Skoltech
Skolkovo Institute of Science and Technology

Jury Member Report – Doctor of Philosophy thesis.

Name of Candidate: Anna Maikova

PhD Program: Life Sciences

Title of Thesis: The CRISPR-Cas system of human pathogen *Clostridium difficile*: function and regulation

Supervisors: Prof. Konstantin Severinov;

Prof. Olga Soutourina, University of Paris-Saclay, France

Chairmen of PhD defense Jury: Prof. Mikhail Gelfand, Skoltech ***Email:*** m.gelfand@skoltech.ru

Professor Harald Putzer, Paris Diderot University, France ***Email:*** putzer@ibpc.fr

Date of Thesis Defense: 30 September 2019

Name of the Reviewer: Konstantin Lukyanov

I confirm the absence of any conflict of interest	**Signature:**  **Date: 29-08-2019**

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 dissertation work by Anna Maikova is devoted to in depth characterization of CRISPR-Cas system of *Clostridium difficile* - one of the major pathogenic clostridia. This work includes three interconnected parts.

The first part describes functionality of *C. difficile* CRISPR-Cas system. The author demonstrated that most CRISPR arrays very efficiently block conjugation of plasmids carrying corresponding sequences. Importantly, all possible PAM sequences in *C. difficile* were experimentally identified. Events of new spacer acquisition were detected although only under conditions of overexpression of Cas1, -2, and -4.

In the second part, regulation of *C. difficile* CRISPR-Cas system was studied. It was revealed that several CRISPR arrays are associated (chromosomal co-localisation) with new type I toxin-antitoxin systems. Functionality of these toxin-antitoxin systems in *C. difficile* was confirmed. Moreover, author showed that in some cases cas operons and the CRISPR arrays are co-regulated with the associated TA systems by stress- and biofilm-related factors (e.g., c-di-GMP).

The third part is focused on development of method of using endogenous CRISPR-Cas system for genome editing in *C. difficile*. The suggested approach allowed to delete a target gene in the genome with rather high efficiency (note that standard methods were unsuccessful in this case).

The main results of dissertation work are published in two papers: in Nucleic Acids Research (impact factor 11.1) – one of the most reputable journals in the field of DNA and RNA studies, and in Applied and Environmental Microbiology (impact factor 2.15) – a journal of American Society for Microbiology.

To summarize, this dissertation work has made significant progress in understanding the structural and functional features of *C. difficile* CRISPR-Cas system, as well as in its practical application for genome editing of this species. However, I have some concerns that should be addressed or at least discussed:

Major points

1. Some histograms on conjugation efficiencies (Figs. 2.5A, 2.6A, 2.14) do not show dispersion.
2. Page 50: “Overall, interference levels of C. difficile 630Δerm CRISPR arrays correlate with their expression levels (Figure 2.5B, C), detected by RNA-seq in the previous work (Boudry et al., 2015)” (also in Discussion, page 66: “Experiments with C. difficile 630Δerm strain demonstrated that defense levels of different arrays correspond to their expression rates, identified previously (Boudry et al., 2015))”. Actually, such correlations are not obvious from Fig. 2.5. RNAseq figures in the panels B and C are very small and contain no Y-axes marks (are they the same for all graphs?). The legend provides almost no explanation (e.g., what do black diamond symbols mean?). Among three low-effective arrays, only CRISPR17 shows low expression level, while CRISPR11 and 12 look quite similar to the efficient arrays CRISPR6, 7, and 10. Possibly, author can choose some other way of data representation (e.g., as a table or a graph for relative expression levels) to convince readers in any correlations between CRISPR array efficiency and expression level. I think that current description does not support it.
3. Page 64: “Adaptation experiments using native endogenous expression levels of Cas proteins were unsuccessful for *C. difficile* 630Δerm (data not shown)”. It would be helpful for readers to describe these experiments in more details. In particular, what stimulus (exogenous DNA) was used to induce adaptation? Can a bacteriophage induce a stronger activation of naïve adaptation (compared to plasmid)?
4. Page 82: “For both TA modules (CD2517.1 and CD2907.1/CD0956.2), the overexpression of the toxins in strain 630/pT led to a significant increase in cell length for about 9% and 5.4% of the cells,

respectively. The length of these cells was above the value of 630/p mean length with 2 standard deviations (10.5 μm) (Figure 3.5D and Figure S3.4 in Supplementary materials).” These Figures show microphotographs; it would be helpful to add some histograms for quantification of cell lengths in these populations (obviously, it is hard to see by eyes just 5-10% differences, especially when intra-sample heterogeneity is high).

5. Page 90: “When the CDIP634 strain grows in medium supplemented with ATc to induce the Ptet, intracellular c-di-GMP levels significantly increase (data not shown)”. Why it is not shown? How it was measured? How large the increase was?
6. Histogram of RT-qPCR in Fig. 3.10 (page 93) does not show dispersion.
7. A general question to the section 3.3.2 “Regulation of C. difficile CRISPR-Cas system by c-di-GMP”: On the base of overlapping of the CRISPR12 array with a c-di-GMP-I riboswitch in genome, the author suggested a plausible model of regulation of CRISPR12 activity by c-di-GMP (Fig. 3.7). At the same time, further experiments showed “that expression of both cas-operons and CRISPR6, 12 and 16/15 arrays increased in high c-di-GMP level conditions (Figure 3.10)”. As only CRISPR12 array overlaps with a c-di-GMP-I riboswitch, these data cast doubt on the proposed model for CRISPR12, suggesting some other general mechanisms of c-di-GMP-dependent expression regulation.

Minor points

Figure design:

- Figure 2.13 (page 64). It would be clearer to place both curves on the same plot to demonstrate its similarity.
- In Fig. 4.1B dashed lines are somewhat misleading as they connect not corresponding ends.
- Fig.4.4B (page 122): The minor tics on Y-axes mark 1/9 of the intervals that is quite unusual for the decimal system.

Misprints/grammar:

- Page 19: “The first ncRNAs (RNAs (tRNAs) and the rRNA ribosomal RNAs) were identified in the 1960s transfer in the 1960s.” --> The first ncRNAs (transfer RNAs (tRNAs) and the rRNA ribosomal RNAs) were identified in the 1960s.
- Page 91: “The difference between CDIP634 and 630Δerm strains was clear only in the plasmid, carrying protospacer, corresponding to CRISPR12 spacer1, case.” --> The difference between CDIP634 and 630Δerm strains was clear only in the case of the plasmid, carrying protospacer, corresponding to CRISPR12 spacer1.”
- Page 125: “The previous work, showed that CRISPR repeats …” --> The previous work showed that CRISPR repeats …

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*