Skoltech
Skolkovo Institute of Science and Technology

Thesis Changes Log

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: Prof. Konstantin Severinov

The thesis document includes the following changes in answer to the external review process.

Comments from **Prof. Mikhail Gelfand**:

1. "The author's contribution should be specified in more detail, not only in the Preface, as in the present version, but at the beginning of each paragraph."
   Response:
   In chapters 3 and 4, short annotations were added describing my contributions to the studies and noted what parts of the studies were conducted by other authors.

Prof. Dmitry Chudakov did not have comments regarding the text.

Prof. Edze Westra did not have comments regarding the text.

Comments from **Prof. Francisco J. Martinez Mojica**:

1. "Please, check the space between lines (e.g., the last paragraph on page 40, the last sentence on page 57)."
   Response:
   Spaces between lines on pages 40 and 57 were corrected.
2. "It's best to use 'prokaryotes' instead of 'bacteria'."
   Response:
   The terminology 'bacteria' was checked in the Introduction section and changed to 'prokaryotes' where it was required.
3. "Make sure that italics are used when appropriated (i.e., in vivo, in vitro, gene names, species names)."
   Response:
   The terminology of such words as *in vivo*, *in vitro*, *in silico*, gene and species names were emphasized with italics through the text.
4. 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.
   Response:
   The sentence on page 3 "RNA-guided immune response against foreign DNA in prokaryotes" and "the proliferation of foreign DNA in a host cell" were changed to "RNA-guided immune response against foreign nucleic acids in prokaryotes" and "the proliferation of foreign nucleic acids in a host cell" respectively.

5. "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".
   Response:
   The abbreviations were corrected according to the comments.
6. Page 20: "destroy foreign genetic elements bearing sequences identical to spacers". Keep in mind that partial complementarity can also result in target destruction.
   Response:
   The sentence "destroy foreign genetic elements bearing sequences identical to spacers" were corrected on "destroy foreign genetic elements bearing sequences that are entirely or partially complementary to the spacers".
7. 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.
   Response:
   The following corrected sentence "In addition to crRNA, in Type II of Class 2 CRISPR-Cas systems the trans-activating CRISPR RNA (tracrRNA) is essential for immunity" were added.
8. Page 24: "… a putative helicase *dinG* gene". Be aware that such activity has already been demonstrated (doi 10.1093/NAR/GKAD546).
   Response:
   The sentence was updated on "a helicase *dinG* gene [150]" with adding the corresponding reference.
9. 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.
   Response:
   The first sentence was clarified by adding the corresponding reference: "The canonical PAM motif recognized by Cas9 effector is sequence 5'-NGG-3' [155]. However, subsequent studies have demonstrated PAM recognition depends on the type of Cas9 [156]."
   The sentence "Cas12 effector complex recognizes the 5′-TTTN-3′ PAM" was changed to "*Acidaminococcus* sp Cas12 effector complex recognizes the 5′-TTTN-3′ PAM" to make it more specific.
10. Page 25: "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.
    Response:
    The sentence was corrected on "Nuclease-inactive Cas9 proteins (dCas9) are applied for gene knockdown [163] and as well as for gene expression activation experiments [164]" with the corresponding reference.
11. 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.
    Response:
    That was corrected on more neutral sentence: "To implement anti-phage protection by CRISPR-Cas system a bacterial cell provides an expression of *cas* genes."
12. "In the type I-E system, it was shown ...". Please, identify the organism being referred to.
    Response:
    The sentence was corrected on "In the type I-E system of *E. coli* K-12, it was shown…"
13. Page 41. Change the letter size/font of "to find 0".
    Response:
    The font was corrected.
14. Page 43. Replace "perfor**e**med as desc**t**ibed" with "performed as described".
    Response:
    The typos were corrected.

15. Page 50: "… the CRISPR-Cas system as was done in experiment shown in Figure 2". Check the figure number (perhaps Figure 2.2).
    Response:
    The figure number was corrected on Figure 2.2.
16. 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.
    Response:
    In general, the same methodology as described for the plasmids is used for the experiments with M13 replication form. Additional methods for the unpublished results listed on page 42-43. The following sentence on page 63 for clarification was added:
    "The transformants were plated on media supplemented with antibiotics for control and on media supplemented with both antibiotics and inducers for CRISPR interference assay as previously described for the experiments performed with plasmids."
17. Page 64. Check quotation for reference 482 (this reference number is not in the bibliography list).
    Response:
    The reference number was corrected on 443.
18. Figure legends 3.2 and 3.3: Replace "bellow" with "right" regarding the position of the kit ranking graph.
    Response:
    The figure legends were corrected correspondingly.
19. Page 108. "2.3.3. Hematological and Biochemical Parameters …". Review heading format and number.
    Response:
    The corresponding section went on page 109. The heading format and number were corrected on "4.2.3.3. Hematological and Biochemical Parameters …"
20. Page 126. Check the quotations for Fig. S1.3. Perhaps you mean Figure S2.3 instead, which is not included as supplementary material.
    Response:
    The corresponding text went on page 127. The figure number was corrected on Figure S2.3. The corresponding figure number in the supplementary on page 155 was also corrected on Figure S2.3.
21. 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?
    Response:
    The more detailed explanation in the Conclusion section was added:
    "During the experiments, we also detected that a small fraction of CRISPN ON colonies after transformation were fluorescent (**Fig. 2.3 a**). The results of sequencing fluorescent CRISPR ON colonies revealed inactive CRISPR-Cas systems (**Table S2.1**) but no escape mutations were detected in the g8 protospacer in the plasmids (**Fig S2.1 a**). The possibility that the minor subpopulation of plasmid-bearing cells in CRISPR ON colony represents cells either bearing inactive CRISPR-Cas system or escape mutant plasmids was ruled out with cross-transformation by compatible plasmids to test the activity of CRISPR-Cas system (**Fig. 2.1 d**) and the transformation by the plasmids extracted from these CRISPR ON colonies to test a presence of escape mutations (**Fig. 2.1 c**). Moreover, the several rounds of reseeding on selective plates demonstrated the persistence of plasmids in a small fraction of CRISPR ON cells (**Fig. 2.2**). Alternatively, if the reseeded CRISPR ON colony contains a

fraction of cells with an inactive CRISPR-Cas system or mutated plasmids, we might expect that the CFU ratio on the antibiotic-supplemented plate and LB-agar plate would align with each other by the second or third round of reseeding. This alignment would occur due to antibiotic selective pressure, as in this case without CRISPR interference due to mutations the majority of the cells remained plasmid-bearing. In addition, the fluorescence of CRISPR ON colonies, as the indicative mark of genetic alterations, generally was not observed during the rounds of reseeding under CRISPR interference conditions. Thus, the reseeding experiments demonstrated the plasmid persistence under CRISPR interference conditions without genetic alterations (**Fig. 2.2**)."

22. 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)?
    Response:
    From my point of view, it is also applicable for plasmids. A potential explanation was added on page 129:
    "Regarding the plasmids, we can suggest that detecting their mutant forms in CRISPR ON colonies is still challenging, as plasmids propagate vertically from a parent cell to the offspring so there is no fast propagation among the CRISPR ON colony."
23. 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.
    Response:
    The text went on page 129. The explanation was extended as follows:
    "Similar to the CRISPR interference assay with plasmids (**Figure 2.1 b**), the efficiency of transformation of CRISPR ON cells by the phage M13 replicative form is at least two orders of magnitude lower compared to the CRISPR OFF control transformation (**Figure 2.7 a**). Thus, the transformation efficiency by the phage M13 replicative form is still detectable, except for the potential fraction of escape mutants in the initial population of the phage M13 replicative form. However, in contrast to the plasmids, in the experiments with phage M13, we revealed CRISPR adaptation (**Figure 2.7 c**) and phage M13 escape mutants in CRISPR ON colonies (**Figure 2.8**) after the transformation. Regarding the plasmids, we can suggest that detecting their mutant forms in CRISPR ON colonies is still challenging, as plasmids propagate vertically from a parent cell to the offspring so there is no fast propagation among the CRISPR ON colony. Therefore, I assume that the transformation predominantly occurred with the wild-type dsDNA replicative form of phage M13, and phage M13 escape mutants appear during rounds of infections and rapidly propagate in CRISPR ON *E. coli* colonies."
24. 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.
    Response:
    The style of references was corrected.
25. Page 153. "Figure S2.2 is put in Chapter6 as Figure 5.1". However, there is no Chapter 6.
    Response:
    The sentence was corrected "Figure S2.2 is put in Chapter 5 as Figure 5.1".

Comments from **Dr. Simon Jackson**:

1. Page 18 "… conjugative plasmids can replace the host TA system with a more…." To which TA system are you referring?
   Response:
   The sentence was corrected on "In *Bacteroides fragilis*, for example, conjugative plasmids can replace the host Type VI secretion systems (T6SS) with a more effective antagonistic secretion system thereby enhancing the host's fitness in intraspecies competition [71]."
2. Page 23 "… K-12 strain harbors an induced type I-E system…" It should be made clear that this is an engineered variant where the Ara/IPTG inducible promoters have been inserted upstream of the *cas* genes."
   Response:
   The following correction was added "A popular model bacterium for molecular biology studies, the *E. coli* K-12 strain was engineered to harbour an inducible type I-E system, with IPTG- and arabinose-inducible promoters, that is one of the best-studied CRISPR-Cas systems [124, 125, 128]."
3. Page 23. In places, some key references are lacking. E.g. Swarts et al 2012 for priming.
   Response:
   The following sentence with corresponding reference was added "The study conducted by Swarts. *et al* has demonstrated that the activated type I-E CRISPR-Cas cured *E. coli* K12 Δ*hns* cells from high-copy plasmids in non-selective prolonged cultivation with the acquisition of new spacers against the plasmids into the CRISPR array [138]."
4. For the bioinformatic analyses presented, the candidate states (p12) that they were responsible for the analyses of the 16S data. However, the methods list a github account belonging to someone else for the 16S data analysis pipeline (p77). The github repository listed is not publicly available, so I am unable to further assess this. It will be necessary for the candidate to clarify their own direct contributions to the analyses.
   Response:
   The following annotation on page 77 was added "*I contributed to updating 16S_snakemake.smk pipeline in the GitHub repository of my colleague D. Sutormin and conducted the computational analysis of 16S rRNA data described in this section. All data related to the 16S analysis is also presented in public at https://github.com/mavic9/16S_rRNA_analysis.*" In addition, short annotations describing my and other authors' contributions on pages 78-95 for each section were added.
5. The author contribution statement (p13) is a little vague, "… I made substantial contributions to the in silico analyses…". It is not clear whether this means the candidate performed the analyses presented in 4.2.1.1, .2, .3, or .4 (and which parts were performed by or alongside other authors).
   Response:
   The following annotation on page 100 for Section 4.2.1 was added: "*The results presented in sections 4.2.1.1-4.2.1.4 were conducted in collaboration with D. Sutormin. I was responsible for genome assemblies and annotations and D. Sutormin assisted with feature analysis of annotated genomes of E. coli strains.*" In addition, short annotations describing my and other authors' contributions to the methodology on pages 118-119 were added.
6. Was a comparison made to genome assemblies generated by short-read sequencing?
   Response:
   Unfortunately, the direct comparison with short-read sequencing was not performed in this work.

Common updates:

1. Page 4. The reference on publication #3 was corrected on:
   Demkina, A., Slonova, D., Mamontov, V., Konovalova, O, Yurikova, D, Rogozhin, D., Belova, V., Korostin, D., Sutormin, D., Severinov, K., & Isaev, A. (2023). Benchmarking DNA isolation methods for marine metagenomics. *Sci Rep* 13, 22138 2023. https://doi.org/10.1038/s41598-023-48804-z.
2. The numerations of headlines were corrected and the table of contents was updated correspondingly.
3. The numeration of the references was corrected.