Skoltech

Skolkovo Institute of Science and Technology

Jury Member Report – Doctor of Philosophy thesis.

Name of Candidate: Tatyana Zyubko

PhD Program: Life Sciences

Title of Thesis: Efficient in vivo Synthesis of Lasso Peptide Pseudomycoidin Proceeds in the Absence of Leader and Leader Peptidase

Supervisor: Prof. Konstantin Severinov

Date of Thesis Defense: 19 December 2019

Name of the Reviewer: Prof Sylvie Rebuffat

I confirm the absence of any conflict of interest (Alternatively, Reviewer can formulate a possible conflict)	**Signature:**  **Date: 18 November 2019**

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

Tatiana Zyubko's PhD thesis aims at deciphering the molecular mechanisms of biosynthesis of a class of ribosomally synthesized and posttranslationally modified peptides (RiPPs) of bacterial origin termed lasso peptides. More specifically, T. Zyubko's studied the structure and biosynthesis pathway of a novel lasso peptide called pseudomycoidin, from the Firmicutes *Bacillus pseudomycoides*. This lasso peptide carries extra posttranslational modification in addition to the formation of an isopeptide bond and presumably acquisition of the lasso topology. T. Zyubko used complementary methods (bioinformatics, microbiology, molecular biology, heterologous expression, peptide purification and peptide chemistry) to decipher the structure of this lasso peptide. It was shown that pseudomycoidin is polyphosphorylated at the C-terminal serine and further glycosylated. Its stability (to temperature and peptidases), the different steps of its biosynthesis, as well as the roles of the leader region of the precursor and the different modification enzymes involved have been studied.

Lasso peptides are a family of RiPPs of 14-21 residues produced by different bacterial phyla, essentially Proteobacteria (Enterobacteriaceae), Actinobacteria, and a few Firmicutes. They are characterized by a unique and highly stable topology where a ring is formed via an isopeptide bond established between the amino group at the N-terminus and the carboxylate of an aspartic or glutamic side chain at position 7 to 9: the ring is threaded by the tail and locked inside via steric hindrance from bulky amino acid side chains located below and above the ring, or didulfide bonds, or both. Some lasso peptides exhibit potent antibacterial properties.

This PhD study takes place in the context of resistance of bacteria to conventional antibiotics and the potential of antibacterial peptides and RiPPs as alternatives to antibiotics. The specific lasso topology cannot be obtain by conventional peptide synthesis methods and requires the bacterial enzymes. Therefore deciphering the biosynthetic pathways for lasso peptides remains an international challenge despite numerous efforts and advances recently acquired. Tatiana Zyubko's PhD takes place in this highly competitive field.

Brief evaluation of the thesis quality and the overall structure of the dissertation. The PhD manuscript (115 pages) is classically organized into three sections, Literature review, Materials and Methods, Results and discussion and a list of references.

The Literature review (30 pages, 183 references) is well documented, including up to date literature and describes the world and issues of lasso peptides in a clear and comprehensive fashion. It addresses successively the characteristics of lasso peptides, the present knowledge on their biosynthesis, the genome mining approaches for identifying lasso peptide gene clusters in bacterial genomes and ends with a brief overview of their biological properties and the perspectives offered for bioengineering.

The Materials and Methods section (7 pages) provides a brief description of the different biochemical protocols used and the mass spectrometry analysis conditions.

The Results and discussion section (34 pages) describes successively (i) evidence for heterologous production of pseudomycoidin in *E. coli*; (ii) roles of the different genes encoding the tailoring modification enzymes (PsmK, PsmN) and the structure of the tailoring modification (a triphosphorylated undefined hexose, with phosphate anchored at the C-terminal serine and the hexose onto the phosphate group); (iii) roles of the genes encoding the proteins/enzymes involved in acquisition of the lasso topology (PsmB1/ RRE, PsmB2/protease, PsmC/lasso cyclase); (iv) analysis of the lasso/branched-cyclic topology of

pseudomycoidin and variants resulting from deletion of different genes in the cluster or from point mutations; (v) analysis of antibacterial activity. The manuscript is closed by a brief conclusion.

The manuscript is clearly written and English standards are mostly respected. The use of litterature is mostly appropriate. The pictures and schemes shed light on the main text and in most cases legends are precise and clear. The supplementary information section provides complementary information on the constructs used.

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 original. Complementary and important results have been successfully obtained using appropriate methods. Those include both fundamental advances and the proof of concept of a promising use of the lasso peptide biosynthesis system, and specifically of the simplified pseudomycoidin gene cluster, for the design of bioactive peptides. Pseudomycoidin is the second lasso peptide discovered from the phylum of bacteria Firmicutes. The candidate shows it shares high similarity of the gene cluster organization and of the leader region in the precursor and identity of the leader-core junction sequence with the first one, paeninodin, which is produced by *Paenibacillus polymixa*. Moreover it is proposed using this lasso peptide system that a lasso peptide, which was found to be in a branched-cyclic form, can be produced from its precursor in the absence of the leader peptide and processing by the leader peptidase, suggesting that an endogeneous generalist protease can ensure this step of processing. This tempers the assumption currently admitted in the RiPP international scientific community that the leader peptide is required in the biosynthesis of RiPPs. This will be interestingly discussed during the PhD defence.

The data are of good scientific value and are well analyzed and globally described in a clear fashion. The resulting conclusions are well argumented and discussed pertinently and afford advances as regard the current state of the art. Questions at the oral presentation will allow going deeper in the different aspects covered by the PhD work, bioinformatics, enzyme biochemistry, RiPP bioengineering, structural analysis, 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 and rely on her own and personal work and what experiments have been done by other people in the laboratory or in the context of a collaboration (purification and characterization of peptides, bioinformatic searches, spectroscopic analysis (MS and NMR)). This will allow identifying the original and personal contribution of the candidate, who appears to have done a great amount of work, based on the manuscript.

Quality of publications. T. Zyubko is co-author of two papers (T. Zyubko et al. 2019, Chem Sci doi: 10.1039/C9SC02370D (IF 9.556); I. Zukher et al. 2019, MBio, 10(2): pii: e00768-19) and is first author for the first one. Both publications are of high quality. The first one describes the results presented in the PhD manuscript. The second paper concerns the biosynthesis pathway of another RiPP family (nucleotide peptides). It describes the role of the length of the precursor on the antibacterial peptide production and activity and shows that increasing the precursor length perturbs different steps of the biosynthesis and results in a production decrease.

As a conclusion, Tatiana Zyubko's PhD can be considered an original piece of work in the field of bioorganic chemistry and chemistry of natural products, combining complementary bioinformatics, microbiology and biochemistry methods, which both affords novel knowledge and opens perspectives for lasso peptide bioengineering.

To my opinion, the PhD manuscript presented by Tatiana Zyubko meets the criteria required for defending her work in a formal thesis defence at Skoltech.

Minor points have to be corrected in the manuscript.

Modifications.

- Figure 1: RiPP biosynthesis uses in many cases bifunctional ABC transporters called PCAT (Peptidase Containing ATP-binding Transporters) or AMS (ABC transporter Maturation Secretion), which ensure both proteolysis (protease) and export (ABC exporter) (see the review Beis et al 2019, Res Microbiol and reference herein). This has to be mentioned and the figure modified accordingly.
- Throughout the manuscript (p. 11, p. 15, p. 23, 24, etc) "small" is used to qualify peptides or peptide precursors of 20 to 50 amino acids, while a peptide considered as "small" contains less than 10 amino acids. This should be corrected. Similar page 16, the description of lasso peptides has not to insist on their "small" size (they contain 14 to 26 amino acids...).
- Throughout the manuscript change "express" to "produce" or "synthesized" when required: "expression" is incorrect for any compound – genes are expressed, whereas compounds are either modified from peptides or synthesized by enzymes and thus "produced" by bacteria and other organisms.
- Table 1 page 19 is a very good point in the chapter as it aims at assembling all lasso peptides discovered and identified until now. To improve it, appropriate references for each peptide have to be included in the Table. In addition some recent lasso peptides, which have been evidenced to adopt a lasso fold have not been included, for instance: subterisin (Kuroha 2017, Tetrahedron Lett 58, 3429-32); specialicin (Kaweewan 2019, Bioorg Med Chem, 26, 6050-55); leepeptin (Gomez-Erscribano 2019, Appl Environ Microbiol doi: 10.1128/AEM.01752-19). Litterature in the domain has to be carefully checked and the missing peptides have to be added.
- Figure 7 page 22 shows a very heterogeneous representation of lasso peptides (color/no color, amino acid numbering/no numbering; moreover the figure seems to show that some lasso peptides adopt a lasso topology (siamycin, paeninodin), while others would be in the branched-cyclic topology, while this has not been assessed (citrulassin which is known to be in the lasso fold and lassomycin which has been shown to adopt a non-lasso fold are represented similarly). This figure has to be modified and clarified and the lasso or cyclic-branched topology has to be specified for the different peptides.
- The paragraph on lasso peptide chemical synthesis, which describes the chemistry of the tentative chemical synthesis of lasso peptides published, is included in the section describing the perspectives opened by using the lasso peptide scaffold for creating novel bioactive peptides (section 1.7 Lasso peptides as an efficient scaffold for molecular grafting). This chemical approach should be mentioned page 23 as a short section entitled "Chemical synthesis of lasso peptides" before section "1.3 Enzymatic biosynthesis of lasso peptides", and only a brief reminder included in section 1.7.
- Page 37 last line, legend to figure 29 page 71: change "rotaxane" to "[1]rotaxane"; page 71 line 5, page 73 lines 5 and 9, specify which type of "rotaxane", [1]rotaxane or "[2]rotaxane", is concerned; (the lasso

topology is a [1]rotaxane and the branched-cyclic non lasso topology where a tail fragment remains blocked inside without covalent bond is a [2]rotaxane); thus specifying which type of rotaxane is involved is essential.

- Page 50 two last lines: m/z values (here MH^+) should not be expressed in Da; this has to be corrected.
- Page 51 third paragraph, the sentence describing the presence of m/z ions typical of phosphorylated pseudomycoidin species is unclear and has to be reworded.
- Page 53 first line and legend to figure 16, the amino acid sequence AGPGTSTPD is not found in pseudomycoidin but in paeninodin. Change to the correct amino acid sequence.
- Pages 78-79: it is surprising that the brief NMR analysis of natural pseudomycoidin and its L21F variant does not describe the typical NOE correlations that should occur between amino acids from the ring and the loop that unambiguously assign a lasso topology. Even if a complete three-dimensional structure analysis of the two pseudomycoidins is not included in the scope of the PhD, this specific point should be added or discussed, as it is critical and the only information discussed in the manuscript only allows to confirm the presence of the macrolactam ring closed between A1 and D9 side-chain. In addition enlargements of the important regions in the TOCSY/NOESY 2D spectra should be added.
- Although the list of references is appropriate, up to date references and particularly reviews published recently on lasso peptides and associated domains, and showing the field is highly competitive and raises high interest, have to be included (on lasso peptides: Hegemann J, ChemBiochem 2019, Tan S, Antibiotics 2019; Cheung-Lee WL, J Industrial Microbiol Biotechnol 2019; on transporters: Beis K, Res Microbiol 2019).
- The list of references is very badly presented. It has to be checked and corrected carefully as it contains a lot of mistakes: page numbering are lacking (for instance [56, 68, 97, 98, 104, 105, 112, 117... and many others]), or are wrong (for instance [72, 85,...]), author names (or part of author names) are lacking (for instance [2, 65, 116 and many others]), names of the journals are lacking (for instance [40, 47, 83, 97,... and many others]); in many references, genus and species names of bacteria and other organisms have to be in italics; papers cannot be "in press if published before 2019 (for instance [49]); some references are in full capitals ([150]. In addition, all author names or at least four or five and not only the first one have to be cited in some references (for instance [60]).
- In supplementary materials, it is required to provide titles to the tables.
- Quality of certain figures has to be improved, particularly Fig 16 and 19 where m/z values are difficult to read, or Fig 20B.

Minor points or typo

- When abbreviations are used they should be defined the first time they appear and then used throughout the manuscript (ex: microcin J25 p. 25, which has been abbreviated since page 15)
- Figure 1: change "cianobactin" to "cyanobactin"
- Throughout the manuscript, do not use capital letter for the the names of peptides (such as p. 13: lantipeptides, page 19 all cited laso peptides, page 25 microcin, etc)
- Page 15 in the subtitle, change "MccC" to "McC", as MccC is not the microcin C but one of the modification enzymes
- Page 16, four lines before the end reword the sentence; - Page 29, add the greek letter required.
- Page 33 line 10 change "by an ATP-binding cassette (ABC) transporters" to "by ATP-binding cassette (ABC) transporters"; - Page 51 change "phosphate residue" to "phosphate group".

☒ *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*