MBU Institute of Microbiology
of the Czech Academy of Sciences

Opponent's Review of the Master's Thesis: Bc. Jan Říha (2024). tRNA Synthetases as Potential RNA Capping Enzymes.

The master's thesis by Bc. Jan Říha deals with the study of dinucleoside polyphosphates production by selected bacterial aminoacyl-tRNA synthetases and a potential role of these enzymes in non-canonical 5′ RNA cap synthesis. The proposed thesis is a continuation of projects currently under investigation in Dr. Cahová's laboratory in which novel 5′ mRNA caps have been identified. The project is original and ambitious, involving a wide portfolio of techniques and methods ranging from molecular biology and biochemistry to *in silico* modeling and prediction. This demonstrates the author's competence in navigating throughout the project with the aim of answering the scientific questions and coping with the arising challenges. The author prepared four expression plasmids and successfully overexpressed and purified aminoacyl-tRNA synthetases (LysU, LysS, MetG, HisS). Their ability to produce dinucleoside polyphosphates was successfully tested *in vitro*. Next, the author investigated the role of lysyl-tRNA synthetase (LysU) in the production of non-canonical 5′ RNA caps and reported that, contrary to published results, it is unable to do so. In addition, the author successfully used purified LysU for synthesis of diadenosine-$^{15}N_{10}$ tetraphosphate, containing stable nitrogen isotope (^{15}N), that can be used as a quantification standard in further studies.

Formal aspects

The thesis has 88 pages and a standard structure. It contains a total of 52 figures most of which were created by the author. These are mostly in a good resolution, clearly conveying the information. In Figures showing agarose and PAGE gels (Figures 24, 25, 27, 28, 38), however, a higher resolution and larger fonts would be desirable. Several figures are not referenced in the text raising a question about their presence in the work (Figures 2, 3, 6, 8, 26, 50). In addition, numbering of the gel lanes would greatly improve the readability of these pictures, as these are referred to in the text ("*…first line after standard…*"). In addition, although this may be a reviewer's preference, I would recommend the author to introduce the figure first in the text and then place it after the text (Figure 5, 6). In Figure 5, the reviewer appreciates the author's own figure, but it appears that it is based on someone else's work, which should be referenced in the legend (Yannay-Cohen *et al.* 2009).

The author cites 127 sources, mainly from the established scientific journals. In two cases (Figures 9, 13), however, the author refers to Wikimedia. In reviewers' opinion, this is not a good source of information. In the case of Figure 9, the reviewer is not sure what this structure represents and how it was created. Since the citation refers to the Wikimedia image, it is difficult to obtain additional information. Next, several statements seem to lack citations or the citations of sources that these statements originate from are further away in the text.

The reviewer would like to acknowledge that the thesis is written in English, which to the reviewer's knowledge is not the author's native language. The text is comprehensible but would benefit from further proofreading (prepositions, punctuation, syntax…, lines vs. lanes, columns vs. rows). When naming organisms that are mentioned for the first time (page 3, first paragraph *E. coli* and *S. venezuelae*), the name of the genus should be written in full and not as an abbreviation, e.g. *Escherichia coli*, *Streptomyces venezuelae*.

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Internal parts of the thesis

The section Introduction contains all the information required to understand the topic. However, the subchapters 4.2. Evolution of tRNA synthetases and 4.3. tRNA processing do not really fit into the flow of the text and the project. On page 19, beginning of the 4th paragraph, a 3D figure showing the catalytic domain(s) of tRNA synthetases would be helpful. I would prefer the section Aims to be longer and include a more detailed description of the What, How and Why questions of the master's project. But this is perhaps a personal preference and a local practice. The Methods section lacks information on plasmid preparation such as PCR amplification and ligation conditions and other details. Figures 24 and 25 lack information on whether the protein sample loads were comparable or how these samples were prepared. When overexpressing genes coding for tRNA synthetases (Figures 27 and 28), it is not clear which fractions were collected after HisTrap or size exclusion chromatography. Which fractions were pooled and used for the analyzes? How was the purity of the isolated proteins assessed? Was the activity of the enzymes tested in any way? In Figures 20-23, it looks like the 6xHis epitope is present at both ends of the expressed protein, is this correct? To further improve the thesis, the reviewer suggests including this information either in the Methods or in the figure legends.

The section Results comprises 24 pages and contains a respectable 35 figures. The reviewer's concern is the lack of controls in the experimental results shown in Figure 29 (page 48). 1) ATP hydrolyzes spontaneously in aqueous solutions. 2) As mentioned by the author, there are several enzymes besides LysU that can produce Ap_4A. When using 6xHis epitopes, it is not uncommon for other proteins to be co-purified. These could be present, albeit in smaller amounts, in the prepared LysU protein samples and thus be involved in Ap_4A production. **Q.** How would author improve this experiment? Suggest controls that could be used.

Similarly, there are also controls missing in the experiments in Figures 30, 32-36. There is a NC (Figure 30) that probably stands for Negative Control, but additional information is missing. In Figure 37, it is unclear whether the column chart is a representation of the chromatograms in Figures 30, 33-35. If this is the case, it should be explicitly noted in the legend. In case of a 1:1 ATP:GTP mixture (Figure 33), however, the sizes of the columns (Figure 37) do not correspond to the peak sizes in Figure 33.

Q. In the legend of Figure 40, the author states that the difference in the migration of the standards (lanes 1 and 2) could be due to the different ionic strength of the solutions used. This makes the interpretation of the results shown quite difficult. In addition, this issue seems not to be a problem in Figure 38 and Figures 42, 43. Was something done differently?

In the section Results: 6. Molecular dynamics simulation of ion binding to LysU (page 60), the paragraph starts with a rhetorical question: "*…why tRNA synthetases are not able to accept RNA as a substrate…*" the reviewer would like to argue that tRNA synthetases bind tRNA molecules very well. Perhaps this should be rephrased a little. The misunderstanding is, however, cleared up after further reading of this paragraph. To shed more light on possible RNA binding, I suggest analyzing the structures of tRNA synthetases bound with tRNAs. These complexes (tRNA synthetase with bound tRNA) could survive the isolation and purification steps. According to the presented model, the tRNA synthetase must bind the mRNA molecule to facilitate the transfer of AMP from the activated amino acid to 5′ triphospate at the 5′ end of the mRNA. If the tRNA synthetase is already bound to the tRNA molecule, the reaction cannot occur. Furthermore, it is possible that this scenario still allows the production of Ap_4A. **Q.** How would author test this hypothesis?

Q. The results shown in Figures 38-40, 42 and 43 indicate the presence of 5′ p_3RNA, which represents the majority of RNA molecules when incubated with LysU. However, only very small traces of p_3ApGp were found in the same set-up in the LC-MS results. Could author comment on these discrepancies?

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The discussion spans 12 pages, but on closer inspection the reviewer finds it repetitive and some parts fit more into the sections Introduction and Results. The reviewer would like to see the following topics discussed as they arise from the work:

Why was AlaS not transformed and thus removed from the analysis?

Q. In Figure 45 (page 58, right side of the panel), in p_3RNA+LysU experimental condition, low amount of p_3ApGp was observed, however, some larger signal is clearly visible on the chromatogram. It seems to be specific to the action of the tRNA synthetase LysU. Could it be an unexpected form of 5′ cap?

It is unclear whether the Ap_4A is an alarmone, toxic metabolite, an energy and purine storage or all the above. What is the relationship between the appearance of Ap_4A and non-conventional caps? Is it a side product, or does it have a physiological role? What is the author's opinion?

Q. In the text, author comments that the experimental conditions when testing the capping abilities of tRNA synthetase LysU were similar to those used by Luciano *et al.* (2019). However, he reached the opposite conclusions. According to the reviewer's quick search, there are several differences which unfortunately were not discussed.

Conclusion

I recommend the master's thesis by Bc. Jan Říha (2024). *tRNA Synthetases as Potential RNA Capping Enzymes* for dissertation defense.

In Prague, 24th of May 2024

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