Oponentský posudek doktorské disertační práce

Uchazeč: **Vitali Bialevich, MSc.**

Název práce: **Domain conformations of the motor subunit of EcoR124I involved in ATPase activity and dsDNA translocation**

The PhD dissertation thesis of Vitali Bialevich focuses on biochemical and physico-chemical characterization of the motor subunit of bacterial type I restriction-modification enzyme EcoR124I. This complex enzyme is interesting as it shows double-edged function in a bacteria cell; it either stabilizes hemi-methylated DNA *via* its methyl-transferase activity, or it cleaves unmethylated dsDNA using its restriction activity.

Candidate employed wide range of experimental techniques in order to obtain new information on molecular mechanism of EcoR124I dsDNA cleavage activity. Besides the conventional methods of biochemistry and molecular biology, he employed numerous enzyme kinetics studies, side-directed mutagenesis and crystallographic analysis to propose dsDNA translocation mechanism, ATPase and endonuclease activity of the enzyme complex.

The thesis is written using classical form and structure, consists of 110 pages of the text without supplements and contain very small amount of typographical and grammatical errors. I appreciate that the thesis introduction contains an exhausting and well arranged review of information relevant on the topic as this helps reader to familiarize with the complex problematic. I have not found Figure number 9, but as there is no reference to it in the text, I believe it is just a skip in the figure numbering.

Results bring new information on the prokaryotic type I restriction-modification systems. For instance, describing very extensive investigation of an important inter-domain protein-protein contact between helical-helicase 2 domains or ATP-dependent translocase activity of the motor subunit. The candidate successfully conducted crystallization experiments obtaining crystals of the functional mutant of the motor subunit. The thesis also benefit from theoretical studies represented by structural analysis and molecular dynamics simulations.

The major portion of the presented data was published as three original articles in respected journals with overall impact factor of 7.584. The remaining results were prepared for publication and are shown as a manuscript. I fully recommend the PhD thesis of Vitali Bialevich for being awarded the PhD degree.

Questions for the defense that should be addressed by the candidate:

1. Is it possible to speculate on mechanistic details how does the enzyme sense the DNA methylation and "decide" whether it will methylate or translocate/cleaves?
2. You used ammonium sulfate precipitation in combination with ion-exchange chromatography for isolation of the pure enzymes after their heterologous expression. Why frequently employed immobilized metal ion affinity chromatography was not employed?
3. Transient protein-protein contacts are frequently based on electrostatic interactions. The discussed inter-domain interactions at the helical-helicase 2 domain interface in the HsdR subunit of EcoR124I restriction-modification complex might also benefit from Coulombic attraction. Would it be possible to present the visualization of the mentioned interfaced as shown in Figure 17, but indicating electrostatic potentials in vicinity of the contact, that are projected on the isolated interacting domains? I mean something like e.g. in Fig. 5 in work of Jeřábek P. et al *Biochemistry* **2014**, *53*, 6695.

In Prague, 10. Feb 2017

Doc. RNDr. Václav Martínek, Ph.D.
Department of Biochemistry
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OPONENTSKÝ POSUDOK

doktorskej dizertačnej práce

(ďalej v anglickom jazyku)

Title:	**Domain conformations of the motor subunit of EcoR124I involved in ATPase activity and dsDNA translocation**
Author (PhD defender):	Vitali Bialevich, MSc.
Supervisor:	Prof. RNDr. Rüdiger Ettrich Ph.D.
Place:	University of South Bohemia, Faculty of Science, České Budějovice
Date of defense:	February, 2017
Opponent:	Dr. Ivana Nemčovičová, Ph.D.

The thesis of Vitali Bialevich entitled *'Domain conformations of the motor subunit of EcoR124I involved in ATPase activity and dsDNA translocation'* is based on various mostly experimental methods. The molecular modeling and dynamics simulations studies are also present but are carried out in close collaboration with experimentalists, allowing for a constant scrutiny of models and hypothesis and providing continuous input from experiments. The present work demonstrates a previously unsuspected role for the helical C-terminal domain of the HsdR motor subunit in DNA translocation, cleavage, and ATPase activities. This domain has been assumed to be involved in assembly with the HsdS/M methylase. A role involving all three of the subunit's activities implicates the C-terminal domain in controlling enzymatic function. Thus a controlling function resides at a nexus of intersubunit communication. Because the observed interdomain interactions contribute to enzymatic function as described in this work, they may be conserved also in other Type 1 restriction-modification systems in which single HsdR subunits have multiple functions.

Vitali's efforts were focused on studies of structural elements of this HsdR subunits by using site-directed mutagenesis, various ATPase and restriction activity assays with combination of *in vitro* and *in vivo* characterization of tested mutant enzymes involving also MTases and by which Vitali proposed additional function in communicating a signal across this enzyme. Therefore, I really appreciate Vitali's work on the domain conformations of the HsdR motor subunit that was probed experimentally (both *in vitro* and *in vivo*) and nicely confronted with computational predictions.

The thesis is based on three published papers (records from PubMed). The PeerJ paper (IF=2.181) that Vitali authored as a joint first author, therefore contributed the most to both experimental and writing tasks; then the Plos ONE paper (IF=3.234) that he participated in minor on performing experiments and partially on data analysis; and the paper in Journal of Molecular Modeling (IF=1.867) that he appeared on the fifth place (individual contributions

are not reported in the final paper). The forth paper was also listed in the thesis, but to my knowledge was not published yet. Those three papers I therefore consider as a core of his PhD work. The papers published by the candidate gained to date only 3 citation records, which is not remarkable yet due to its freshness. However, I would say there is a reasonable potential that papers will be cited well based on previous work published from the research group. Therefore, the topics of this PhD thesis is obviously actual with international recognition.
The thesis has all together 111 pages divided into main 5 chapters and the supplementary material. The individual chapters are quite well organized and the two main chapters *‚Materials and Methods'* and *‚Results and Discussion'* are also well distributed. The text itself contains a reasonable number of typing and spelling errors (e.g. p2, p55, p87..) and candidate is probably aware of it. The description of the methods in chapter 2 (e.g. p33-38) could be polished more in respect to details and language. Some citations listed are not the primary citation expected (e.g. missing the primary reference for 'who first divided RM systems into four types'). Not all of the used abbreviations are listed and properly explained (e.g. standard RESP procedure,..) Regarding the tables and graphs presented within the thesis, those are treated appropriately. Regarding experimental part, Vitali obviously contributed extensively to the preparation of enzymes, site-directed mutagenesis, expression and purification of wild-type and mutant proteins, he also tested purified enzymes *in vitro* and *in vivo*, as well as performed restriction and ATPase activity assays. It is obvious, that Vitali got exposed to various experimental techniques by his own hands. I would like to also appreciate Vitali's contribution in writing of the manuscript. All those are good attributes to become an independent scientist. Therefore, the thesis overall provides a sufficiently comprehensive investigation of the topic; the results are suitably set out and accompanied by adequate exposition and interpretation. Therefore, the thesis as a whole constitutes a substantive original contribution to the knowledge of restriction modification enzymes.

Questions for the defense that should be addressed by the candidate:

1. How limited proteolysis is usually being used in molecular biology and for what purpose? What was the original reason to use the Trypsin proteolysis of MTase and HsdR in this thesis?
2. What are the differences in between two ATPase activity assays used within the thesis? Malachite green method vs. [λ-P^{32}] ATP method; the Pros and Cons.

Finally, I can clearly state that the candidate fulfills all criteria for being awarded a PhD degree. Therefore, **I can fully recommend Vitali Bialevich's thesis for the PhD defense**.

(Záver: Vitali Bialevich vo svojej práci jasne preukázal svoje tvorivé schopnosti i vedomosti a preto táto práca bezpochyby spĺňa požiadavky kladené na dizertačné práce v tomto odbore. **Odporúčam prácu k obhajobe**.)

In Bratislava, 09.02.2017

Ivana Nemčovičová

Dr. Ivana Nemčovičová, PhD
Biomedical Research Center
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University of South Bohemia in Ceske Budejovice

Head of the Committee of PhD studies in Biophysics

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JOHANNES KEPLER
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Gruberstrasse 40
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Linz, 7.2.2017

Dear committee for PhD studies in Biophysics, Faculty of Science, University of South Bohemia, dear Prof. Polivka,

In response to your request for a review of the PhD thesis of Vitali Bialevich MSc, I am pleased to submit this evaluation. Based on the PhD thesis of Mr. Bialevich MSc. and his publications I am impressed by his excellent work on the restriction-modification system type I of bacteria. The restriction-modification system is an essential bacteria system to detect foreign DNA and subsequently cleave this DNA to protect the bacteria. The PhD candidate used the first crystal structure of the HsdR subunit of the type I restriction-modification system EcoR124I that binds an ATP molecule to determined essential domains that affect ATPase activity, the translocation activity of DNA and the endonuclease activity.

To target these essential functions of the restriction-modification system type I, Mr. Bialevich MSc investigated specific contact sites between domain subunits of this motor subunit. As EcoR124I coordinates several enzyme functions, the structure needs to undergo several conformational changes. Consequently, Mr. Bialevich MSc. focused on hydrogen bonds between EcoR124I subunits: helicase II and helical as well as helicase motif III and its extended region. The contact sites that were detected based on MD simulations of EcoR124I were mutated from charged to non-charged amino-acids in EcoR124I. The PhD candidate succeeded in expression and purification of several EcoR124I mutants. It should be noted that the successful expression and purification of 120 kDa EcoR124I mutants is a challenging task, underlining the extraordinary skillful work of Mr. Bialevich MSc. The PhD candidate. identified Lys 527 and Tyr 736 as key residues at the contact site of helicase II and helical. These two generated EcoR124I mutations failed to largely cleave two-site linear DNA and largely decreased ATPase activity in comparison to wild-type EcoR124I.

For extended region of motif III, Mr. Bialevich MSc. generated several point mutations and successfully expressed them. Out of these single point mutations, EcoR124I G436A is of special note as this rather conservative mutation was able to interfere with ATPase activity and are restriction-deficient and hence cannot resist phage infection anymore. Moreover, Mr. Bialevich MSc. managed to get crystals from the G436A mutant and his pioneer work might lead to identification of another conformational state of EcoR124I.

In addition to this important work on EcoR124I, Mr. Bialevich MSc. has expressed and purified several other mutations within this protein and also a methyltransferase for two additional publications. His PhD thesis is excellent written and perfectly connects his cutting-edge science with an informative overview of the field.

In all, Mr. Bialevich MSc. has been extremely productive and is a main contributor to the understanding of EcoR124I function as essential part of the restriction-modification system of bacteria. I therefore evaluate the PhD thesis of Mr. Bialevich MSc. as excellent.

For the defense of the excellent PhD thesis of Mr. Bialevich MSc., I'd like to ask the candidate several questions:

1) The current work identified critical sites that can regulate conformational changes in EcoR124I. In your thesis you have mentioned the FRET (Fluorescence resonance energy transfer) technique as key methods to determine such conformation changes within the restriction-modification system. Can you please give an overview how this FRET technique works?

2) The restriction-modification system type II is frequently used in labs for molecular biology cloning strategies. Please compare the function of the type I and type II systems and comment if you also see a potential of the restriction-modification system type I to be helpful for life science techniques?

3) Specifically in your work on contact site of helicase II and helical within EcoR124I you have evaluated a hydrogen bond between Lys 527 and Asp 796. In your results the alanine mutation of Lys 527 is much more critical than an alanine mutation of Asp 796 based on your results on ATPase activity or DNA cleavage. Please explain this predominant effect of the Lys 527 Ala mutation based on your insights from molecular dynamics simulations.

Best wishes

Rainer Schindl

PD Dr. Rainer Schindl
University of Linz