ABSTRACT

For the dissertation for Doctor of Philosophy (PhD) degree
on the speciality "8D05104 – Genetics" of
BAKIYEV SERIK SAMIGULLOVICH
on the theme
Construction of bacteriophage endolysins and evaluation of their effectiveness in the inactivation of bacterial pathogens of sturgeons

General description of the dissertation. The dissertation work is aimed at studying the antibacterial activity of endolysins of bacteriophages against bacterial pathogens that cause diseases of sturgeons grown in conditions of recirculating aquaculture systems (RAS).

Relevance of the research topic. Growing sturgeon in aquaculture is one of the important actions aimed at solving the problem of declining populations of sturgeon fish species. In addition, sturgeon aquaculture production has increased significantly in recent years due to the high demand for black caviar in the world market. However, the rapid development of aquaculture is accompanied by outbreaks of diseases caused by bacterial infections, which lead to high mortality and catastrophic economic losses. The most severe bacterial diseases in sturgeon aquaculture are infections caused by bacteria of the genus *Aeromonas* and *Pseudomonas*, fish mortality from these bacterial pathogens reaches up to 100%.

In Kazakhstan, most sturgeon species are farmed in aquaculture and bacterial pathogens are known to be the main causes of mortality in these fish, although information on sturgeon disease and health control is limited to date. Currently, due to the widespread and often uncontrolled use of antibiotics, the number of antibiotic-resistant bacteria has increased dramatically and is a leading cause of morbidity and mortality. This phenomenon may not only lead to failure of antimicrobial therapy, but also raise concerns about the safety of fish products. For this reason, new strategies to combat these drug-resistant pathogens are urgently needed. Bacteriophage therapy is being considered as one of these alternatives. Phage therapy experiments have shown promising results in killing several pathogenic bacteria in aquaculture. However, the effectiveness of phage therapy in aquaculture depends on environmental factors such as salt concentration, pH, temperature etc. In addition, bacteria may also develop resistance mechanisms to phage infection.

Currently, endolysin therapy is considered a very promising alternative for the treatment of complex infections. Endolysins are phage-encoded enzymes that possess peptidoglycan hydrolase activity and therefore capable of destroying the bacterial cell wall, allowing the phage to leave the host cell after replication. Experimental data were obtained under *in vitro* and *in vivo* conditions demonstrating the impressive effectiveness of endolysins in killing bacterial cells, including multidrug-resistant bacteria. Unlike antibiotics and bacteriophages, bacterial strains do not develop resistance to endolysins.

The field of endolysin research has accelerated significantly over the past ten years. Several endolysins, developed by various companies, including chimeric endolysins, primarily against Gram-positive and Gram-negative pathogens of humans and animals, are currently in preclinical and clinical trials. However, the potential of endolysins as antibacterial agents has not yet been explored in the field of aquaculture, although cultured fish, like other animals and humans, are constantly under threat of microbial attack.

The purpose of the research. Determination of the effectiveness of the use of bacteriophage endolysins in the inactivation of bacterial pathogens that cause diseases of sturgeons in industrial aquaculture conditions.

Research objectives:

1. Physiological, biochemical and molecular identification of pathogenic bacteria of the genus *Aeromonas* and *Pseudomonas* isolated from infected sturgeons.
2. Cloning and functional expression of 6xHis-tagged bacteriophage endolysins in *E. coli* and purification of recombinant proteins.
3. Construction of chimeric endolysins with increased lytic activity against pathogenic bacteria that cause diseases of sturgeons in aquaculture.
4. Characteristics of antibacterial activity *in vitro* and *in vivo* parental and constructed new chimeric endolysins.
5. Determining the possibility of endolysin therapy for skin lesions of diseased *A. baerii*.

The research objects. Endolysins (OBPgp279, Gp110, LysPA26 and their chimeras) of bacteriophages and pathogenic bacteria of the genus *Aeromonas* and *Pseudomonas* of sturgeons.

Subject of the research. Antibacterial effect of endolysins against bacterial pathogens of sturgeons.

Research methods. When conducting research as part of the dissertation work, the following methods were used: microbiological, biochemical, molecular genetic.

The scientific novelty of the research. Currently, the potential of endolysins as antibacterial agents has not yet been explored in the field of aquaculture, although cultured fish, like other animals and humans, are constantly under threat of microbial attack. The presented research work is aimed at studying the therapeutic potential of endolysins representing different types of domain organization and having different origins. And also, the development of new effective chimeric endolysins with increased lytic activity against Gram-negative and antibiotic-resistant bacteria, which are the main cause of sturgeon diseases in aquaculture.

Theoretical and practical significance of the research. It has been established that the endolysin Gp110, as well as the chimeric endolysins we constructed, have pronounced antibacterial activity against pathogens of the genus *Aeromonas.* Based on the proposed chimeric endolysins, finished drugs with high antibacterial activity can be developed, including against antibiotic-resistant strains of the genus *Aeromonas* and *Pseudomonas.* A significant scientific and technical basis has been created to initiate pharmaceutical development.

The main provisions for the defence:

1. *A. hydrophila* AB005 and *A. salmonicida* AB001 are highly virulent strains from isolated and identified bacterial isolates, capable of causing severe diseases of sturgeons, characterized by 100% mortality.
2. Highly virulent strains *A. hydrophila* AB005 and *A. salmonicida* AB001 contain seven genes out of 10 virulence genes studied.
3. All isolated bacterial isolates are characterized by multidrug resistance to antibiotics. Among the bacterial strains studied, *P. parafulva* AB004 and *P. protegens* AB006 strains are resistant to 15 of the 19 antibiotics tested.
4. Recombinant parental and chimeric endolysins were obtained by microbiological synthesis with chromatographic purification.
5. Recombinant parental and chimeric endolysins exhibit activity against *Aeromonas* species, including those with antibiotic resistance.
6. Recombinant endolysin Gp110 and chimeric endolysin Gp110 (CWBD) / LysPA26 (CD) showed the most pronounced antibacterial effect against *Aeromonas* species under *in vitro* and *in vivo* conditions.
7. The effectiveness of endolysin therapy for skin lesions of diseased *A. baerii* caused by aeromonosis has been shown.

Main research results and conclusions:

1. Based on phenotypic and biochemical properties, six cultures of gram-negative bacteria of the genus *Aeromonas* and *Pseudomonas* were isolated and identified from diseased sturgeons (*A. baerii*), capable of growing in a wide temperature range from 13 to 42 ºC, with a pH value of 5.0– 9.0 and NaCl concentrations from 0 to 5%
2. Based on the results of genotyping, determination of phenotypic and biochemical properties, the species affiliation of the isolates was determined. The following bacterial species have been identified: *A. hydrophila* AB005 (OK634406, 16S rRNA; ON124027, *gyr*B), *A. salmonicida* AB001 (OK634025, 16S rRNA; ON124026, *gyr*B; OQ144653, *rpo*D; OQ144652, *fla*A), *A. veronii* AB003 (OK634393, 16S rRNA), *P. parafulva* AB004 (OK634400, 16S rRNA), *P. protegens* AB006 (OK635331, 16S rRNA), all obtained nucleotide sequences are included in the NCBI GenBank database.
3. It has been shown that strains *A. hydrophila* AB005, *A. salmonicida* AB001 and *A. bestiarum* AB002 are highly virulent and capable of causing severe diseases accompanied by pallor of the gills, extensive hemorrhages in various parts of the body, circulatory disorders and necrosis of internal organs in *O. niloticus*, *O. mossambicus*, *A. baerii* and *A. ruthenus*. The mortality rate of experimental fish reaches 100%. The *A. veronii* strain turned out to be less pathogenic with a 30% mortality rate. In contrast to the genus *Aeromonas*, the isolated bacteria of the genus *Pseudomonas* did not show pathogenicity.
4. Analysis of virulence genes in the bacterial genome confirmed the presence of the largest number of virulence factors in strains *A. hydrophila* AB005 and *A. salmonicida* AB001, seven genes each (*aer*B, *ast*, *pla*, *ahe2*, nucl, *gca*T, *aer*A; *hly*A, *aer*A, *alt*, *ahp*B , *gca*T, *pla*, *ahe2*, respectively) of the 10 studied, as well as six genes

found in *A. bestiarum*, the *A. veronii* isolate was characterized by the presence of only 3 virulence genes.

5. It was shown that the most resistant to antibiotics among the studied bacterial strains were the strains *P. parafulva* AB004 and *P. protegens* AB006, which showed resistance to 15 of the 19 antibiotics studied. The strains *A. hydrophila* AB005, *A. salmonicida* AB001, and *A. bestiarum* AB002 were characterized by the least resistance to antibiotics; for these isolates, 9 antibiotics were sensitive.

6. The endolysin genes Gp110, OBPgp279 and LysPA26 of bacteriophages were cloned, as well as the genes of four new chimeric endolysins constructed. Functional expression of histidine-terminated genes in *E. coli* and affinity purification of recombinant endolysins were carried out. Using the cell walls of *A. hydrophila* strain AB005 as a substrate, the peptidoglycan hydrolyzing activity of purified recombinant endolysins was determined.

7. Gp110, OBPgp279 and LysPA26 recombinant endolysins showed a broad but varied spectrum of bactericidal activity depending on the pathogen species used. All three endolysins showed increased activity against *A. veronii*. Relatively low activity of all enzymes was observed against *A. salmonicida*. No antibacterial activity was observed against the Gram-positive bacteria *S. aureus*.

8. The most pronounced effect was observed for Gp110. The results obtained *in vitro* were confirmed by the results of *in vivo* assays, as the survival of infected *O. niloticus* was higher when *O. niloticus* was treated with endolysin Gp110 than when treated with OBPgp279 or LysPA26 alone.

9. Chimeric endolysins Gp110 (CWBD) / OBPgp279 (CD), Gp110 (CWBD) / LysPA26 (CD), OBPgp279 (CWBD) / LysPA26 (CD) showed high efficiency against bacterial species of the genus *Aeromonas.* Among the chimeras, the novel chimeric endolysin Gp110 (CWBD) / LysPA26 (CD) showed higher antimicrobial activity than its parent endolysin LysPA26.

10. Therapeutic trials of endolysins in the Nile tilapia model showed that recombinant endolysins Gp110 and Gp110 (CWBD) / LysPA26 (CD) provided 100% survival of infected *O. niloticus*. The possibility of endolysin therapy for skin lesions of diseased *A. baerii* with aeromonosis has been determined. The percentage of wound closure in the fish treated with Gp110 was 41.8% on the 6th day, 79% on the 12th day, and 95.7% on the 25th day, respectively. Our results indicate that Gp110 and Gp110 (CWBD) / LysPA26 (CD) are promising candidates for the development of therapeutics against *Aeromonas* infection in aquaculture.

Personal contribution of the author. The author carried out the selection of diseased sturgeons into quarantine basins, collection of biological materials for the isolation of bacteria, identification of bacterial pathogens, determination of the pathogenicity of isolated strains, submission of the obtained information on bacterial isolates to the NCBI database, cloning, obtaining constructs and purification of endolysin proteins, analysis of the antibacterial activity of endolysins *in virto* and *in vivo* conditions, writing theses and scientific articles, writing a dissertation according to an established plan agreed with scientific consultants.

Connection with the plan of basic scientific work. The dissertation work was carried out within the framework of the grant funding project of the Committee of Science MSHE (MES) of the Republic of Kazakhstan for 2021-2023 AP09259735 “Development and evaluation of bacteriophage chimeric endolysins to combat multidrug-resistant Gram-negative pathogens of sturgeon fish”

Approbation of works. The materials of the dissertation work were reported and discussed at the following conferences: International scientific conference of students and young scientists “Farabi Alemi” (2020-2023, Almaty, Kazakhstan); VIII International conference “Modern biotechnology for science and practice” (22-23 April 2021, St. Petersburg, Russia); The 5th Symposium on EuroAsia Biodiversity (1-3 July 2021, Mugla, Turkey; Almaty, Kazakhstan).

Publications. The main content of the dissertation is reflected in 14 published works, including 3 articles in publications included in the first (Q1) and second (Q2) quartiles of the Scopus database; 3 articles in journals included in the list of CQAFSHE MSHE RK; 8 theses in materials of international conferences.

Scope and structure of the dissertation. The dissertation includes an introduction, literature review, research materials and methods, results of own research, conclusions, list of references and applications. The dissertation is presented on 166 pages of computer text, designed in compliance with the required standards, includes 12 tables, 64 figures. The list of used literature contains 333 sources.