Skoltech
Skolkovo Institute of Science and Technology

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: Professor Sylvie Rebuffat

I confirm the absence of any conflict of interest (Alternatively, Reviewer can formulate a possible conflict)	**Signature:** **Date: 28-07-2022**

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

Dmitrii Travin's PhD thesis sets in the context of nitrogen-fixing symbiosis established by leguminous plants with rhizobium bacteria. This symbiotic system has a major role in the nitrogen cycle, and nitrogen fixation by rhizobia in symbiosis with legumes is economically and ecologically important.

D. Travin's study aims at deciphering the molecules and molecular mechanisms involved in the complex symbiont/host relationships established by bacteria of the genus *Sinorhizobium* which develop root nodules in leguminous plants of the *Medicago* genus (Fabaceae).

D. Travin identified a biosynthetic gene cluster (BGC) coding for a novel "linear azol(e)ine containing peptide" (LAP) named phazolicin (PHZ) in the genome of *Rizobium* sp. Pop5 by genome mining. PHZ exhibits an antirhizobial activity. The LAP structure and its translation-inhibiting mechanism of action were determined. The two transporters involved in PHZ uptake into susceptible cells were identified. Perspectives are opened thanks to the identification of PHZ homologues produced by other rhizobia and more largely in many other classes of bacteria, which opens roads for both understanding the symbiosis system and discovering novel families of LAPs, thus expanding their chemical diversity and pharmacological potential.

Context of the PhD work.

Dmitrii's PhD work takes place in the two highly competitive scientific domains of symbiosis and ribosomally synthesized and posttranslationally modified peptides (RiPPs).

LAPs belong to the wide family of the RiPPs natural products, which arise from modifications of ribosomally synthesized peptide precursors by dedicated enzymes encoded in the RiPP BGCs. Despite numerous efforts and advances recently acquired in recent years, deciphering the mechanisms explaining the biosynthetic pathways and mechanisms of action of RiPPs still constitutes an international challenge. Moreover, their roles in various physiological and ecological functions remains poorly understood.

Symbioses are essential for eukaryotic life. Nitrogen-fixing symbiosis, which involves leguminous plants and proteobacteria collectively called Rhizobia is of major ecological importance. It occurs on all continents and accounts for a fourth of the nitrogen fixed annually on earth. Rhizobia can install symbiotic interactions with legumes via the formation of nodules, where they live intracellularly as bacteroids, a form that can reduce atmospheric nitrogen via a complex process called biological nitrogen fixation (BNF) involving several enzymes and cofactors. By this nutritional symbiosis process, the plant receives ammonium and the bacterium benefits from the nutrients and sources of energy and carbon from the plant. A complex cross-talk involving a panel of molecules from the *Rhizobium* and the plant establishes between the two partners. The terminal differentiation of bacteria to the bacteroid state is triggered by a family of molecules produced by the symbiotic nodule cells called nodule specific cysteine rich peptides (NCRs). NCRs share structural characteristics with antimicrobial peptides (AMPs) and some can kill bacteria and in some cases fungi. To withstand the NCRs, the rhizobia use different tactics and in particular peptide transporters that expell the peptides or exopolysaccharides to protect the rhizobia. In the *Sinorhizobium meliloti/Medicago* plant model selected here, the symbiont uses a complex multifactorial strategy involving a transporter, the lipopolysaccharide outer membrane and a stress response regulator.

Evaluation of the thesis quality and the overall structure of the dissertation.

The PhD manuscript (174 pages) is organized into three main sections that follow a brief introduction on the search for natural products: Literature review (Chapter 2), Materials and Methods (Chapter 4), Results and discussion (Chapters 5 to 8). It is completed by a description of the thesis objectives (Chapter 3) and a list of references.

The Literature review (50 pages, 135 references) provides a comprehensive description of LAPs in the context of RiPPs, and of rhizobia symbiosis. It is well documented, including up to date literature. It is clear and concise and gets to the point.

The Materials and Methods section (20 pages) provides a detailed description of the different complementary methods used, including bioinformatics methods, molecular biology protocols (cloning, construction of a transposon library, selection of mutants, genome sequencing…), peptide/protein purification procedures, mass spectrometry analysis conditions (MALDI-TOF, HR-MS and MS/MS), bacteriology methods including the diverse *in vitro* and *in vivo* assays to probe PHZ bioactivity (antibacterial assays and MIC determination, competition experiments, soil assays, nodulation assays and analysis of nodules), cryo-EM measurements for studying the PHZ/ribosome interaction.

The Results and discussion section (Chapters 5 to 8, about 90 pages) affords a very complete picture of phazolicin (PHZ), the novel LAP produced by *Rhizobium* sp. strain Pop5, and opens important perspectives.

Chapter 5 is a major piece of work. It describes the discovery and characterization of PHZ, from the genome-mining guided discovery of its BGC, to its purification procedure from the complex mixture of different forms obtained in the cultivation broth, and complete structure determination by MS/MS analyses. PHZ was identified as a 27 amino acid peptide including eight azole rings resulting from posttranslational modifications. It also affords an unambiguous correlation between the LAP product and the BGC and a description of the PHZ narrow spectrum antimicrobial properties, strictly directed against related *Rhizobium* species. Finally, PHZ was shown to act as an inhibitor of translation, and the detailed mechanism of action was deeply studied and deciphered. The azole cycles of PHZ are essential for binding to the 23SrRNA. The cryo-EM structure of the PHZ-bound *E. coli* 70S ribosome complex allowed identifying the PHZ location in the ribosome, in the tunnel formed by loops uL4-uL22. It showed that similar to klebsazolicin (another LAP produced by *Klebsiella pneumoniae*), PHZ inhibits the elongation step of prokaryotic translation through the obstruction of the nascent peptide exit tunnel (NPET) of the large ribosomal subunit. But, PHZ binds in a significantly different mode compared to klebsazolicin, which involves especially several cationic amino acid side chains. Finally, PHZ specificity was shown to depend on the presence of specific amino acids in the ribosome uL4 loop, which distinguishes it from many other cases where specificity relies only on the import systems.

Chapter 6 completes the mechanistic information on PHZ provided in Chapter 5 by studying the transporters involved in import of PHZ into susceptible bacteria. Two very different types of transporters were shown to be required for PHZ uptake into its rhizobia targets. The first one that is located at the outer membrane (BacA) permits transport from the external environment to the periplasmic space. The other one is anchored at the inner membrane (YejABEF) and ensures internalization into the cytoplasm. YejABEF is an ABC transporter. BacA is a novel type of transporter recently described (SbmA-like peptide transporter; SLiPT) that resembles ABC

transporters but uses the proton motive force rather than hydrolysis of ATP to gain energy. Both BacA and YejABEF transporters are very promiscuous. Bac A also transports NCR peptides. YejABEF is also used for uptake of other AMPs and in particular of another RiPP, the peptide nucleotide microcin C, and contributes to NCR uptake. Both transporters can internalize bleomycin, another thiazole-containing but unrelated antibiotic, and conversely homologues of BacA and YejABEF can ensure uptake of PHZ. This lack of specificity is in contrast with other RiPP uptake systems (such as iron-siderophore receptors), which show strict specificity and explain the restricted spectrum of activity of the imported antimicrobial compounds. Interestingly, the requirement of two transporters for PHZ internalization seriously decreases the level of acquired resistance of target bacteria to PHZ. Unfortunately, the X-ray structure of the PHZ /YejA complex could not be obtained due to multiple unspecific associations with small peptides.
Taken together, the results from Chapters 5 and 6 point that the high PHZ specificity to *Rhizobium* susceptible bacteria tightly related to the producer *Rhizobium* sp. Pop5 only relies on the structure of the target (the ribosome), and that the uptake machinery does not serve as a filter to select bacteria susceptible to PHZ.

A genome mining approach in genomes of plant-associated bacteria and a preliminary study of the ecological role played by PHZ in competition against other rhizobia is provided in Chapter 7. It shows that other LAPs could be produced by geographically distant bacteria mainly isolated from root nodules or plant material over the world. PHZ production can eliminate a PHZ-sensitive strain in co-cultivation, suggesting a role in competition with other rhizobia for nodulation, but this result could not be reproduced in field-like conditions, such as in soil samples or experimentally obtained nodules, pointing the requirement for improving such approaches.

Finally, a systematic genome mining search in publicly available bacterial genomes was used in Chapter 8 to identify novel BGCs encoding putative LAPs. It allowed predicting groups of novel LAPs in many other classes of bacteria (Actinobacteria, Firmicutes, lactic acid bacteria, Pseudomonadaceae, Flavobacteriaceae). This chapter opens doors to explore and increase the chemical and biological diversity of LAPs.

The manuscript is closed by a brief conclusion, which clearly points the major results of the study and advances that emerge, but is particularly brief. Moreover, although numerous perspectives are predictable, a perspective section is lacking.

Global evaluation of the manuscript. Overall, despite this very short conclusion, the manuscript is well organized and clearly and concisely written in a fluent style. English standards are mostly respected throughout the manuscript. The use of litterature is appropriate. The pictures and schemes shed light on the main text and the legends are clear and include sufficient details. The Appendix section provides complementary information on the strains, vectors and oligonucleotides used, on cryo-EM and crystallographic data, and on characteristics of experimentally validated BGCs for LAPs in various bacteria.

Quality of the publications. Dmitrii Travin is co-author of two original papers describing - the structure and mechanism of action of phazolicin (D. Y. Travin et al. 2019, *Nat Commun* 10(1): 4563; doi: 10.1038/s41467-019-12589-5; IF 14.919; for this paper, D. Travin is first author of twelve) and - the involvement of a cascade of events allowing the symbiont *S. meliloti* residing inside *Medicago* cells and ensuring its functions (Q. Nicoud et al. 2021, *MBio*, 12(4): e00895-21; doi: 10.1128/mBio.00895-21; IF 7.867; D Travin is 5th author of sixteen).
Both original publications are of high quality.

D. Travin is also first author of two review papers (D. Travin et al. Front Genet. 2020, 11: 226, doi: 10.3389/fgene.2020.00226, IF 4.772; D. Y Travin et al. RSC Chem Biol. 2021, 2(2): 468-485, doi: 10.1039/d0cb00208a, IF 2.7) that describe (i) RiPPs acting as aminoacyl-tRNA synthetase inhibitors and (ii) LAPs targetting the ribosome to inhibit susceptible bacteria.
He also presented two communications at international conferences (1st international RiPP conference, Granada, Spain, 2019; 12th International Multiconference Bioinformatics of Genome Regulation and Structure/Systems Biology, Novosibirsk, Russia, 2020).
In addition to these papers directly connected to his PhD work, D. Travin is also co-author of three other papers describing the biosynthesis pathways and mechanisms of action of other related LAPs, klebsazolicin (Nat Chem Biol 2017, 13(10), 1129-1136; J Am Chem Soc 2018, 140(16), 5625-5633) and microcin B17 (Mol Cell 2019, 73(4), 749-765).

Globally, the quality of the published publications to which D. Travin contributed can be considered as excellent.
The currently unpublished results presented in the manuscript should lead to another article.

Relevance of the topic of dissertation work to its actual content. Relevance of the methods used in the dissertation. Scientific significance of the results and compliance with the international level and current state of the art.

Overall, the study is self-consistent and fully original. The data are of excellent scientific value. Complementary and important results have been successfully obtained using appropriate methods. They provide an impressive trajectory describing the characteristics, mechanisms and properties of a rhizobial LAP by using state-of-the art methods from genome mining to up-to-date analytical methods, molecular biology and bacterial genetics, to biological assays.
The results are well analyzed and clearly described in a sufficient level of details. The resulting conclusions are well argumented and discussed appropriately. They afford both fundamental advances and perspectives for a promising use of LAPs for different application domains.
Globally, the study affords advances as regard the current state of the art in both the domains of RiPPs and symbiosis.

As a conclusion, I consider that Dmitrii Travin's PhD constitutes an original piece of work in the field of bioorganic chemistry and chemistry of natural products that combines complementary bioinformatics, microbiology and biochemistry approaches. It affords novel knowledge and opens up new opportunities for RiPP bioengineering and symbiosis understanding.
The results are of high international level and in my view, the PhD manuscript presented by Dmitrii Travin fully meets the criteria required for defending his work in a formal thesis defence at Skoltech.

Summary of issues to be addressed before/during the thesis defense.

The PhD work affords an important number of solid conclusions, but also raises an important number of questions for future studies that will be debated at the oral presentation. Questions will also allow going deeper in different aspects covered by the PhD work, and help confirm the good evaluation based on the manuscript. Moreover, it will be the occasion to clarify which parts of the study have been designed by D. Travin and rely on his own and personal work and what experiments have been done by other people in the laboratory or in collaboration (bioinformatic searches, peptide purification and characterization, spectroscopic analyses (MS, cryo-EM)). This

will allow identifying the original and personal contribution of the candidate, who appears to have done a major part of the work, based on the manuscript.

Minor points or typos have to be corrected in the manuscript, as detailed below.
- Page 27: Figure 2.1.2, the α and β carbons should be pointed on the figure to better show the difference between lanthipeptides and sactipeptides.
- Page 28: Figure 2.1.3 and its legend: - examples of tailoring modifications in the group of lasso peptides could be interestingly added as supplementary examples (such as phosphorylation at a C-terminal Ser in paeninodin, Arg deimination in citrulassin, or acetylation at a Lys side chain in albusnodin).
- Pages 28-38, legends to Figures 2.1.3 to 2.1.7: literature reference numbers for the diiferent RiPPs cited as examples (microviridin J, plantazolicin, azolemycin, YM-216391, GE2270, …) should be added in the legends.
- Page 101, line 8-9: the sentence "such molecules often rely on the activity of non-specific peptide transporters for internalization" has to be qualified, as certain RiPPs use highly specific transporters (such as iron-siderophore transporters), while others use for instance porins which are unspecific.
- Page 127, legend to Figure 7.2.2 line 4, change "Residues converted into azoles in PHZ and those matching them are blue, positively charged residues are red" to "Residues converted into azoles in PHZ and those matching them are red, positively charged residues are blue".
Typing mistakes - Page 23 line 6: "unites"? - Page 28, legend to figure 2.1.3 change "oxyme" to "oxime", change "dehydrobutirine" to "dehydrobutyrine"; - Page 29, legend to Figure 2.1.3, change "cynnamicin" to "cinnamycin"; Page 38 legend to Figure 2.1.7 change "adopted" to "adapted"; Page 101, change "one or two membranes…, respectively" to "one and two membranes…, respectively".

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*