Summary of Ph.D. Thesis

Phage infection reinstates antibiotic sensitivity in MDR *Pseudomonas aeruginosa:* A study on phage and bacterial evolution.

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1.0. BACKGROUND

1.1 *Pseudomonas* aeruginosa

Pseudomonas aeruginosa is a ubiquitous Gram-negative bacterium occurring almost everywhere in the environment. This bacterium can infect a wide range of hosts, including humans, animals and plants. In humans, it is one of the leading pathogens that cause infections in vulnerable patients. These infections include cystic fibrosis (CF) and obstructive pulmonary diseases in patients who have permanent bladder catheters or who have been intubated for a long period of time. Similarly, *P. aeruginosa* is a frequently occuring pathogen in burn wounds, diabetic foot ulcers, otitis media and keratitis.

In 2018, the WHO warned of a new wave of Gram-negative pathogens that are resistant to multiple antibiotics, listing one of them as *P. aeruginosa*. A recent survey by the European Antimicrobial Resistance Surveillance Network (EARS-Net) raised the alarm about eight increasingly prevalent drug-resistant bacteria species in the EU.

Nearly all clinical cases of *P. aeruginosa* infection can be correlated with the compromise of host defenses. *P. aeruginosa* infection has acquired a special status because it has become the leading cause of morbidity and mortality in CF patients. A recent study revealed a great prevalence of *P. aeruginosa* infections in ICU (Intensive Care Unit) patients in Europe. Beyond the striking virulence, it has intrinsically evolved resistance to 'drugs of last resort,' resulting in emergent strains that are pan-drug-resistant. Infections caused by this strain of bacteria are challenging to treat because of the strain's physical adaption to its host and its genomic plasticity. Designing and developing new alternative solutions is the only way to stop the rising crises related to drug-resistant bacteria. One of the oldest and most efficient alternative solutions is the use of bacteriophage (or phage) therapy. Bacteriophage-based therapy exploits the ability of phages to selectively infect compatible bacterial hosts.

Phages can conduct precise bactericidal activity by killing only a particular host with low inherent toxicity. They are the only auto-dosing antibacterial agent capable of increasing their numbers in the specific site where the host is located. Compared to other therapeutic agents, a single infection can produce an exponential pattern, showing a continuous killing effect. The rise of antimicrobial resistance and the paucity of new antibiotics led to the rediscovery of phage therapy.

2.0. GOALS AND OBJECTIVES

As antibacterial agents, phages have several advantages that make them convincing alternatives to antibiotics. Nevertheless, not all phages make for promising therapeutics. A decent therapeutic phage should have a high potential to reach and overcome bacterial virulence, the bacterial resistance or defence shields discussed above. The existence of these characteristics can be reasonably ascertained by a detailed study on bacteria and phages.

The main goals are as follows:

1) To isolate and characterize drug-resistant *P. aeruginosa* from clinical samples.
2) To isolate and characterize potential phages that target MDR *P. aeruginosa.*
3) To evaluate the efficacy of isolated phages against *P. aeruginosa* biofilm.
4) To isolate and characterize phage-resistant strains.
5) To reveal the relationship between phage resistance and antibiotic sensitivity in *P. aeruginosa.*
6) To disclose the molecular background and evolutionary reasons for the formation of two types of mutants.
7) To assess the efficacy of phage and antibiotic combinational therapy *in vitro*.
8) To ascertain the efficacy of combinational therapy *in vivo* using a mouse lung infection model.
9) To establish the role of PAK-like phages in bacterial genome deletions.
10) To isolate and characterize PAPSZ1 mutants that can block/suppress bacterial resistance.

3.0. MATERIALS AND METHODS

3.1. Bacterial strains and Bacteriophage isolation

Bacterial isolates were acquired from the Clinical Microbiology Laboratory, University of Szeged over the course of a year. The strains were identified by MALDI-TOF MS and 16S ribosomal RNA gene sequencing. We assayed different clinical sources, including swabs from an infected wound and blood samples, for *P. aeruginosa*–specific bacteriophages. Phage DNA isolation was carried out and sequenced the genomes on the Illumina MiSeq platform.

3.2. Biofilm inhibition assay

For the light microscopy and confocal light scanning microscopy (CLSM) study, the biofilm was grown on glass slide pieces (1 x 1 cm) placed in 24-well polystyrene plates. The stained cells were detected using a 488 nm argon laser and a 500e640 nm bandpass excitation filter. The biofilm stack images were then analysed using COMSTAT software.

3.3. Determination of PIAS phage infection efficiency.

The PIAS phage infection efficiency was assayed in 96-well plates for 24 h. We treated bacterial samples with of phage stock with different multiplicity of infection (MOI) (0.1, 1.0, and 10.0). We measured the OD_{600nm} every 3 h for 24 h using a Thermo Multiskan Ascent Plate Reader. The latent period, burst size, adsorption assay and mutant frequency were determined.

3.4. Isolation of phage mutants (E-PIAS and PAPSZ1 derivatives).

We used the double agar plaque assay method for phage mutants isolation. We mixed 200 µl cultures with the same number of PIAS (MOI 1.0). After 20 min incubation at room temperature, the suspension was mixed with 5 mL warm, soft agar (4 g/L) and poured onto LB agar. Following 64 h incubation at 37°C, the plate's soft agar was scraped and homogenized in 5 mL of phage suspension buffer. This suspension was centrifuged and filtered through 0.22 µm pore-size filters. Finally, we diluted the phage suspension in tenfold increments and plated them with overnight bacterial mutant culture (SNP mutant). After 24 h, we isolated single plaques and carried out purification and genome sequencing as described above.

3.5. Elimination of bacteria with combined PIAS phage and antibiotic therapy in vitro

We mixed 100 µl from an overnight-grown sensitive strain with PIAS (MOI 1.0) and 3 mL of the molten soft agar and overlaid it on the surface of the solidified LB agar. We allowed each overlay to solidify for 20 min and incubated them at 37°C for 48 h. To investigate the combination therapy, we plated the above mixture on solid antibiotic agar with soft agar containing various antibiotics (FSF, GMN, TET and CAZ). In addition, we spread culture without phage as control onto the antibiotic-containing agar plates.

3.6. In vivo rescue experiments in mouse lung infection model

P. aeruginosa clinical strain PA16 was used as the host bacterium. We used PIAS and FSF as therapeutic agents. We assayed the potential therapies in a lung infection model using 8–10-week-old BL67 black female mice (C57/BL67 weight: 18–25 g, origin: Charles River Laboratories). PA16 grew up to OD_{600nm} ≈0.5 in 50 mL of LB broth at 37°C with shaking at 120 rpm. The bacterial suspension in the log phase was centrifuged (for 1 min at 12,000 rpm), washed with PBS, and the OD_{600nm} was set to 1.0. The suspension was diluted to 5× and 15×diluted suspension was administered through the nostrils of intraperitoneally anesthetized mice. Five days after the bacterial challenge, we administered PBS, FSF, PIAS phage, and their combinations for both the control and the treatment groups once intraperitoneally.

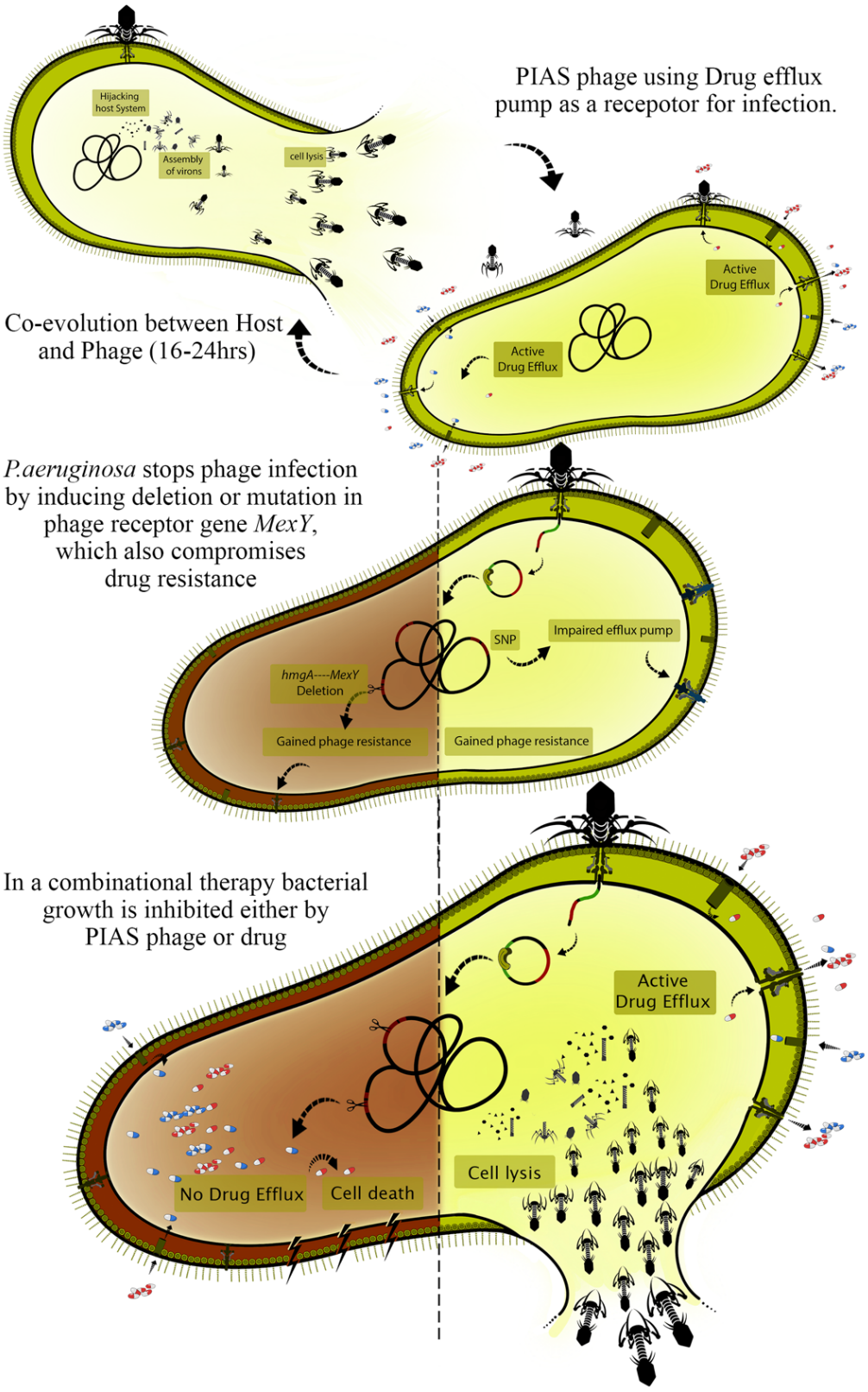
4.0. RESULTS

This thesis was written based on three projects carried out at the University of Szeged, Department of Biotechnology. The study began with the isolation and characterization of novel phages against MDR *Pseudomonas aeruginosa*. This process was followed by an investigation of efficacy of these phages against *in vitro P. aeruginosa* biofilm. The isolated phages were later characterized based on their morphologies, genomes and infection spectra. During this study, we isolated two novel PAK-like phages capable of provoking single mutations and genomic deletion in MDR *P. aeruginosa.* Further study on these two phages has uncovered new insights into phage and bacterial coevolution. This window was used to explore the possibilities of treating MDR resistance infection in an *in vivo* mouse model.
The following points summarize the primary findings of my research:

1) We isolated and identified 25 clinical *P. aeruginosa* strains. The highest antimicrobial susceptibility, which was 100%, pertained to polymyxins. The lowest susceptibilities belonged to meropenem (3%) and fosfomycin (5%).
2) We isolated eight lytic phages (PIAS, PAPSZ1, PAPSZ2, PAPSZ3, PAPSZ4, PAPSZ5, PAPSZ6 and PAPSZ7).
3) The phages' biofilm infection efficiency was studied using confocal laser-scanning microscopy. *In vitro* biofilm study showed that PAPSZ2 could disrupt biofilm most effectively.
4) Further study on PIAS and PAPSZ1 phage infections led to the sequential appearance of phage-resistant colonies with two phenotypes. The initial set of colonies was green (pale). These colonies turned brown in the presence of phages. The colonies were isolated and screened against phages and antibiotic sensitivity. The isolated mutants exhibited resistance to phages, but they had decreased MIC for gentamicin, ceftazidime, tetracycline and fosfomycin.
5) Genome analysis of these mutants revealed they had undergone significant mutations. The PIAS-phage infection resulted in green colonies harbouring SNPs and relatively small deletions (20–80 kbp**)**. The PAPSZ1 phage provoked only SNPs. Phage-provoked brown mutants had larger genome deletions (274–417 kb), however. After PIAS infection, a closer investigation of several genomic data revealed that SNPs change in the *mexY* gene is responsible for phage resistance and antibiotic sensitivity.

6) We investigated the evolutionary explanation for the two types of mutants: green (pale) and brown. We uncovered phage mutants capable of infecting green mutants from previously infected plates. Comparison of their genomes and that of the wild-type PIAS revealed that phages had SNPs in the phage tail fiber protein, which enabled them to infect the green bacterial mutant.
7) After achieving this new insight into the evolutionary arm race between hosts and phages, we comprehensively eradicated mutants by treating MDR strain with an antibiotic (fosfomycin, gentamycin, tetracycline and ceftazidime) combined with PIAS. The *in vitro* study with PIAS-antibiotic combination completely prevented the formation and growth of mutants after a 48-h incubation period. We confirmed this finding in a broth susceptibility assay, and we found that combination therapy outperforms either phage or antibiotic treatments alone.
8) After a successful *in vitro* experiment, we experimented with same strategy in an *in vivo* rescue mouse lung infection model. Combinational therapy, combined PIAS (MOI 1 and 2) with FSF, saved 5% of the animals. In the case of MOI 5 applied with FSF, 75% of the animals survived. Combination therapy ensured a 100% survival rate among mice in groups in which the bacterial challenge was performed with the 15-time diluted bacterial inoculum.
9) The study on PIAS phages revealed that PAK-like phages can undergo rapid mutation to overcome temporary bacterial resistance. When we compared the frequencies of the green and brown mutants formed after PIAS and PAPSZ1 infection. The PAPSZ1 infection yielded lower mutant frequency than PIAS. Additionally, PAPSZ1 had a broader host specificity compared to PIAS. Considering these properties, we selected PAPSZ1 over PIAS to investigate whether phage mutants can prevent/suppress the formation of bacterial resistance. We intently isolated multiple PAPSZ1 mutants after a continuous evolutionary infection cycle.
10) The phage mutants were classified into four categories based on the nature of their mutations. The PAPSZ1/M1 mutant category had single SNP change in the *tfp* (tail fiber protein). The PAPSZ1/M2 mutant contained two mutations in *tfp* as well as an SNP in phage tail length tap-measure protein, *tltmp* gene. PAPSZ1/M3 had 4 SNPs *tfp*, one SNP in *tltmp* and 1-1 insertion in the HP and the BPP gene. Compared to PAPSZ1/M3, PAPSZ1/M4 harboured two extra mutation in the *tfp* gene. Further study showed that the phage mutants PAPSZ1/M3 and PAPSZ1/M4 overcame bacterial phage resistance.

Summary Diagram



Overview of the phage-induced coevolutionary process leading to phage resistance and antibiotic sensitivity.

6.0. LIST OF PUBLICATIONS

LIST OF PUBLICATION RELATED TO THIS THESIS

MTMT Author ID: 10053210

Cumulative impact factor: 17.167

Koderi Valappil, S., Shetty, P., Deim, Z., Terhes, G., Urbán, E., Váczi, S., Patai, R., Polgár, T., Pertics, B. Z., Schneider, G., Kovács, T., & Rákhely, G (2021). Survival Comes at a Cost: A Coevolution of Phage and Its Host Leads to Phage Resistance and Antibiotic Sensitivity of *Pseudomonas aeruginosa* Multidrug Resistant Strains. *Frontiers in Microbiology* 12, 1–17. DOI:10.3389/fmicb.2021.783722. (IF_{2021}: 5.640)

Horváth, M., Kovács, T., **Koderi Valappil, S**., Ábrahám, H., Rákhely, G., and Schneider, G. (2020). Identification of a newly isolated lytic bacteriophage against K24 capsular type, carbapenem resistant *Klebsiella pneumoniae* isolates. *Scientific Reports* 10. doi:10.1038/s41598-020-62691-8. (IF_{2020}: 4.130)

OTHER PUBLICATIONS

Molnár, J., Magyar, B., Schneider, G., Laczi, **Koderi Valappil, S**. K., Kovács, Á. L., Nagy, I. K., Rákhely, G., & Kovács, T. (2020). Identification of a novel archaea virus, detected in hydrocarbon polluted Hungarian and Canadian samples. *PLoS ONE* 15. DOI:10.1371/journal.pone.0231864. (IF_{2020}:3.240)

Deim, Z., Dencső, L., Erdélyi, I., **Koderi Valappil, S**. K., Varga, C., Pósa, A., Makrai, L., & Rákhely (2019). Porcine circovirus type 3 detection in a Hungarian pig farm experiencing reproductive failures. *Veterinary Record* 185, 84. DOI:10.1136/vr.104784. (IF_{2019}:3.357)

Kovács, T., Molnár, J., Varga, I., Nagy, I. **Koderi Valappil, S**. K., Papp, S., Vera Cruz, C. M., Oliva, R., Vizi, T., Schneider, G., & Rákhely, G. (2019). Complete Genome Sequences of 10 *Xanthomonas oryzae pv. oryzae* Bacteriophages. *Microbiology Resource Announcements* 8. DOI:10.1128/mra.00334-19. (IF_{2019}:0.800)

CONFERENCE ABSTRACTS

Koderi Valappil, S., Shetty, P., Deim, Z., Terhes, G., Urbán, E., Váczi, S., Patai, R., Polgár, T., Pertics, B. Z., Schneider, G., Kovács, T., & Rákhely, G. (2018). Characterization of Novel Lytic Bacteriophages Against *Pseudomonas aeruginos*a Biofilm. The 5th World Congress on Targeting Infectious Diseases: Targeting Phage & Antibiotic Resistance 2018**,** Florence, Italy in May 17-18, 2018. Suppl. 1 Pp. 123. (2018)

Rákhely, G., Varga, I., Molnár,J., Gazdag, A., Szűcs, D., Doffkay, Z., **Koderi Valappil, S.,**, Papp, S., Pintér, R., Cruz, C., Vizi, T., Schneider, G., Kovács, T. Comparative Genomics Of Xop2-Like *Xanthomonas Oryzae Pv. Oryzae* Bacteriophages Acta Microbiologica Et Immunologica Hungarica 64 : Suppl. 1 Pp. 160-161. , 2 P. (2017)

Koderi Valappil, S., Shetty, P., Deim, Z., Terhes, G., Urbán, E., Váczi, S., Patai, R., Polgár, T., Pertics, B. Z., Schneider, G., Kovács, T., & Rákhely, G. Characterization Of Novel Lytic Bacteriophages Against *Pseudomonas aeruginosa* Clinical Isolates Acta Microbiologica Et Immunologica Hungarica 64 : Suppl. 1 Pp. 186-186. , 1 P. (2017)

7.0. CO-AUTHOR WAIVER

Hereby, I certify that I am familiar with the thesis of the PhD. candidate, **Sarshad Koderi Valappil.** Regarding our jointly published results used for this PhD. desertion, I declare the applicant's contribution was prominent in obtaining the results, and these papers were not used for PhD defense and will be used for at most one PhD dissertation in the future. Furthermore, the Koderi et al. *Frontiers in Microbiology* (DOI:10.3389/fmicb.2021.783722) article will not be used as first but at the most as a co-author paper by anyone of the authors.

List of publications:

Koderi Valappil, S., Shetty, P., Deim, Z., Terhes, G., Urbán, E., Váczi, S., Patai, R., Polgár, T., Pertics, B. Z., Schneider, G., Kovács, T., & Rákhely, G (2021). Survival Comes at a Cost: A Coevolution of Phage and Its Host Leads to Phage Resistance and Antibiotic Sensitivity of Pseudomonas aeruginosa Multidrug Resistant Strains. *Frontiers in Microbiology* 12, 1–17. DOI:10.3389/fmicb.2021.783722.

Horváth, M., Kovács, T., **Koderi Valappil, S**., Ábrahám, H., Rákhely, G., and Schneider, G. (2020). Identification of a newly isolated lytic bacteriophage against K24 capsular type, carbapenem resistant Klebsiella pneumoniae isolates. *Scientific Reports* 10. DOI:10.1038/s41598-020-62691-8.

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