affect MmPheRS aa discrimination. To study the effects of these amino acid replacements, we generated MmPheRS enzyme variants by changing the residues to their highly conserved counterparts using site-directed mutagenesis. For example, D207 is shown to be a glutamate throughout all of the organisms we examined except Mycoplasma sp., hence we generated a MmPheRS variant D207E
(Fig. S1). This resulted in T166S, D207E, S267C, L287F, W289F and the double mutant L287F/W289F (Fig. 1B) as this FAF motif seems to be highly conserved among various bacterial species, and the Ala flanked by these two hydrophobic residues is known to be crucial for amino acid discrimination [26] (Fig.1A and S1). Interestingly, all the residues we identified are replaced with similar amino acids. For example, Thr and Ser are both polar amino acids and only differed by a methyl group; Asp and Glu are both large acidic amino acids while Phe, Leu and Trp are all hydrophobic amino acids. These observations suggested that evolutionarily the properties of these residues may be crucial for the enzyme's function.

*MmpheRS does not efficiently exclude m-Tyr at the activation step*

To elucidate how amino acid replacements in the MmpheRS active site affect amino acid activation catalysis, we performed pyrophosphate exchange (ATP-PPi exchange) to determine the steady state amino acid activation kinetics of MmpheRS. The discrimination factor is defined by the ratio of $k_{cat}/K_M$ of cognate over noncognate amino acid, hence is an indication of how well the non-cognate substrate is differentiated from the cognate amino acid. Six enzyme variants as mentioned above were examined along with the WT enzyme in the presence of cognate amino acid Phe, and non-cognate amino acids m-Tyr and p-Tyr to study substrate discrimination. The results showed that the WT enzyme has a 6 fold higher discrimination against p-Tyr compared to m-Tyr, and this overall trend of m-Tyr being a better non-cognate substrate is observed in all of the enzyme variants except T166S and S267C, where both non-cognate substrates were excluded poorly (Table 1). Compared to existing *E. coli* data [16], MmpheRS discriminates m-Tyr with similar efficiency, showing that m-Tyr is not efficiently excluded from the active site.

All the variants except T166S and S267C showed poorer catalytic efficiencies toward p-Tyr compared to WT. For D207E, L287F, L287F/W289F, the activation of p-Tyr were undetectable at the highest p-Tyr concentration tested (3mM). Due to the low solubility of p-Tyr, we were not able to increase the substrate sufficiently concentration to reach $V_{max}$, hence the discrimination factors were determined indirectly as shown in Table 1. Among these enzyme variants, L287F showed significant
discrimination toward both \(m\)-Tyr and \(p\)-Tyr, indicating a hyper accurate mutation. However, this mutation compromises the catalytic efficiency toward Phe by a \(\sim 12\) fold increase of \(K_M\) with no significant change in \(k_{cat}\), showing that L287F alters substrate accommodation of MmPheRS but not its overall catalytic activity.

\textit{m-Tyr is not subjected to MmPheRS editing}

Since \(m\)-Tyr is not efficiently excluded from the active site of MmPheRS, we investigated if MmPheRS is able to edit this non-canonical amino acid. One way to determine if aaRS proofreading occurs is to monitor the level of ATP consumption as described previously [12]. Since one ATP molecule is used for every AA-AMP formation, the hydrolysis of misactivated AA-AMP leads to the release of amino acid, resulting in iterative rounds of amino acid activation and thus the futile cycling of ATP hydrolysis. To understand both tRNA dependent and tRNA independent editing activities, the assays were carried out with and without the presence of tRNA\(^{Phe}\). In addition to MmPheRS, EcPheRS was included as a positive control because its post-transfer editing activity of \(m\)-Tyr-tRNA\(^{Phe}\) is well characterized [16,28]. The results failed to show ATP-futile cycling with MmPheRS, neither with nor without the presence of tRNA, indicating that MmPheRS does not edit \(m\)-Tyr (Fig. 2)

\textit{m-Tyr does not impair the growth of M. mobile in Aluotto media}

Previous report has determined that a PheRS editing-deficient \textit{E. coli} strain is hyper sensitive to \(m\)-Tyr toxicity[16], prompting us to investigate the possibilities of active site discrimination as well as editing activities of MmPheRS toward \(m\)-Tyr. Since our results showed that \textit{M. mobile} PheRS lacks quality control pathways to exclude \(m\)-Tyr from the translational machinery, we decided to test if \textit{M. mobile} is susceptible to external \(m\)-Tyr stress. Although \textit{M. mobile} is a fish pathogen and relies on its host for survival, it can be cultured in rich media, such as Aluotto medium, both in liquid broth and solid agar as previously reported [29,30]. To understand if \(m\)-Tyr addition contributes to any growth defects in \textit{M. mobile}, we performed spot assays where cells were spotted at different dilutions on Aluotto plates containing designated cognate or non-cognate amino acids to observe colony growth. Among all replications regardless of aa addition, we were not able to detect any phenotypic differences between various growth conditions (Fig. 3A). To further confirm this phenotype, we
carried out a growth assay in liquid Aluotto medium for over two weeks, in which we failed to observe any significant growth defects as well, showing that m-Tyr does not impair the growth of *M. mobile* in Aluotto media (Fig. 3B). These results suggested that mistranslation frequencies did not significantly increase by the addition of 1mM m-Tyr and p-Tyr when the cells are grown in rich media.

Since the optimal growth temperature for *M. mobile* is 25°C and the activation kinetics showing in Table 1 were carried out at 37°C, we sought to explore if temperature alteration affects the catalytic efficiencies of MmPheRS, which may be one explanation for *M. mobile*’s lack of growth defects when external m-Tyr is added. Thus, ATP-PPi exchange was carried out at 25°C, 37°C and 42°C using WT MmPheRS. The resulting kinetic parameters showed no considerable variations between different temperatures; hence, it is unlikely that at its native growth temperature MmPheRS is able to exclude m-Tyr from its active site (Table S2).

**MmPheRS expressing E. coli stains are susceptible to external m-Tyr**

Despite not being able to exclude m-Tyr during activation and the lack of editing, external m-Tyr stress does not impose any growth defects on *M. mobile*. This is presumably due to the abundance of cognate amino acids in rich media, in which cognate amino acids may dilute out the additive amino acids. Hence, we sought to proceed the growth assays in a system where defined minimal media can be used for understanding the phenotypic consequences of MmPheRS induced mistranslation. For this purpose, we utilized a previously constructed *E. coli* pheT (βG318W) editing-deficient Phe auxotrophic strain (*ApheA::kan*) to host various plasmid-encoded PheRSs [21]. The pheT editing-deficient strain was chosen so that mischarged tRNA^{Phe} would not be robustly hydrolyzed and the phenotypic consequences of misaminoacylation can be observed. Note that this mutation (βG318W) renders *E. coli* PheRS editing-deficient but does not completely abolish editing. Additionally, Phe auxotrophy was acquired to resemble the external Phe dependency of *M. mobile* [31]. Although our results revealed no editing activity of MmPheRS towards m-Tyr in the presence of Mm tRNA^{Phe} (Fig. 2), the editing assay was performed again using Ec tRNA^{Phe} to ensure that unexpected editing activities of MmPheRS do not occur in *E. coli*. Similar to what was shown in Fig 2, MmPheRS
showed no editing activity towards \textit{m-Tyr} regardless if \textit{Ec tRNA}^{	ext{Phe}} is in the presence or not (Fig. S2), excluding the possibilities of \textit{m-Tyr} editing in \textit{E. coli}.

To understand if active site discrimination of MmPheRS is sufficient to defend the cells from \textit{m-Tyr} induced toxicity, we examined not only WT MmPheRS but also selected active site variants. Specifically, we are intrigued if the hyper accurate variant L287F is able to support cellular growth upon \textit{m-Tyr} stress. MmPheRS editing domain variant $\beta$Q306A was previously reported to be able to reduce the level of mischarged $p$-Tyr-tRNA$^{\text{Phe}}$ presumably due to active site discrimination, as it does not demonstrate ATP-futile cycling [10]. Hence $\beta$Q306A was also included in this study as a comparison. Additionally, editing capable EcPheRS was also examined as a positive control, along with empty vector and editing-deficient EcPheRS ($\beta$G318W).

Our results showed that, when no \textit{m-Tyr} is added, MmPheRS expressing strains demonstrated a slight doubling time defect compared to the strains expressing EcPheRS WT, $\beta$G318W and empty vector, indicating a possible intrinsic disadvantage for \textit{E. coli} to express MmPheRS even when no \textit{m-Tyr} stress is imposed (Fig. 4A). Although it has been reported that MmPheRS aminoacylates \textit{E. coli} tRNA$^{\text{Phe}}$ with lesser efficiency compared to the \textit{E. coli} counterpart [10], empty vector still grows better than the MmPheRS expressing strains. This suggested that the intrinsic defect may result from something independent of an aminoacylation defect, such as a tRNA-sequestering effect, where the expression of MmPheRS may decrease the availability of chargeable tRNA$^{\text{Phe}}$. It is also possible that the observed effects, or lack thereof, may also result from minimal or variable expression of the MmPheRS variants. We attempted to investigate this possibility by immunological detection and quantification of MmPheRS levels using anti-His antibodies, however no signal was detected in test or control samples even after extensive concentration of cell-free extracts.

Under \textit{m-Tyr} stress, all strains with editing-deficiency showed considerable growth defects compared to editing-capable EcPheRS (Fig. 4B). Our results also demonstrated that EcPheRS $\beta$G318W expressing strain grows better than empty vector and MmPheRS under \textit{m-Tyr} stress, corresponding to previous biochemical findings where EcPheRS $\beta$G318W deacylates mischarged tRNA$^{\text{Phe}}$ at a higher extent than MmPheRS [10]. The observed \textit{m-Tyr} dependent growth defect revealed that MmPheRS expression in \textit{E. coli} renders the cells susceptible to external \textit{m-Tyr}, supporting previous finding
where PheRS editing is crucial for cellular survival under $m$-Tyr stress. Apart from our expectation, no growth differences between our MmPheRS variants were observed, even for $\beta$Q306A, indicating that active site discrimination may be insufficient to generate growth advantages or overcome toxicity in our system where $m$-Tyr is in excess. As with the data described above (Fig. 4a), the possibility also exists that the observed effects are impacted by differences in the levels and/or stability of the MmPheRS variants.

Discussion

A hyper accurate active site may not be evolutionarily preferred in MmPheRS

In this study, we identified a hyper accurate mutation which allows MmPheRS to exclude non-cognate amino acids from its active site at the cost of lowering the affinity to Phe. This residue, L287 (F293 in *E. coli*), has never been reported to affect amino acid discrimination in organisms with existing PheRS crystal structure such as *E. coli* [25] and *T. thermophilus* [24]. Because this residue is not in close proximity with the substrates (Fig. 1B), it is likely that L287F alters the orientation of adjacent residues, resulting in an indirect change of the aa activation pocket. However, the possibility that *M. mobile* utilizes a different conformation for the Phe activation site cannot be ruled out. This hyper accurate enzyme variant also sheds lights on the evolution of the MmPheRS active site, as it is possible that high discrimination is not evolutionarily favored because the activation efficiency for cognate amino acid would be compromised. As a parasitic bacterium which depends on nutrients provided by its host for survival, *M. mobile* may opt for this error-prone PheRS as efficiency is more important than accuracy.

In addition to *M. mobile*, many intracellular or host-restricted organisms including both bacteria and eukaryotes were found with degenerated aaRSs, in which crucial residues or motifs in editing are mutated [32,33]. An extensive bioinformatic study examined the aaRS editing domains of 11,423 bacteria and found degenerated editing sites in all major bacterial phyla, primarily within host-restricted species [32]. Although the editing capacities of these aaRSs were not experimentally verified, these data suggested that genome reduction is associated with aaRS editing degeneration as...
the organism's lifestyle transitioned from free-living to host-dependent. It is worth-noting that erroneous protein synthesis is not merely a consequence of amino acid misactivation or failure in editing. Previous proteomic analysis of Microsporidia, a group of parasitic fungi with LeuRS editing-deficiency, showed that 5.9% of leucine codons were mistranslated with not only known LeuRS non-cognate substrates Met, Val, Nva and Ile, but also all other amino acids, suggesting other promiscuous machineries such as tRNA charging or ribosomal decoding exist in addition to aa activation errors [33].

The divergent translational machinery of Mycoplasma

Apart from the loss of aaRS editing activities, the translational machinery of *Mycoplasma* is largely different from other bacteria as a consequence of their reductive evolution, and half of the translational proteins were lost compared to model organisms such as *E. coli* and *B. subtilis* [34]. Proteins homologs for trans-editing factors such as DTD, Ybak, ProX and AlaX were also missing in *Mycoplasma* [34]. Furthermore, the ProRS in *Mycoplasma* is Eukaryote/Archaeon-like [35,36], which lacks the intrinsic editing domain, suggesting potential Pro codon specific mistranslation unless other unknown quality control mechanisms exist [37]. Previous phylogenetic studies of PheRS revealed the existence of a coding region consisting of partial PheRS β subunits (β1-β3) named pFRS [38,39]. Although partial β3 is included, the editing domain is not a part of the pFRS sequence, hence it is not a trans-editing factor. This putative protein was identified exclusively in Gram positive bacteria with low GC% and showed homology to EMAP II domains [38]. Since *M. mobile* is a G+ bacteria with only 25% GC, we performed a BLAST search to look for pFRS and identified the coding region (NCBI accession # WP_011265051). The EMAP II domain is presumed to have tRNA binding activity, and has been identified in proteins involved in tRNA interactions, including mammalian TyrRS [40], plant MetRS [41] and yeast Arc1p [42]. Interestingly, it was reported that Arc1p, when acting in a complex with MetRS and GluRS, facilitates the selection of cognate tRNAs, suggesting a positive role on translational fidelity regulation [43]. As the function of pFRS remains unclear, it may be worth investigating if similar mechanisms in coordinating protein synthesis accuracy also exist in *Mycoplasma*. 

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The effect of oxidative stress towards MmPheRS is still understudied

In this work, we revealed that MmPheRS does not exclude m-Tyr from its active site nor does it edit this non-canonical amino acid. Although external m-Tyr and p-Tyr supplementation did not result in M. mobile growth defects in Aluotto media, we demonstrated that this may be a consequence of using rich media as expressing MmPheRS in M9 renders the cells susceptible to m-Tyr treatment (Fig. 4). Since m-Tyr accumulates upon oxidative stress, it is likely that MmPheRS would also be subjected to reactive oxygen species (ROS) when intracellular m-Tyr concentration increases. Previous reports have shown that oxidized PheRS presents different activities compared to its unmodified counterpart. For example, when oxidized, the editing activity of Salmonella PheRS increased due to an overall conformational change [28]; While in yeast, the activity of mitochondrial PheRS is reversibly inactivated due to the formation of disulfide bond [44]. Although the host-pathogen relationship is not well characterized in M. mobile, as a pathogenic bacterium it may still be subjected to oxidative stress as a part of the host’s immune response. Hence, it may be informative to investigate the activation kinetics between oxidized MmPheRS and m-Tyr to provide a more complete picture of the effect of oxidative stress on MmPheRS discrimination and activity.

In conclusion, we characterized the activation site of the naturally editing-deficient MmPheRS and identified a previously unknown hyper accurate mutation. Our work also showed that MmPheRS lacks active site discrimination and editing to prevent m-Tyr from entering the translational machinery. Overall, novel features of MmPheRS have been revealed and more extensive studies are required to further understand the impact of oxidative stress on the translation integrity of M. mobile.

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Conflict of interest

The authors declare no conflict of interest.

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Data availability
Data supporting the findings in this work can be found in Table 1, Figures 1 to 4 and the supporting material.

Author contributions
Conceptualization, Methodology, and Formal Analysis N.H., A.K. and M.I.; Investigation and Data Curation, N.H. and A.K.; Writing—Original Draft Preparation, N.H.; Writing—Review & Editing, N.H., A.K. and M.I.; Supervision, M.I.; Project Administration, M.I.; Funding Acquisition, M.I.
1 Gomez MAR & Ibba M (2020) Aminoacyl-tRNA synthetases. RNA.

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Table 1. Amino acid activation kinetics of MmPheRS enzyme variants

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<tr>
<td></td>
<td>$K_M$ (µM)</td>
<td>$k_{cat}$ (s$^{-1}$)</td>
<td>$k_{cat}/K_M$</td>
<td>$K_M$ (µM)</td>
</tr>
<tr>
<td>WT</td>
<td>12 ± 1</td>
<td>6 ± 1</td>
<td>0.5</td>
<td>530 ± 120</td>
</tr>
<tr>
<td>D207E</td>
<td>44 ± 12</td>
<td>10 ± 1</td>
<td>0.23</td>
<td>310 ± 50</td>
</tr>
<tr>
<td>L287F</td>
<td>140 ± 30</td>
<td>4 ± 1</td>
<td>0.03</td>
<td>ND</td>
</tr>
<tr>
<td>W289F</td>
<td>12 ± 1</td>
<td>9 ± 1</td>
<td>0.75</td>
<td>440 ± 80</td>
</tr>
<tr>
<td>L287F/W289F</td>
<td>130 ± 30</td>
<td>12 ± 2</td>
<td>0.09</td>
<td>3800 ± 2</td>
</tr>
</tbody>
</table>

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Since $[S] \ll K_M$, $k_{cat}/K_M$ was determined by $V = [E][S] k_{cat}/K_M$.

\(^a\) No $p$-Tyr activation signals were detectable even at the highest $[S]$, hence the discrimination factor was estimated using the lowest detectable $k_{cat}/K_M$, which is $2 \times 10^{-5}$.  

\[ \begin{array}{|c|c|c|c|c|c|c|c|}
\hline
 & 97 \pm 19 & 14 \pm 1 & 0.15 & 1400 \pm & 3 \pm 0.4 & 0.004 & 300 \\
\hline
T166S & 230 & ND & ND & 0.003 \pm & 0.001^a & 42 & 45 \\
\hline
S267C & 30 \pm 9 & 14 \pm 1 & 0.46 & 710 \pm 110 & 4 \pm 0.4 & 0.01 & ND \\
\hline
 & 3 \pm 0.4 & ND & ND & 0.005 \pm & 0.001^a & 88 & 92 \\
\hline
\end{array} \]
Figure legend

**Figure 1. Identify residue replacements of MmPheRS active site**

(A) A representative image to show the results of our MSA, the complete alignment result can be found in Fig S1. *M. mobile* PheS sequence was used as reference in Jalview for easier data presentation, and the FAF motif is highlighted with arrows. (B) EcPheRS crystal structure with Phe and ATP (PDB: 3PCO) was analyzed using PyMOL and residues selected for this study are highlighted in magenta. *E. coli* numberings are shown in cyan while *M. mobile* numberings are shown in black.

**Figure 2. MmPheRS does not edit m-Tyr**

(A) ATP consumption assays were carried out to estimate the occurrence of ATP-futile cycling. 1 mM Phe and m-Tyr were used in the presence of 1 uM PheRS. *E. coli* PheRS was also examined as a positive control. For the experimental groups carried out with EcPheRS, *E. coli* tRNA^{Phe} was used, and *M. mobile* tRNA^{Phe} was used in the ones carried out with MmPheRS to ensure optimal interaction between the enzymes and tRNAs. At least three individual replicates were performed, and the results were analyzed using ImageJ before plotting by Prism. To allow better visualization, (B) shows the results in (A) minus the experimental condition of EcPheRS+tRNA+m-Tyr.

**Figure 3. Supplementing non-cognate amino acids in Aluotto media does not impair the growth of M. mobile**

*M. mobile* growth was monitored at 25 °C with 1mM designated external amino acids as described in the method section. The dilution factors for each spot were labeled in red. Phe supplementation was also carried out as a negative control. (A) Results for spot assays were recorded on day 12 and (B) liquid culture growth was monitored for over 14 days. Both assays showed no significant growth differences between each condition, showing that the supplementation of external m-Tyr and Tyr does not impair *M. mobile* growth in rich media.
Figure 4. MmPheRS expressing *E. coli* stains are susceptible to external *m*-Tyr
MmPheRS variants were expressed in the Phe-auxotrophic editing-deficient *pheT* (βG318W) backgrounds and the growth was monitored in M9 minimal media supplemented with constant concentration of Phe (3μM) with varied concentrations of *m*-Tyr. (A) No *m*-Tyr and (B) 360 μM *m*-Tyr supplementation in addition to Phe. Cells were incubated at 37°C and the growth was recorded every 30 minutes. Three biological replicates were conducted and the error bars represent the standard deviation between each data set.