REVIEW of the official opponent
on the dissertation presented by Wang Yueqi for the degree of Candidate of Biological Sciences (Ph.D.) entitled: "Features of the structural organization and interaction of tailed bacteriophages with biofilm-forming bacteria" in the specializations
1.5.2 Biophysics and 1.1.10 Biomechanics and Bioengineering

Relevance of the research topic. The problem of antimicrobial resistance is currently recognized as one of the most serious threats to global public health. The spread of multidrug-resistant bacteria leads to the ineffectiveness of standard antibiotic therapy, increased duration of hospitalizations, higher mortality rates, and enormous economic losses. According to World Health Organization estimates, antimicrobial resistance causes more than 700,000 deaths annually, and in the absence of systemic measures, this figure could reach 10 million per year by 2050. Among the most problematic pathogens, WHO identifies *Acinetobacter baumannii* and *Pseudomonas aeruginosa*—microorganisms that not only possess multidrug resistance but are also capable of forming biofilms, which significantly enhances their survival in healthcare settings and protects bacteria from antimicrobial agents and host immune system factors.

In recent decades, there has been a resurgence of interest in alternative antimicrobial agents, particularly bacteriophages—viruses that selectively infect bacterial cells. Phage therapy is considered one of the promising approaches in combating resistant infections. Bacteriophages have several advantages: high specificity, lack of cross-resistance with antibiotics, ability to self-replicate at the site of infection, and the capacity to penetrate and disrupt biofilm structures. Nevertheless, the widespread clinical implementation of phage therapy is hindered by a range of challenges: the absence of standardized regulatory mechanisms for live biotherapeutic products; a deficit of fundamental knowledge about how different bacteriophages are organized at the molecular level and how their structural features correlate with infection efficiency; insufficient understanding of phage interactions with bacterial biofilms, which are a major cause of chronic infection; the rapid development of bacterial resistance to phages; limited stability of pharmaceutical formulations and the

lack of convenient delivery systems; narrow specificity requiring personalized selection; and a lack of high-quality clinical data on efficacy and safety.

Thus, the aim of the dissertation work—to determine the features of the structural organization and antibacterial action of tailed bacteriophages, as well as to develop bioengineering constructs for their controlled delivery—is relevant from both fundamental scientific and practical healthcare perspectives.

Scientific novelty of the dissertation work. The scientific novelty of the work is determined by the combination, within a single study, of methods from structural biology, microbiology, and bioengineering to investigate the interaction of tailed bacteriophages with biofilm-forming pathogens. This integrative approach made it possible to demonstrate that the effectiveness of phage-biofilm interaction is inseparably linked to the features of their structural organization. The obtained results expand fundamental understanding of the mechanisms of antibacterial action of phages and open up opportunities for the development of new practical strategies to overcome antimicrobial resistance.

Reliability of the obtained results. The reliability of the results of the dissertation work is ensured by the representative volume of experimental studies conducted, the use of modern and complementary methods (cryo-electron microscopy and tomography, scanning electron microscopy, Raman spectroscopy, and microbiological approaches), as well as by statistical processing of the obtained data where required by the experimental design. All key results have been published in peer-reviewed scientific journals indexed in international databases, which confirms their independent expert evaluation. The conclusions of the dissertation logically follow from the presented experimental data.

Structure of the dissertation work. The dissertation work of Wang Yueqi is written in English and is accompanied by an abstract written in Russian. The work is presented on 149 pages and includes the following sections: "List of abbreviations", "Introduction", "Literature review", "Materials and methods", "Results", "Discussion", "Conclusion", "Conclusions", "Acknowledgments", "References", and

"Supplementary materials". The dissertation contains 57 figures and 3 tables. The reference list includes 124 sources.

The Introduction describes the relevance of the research, formulates the aim and objectives of the work, presents the scientific novelty, theoretical and practical significance of the work, provides data on the methodology and research methods, formulates the provisions submitted for defense, and also indicates the degree of reliability and the results of the research.

The "Literature review" chapter discusses current concepts of bacteriophage diversity and classification, their structural organization and antimicrobial potential, including medical applications for the treatment of wound infections and biofilm-associated infections. The mechanisms of phage interactions with bacterial biofilms are analyzed in detail, including enzymatic degradation of the matrix, diffusion through water channels, modulation of quorum sensing systems, and overcoming bacterial defense systems. The review also characterizes the main methods for studying phage-bacterium interactions, including direct visualization and quantitative approaches.

The "Materials and methods" chapter describes the bacterial strains used (including reference strains and clinical isolates) and bacteriophages, and presents protocols for phage cultivation, purification, and titration. The methods of cryo-electron microscopy and tomography are detailed, including sample preparation, data collection, and image processing using RELION, AlphaFold, and PHENIX software for atomic model building. The procedures for obtaining silk fibroin-based scaffolds with various modifications (gelatin, chitosan, polyethyleneimine), their functionalization with bacteriophages, as well as methods for assessing swelling, degradation, pore morphology, Raman spectroscopy, and phage diffusion are also provided. Approaches to biofilm visualization using scanning and transmission electron microscopy, as well as methods for quantitative assessment of biofilms and cell viability evaluation, are described.

The "Results" chapter presents four blocks of experimental data. The first block describes the structure of the head-tail complex of the *A. baumannii* phage TaPaz at 3.18 Å resolution and presents an atomic model of the portal complex. In the second

block, electron tomography was used to visualize the early stages of phage phiKZ infection, including the formation of vesicles protecting viral DNA, and a three-stage model of early infection is proposed. The third block examines the influence of biofilm architecture and pH on phage efficacy using *Bacillus subtilis* and *Pseudomonas aeruginosa* as examples. The fourth block characterizes the developed silk fibroin-based scaffolds functionalized with PEI and bacteriophages AR9 and PB1.

The "Discussion" chapter provides additional discussion of the obtained results and offers the author's interpretation.

Questions and comments. First of all, it should be noted that the dissertation work of Wang Yueqi is carried out at a high multidisciplinary level using modern research methods. The author has obtained extensive and diverse experimental material, and overall the work gives the impression of a thorough and qualified study. However, as personal experience shows, such a wide range of methods is often accompanied by difficulties when attempting to integrate the obtained data into a single coherent conceptual model. Turning to this dissertation, it should be noted that its title is formulated extremely broadly—"Features of the structural organization and interaction of tailed bacteriophages with biofilm-forming bacteria"—and, in essence, covers the entire diversity of tailed phages and all biofilm-forming bacteria, which, of course, does not correspond to the actual scope of the presented material. In this regard, I have the following conceptual questions concerning primarily the formulation of the aim and the structure of the work.

1. The dissertation consists of several sections: a structural study of the TaPaz phage, an investigation of the early stages of infection by the phiKZ phage, an examination of phage interactions with biofilms, and the development of delivery scaffolds. Each of these sections is noteworthy in its own right; however, the connections between them are not clearly articulated in the work. The structural study was performed on the TaPaz phage, which infects *A. baumannii*, while the biofilm experiments and scaffold development were conducted using other phages (phiKZ, PB1, AR9) and other bacteria (*P. aeruginosa*, *B. subtilis*). It remains unclear how knowledge of the portal complex structure of a single phage can be used to understand

the antibacterial activity of other phages or to design delivery systems. Why was the TaPaz phage not used for the biofilm and scaffold experiments, and how, in the author's opinion, do the structural data on TaPaz relate to the rest of the work? The same applies to phiKZ: what was the rationale for choosing this particular phage for the study, and how does this section fit into the overall concept of the work?

2. The paper includes a section devoted to the interaction of phage AR9 with *B. subtilis* biofilms. It should be noted that this is a rather elegant experiment, demonstrating both the influence of the medium on biofilm formation and the dynamics of its formation over time. Modern studies often overlook such patterns. Nevertheless, this section is practically not described in terms of methodology: some important parameters are missing from the relevant chapters (for example, the composition of the NB and DSM media). Furthermore, the results for this section are not reflected in either the abstract or the conclusions of the dissertation. This raises several questions. First, why is the experiment limited to 6 hours for the dense biofilm, whereas the loose biofilm is monitored for up to 20 hours? Was a control experiment conducted with a five-day-old biofilm incubated for 20 hours without phage to rule out its natural degradation due to cell aging? Second, was cell viability assessed, or was the analysis limited to SEM morphology alone?

3. The study describes scaffolds made of silk fibroin, functionalized with PEI and bacteriophages, and demonstrates their antibacterial activity against planktonic cultures of *P. aeruginosa* and *B. subtilis*. However, biofilms, which are the main cause of chronic infections in wounds and on implants, have a fundamentally different architecture and resistance to external influences. This raises the question: are there plans to test the developed scaffolds on biofilm models *in vitro* or *in vivo*, and how, in the author's opinion, do the results obtained on planktonic cultures correlate with the potential application of the scaffolds for treating infections associated with biofilms?

Below are a number of comments that are primarily of a technical and terminological nature.

1) As far as can be judged, the author does not fully master the terminology. For example, it is somewhat strange to read about the classification of bacteriophages where there is no reference to the International Committee on Taxonomy of Viruses, and bacteriophages of the class *Caudoviricetes* are classified under an order (even though this is a class). This remark also applies to other sections. Unfortunately, it should be noted that the "Discussion" section is more of a summary of results than a thorough comparison of the author's data with those of other researchers.

2) In Chapter 3.5, devoted to the interaction of phages with bacterial biofilms, subsection 3.5.6 describes how phages overcome restriction-modification and CRISPR/Cas systems. These mechanisms are not specific to biofilms and operate regardless of whether bacteria are in a planktonic state or within a biofilm. The inclusion of this material in the section on biofilms disrupts the logical structure of the review. Furthermore, the issue of bacterial resistance to bacteriophages is a “hot” topic in modern microbiology and is not limited to the examples provided;

3) The paper lacks a summary table listing the bacteriophages and bacterial strains used, which significantly hinders the comprehension of the material. The names of phage taxa are given in the text, but there are no references to genomic databases (e.g., GenBank, INPHARED), which prevents readers from examining their genomic organization and conducting a comparative analysis. For the TaPaz phage, whose structure has been solved at high resolution, accession numbers for the amino acid sequences of individual open reading frames (ORFs) are not provided. It remains unclear whether the genome annotation was improved based on the obtained atomic model, and if so, what specific changes were made.

4) For clinical isolates of *P. aeruginosa*, lysis zones on agar plates and a quantitative assessment of biofilm disruption (Crystal Violet) are shown. However, I did not find a comparison of phage titer on the studied strains relative to the host strain in the paper. Without this data, it remains unclear whether, for example, the high resistance of Ur14 is due to problems with penetration through the EPS or whether the strain itself poorly supports phage replication.

At the same time, these comments do not diminish the significance of the dissertation research. The dissertation meets the requirements established by Lomonosov Moscow State University for works of this kind. The content of the dissertation corresponds to the specializations 1.5.2 Biophysics and 1.1.10 Biomechanics and Bioengineering (in biological sciences), as well as the criteria defined in paragraphs 2.1-2.5 of the Regulations on the Awarding of Academic Degrees at Lomonosov Moscow State University. The dissertation research is formatted in accordance with the requirements of the Regulations on the Dissertation Defense Council for the Degree of Candidate of Sciences and for the Degree of Doctor of Sciences at Lomonosov Moscow State University.

Thus, the candidate Wang Yueqi deserves the award of the degree of Candidate of Biological Sciences in the specializations 1.5.2 Biophysics and 1.1.10 Biomechanics and Bioengineering.

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