REVIEW

by Academician Prof. Ivan Georgiev Ivanov, DSc.
of the thesis of Georgi Yordanov Miloshev *„Design and application of functional nucleic acids for synthetic control of gene expression"*, presented for the awarding of the educational and scientific degree Doctor (PhD) in the Field of Higher Education 4. Natural Sciences , mathematics and informatics, Professional direction 4.3. Biological Sciences, Doctoral Program: Genetics - Bioinformatics

1. General part

Mr. Georgi Yordanov Miloshev has elaborated his doctoral thesis at the Department of Genetics, Faculty of Biology (FB), Sofia University "St. Cl. Ohridski" (SU) in the doctoral program "Genetics and Bioinformatics" under the supervision of Prof. Dr. Robert Penchovski. His thesis, reported and approved at an extended Departmental Council on 13/07/2023, contains 131 pages, 61 figures, 4 tables and 184 references. The overview of the documents shows that the procedure for enrolling and training of the PhD student has followed the requirements of the Law on Development of the Academic Staff in the Republic of Bulgaria (LDASRB) and the Rules for its implementation at the BAS.

2. Brief biographical data

Mr. Georgi Yordanov Miloshev is a graduate of the BF of SU. In 2017, he obtained a BS degree in "Molecular Biology", in 2019 a MS degree in "Genetics and Genomics". Soon after that he joined the BF PhD program of Genetics and Bioinformatics. He has published 4 scientific papers, 2 of which are related to his PhD thesis. He has also participated in the development of 3 research projects funded by the Bulgarian National Research Fund. Mr. Miloshev is the recipient of the "PhD Student of the Year" award of the SU for 2022.

3. Relevance of the topic

Mr. Miloshev's dissertation is dedicated to an important and relevant for modern infectology and bacterial genetics topic - antibiotic resistance of bacteria and search for new approaches to combat resistant pathogens. Taking into consideration the great social significance of antibiotic resistance for modern medicine, the relevance of the thesis topic is obvious.

4. Familiarity with the problem

Judging by the literature review, Mr. Miloshev is well acquainted with the literature in his research field. The review covering 50 print pages is devoted to allosteric ribozymes and small RNAs as a tools for regulation, modulation and inhibition of gene activity. It consists of 5 sections dedicated respectively to the: role of allosteric ribozymes in synthetic biology; approaches and methodology for identifying small RNAs; application of allosteric ribozymes for exogenous control of gene activity; engineering and design of allosteric ribozymes and antisense oligonucleotides, etc.

The review is written in understandable scientific language and impresses with its thoroughness and profound literature analysis. Due to the latter, the student has found his research niche and has formulated clearly the goals of his thesis. In brief, he aims to create a new universal method for inhibiting gene expression in *Escherichia coli* based on the use of hammerhead ribozymes interacting with synthetic antisense oligonucleotides, using the *LacZ* gene as a model. For achieving this goal, he has formulated six specific tasks.

5. Research methodology

To develop his PhD thesis Mr. G. Miloshev's has employed diverse and modern methodology. The methods can be classified roughly as *bioinformatic* and *experimental*. Bioinformatic methods include different software packages for screening of publicly available DNA and RNA databases such as NCBI, Kyoto Encyclopedia of Genes and Genomes, Rfam 14.1.,

Rswitch, etc., and also for the design of antisense oligonucleotides. The experimental methods are described in detail, which allows to be reproduced without using additional methodological literature. By mastering such a rich methodology, the student has achieved one of the goals of his doctoral education, i.e. acquiring experimental knowledge and skills for independent research.

6. Evaluation of the obtained results

The "Results" section covers about 30 pages. It is structured in 6 sections corresponding to the set of 6 tasks, namely: design of antisense oligonucleotides for inhibition of gene expression in *Escherichia coli*; cloning the constructs for gene expression control into a plasmid bearing *LacZ* reporter gene; strategy design for OFF Switch control; design of synthetic antisense oligonucleotides for OFF Switch control of gene expression; design of synthetic hammerhead ribozymes for OFF Switch control of gene expression; experimental verification of the effeciency of antisense oligonucleotides and hammerhead ribozymes in *Escherichia coli*.

To achieve the thesis goals, Mr. Miloshev created an original test system based on the expression plasmid pRS414 in which he cloned an expression cassette containing the bacteriophage λ PL promoter, a strong SD sequence and an initiator (ATG) codon in frame with the beta-galactosidase *lacZ* gene. This system allows evaluation of the influence of antisense oligonucleotides and ribozymes on gene expression by measuring the expression level of beta-galactosidase. A 19-meric oligonucleotide designed to cleave the corresponding mRNA in the region of the SD site was used as an antisense molecule. In order to increase its stability in bacterial cells, the central 11 nucleotides long zone was chemically modified and covalently linked to a cell-penetrating peptide. I am highly appreciating the originality of this system since it allows an easy estimation of the ASO concentration dependence of the *lacZ* gene expression. The results showed that at 2238 μg/ml of ASO bacterial growth was suppressed by 96%. Examining the combined effect of two ASOs one of which is normal and the other one is mutated, Mr. Miloshev verified in full his working hypothesis.

The application of antisense oligonucleotides and synthetic ribozymes as an alternative to antibiotics in fighting pathogenic bacteria is a relatively new field of bacteriology whose success depends on the right selection of target site and the correct design of ASOs. All this requires a good knowledge in both molecular genetics and bioinformatics. Judging by the presented results, MR. G. Miloshev has acquired the necessary knowledge and skills in both areas.

Using the *lacZ* reporter system, the PhD student has observed high levels of beta-galactosidase at all tested time intervals (15, 20 and 30 minutes), which proves the effeciency of the OFF Switch strategy for synthetic control of gene expression based on ASOs of first and second generation. It also proves the correct design of the 19-meric oligonucleotide with 11 central nucleotides modified. The effectiveness of the hammerhead ribozyme working with the 18-meric ASO (5′- CTATTTGGGACTCATCAG - 3′) was also experimentally proven.

Investigating the secondary structure of the hammerhead ribozyme, Mr. Miloshev concluded that the mutations in ASO deteriorated its hybridization with the ribozyme and therefore impaired its efficiency in regulating gene expression. To verify this judgment, he synthesized an oligonucleotide with 5 point mutations, which would give a duplex after hybridization with the hammerhead ribozyme of thermodynamic stability equal to -6.43 kcal/mol. The obtained results show that the mutant oligonucleotide is indeed significantly less effective than the original 18-mer.

Although the gene expression experiments were carried out in *Escherichia coli* and *LacZ* gene only, the new method for specific inhibition of gene expression (by specific ACOs hybridizing with hammerhead ribozymes) could also be applied to the regulation of the expression of other bacterial genes, and may be in eukaryotes?!

The results obtained by Mr. G. Miloshev are illustrated with appropriate figures and tables and are critically discussed in the light of literature data in the Discussion section.

7. Publications related to the thesis

Two articles are published in refereed journals (total IF 0.45) in connection with Mr. Miloshev's thesis, in one of which he is the lead author.

8. Abstract, conclusions and contributions

The thesis abstract adequately reflects the content and achievements of the reviewed dissertation. The formulated conclusions and contributions are objective and correspond to the obtained experimental results.

9. Critical remarks

I have no objections to the peer-reviewed version of Mr. Miloshev's dissertation. I had some for the working version presented to the internal defense and now I find that they are all taken into consideration.

CONCLUSION

Georgi Miloshev's PhD thesis is dedicated to an important and relevant problem for modern infectology and bacteriology - antibiotic resistance in bacteria and the search for new approaches for fighting resistant and pathogenic bacteria. Employing bioinformatics methods, he managed to design and create original synthetic genetic constructs based on the *LacZ* gene for specific inhibition of gene expression using antisense oligonucleotides and hammerhead ribozymes. Their effectiveness have been proven experimentally.

In connection with the peer-reviewed dissertation, 2 articles are published in refereed journals with a total IF of 0.45. Thus the student satisfies the formal minimal requirements of the law to be admitted to defense.

To my opinion, Mr. G. Miloshev is an experienced investigator in the field of molecular genetics, satisfying in full the requirements of the LDASRB for the acquisition of the educational and scientific degree “Doctor" (PhD) in Genetics and Bioinformatics and I am strongly recommending the esteemed Scientific Jury to award it to him.

22/08/2023

Reviewer:

/ Acad. Ivan G. Ivanov/