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Jury Member Report – Doctor of Philosophy thesis.

Name of Candidate: Viktor Mamontov

PhD Program: Life Sciences

Title of Thesis: Escape mechanisms of mobile genetic elements against CRISPR-Cas system and diversity in microbial communities

Supervisor: Professor Konstantin Severinov

Name of the Reviewer: Francisco J. Martínez Mojica

I confirm the absence of any conflict of interest	**18-12-2023**

Reviewer's Report

The PhD thesis is structured in the following main sections: general abstract; complete list of abbreviations; introductory Chapter 1 covering the state of the art of the main topics of the dissertation; Chapters 2 and 4 presenting the results of two published manuscripts coauthored by the candidate (Chapter 2 also includes unpublished results); Chapter 3 on a submitted manuscript under revision; Chapter 5 highlighting the main conclusions of the thesis; and a section with the list of references.

The thesis is well-written and correctly organized, including the relevant data and required supporting information.

The candidate's attention in detailing his contributions to the research process for each chapter is remarkable. To note, he also acknowledges the limitations of the study and suggests ways to overcome them.

The methodology employed involves microbiology techniques, advanced molecular biology tools, sequencing data analyses and mathematical modelling. The strategies used are perfectly suited to the aims of the dissertation.

The results of the thesis have been published in two international scientific journals ranked Q1 (Science Citation Index):

Mamontov *et al.* (2022). Persistence of plasmids targeted by CRISPR interference in bacterial populations. Proceedings of the National Academy of Sciences. PNAS has a very high impact factor (JIF: 11.1), being ranked in position 8/73 within the Multidisciplinary Sciences subject area (SCIE edition).

Mihailovskaya, … Mamontov *et al*. (2023) Bacteriocin-Producing *Escherichia coli* Q5 and C41 with Potential Probiotic Properties: In Silico, In Vitro, and In Vivo Studies. International Journal of Molecular Sciences. The journal has a high impact factor (JIF: 5.6), being ranked in position 66/285 within the Biochemistry & Molecular Biology subject area (SCIE edition).

The interest and scientific quality of these two papers as well as the manuscript under-revision (Demkina, A., Slonova, D., Mamontov, *et al.* Benchmarking DNA Isolation Methods for Marine Metagenomics Studies) dealing with the diversity and dynamics of microbial communities, is beyond doubt. To highlight, the study of escape strategies used by MGEs against CRISPR immunity expands our understanding of their interaction with the host microbes. The arms-race between prokaryotes and MGEs is a hot topic, with many biological and applied consequences (i.e., control of pathogenic bacteria, implementation of molecular tools). Moreover, the comprehensive analysis of commercial DNA isolation kits carried out will allow researchers for the selection of the most appropriate kit for different types of samples and provides valuable information for further improving related techniques. Finally, *E. coli* strains that produce bacteriocins have been identified in this work as potential probiotics to prevent infection from pathogenic bacteria.

In my opinion, the thesis meets all the requirements for defence as it is. Below are a some questions and recommendations.

Please, check the space between lines (e.g., the last paragraph on page 40, the last sentence on page 57).

When discussing both archaea and bacteria, it's best to use 'prokaryotes' instead of 'bacteria'.

Make sure that italics are used when appropriated (i.e., *in vivo*, *in vitro*, gene names, species names).

Bacterial species names should be abbreviated once the corresponding full name has been already provided.

Page 3: “proliferation of foreign **DNA** in a host cell” should be replaced with “proliferation of foreign **nucleic acids** in a host cell” to include RNA MGEs.

Abbreviations list: Please, replace “**CRISPR-**Cas – CRISPR Associated” with “Cas – CRISPR Associated”; incorporate the “PAPS” acronym and its description; remove “g – gram”; replace “LB – Luria-Bertani **medium**” with “LB – Luria-Bertani”.

Page 20: “destroy foreign genetic elements bearing sequences **identical** to spacers”. Keep in mind that partial complementarity can also result in target destruction.

Page 21: “In Class 2 CRISPR-Cas systems the trans-activating CRISPR RNA (tracrRNA) is essential for immunity”. Please note that many Class 2 systems do not require tracrRNA.

Page 24: “… a putative helicase *dinG* gene”. Be aware that such activity has already been demonstrated (doi 10.1093/NAR/GKAD546).

Page 25:

“The canonical PAM motif recognized by Cas9 effector is sequence 5’-NGG-3’”. “Cas12 effector complex recognizes the 5’-TTTN-3’ PAM”. It should be clarified that NGG and TTTN are not the universal Cas9 and Cas12 PAMs, respectively.

“Nuclease-inactive Cas9 proteins (dCas9) are applied for gene knockdown experiments”. Please, make it clear that, apart from knockdown, dCas9 has been utilized in other types of experiments.

Page 27:

Review the sentence “To maintain anti-phage protection a bacterial cell provides a **permanent** expression of *cas* genes” considering that the *cas* genes of many bacteria (and prokaryotes in general) are not “permanently” expressed, at least at a detectable level.

“In the type I-E system, it was shown ...”. Please, identify the organism being referred to.

Page 41. Change the letter size/font of “to find 0”.

Page 43. Replace “perfor**e**med as desc**t**ibed” with “performed as desc**r**ibed”.

Page 50: “… the CRISPR-Cas system as was done in experiment shown in Figure 2”. Check the figure number (perhaps Figure 2.2).

Preliminary results related to those of the paper published in the PNAS journal are succinctly presented in Section 2.3.6. Further details, particularly on the methodology used, such as how *E. coli* cells transformed with the M13 replication form were selected and what controls were included in these experiments, would be appreciated.

Page 64. Check quotation for reference 482 (this reference number is not in the bibliography list).

Figure legends 3.2 and 3.3: Replace “bellow” with “right” regarding the position of the kit ranking graph.

Page 108. “2.3.3. Hematological and Biochemical Parameters …”. Review heading format and number.

Page 126. Check the quotations for Fig. S1.3. Perhaps you mean Figure S2.3 instead, which is not included as supplementary material.

In the Conclusions section, regarding the experimental results presented in chapter 2, it would be appreciated a more detailed discussion on why inactivation of CRISPR-Cas system or accumulation of escape mutations were ruled out as possibilities to account for pG8 plasmid maintenance in CRISPR ON colonies. The detection in CRISPR ON colonies of cells with mutations (in *cse1* gene and/or the *araBp8* promoter) along with the fact that a small number of plasmid-bearing cells might support the growth of the most abundant plasmid-less cells, could in principle suggest that these mutants (with inactive CRISPR-Cas) are the only cells in the colonies that carry the target plasmid. Could you list the results that reject this possibility?

Related to this, on page 128 you argue that "the sequencing results of the initial population of phage M13 showed no mutations in the protospacer, or if present, these mutations were undetectable by sequencing". Is this also applicable (i.e., mutations could be present, but were not detected) to your experiments involving plasmids where you assume that there are not mutations in the protospacers based on sequence logos (Fig. S2.1)?

Page 128. "… the potential fraction of escape mutants in the initial population of the phage M13 replicative form does not align with the transformation efficiency of ~0.01 compared to the control transformation". It would be great if you could explain this reasoning in more detail.

Bibliography section (pages 131-152). In some cases, the year of publication is placed after the authors' names, either in brackets or not, and in others it is placed following the page number. Similarly, some journal names are abbreviated and/or italicized, while others have the full name and/or are not italicized. Please, be consistent throughout the text.

Page 153. "Figure S2.2 is put in Chapter6 as Figure 5.1". However, there is no Chapter 6.

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*