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Review of the PhD thesis of Krzysztof Kubiak
entitled
"Mechanism of ColE1 plasmid replication regulation by the Hfq protein of *Escherichia coli*"

The PhD thesis of Krzysztof Kubiak has been prepared at the Faculty of Biology of the University of Gdańska, under the joint supervision of Prof. Grzegorz Węgrzyn (University of Gdańsk), and Prof. Veronique Arluison (Université de Paris). The topic of the thesis is the contribution of the chaperone protein Hfq to the regulation of replication of plasmid ColE1. This study is a continuation of previous work from the collaborating labs (Cech, G.M. *et al.*, Plasmid 2014). Hfq is a global RNA binding protein in numerous bacteria, and has pleiotropic effects on the physiology of the bacterial cell. Together with other RNA-binding proteins it plays essential roles in RNA dependent regulation, because it affects RNA stability, assists in RNA-RNA matchmaking, and recruits other protein factors to RNA molecules. Overall, it is thought that Hfq serves as a platform, on which various RNA-dependent processes converge. The studies of the mechanisms involving the Hfq protein are a challenging subject, because Hfq can interact with RNA molecules in different ways using its three distinct RNA-binding sites, and it can have both direct and indirect effects on events occurring in the cell. Hence, it is impressive that the PhD candidate chose this important, but demanding research topic as the object of his research.

The PhD dissertation consists of 5 publications, two of which are research papers (in *International Journal of Molecular Sciences* and *Nucleic Acids Research*), two are

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describing methodologies (in *Methods in Molecular Biology* and *Applied Sciences*), and one is a review (*Frontiers in Molecular Biology*). Krzysztof Kubiak was one of two joint-first authors in the paper in *International Journal of Molecular Sciences*, one of four joint-first authors in the paper in *Methods in Molecular Biology*, and a middle co-author in the other 3 papers. Contributions of co-authors are appropriately described in the included documents. Four of the papers were published in very good international journals, and one in a book published by an international publisher (Methods in Molecular Biology).

The set of publications contained in the thesis is preceded by a short summary of the most important achievements. I do appreciate its presence, but it is quite short. Because the subject of the thesis is " The mechanism … ", and the thesis consists of 5 papers with differing contributions of the PhD Candidate, it would be helpful to provide more in-depth introduction that would explain what is the proposed mechanism, possibly with help of a unifying picture, and how His distinct contributions to the 5 publications helped build this hypothesized mechanism.

The publication by Gaffe, Kubiak *et al.* (*International Journal of Molecular Sciences*, 2021) is focused on analyzing how the antibiotic resistance of bacteria, containing plasmids with different resistance genes, is affected by wild type Hfq, by Hfq with deletion of a 30-aa C-terminal unstructured region, and by the absence of Hfq. The four resistance plasmids tested differed in copy numbers per cell, and carried resistance to chloramphenicol, ampicillin and tetracycline. The data showed that strains with wt Hfq or with C-terminally truncated Hfq, and chromosomally encoded resistance to kanamycin, were susceptible to kanamycin, while the growth of the *hfq* deletion strain was little affected by this antibiotic. The data also showed differences in transformation efficiency between middle copy number plasmid pBR322 and it mutant with a deletion of *rom* gene. While the transformation of pBR322 was negatively affected by truncation of Hfq or the deletion of the whole *hfq* gene, the transformation of pBR322 delta *rom* was increased in these backgrounds. The antibiotic resistance carried by the four plasmids was analyzed by the authors in two ways, either by monitoring colony numbers on plates, which was

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performed by the PhD Applicant, or by monitoring growth in liquid culture. The experiments in liquid culture consistently show weaker antibiotic resistance of *hfq* deletion mutant and *hfq* truncation mutant strains for plasmids with resistance genes to chloramphenicol (1 plasmid), and tetracycline (3 plasmids). In contrast, experiments on plates show differing effects of *hfq* deletion and *hfq* truncation for antibiotic resistance in the same strains. Additional experiments measuring stability of mRNAs for tetracycline and kanamycin resistance seem to show greater mRNA stability in *hfq* deletion strain than in the strain with wt Hfq or with truncated Hfq.

My questions to the PhD Applicant concerning this part of the thesis are as follows. (1) Could you hypothesize what could be the reasons for observed differences in antibiotic resistance versus the *hfq* strain used for plate experiments versus liquid culture experiments? (2) It is not clear for me, if C-terminally truncated hfq gene and kanamycine resistance gene are expressed together as a fusion protein or as independent genes? It is not clear for me from Figure 2. (3) Could you hypothesize what could be the overall mechanism of Hfq involvement in antibiotic resistance for these resistance genes? Would it be one mechanism or several mechanisms specific for individual resistance genes? Do you expect Hfq to be directly involved in the regulation or are there likely to be indirect effects?

In the publication by Malabirade, Jiang, Kubiak *et al.* (*Nucleic Acids Research* 2017) Krzysztof Kubiak performed gelshift experiments to measure the binding of wt Hfq, C-terminally truncated Hfq, and the isolated C-terminal region of Hfq to 1000-nt long DNA molecule. The measured K_d value for wt Hfq was at 1.5 μM, and the binding of both mutants was much weaker. It is not clear for me how the value for wt Hfq was obtained because in the corresponding panel of Figure 6A two complexes are visible on the gel, but their fitting is not clear. Could you clarify?

Additionally, Krzysztof Kubiak contributed to preparing sections on measurement of mRNA levels and on determination of antibiotic resistance in a methodological paper (*Methods in Molecular Biology*, 2022), and designed nucleic acid molecules used in synchrotron radiation CD experiments, as well as prepared figures in the second

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methodological paper (*Applied Sciences*, 2022). He also contributed to preparing a review paper on a related topic (*Frontiers in Molecular Biology*, 2016). All three publications are closely related to the topic of the PhD dissertation and analyze additional aspects of this subject.

To summarize, the PhD dissertation prepared by Krzysztof Kubiak analyzes important topics related to functions of RNA-binding proteins, which are involved in gene expression regulation in bacteria. The results of His studies have been published in very good international scientific journals. The presented work fulfills all requirements required of a PhD dissertation. Hence, I am applying to the Council of the Discipline of Biological Sciences of the Faculty of Biology of the University of Gdańsk to award Krzysztof Kubiak the title of PhD in Biological Sciences.

Because of the high scientific impact of the research results obtained by Krzysztof Kubiak and published in renowned international scientific journals I apply to the Council of the Discipline of Biological Sciences of the Faculty of Biology of the University of Gdańsk to award the PhD dissertation with distinction. The particularly important aspect of His work justifying the application to distinguish the PhD dissertation are his detailed studies of the role of Hfq in the regulation of bacterial antibiotic resistance using specifically designed bacterial strains, which contributed to the elucidation of biological roles played by this important RNA chaperone protein.

Sincerely,

Mikołaj Olejniczak

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