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Jury Member Report – Doctor of Philosophy thesis.

Name of Candidate: Karyna Karneyeva

PhD Program: Life Sciences

Title of Thesis: Exploring type III CRISPR-Cas immunity in *Thermus thermophilus*

Supervisor: Professor Konstantin Severinov

Name of the Reviewer: Olga Soutourina

I confirm the absence of any conflict of interest (Alternatively, Reviewer can formulate a possible conflict)	 **Date: 25-02-2024**

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

The work of Karyna Karneyeva, supervised by Pr. Konstantin Severinov, focuses on the characterization of type III CRISPR-Cas systems in *Thermus thermophilus*.

The manuscript of 143 pages is organized in 6 chapters including the introduction, the review of the literature, materials and methods, results with two parts on type III CRISPR-Cas immunity against phages and on the interference requirements in this system and conclusions. The individual contributions of the candidate as well as collaborators are specified before each results part. The introduction section underlines the significance of the work, clearly states the research aims and explains the main focus of the study. The dissertation contains a short review of the literature on the life cycles of bacteriophages, defense strategies of microorganisms against phages and other mobile genetic elements with detailed description of type III CRISPR-Cas system as a subject of the thesis. The last part of this chapter is devoted to the anti-CRISPR-Cas strategies, a citation to an updated list of the anti-CRISPR proteins could be included in this part, as well as the strategies for their identification. This chapter is well written and well structured, but contains only two illustrations on type III CRISPR-Cas from previously published review paper with participation of the candidate. On my opinion the addition of figures describing different cycles of phages (it would be interesting to evoke alternative cycles), diversity of defense systems and general features of CRISPR-Cas systems including for example classification and PAM definition before focusing on type III CRISPR-Cas features would be helpful for the readers. The manuscript in general is well written; I would suggest just an additional round of proofreading to include minor modifications. A complete list of suggested modifications is provided to the candidate, this includes for example a reference 44 to check, some sentences to improve, gene/mutation names to put in italic in the *E. coli* strain genotype description.

In the chapter 3, the materials and methods section details the main biological material and experimental procedures used during this work.

The first results and discussion section describes the main findings on the characteristics of type III CRISPR-Cas defense against phages in *T. thermophilus*. A part of these results was published in "***Nucleic Acids Research***" in 2020 with the participation of Karyna Karneyeva as a second author. The interactions of *T. thermophilus* with two phages phiKo and phiFa were investigated and the efficient defensive function of type III-A and type III-B CRISPR-Cas was demonstrated. An intriguing point on the particular distribution of protospacers located only in the LTR region of phiFa phage genome has been explored with several hypotheses to explain this unequal location. An elegant RNA-seq experiment during phiKo phage infection demonstrated a gradual accumulation of phage reads and defined the three temporal classes for phage gene expression. No correlation between targeted protospacer gene temporal classes and CRISPR-Cas protection has been revealed. Individual contribution of type III-A and type III-B CRISPR-Cas for the protection against both phages is clearly demonstrated. Interestingly, experimental evidence for the emergence of escaper phages has been provided with deletion in CRISPR-targeted regions. This new observation contradicts previously postulated lack of phage escapers against type III CRISPR-Cas that differ from other systems by the absence of PAM-dependency and high tolerance rate for mutations in protospacers. An interesting application potential is discussed for a plasmid-based system to generate deletions in targeted phage genomes. Recombination events between CRISPR repeats leading to large deletion of *cas* operons have been observed and could be at the origin of CRISPR-Cas elements mobility.

The second part of the results explores the target recognition by the effectors of type III CRISPR-Cas during plasmid transformation with detailed analysis of mismatches between crRNA spacers and protospacers impacting the efficiency of CRISPR protection. The results are in line with previous

observations on the importance of target abundance that determines the tolerance to mismatches. Comparison of type III-A and III-B systems showed that type III-B system associated with less abundant effector proteins is less efficient and tolerates less mismatches than type III-A system. A thermodynamic model describing the correlation between the target RNA abundance and the minimal duplex length for type III CRISPR-Cas interference is provided in the discussion section. Defense mechanism of this particular CRISPR-Cas system includes target transcript degradation, target-dependent DNA cleavage, and cOA-dependent activities of accessory proteins. The contribution of Cas10 HD domain to the CRISPR-Cas interference has been analyzed showing its crucial role for effective interference against plasmids for type III-A CRISPR-Cas immunity, in contrast to the active type III-B lacking HD domain-containing proteins.

Finally, the major findings of the work and their potential applications are summarized in the last conclusions section. On my opinion, the perspectives of the work and future directions could be discussed in more detail in this part. The figure summarizing the main findings of the thesis with the comparison between type III-A and type III-B CRISPR-Cas could be also included.

I suggest to discuss during the thesis defense the intriguing presence of four different CRISPR-Cas subtypes encoded in the chromosome and megaplasmid in *T. thermophilus* as well as potential interplay between these systems and their differential contribution to the efficient defense. Unequal distribution of protospacers observed for two phages tested could be also discussed. The combination of various activities (RNA and DNA targeting, activation of auxiliary nucleases) contributing to type III CRISPR-Cas function will be interesting to cover during thesis defense session as well as a question on the significance of functional redundancy between type III-A and type III-B CRISPR-Cas in this model.

In conclusion, the results of Karyna Karneyeva represent an important scientific contribution to better understand the particular characteristics of extremely complex type III CRISPR-Cas system. Karyna Karneyeva signs an article published in international peer-reviewed journal "***Nucleic Acids Research***" in 2020 as second author describing the spacer acquisition by type III CRISPR-Cas during phage infection. The results on the interference requirements of type III CRISPR-Cas of *T. thermophilus* are described in the paper published in 2024 in «***Journal of Molecular Biology***» that Karyna Karneyeva signs as the first author. Other results presented in the dissertation are promising and should bring to additional publications. The candidate also contributed as a third author to a review article published in "***Biochemistry***" in 2021. The thesis work represents a high quality set that opens many perspectives for the future developments. The thesis manuscript is pleasant to read and clearly outlines the intellectual path and approaches used during the work. It is worth noting the impressive volume of work done by Karyna Karneyeva with a broad spectrum of *in vitro*, *in vivo* and *in silico* approaches. For all these reasons I consider that Karyna Karneyeva fully deserves to present her results in order to obtain a Ph.D. degree.

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*