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In Prague on 9th December 2015

Topic: Statement of the Opponent of the Ph.D. Thesis of "Katsiaryna Shamayeva: Involvement of the endonuclease domain of the EcoR124I restriction-modification complex in interdomain communication"

The presented Ph.D. thesis is devoted to a structural study of the restriction enzyme EcoR124I. Type I endonucleases play an important role in bacterial cells during bacteriophage attacks. Its multi-subunit structure is complicated and its functional behavior is essential for full understanding of endonuclease activity. From this point, the thesis topic is of broad scientific interest.

Summary of the thesis is divided out standard parts. It starts by theoretical introduction of restriction modification enzymes in general followed by more detailed introduction of EcoR124I restriction modification complex. Subsequently, materials and methods are briefly presented. The results, supported by two published papers – given in the section 5, extend experimental and theoretical work already published. Conclusions mostly repeat already published. The applicant clearly present what is her work and which experiments (i.e., theoretical calculations) were performed by other members of the group. The work of applicant was devoted to experimental work in the field of molecular biology and biochemistry. She performed site directed mutagenesis (single, double point mutations or deletion), expression and purification of EcoR124I subunits – HsdR, Methyltransferase, and measurements of restriction and ATP activity assays of mentioned proteins.

In spite of huge laboratory work, the applicant is co-author of only two scientific papers in impacted journals that is a required minimum. It seems to be a pity that she did not include the third submitted manuscript (in *BMC Biophysics*) into the thesis that could

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enrich the thesis. It is not clear which part of the thesis is connected with the manuscript in preparation (for *Nucleic Acids Research* journal).
Moreover, the presented thesis shows unripe ability of applicant for carefully manuscript preparation. Huge amount of typing errors could be maybe tolerated in MSc. thesis but it is unacceptable in Ph.D. thesis, e.g., page X missing abbreviations DLS, SPR and "Deoxyribonucleic acid Acid" – two times word acid; page 1, 5 citation "Bertani and Weige, 1953" has to be "Bertani and Weigle, 1953"; page 5 citation "Arber and Dissoix, 1962; Smith et al., 1972" has to be "Arber and Dussoix, 1962; Smidt et al., 1972". It is followed by wrongly formatted journal references full of typing errors, e.g., page 96 Bickle, T.A. (1993) pp. 35±88; or several forms of the journal title: Nucleic Acids Research, Nucleic Acids Res., Nucl. Acids. Res. Missing citations for the references mentioned in the text are another example of free reference treatment, e.g., Studier and Bandyopadhyay, 1988; Kelleher and Raleigh, 1994; Strick et al., 1998. It seems marginal but it will not be accepted by journals during submission process in an independent scientific path.

This inconsequent work is also observable in handling of figures. It is hard to distinguish between red and orange color of amino acids in the Figure 2 at page 12. Chromatography graphs are in figures very small, so no one can read labels. Figures of gels have labels inside the picture, they are sometimes very small and sometimes markers values are missing or overlapping (Figures 18, 22, 23, 32). Thus, more accurate scientific work, including presentations of results, is appropriate for the applicant.

Finally, I would like to kindly ask the applicant for answering following questions:

1.) Did you prepare plasmids on the basis of published articles – plasmid pTrcR124 with mutation Arg218Ala+Lys884Glu and plasmid pACR124 with double mutants Glu165His+Asp855Ala and Glu165His+Arg858Ala?

2.) Why did you use smaller markers than yours samples mentioned in the Figure 18?

3.) Produced proteins were stored in 50% glycerol at -20°C. Which effect did you observed at these conditions on protein's stability and function?

Even if the applicant thesis represents required minimum for a Ph.D. thesis, the author showed ability of independent scientific work that is supported by two published papers in good-class journals. Thus, I advice the Ph.D. thesis for the defense.

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INSTITUTE OF VIROLOGY
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OPONENTSKÝ POSUDOK

doktorskej dizertačnej práce

(ďalej v anglickom jazyku)

Title:	**Involvement of the endonuclease domain of the EcoR124I restriction modification complex in interdomain communication**
Author (PhD defender):	Katsiaryna Shamayeva, MSc.
Supervisor:	Prof. Rüdiger Ettrich Ph.D.
Place:	University of South Bohemia, Faculty of Science, České Budějovice, Czech Republic
Date of defence:	December 18, 2015
Opponent:	Dr. Ivana Nemčovičová, Ph.D.

Katsiaryna Shamayeva, within her PhD thesis, makes an attempt to understand the involvement of the endonuclease domain of the EcoR124I restriction-modification complex in interdomain communication within the HsdR motor subunit, in particular, focuses on the potential role of Lysine 220 and the QxxxY motif on the 180s loop in coupling of translocation and endonuclease function. In addition, Katsiaryna hypothesizes that this loop might participate in a signal transfer across the motor subunit, connecting the 220s loop and the catalytic site. This study also involves the probing of various other interdomain contacts of the endonuclease domain to the helical and the helicase 1` domain to determine a potential co-contribution. It is obvious, that studies of structure-functional interrelationship in the motor subunit of the EcoR124I RM complex present a complex task as only the fully assembled EcoR124I RM complex exhibits the individual enzymatic function. The one on the methyltransferase, as well as the variety of functions accommodated in the 120 kDa HsdR subunit, such as ATPase translocation and endonuclease which are assigned to helicase 1 and 2 domains and the endonuclease domain, respectively. Katsiaryna's efforts were focused on studies of structural elements of this endonuclease domain by using site-directed mutagenesis with combination of *in vitro* and *in vivo* characterization of tested mutant enzymes by which Katsiaryna assigned additional function for Q179xxxY183 motif via Arginine 182 residue in communicating a signal across the enzyme about its ATP-ligation status. Therefore, I really appreciate Katsiaryna's work on the interdomain communication within the HsdR

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motor subunit that was probed experimentally (both *in vitro* and *in vivo*) and nicely confronted with computational predictions.

The thesis is based on two published papers. The Journal of Molecular Modeling paper (IF=1.867) that Katsiaryna Shamayeva authored as a joint first author and Plos ONE paper (IF=3.534) with minor Katsiaryna's contribution; both of which I therefore consider as a core of her PhD work. These two papers published by the candidate gained to date only 1 citation, 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. There have been two more papers related to the main topic of her PhD work (not included in the thesis) but have not been published yet. However, the topics of this PhD thesis is obviously actual with international recognition.

The thesis has all together 112 pages divided into main 6 chapters. The individual chapters are quite well organized and the two main chapters ‚Materials and methods' and ‚Results' are also well distributed. The text itself contains a reasonable number of typing and spelling errors (e.g. p3, p45,..) and candidate is probably aware of it. However, the slang should be avoided in the scientific publications, e.g. the phrases as of „a bit better", or „doing better", or „quite good"; the usage of those is not in place and could be easily replaced by exact scientific jargon. In regards to the tables and graphs presented within the thesis, many of them have not been appropriately treated (e.g. p45-48, or p72). When presenting e.g. elution profiles or other curves, or experimental gel; the axis must be labeled, the digits and labels have to be of read-able size, markers (protein or DNA) need to be labeled correctly, ..etc. However, I appreciate the ‚Materials and methods' section that goes beyond the information given in the individual papers. Regarding experimental part, Katsiaryna contributed extensively to the site-directed mutagenesis, expression and purification of wild-type and mutant proteins, she also performed *in vitro* and *in vivo* tests on purified enzymes. It is obvious, that Katsiaryna got exposed to various experimental techniques by her own hands. I would like to also appreciate Katsiaryna'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; and the methods and techniques adopted are appropriate to the subject matter and they are properly justified and applied.

The results are suitably set out and accompanied by adequate exposition and interpretation. The conclusions and implications are appropriately developed and clearly linked to the nature and content of the research framework and findings. Therefore, the thesis as a whole constitutes a substantive original contribution to the DNA knowledge in the area of restriction modification enzymes.

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

1. Describe how does the site-directed mutagenesis works. The principle.
2. How different were expression and isolation conditions for mutant HsdR subunits in compare to the wild-type subunit and how this contributed or affected *in vitro* activity assays?
3. What kind of MD simulation in the wild-type HsdR predicted that the Arginine 182 alternates between Aspartic acid 881 and 220-loop? How did you therefore used this information for your experiments? And if the Aspartic acid 881 substitution therefore influenced the translocation activity?

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4. How sensitive is the helical domain for changes raised from point mutations? Could this potentially lead to problematic protein packing during expression?
5. The thesis describes in detail a variety of experimental and theoretical methods ranging from molecular biology; expression, purification,.. via activity assays both *in vivo*, and *in vitro* to targeted MD and QM/MM calculations. I would be glad if the candidate could describe a bit to which degree she manages the various techniques and where she sees her main strength.
6. I am curious, what are the future career plans of candidate (after PhD).
7. One paper has been submitted to BMC Biophysics recently. Is there already any feedback from the reviewers?

In summary, this is a well-conducted and well-presented study. Any minor deficiencies in conceptualization, measurement, design or execution are counterbalanced by positive qualities and a thorough discussion of methodological and experimental limitations while working in such difficult biological field. Finally, I can clearly state that the candidate fulfills all criteria for being awarded a PhD degree. Therefore I can fully recommend Katsiaryna Shamayeva for being awarded the PhD degree.

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

Bratislava, 4.12.2015

Ivana Nemčovičová

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Opponent's Review of the Ph.D. Thesis

Katsiaryna Shamayeva: Involvement of the endonuclease domain of the EcoR124I restriction-modification complex in interdomain communication

The aim of the work of Katsiaryna Shamayeva was to study the EcoR124I restriction-modification complex from *E. coli*, which is a part of the protection system of this organism against host DNA. Specifically, she focused on the study of the potential involvement of Lys220 and residues in QxxxY motif of the endonuclease domain in coupling of translocation and endonuclease activity. Her work involved both experimental and computational approach.

Ph.D. thesis of Katsiaryna Shamayeva has 112 pages, and is divided into following parts: Introduction, Materials and Methods, Results, Conclusions, Publications and References. In the Publication section, the work contains 2 attachments with full texts of original papers. Katsiaryna Shamayeva is also co-author of other two papers, which are not included as part of this Ph.D. thesis. It was quite interesting for me to read the whole thesis and accompanied papers, since they represent quite a comprehensive approach using a broad range of methods to answer some problems of enzymology and structural biology. The work can be useful, in a similar way, even for the younger colleagues in her laboratory.

The most important results of this work are, in my opinion, those suggesting that the QxxxY motif in HsdRs gained a new function here, (perhaps to communicate the signal about ATP-ligation status of the protein to other parts of the molecule), together with the hypothesis about the putative partner of this motif. This brings many new questions (maybe more than answers) and the whole coupling of enzyme activities in the complex is still waiting for the complete detailed characterization. However, this work represents an important step forward.

Comments:

The overall quality of the English language seems to be quite good. But there are also some exceptions, like the second sentence of the Annotation.

The part Introduction is very good, detailed and figures are well prepared.

In the part Materials and Methods, RPM are used for the characterization of centrifugation instead of the relative centrifugal force (given in g). This is quite a typical mistake of this section in many works.

The number of results in this Ph.D. thesis is indisputable. However, their presentation in figures in the Results section was perhaps influenced by the time pressure and the quality (graphical quality and description) of many is poor and quite insufficient; (for example on pages 44 – 47).

The part "Discussion" seems to be missing, but in fact, the part Results involves discussion as well. (The title "Results and Discussion" would be more suitable here.) The discussion paragraphs in Results section are written in a very good way and are able to put obtained results into the context of other published works.

I have these questions:

1. You analyzed the translocation activity by measurement of inorganic phosphate released. Would it be possible to characterize this enzyme activity in a more detail (K_m, V_{max}), using this type of enzyme assay? Is it possible at least to estimate k_{cat}?

2. If I understand well, the translocation activity of Type I R-M enzymes has a quite high ATP consumption. Is it possible to speculate why the organism pays such a high cost for that?

3. Do you have any potential candidate for the partner of the QxxxY motif?

I don't want to diminish the value of this work by all previous comments. I think that this work fulfills the requirements for Ph.D. thesis and I recommend it for the defense.

Prague, 4th December 2015

RNDr. Jiří Pavlíček, Ph.D.