

Jury Member Report – Doctor of Philosophy thesis.

Name of Candidate: Dmitrii Travin

PhD Program: Life Sciences

Title of Thesis: Phazolicin — a novel azole-modified peptide antibiotic: structure, mechanisms of action, transport, and biosynthesis

Supervisor: Professor Konstantin Severinov

Name of the Reviewer:

I confirm the absence of any conflict of interest (Alternatively, Reviewer can formulate a possible conflict)	Signature: Date: 08-08-2022
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The purpose of this report is to obtain an independent review from the members of PhD defense Jury before the thesis defense. The members of PhD defense Jury are asked to submit signed copy of the report at least 30 days prior the thesis defense. The Reviewers are asked to bring a copy of the completed report to the thesis defense and to discuss the contents of each report with each other before the thesis defense.

If the reviewers have any queries about the thesis which they wish to raise in advance, please contact the Chair of the Jury.

Reviewer's Report

Reviewers report should contain the following items:

- Brief evaluation of the thesis quality and overall structure of the dissertation.
- The relevance of the topic of dissertation work to its actual content
- The relevance of the methods used in the dissertation
- The scientific significance of the results obtained and their compliance with the international level and current state of the art
- The relevance of the obtained results to applications (if applicable)
- The quality of publications

The summary of issues to be addressed before/during the thesis defense

Ribosomally synthesized and posttranslationally modified peptides (RiPPs) are a large class of natural products with diverse structures and activities. Topic of this thesis is the genome-based identification of a new group of bacterial RiPP antibiotics based on genomic predictions and further characterization of biosynthetic, ecological, and pharmacological features.

After a short summary in Chapter 1, Chapter 2 presents the background of this thesis, explaining biosynthetic principles of RiPPs, examples of posttranslational modifications, with a special focus onazole-containing peptides and rhizobia in the later sections, as these topics are important for the thesis. Due to their vast biosynthetic diversity, a comprehensive overview of RiPPs would be out of the scope of this chapter, and the examples selected by Dmitrii are well-chosen and scholarly presented. This chapter is followed by Chapters 3 and 4 on goals and experimental methods.

Chapter 5 prepresents the first results section. Based on the previous discovery of the ribosome-targeting klebsazolicin antibiotics, a bioinformatic search was conducted for related biosynthetic gene clusters (BGCs). The sequence data suggested several BGCs encoding precursors distinct from the *klb* system, leading to the hypothesis that the RiPPs might exhibit a new mode of action. The *phz* (phazolicin) BGC from a *Rhizobium* sp. was selected for further study. Candidate compounds were detected in strain extracts by MALDI-MS, purified by HPLC, and characterized by MS. Antibiotic assays revealed phazolicins (PHZ) as narrow-spectrum antibiotics, and knockout of the gene *phzD* functionally linked the BGC to the peptides. Unequivocal biosynthetic proof was ultimately provided by heterologous expression in *E. coli*, resulting in completely processed klebsazolicins, nicely completing the discovery part.

To study the mechanism of action, Dmitrii used a previously developed reporter assay for translation inhibition, which suggested the compounds to interfere with protein biosynthesis. Further experiments including an *in-vivo* translation assay supported this mechanism. The mechanism was confirmed by structural biology studies. While crystallization experiments failed, Dmitrii was able to show binding of PHZ to the ribosome by cryo-EM and to identify the binding site, revealing some differences to KLB binding. Furthermore, structural and mutational studies suggested a rationale for the unusually narrow-spectrum activity of PHZ.

Chapter 6 focuses on the mechanisms resulting in PHZ transport into susceptible bacteria. Since approaches relying on spontaneous resistance development were unsuccessful, Dmitrii pursued a transposon-based strategy to identify resistance determinants, mapped by sequencing to two different genes implied in transport, both of which contained loss-of-function mutations in each strain. Based on these data, Dmitrii then showed in a broader analysis of homologous proteobacterial transporters that most were able to import PHZ. In addition, a role of the *S. meliloti* transporter in the uptake of the unrelated nonribosomal peptide antibiotic bleomycin was shown. These studies were further enriched by crystallographic experiments that showed the structure of the peptide-binding protein YejA, although attempts to obtain a co-crystal structure containing PHZ were not successful.

Chapter 7 presents an updated bioinformatic BGC analysis, revealing *phz*-type clusters in a wider range of bacteria. Preliminary experimental data on a *Mesorhizobium* strain supported production of an antibiotic derived from the cluster. To study ecological roles of PHZ, Dmitrii conducted competition and root infection experiments of the producer and competing bacteria. Unexpectedly, these revealed a lower competitiveness of the PHZ producer in nodule formation, and PHZ could not be detected in nodules by MALDI-MS.

In Chapter 8, a large-scale computational analysis provides general insights into linear azole-type BGCs. Five new groups are proposed, which sets the stage for targeted peptide discovery.

This is outstanding work in which Dmitrii addressed the thesis topic in a comprehensible fashion, using an unusually broad range of methods including bioinformatics, natural product discovery, bioassays, structural biology, and ecological studies. These provide detailed insights into the phazolicin pathway and mode of action and the broader potential of LAPs for genome mining and antibiotic discovery. The competition experiments did not provide clear information on the ecological function of phazolicin, but it should be pointed out that nodulation experiments are time-consuming and it can be challenging to conduct a larger number of repeats as a side project among an already large body of other work. The written thesis is clear, scientifically sound, nicely structured, and understandable also for non-experts. The work of this thesis generated an impressive publication output, with three of four papers published as a first author, a particular highlight being a *Nature Communications* paper on the mode of action of phazolicin. I have only a few minor suggestions for improvement of this excellent thesis:

1. I am not familiar with the thesis structure at this university. In case an overarching discussion at the end of the written thesis is necessary, it should be provided. Likewise, some of the work involves contributions by others. Is it customary to declare the specific contributions by the PhD candidate?
2. Please add spaces before units (T, V). Use x g instead of rpm or, e.g. "15000g". Some abbreviations are defined inconsistently (some are introduced in the text, others aren't).
3. "To confirm" (e.g.: p. 85, 87, 104) implies a bias in testing hypotheses. This should be rephrased.
4. p. 86: "unprecedented diversity of MoAs for LAPs": clarify unprecedented compared to what.
5. p. 89: typo in *Thermus thermophilus*; ...could not observe electron density **that**...
6. p. 94: PTZ is not a drug.
7. p. 108: change alfa to alpha

Provisional Recommendation

I recommend that the candidate should defend the thesis by means of a formal thesis defense

I recommend that the candidate should defend the thesis by means of a formal thesis defense only after appropriate changes would be introduced in candidate's thesis according to the recommendations of the present report

The thesis is not acceptable and I recommend that the candidate be exempt from the formal thesis defense