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Jury Member Report – Doctor of Philosophy thesis.

Name of Candidate: Julia Gordeeva

PhD Program: Life Sciences

Title of Thesis: Recognition strategies of Type I and Type V BREX systems

Supervisor: Professor Konstantin Severinov

Name of the Reviewer: Francisco J Martinez Mojica

I confirm the absence of any conflict of interest	**Date: 12-08-2022**

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

The PhD thesis is very well structured, including Abstract, Introduction, Chapter 1 reviewing the literature, Chapter 2 on materials and methods used, Chapter 3 presenting the results obtained, a section highlighting the main conclusions and a final section discussing results and future perspectives. A very useful symbols and abbreviations list is incorporated at the beginning of the thesis and an updated list of references at the end. Even though a dedicated section listing the objectives of the research performed is not incorporated as such, the goals of the thesis are clearly established in the Introduction section. The literature review is accurate, updated and well written, covering the vast diversity of prokaryotic defense systems. Although many of these systems are unrelated to the topic of the thesis (*i.e.*, BREX systems), the information provided is quite helpful for the interpretation of the results obtained in this work. Material and Methods section is well organized, and information provided would allow for the reproduction of the experiments. Chapter 3 comprises both unpublished (the type V BREX system of the archaeon *Haloarcula hipanica*, $BREX^{HAR}$) and published (the type I BREX system of the bacterium *Escherichia coli* - $BREX^{Ec}$ -; Gordeeva et al. Nucleic Acids Research, 2019) results. Remarkably, the candidate is very careful including only her own results and acknowledges the researchers (e.g., Dr Siksnys and Dr Isaev) responsible for closely related experiments mentioned in the thesis. The candidate extensively discusses on the research and clearly discerns among speculations and what is proven after the results obtained. Also, she proposes

well substantiated explanations for unexpected or uncertain findings, suggesting further experimentation to clarify them. In general, the thesis is clearly written, with few typos and grammatical errors, and both organization and presentation are correct.

The biochemical and functional characterization of prokaryotic defense mechanisms is a hot topic at present, fueled by the recent discovery of novel systems that offer protection against viruses and by the applications that have been deployed out of them in the past, notably, molecular biology tools for nucleic acids manipulation derived from Restriction-Modification (R-M) and CRISPR-Cas systems. One of these new, yet uncharacterized immune systems is BREX (Bacteriophage Exclusion), classified in 6 types (I-VI) based on the identity of the associated genes. BREX research is in its infancy: only type II (one system) and type I (four systems) had been explored before this thesis work, providing just a few tips on the respective mechanism which altogether reveal a wide variety of performances. Exploring in depth representatives of each BREX type is of great biological (i.e., understanding the arms-race between viruses and prokaryotes, and its consequences) and applied (i.e., control of pathogenic bacteria, implementation of molecular tools) interest.

Methods comprise microbiology techniques, applied to the study of both bacteria and archaea as well as their viruses, and advanced molecular biology tools. The approaches used are well adapted to the achievement of the aims of the dissertation. Remarkable fact, when unexpected experimental problems arose, they were circumvented using elaborated alternatives, demonstrating the candidate's resourcefulness.

The contributions to the characterization of the BREX systems are numerous and meaningful, adding new instrumental perspectives in the field on a global scale. The results presented are of great impact for the progress of knowledge on prokaryotic defense systems. Here are some of the main accomplishments of the dissertation. Results show, for the first time, that BREX defense is ensured by epigenetic modification (i.e., methylation). They also target the $BREX^{Ec}$ and $BREX^{HAR}$ genes involved in each of the two main stages of the mechanism (methylation and defense), discern the role played by some of them and identify modification sites. In addition, results demonstrate that $BREX^{Ec}$ elicits phage genome degradation and strongly suggest that it acts on the very early stages of viral infection, targeting unmodified double-stranded DNA. Mechanisms used by the virus to evade $BREX^{Ec}$ defense (cytosine glycosylation of target sites and protein Ocr) are also revealed.

The results obtained certainly open up new grounds for the implementation of molecular tools based on the restriction (DNA cleavage at specific sites) and modification (restriction protection) BREX proteins, equivalent to the widely used R-M enzymes. Given the basic nature of the research performed, additional applications are unpredictable.

The results of the thesis have been published in two original articles:

> Gordeeva J, Morozova N, Sierro N, Isaev A, Sinkunas T, Tsvetkova K, Matlashov M, Truncaite L, Morgan RD, Ivanov NV, Siksnys V, Zeng L, Severinov K. (2019) BREX system of Escherichia coli distinguishes self from non-self by methylation of a specific DNA site. *Nucleic Acids Research*. 47:253-265.
>
> Isaev A, Drobiazko A, Sierro N, Gordeeva J, Yosef I, Qimron U, Ivanov NV, Severinov K. (2020) Phage T7 DNA mimic protein Ocr is a potent inhibitor of BREX defence. *Nucleic Acids Research*. 248:5397-5406.

Both manuscripts are published in the prestigious journal *Nucleic Acids Research* (NAR). NAR has a very high impact (Impact Score: 19.33; SCImago Journal Rank (SJR): 8.241; H-index: 569), being ranked in Q1 within the Genetics subject area.

Over fifty articles have cited the main paper related to the thesis (Gordeeva et al 2019) and more than 30 the other one (Isaev et al 2020) in which the candidate had a lesser involvement. The scientific quality of the papers is outstanding.

In my opinion, the thesis meets all the requirements for defense as it is. Below are a few questions and minor formal/grammar recommendations that the candidate may wish to address:

- Check if every time the term "bacteria" is used, you really mean members of the Bacteria domain or bacteria + archaea (i.e., prokaryotes). Similarly, "bacteriophages" should only apply to viruses that infect bacteria, not archaea.
- Quote the origin of all the figures (in their captions) that are taken or modified from published material, including those from Gordeeva et al 2019.
- Confirm that, as stated on page 17, R-M systems are the most abundant defense mechanisms (see for instance doi: 10.1186/s12862-017-0942-y).
- Each figure/table should be better placed after (not before, as for example Tables 4 and 5, Figures 4 and 14) the first time it's cited in the text.
- Be consistent using either "Type" or "type" of systems
- Page 28 (last paragraph). Elaborate further on the *E. fergusonii* type I system (BrxU appart) clarifying if the conclusion in the last sentence is fully demonstrated or just hypothetical.
- Page 30. Replace "mutations in the PAM region or in the protospacers result in avoidance of CRISPR defense", with "mutations in the PAM region result in avoidance of CRISPR defense"
- Page 32. Define "effector modules" (sentence "participating the **effector modules**, crRNA biogenesis, as well as target recognition and cleavage") and "PLE" (sentence "with a spacer targeting host antiphage system PLE").
- Define "Cascade" (page 36)
- Page 43, replace "Inoue tbuffer" with "Inoue tbuffer"; "…thus work…" with "…this work…" and "Dh2α" with "DH5α" (use DH5α instead of Dh5α throughout the entire text).
- Page 44. Replace "Ligation mixture were…" with "Ligation mixtures were…"
- Be consistent across the text regarding temperature format (e.g., "37°C" or "37 °C")
- Change "efficiency of plating" to "efficiency of plaquing" and use EOP after the first time it is defined in the text.
- Page 47. Clarify how phage titers are inferred from the drop method (i.e., are plaques counted in each drop of phage lysate/dilution? On the same page, justify the addition of uracil.
- Please consider specifying (e.g., adding "*E. coli*" to the subsection title) that subsections 2.9-2.12 deal with *E. coli* experiments only.
- Indicate whether and how genetic deletions generated in this work were confirmed. As several halophilic archaea have been shown to be polyploid, confirmation of a complete deletion of the target gene (i.e., in all the chromosomal copies) is particularly relevant in the case of *H. hispanica*. Still, the absence of BREX-mediated modifications in *brx*-deleted derivative strains (Table 3) supports complete deletion.
- Page 51. Clarify if *E. coli* K12 strain BW25113 carries putative BREX encoding genes (i.e., "lacking endogenous *brx* genes")
- Figure 11d. "BREX-, MOI=0.001" is not identified in the figure legend; it seems to be the dark gray line but "BREX+, MOI=0.001" is labelled instead. The "no phage" lines are not visible (apparently are masked by other lines) in the graph; clarify in the caption where they are located.
- Page 53. The conclusion "Since lysogenization does not require phage DNA replication (96), the result indicates that the defensive action of $BREX^{Ec}$ manifests itself either at the stage of phage adsorption or during injection of phage DNA", needs to be further explained, either here or in the Discussion section. For instance, what outcome is anticipated if $BREX^{Ec}$ would manifest itself at a later stage of the phage cycle (i.e., after phage DNA replication)? Are the two possibilities mutually excluding?

- Page 53. Clarify that the λ phage lysates used in the live fluorescence microscopy experiments are dam-methylated (they come from a *dam*+ strain).
- Page 57 and Fig. 15d. It's important to mention that methylation at GGTAAG sites was not found in the case of BREX- cells.
- Page 60, first paragraph. Change "Fig. 16c" to "Fig. 16d".
- Page 60-61. Check/justify the explanation "It may happen due to problems with transcription or translation of *brxB* as the stop codon of BrxA overlaps with the start codon of BrxB".
- Page 61. Discuss why "No colonies with deleted *brxC* gene were found in the complemented system"
- Page 60-61. Explain why deletions of specific *brx* genes in pBREXAL lead to mutations in other *brx* genes on the same plasmid but not when the two plasmids separately expressed *brxABC* and *brxXZL*. In other words, which is the rational for using the two-plasmid system? Does it have something to do with control of *brx* genes expression?
- Please consider specifying (e.g., adding "*E. coli*" or BREX^{Ec} to the subsection title) that subsections 3.3-3.6 correspond to the *E. coli* BREX system.
- Figure 20c. Change "linar" to "linear"
- Page 66. Replace "Table 2" with "Fig. 21a"?
- Page 66. Could the specific action of BREX^{Ec} on dsDNA (compared to ssRNA and ssDNA) at an early stage of phage infection be inferred after your results with M13 and Qβ phages?
- Page 66. Can you think of a possible explanation for the "lack of dependence of (BREX^{Ec}-dependent) restriction on the number of GGTAAG methylation sites"?
- Page 69. No decline in *H. hispanica* growth was observed up to 10 hours after HHPV3 infection. This is attributed to the non-lytic lifestyle of the virus. However, in principle, a growth delay should be expected in sensitive hosts. Considering the long generation time of haloarchaea, a decrease in growth rate might be observed later (according to reference 106, between 10 and 15 hours after addition of the virus). Similarly, an increase in PFU could still happen in BREX+ cells after the last time point of the experiment. Please, bear these aspects in mind for discussion (e.g., a decline in OD for BREX^{HAR}-carrying cells might occur - after 10 hours in this case - as found for BREX^{Ec}).
- Page 73. Delete "there" from "Inside the *brx* cassette there a gene encoding…"
- Table 6 is not mentioned in the text.
- The candidate might consider including the main findings on the BREX mechanism of previously studied type I systems in table 6 within the Discussion section.
- Page 77. Explain how alternating the proportion of BREX+/- can help withstand phage predation.
- Page 78. In the sentence "its (BrxL) toxicity is responsible for invader's elimination", are you thinking on cell toxicity preventing phage spread like Abi systems?
- Page 78. "The presence of large numbers of non-methylated *brx* sites in bacterial genomes raises a question of how self-destruction of BREX+ cells is avoided" Might it be related to a requirement for cleavage of several non-modified sites? This could be alike the situation reported for PT modification systems mentioned in Chapter 1 "complete phosphorothioate modification of host genome is not necessary for self-protection. The relative geometry of sites is thought to dictate the modification state"
- Page 78. Replace "thus genomes of cells with deletions of individual methyltransferases only one brx motifs…" with "thus, in genomes of cells with deletions of individual methyltransferases only one *brx* motif…"
- Bibliography. Some references are incomplete and contain errors.

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*