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Doctoral (PhD) thesis

Characterization of Klebsiella and Staphylococcus bacteriophages and their depolymerizing enzymes and examination of their potential antibacterial effect

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INTRODUCTION

1. Examined bacteria

Klebsiella pneumoniae is part of the normal human microbiome which colonizes mucoid surfaces, from where it can disperse into various tissues, causing serious infections, mainly in case of hospitalized patients with a repressed immune system, hence *K. pneumoniae* is regarded as an opportunistic, nosocomial pathogen. Nowadays it is responsible for healthcare-associated (HA) pneumonias, urinary tract, wound, catheter-related and blood infections, which often lead to septic shock and other life-threatening conditions (Shon *et al*., 2013; Corsaro *et al*., 2005; Podshun & Ullmann, 1998; Paczosa & Mecsas, 2016). Besides HA, in the last few years, community-acquired (CA) infections have started to increase due to hypervirulent strains, such as pyogenic liver abscess (PLA), metastatic meningitis and endophthalmitis (Shon *et al*., 2013; Hsu *et al*., 2013).

Most *K. pneumoniae* isolates are multiresistant, which means they are resistant to at least three antibiotics categories. Besides classical *K. pneumoniae* (cKP), hypervirulent (hvKP) strains that emerged in the last decades have an even broader resistance and they often result in fatal outcomes in case of originally non-fatal infections, due to the ineffectiveness of antibiotic treatment (Shon *et al*., 2013).

One of the most important virulence factor of *K. pneumoniae* is the capsule (capsular polysaccharide, CPS) (Corsaro *et al*., 2005), which defends the bacteria against host immune system (Tomás *et al*., 2015; March *et al*., 2013), desiccation, lyzozymes, bacteriophages, antibiotics; it facilitates adhesion to surfaces and it is the bulk of biofilm formation, which allows the development of a multicellular prokaryote community, in which cells are operating adhered to one another or to a surface. In opposition to the form of their planktonic counterparts, cells in a biofilm have basically different physiological conditions, which means enhanced protection against environmental effects. For this reason, biofilm formation have a major role in human diseases (Archer *et al*., 2011; Lister & Horswill, 2014).

Out of approximately 80 different capsular serotypes (K antigens) of *K. pneumoniae*, K1 and K2 is the most frequently isolated from patients, their resistance and virulence is prominent, usually defined as hypervirulent (hvKP) (Nassif *et al*., 1989; Lin *et al*., 2004).

Staphylococcus aureus is also part of the normal microbiome which colonizes the 50% of human adult population, providing a source for systemic infections, mainly in patients with a compromised immune system. (Mehraj *et al*., 2016; Weidenmaier *et al*., 2012; Archer *et al*., 2011). *S. aureus* is a pyogenic bacterium, causing skin and soft tissue infections (impetigo, folliculitis, abscesses, wound infections), bloodstream infections, endocarditis, pneumonia, bone marrow inflammation, septic joint infections. Toxin-mediated infections are also possible, leading to toxic shock syndrome (Mehraj *et al*., 2016; Chambers & DeLeo, 2009; Lakhundi & Zhang, 2018).

From the clinical point-of-view, the most threatening issue with *S. aureus* is that it can gain

outstandingly high level of resistance to different groups of antibiotics, thanks to its several virulence factors which can be expressed in various combinations. Some of these are bound to the cell surface facilitating adhesion and inhibiting phagocytosis, e.g. parts of the cell wall (peptidoglycan, PG), which are pyogenic and complement activators causing abscess formation and dissolving platelets (Lowy, 1998; Choi *et al*., 2014).

Resembling *Klebsiellae*, *S. aureus* is also able to colonize biotic (skin, mucosa) and abiotic (catheters, implants) surfaces. It can form a multilayer biofilm which is embedded into the glycocalyx or a slime layer. *S. aureus* biofilm-related infections are osteomyelitis, medical implant-related infections, periodontitis, ophthalmitis (Archer *et al*., 2011; Lister & Horswill, 2014; Gutierrez *et al*., 2014).

Both above-mentioned pathogens are typically **multiresistant**, **nosocomial** and **biofilm-forming**. These traits are related to each other: biofilms adhering to different organic or artificial surfaces provide the bacteria a defensive layer which is impenetrable to antibiotics, dedicated to act on different metabolic pathways or structures of the bacterial cell.

Therefore due to the spread of antibiotic resistance, the value of alternative antibiotic agents is escalating. The antibacterial use of bacteriophages and their recombinant enzymes is a developing field with huge potential to battle different multiresistant and hypervirulent bacterial strains.

2. Bacteriophages

Bacteriophages (shortly: phages) are viruses of bacteria (Abedon *et al*., 2011). Tailed phages („*Caudovirales"*) possess a binal virion: the protein capsid builds up from a head and a tail, enveloping the double-stranded DNA, and helping in the attachment of the virion to bacteria and injecting the nucleic acid into the host cell, respectively.

Regarding their cycle, phages are either virulent or temperate. Obligate virulent phages employ the lytic phage cycle, which eventually leads to the destruction of the host bacterial cell (Parasion *et al*., 2014; Yan *et al*., 2014). Temperate phages may apply the lysogenic route by integrating their genome into the bacterial chromosome (lysogen) and replicate with the bacteria as a prophage. Stress or other factors can induce the prophage to turn back to be lytic (Cieslik *et al*., 2021).

Phage therapy has many advantages over antibiotics. For instance, isolating phages is way cheaper and swifter than synthetizing antibiotics, especially against resistant strains. Obligate lytic phages are bactericidal in contrast with many bacteriostatic drugs, which are only repressing bacterial growth. In this regard, phage amount is self-regulated: due to self-replication, it is dose-independent. Moreover, phages have altogether higher specificity, thus they do not eradicate normal microbiome, and are able to specifically target one strain or degrade biofilms. Phage application is flexible: their use in combination with other phages or antibiotics is possible and provides many opportunities. Last but not least, they are natural agents: they are originally participants of the arms' race with bacteria, and their use does not pollute the environment by enabling the spread on antibiotic resistant strains (Loc-Carillo

& Abedon, 2011; Vázquez *et al*., 2022).

3. Depolymerizing phage enzymes

A whole arsenal of enzymes are employed by the phage to recognize and degrade different bacterial structures during different phases the lytic phage cycle (Yan *et al*., 2014; Roach & Donovan, 2015).

To overcome encapsulated bacteria, the first layer of defence to break is the capsule (capsular polysaccharide, CPS), ore the less dense slime layer (exopolysaccharide, EPS) before attachment to the cell is possible. Some phages have capsular depolymerase enzymes to degrade these structures. These proteins with hydrolase or lyase activity break up capsular polymers to oligo- or monosaccharides (Parasion *et al*., 2014; Pires *et al*., 2016). Depolymerases can be **virion-integrated structural proteins**, which are usually tail proteins, fibers or spikes, but can be also present on the phage neck or head. Depolymerases can also be produced as **originally soluble proteins** during lysis. Both forms are able to diffuse freely in an agar matrix (Nobrega *et al*., 2018; Blundell-Hunter *et al*., 2021; Knecht *et al*., 2020).

Destruction of peptidoglycan (PG) cell-wall is conducted by peptidoglycan hydrolases, shortly lysins. In the beginning of the phage infection, after attachment, **virion-associated lysins (VALs)**, locally penetrate the cell wall (lysis from without) to inject viral DNA into the cell. At the end of the phage cycle, **endolysin**s are employed to destruct the cell wall non-locally (lysis from within) to release progeny phage particles (Latka *et al*., 2017; Fernandes & Sao-José, 2018; Sao-José, 2018).

Recombinant phage proteins with degrading enzymatic activity have a great therapeutic potential. Every drawbacks of phage therapy can be avoided by application of such proteins. Characterizing proteins structurally, pharmacologically and to produce and purify an uniform antimicrobial agent is a way more controllable and foreseeable process than in case of whole phages. Recombinant VALs and endolysins are expected to successfully combat infections caused by Gram positive bacteria (e.g. MRSA) Against Gram negative hypervirulent strain (e.g. *K. pneumoniae*) capsules and biofilms, depolymerases can be resourceful weapons (Nelson *et al*., 2012; Rodríguez-Rubio *et al*., 2012; Kashani *et al*., 2017; Oliveira *et al*., 2018; Drulis-Kawa *et al*., 2012; Fenton *et al*., 2010; Schmelcher *et al*., 2012)).

GOALS OF THE STUDY

Our goal was to isolate and analyse such bacteriophages, which are effective against *Klebsiella pneumoniae* with the K2 capsule serotype, and based on the same guidelines, isolation and characterization of phages, effective against *Staphylococcus aureus*. We aimed to examine the potentials of the antibacterial use of the phages.

For this, we performed the following steps:

- Basic characterization of phages: morphology, adaptation limits, growth and molecular properties.
- Host spectrum of the phages, testing specificity and mapping receptors.
- Effect of phages on biofilm formation and eradication.
- Expressing and characterizing phage enzymes: depolymerases against *K. pneumoniae* capsule, and lysins against *S. aureus* peptidoglycan.
- Examining the combined effect of phages and recombinant phage enzymes.

MATERIALS AND METHODS

1. Isolation and purification of bacteriophages

Phages were isolated with the traditional method (Twest & Kropinski, 2009), Klebsiella phages (B1, 731) from wastewater, Staphylococcus phages (A1, R4) from soil samples. Host of Klebsiella phages B1 and 731 were *K. pneumoniae* 52145 strain with K2 serotype, and capsule-deficient mutant 52145-Δ*wca*$_{K2}$, respectively. Host of Staphylococcus phages A1 and R4 were *S. aureus* JSNZ and an MRSA with code 06-01019, respectively. Phage presence and activity was detected by titrating the phage suspensions and spotting diluted samples onto the host bacterial lawn (spot test), where phage activity was visible by a clear lysis. Phage suspensions were purified by agar overlay method, individual plaques from the lawn were excised, co-incubated with the host, and purified (centrifugation, chloroform-treatment).

2. Morphology

Transmission electronmicroscopic (TEM) images were captured by a JEM-1200EX II (JEOL USA Inc., Peabody, MA, USA) microscope in UPMS Central Electron Microscope Laboratory. Individual plaque morphologies were examined by agar overlaying the phages on their hosts.

3. Examination of growth properties

For phage growth curves, burst size determination, and phage adsorption assay, a previously published method was used (D'Andrea *et al*., 2017). Latency is the time between the phage-infection and the appearance of the first phage progeny particles. Burst size is the number of phage particles under the plateau phase, divided by the initial number of infected bacteria.

4. Phage DNA extraction, genome sequence and bioinformatic analysis

Phage DNA was extracted from suspensions with 10^8-10^9 PFU/ml titer, using our own protocol. Sequencing was performed at the laboratory of Enviroinvest Corporation (Pécs, Hungary).

Raw nucleotide sequences were annotated, homologies, basic genome analysis and phylogenetic properties were examined on different online platforms.

5. Phage adaptation limits

Phages were exposed to different temperatures to test the durability of their activity. For detergent-tolerance, phages were mixed with different agents: Tween 80, Tween 20, Triton X-100, 10% SDS.

6. Host range, resistance, receptor mapping

Host range of the phages was determined by spot testing the concentrated phage suspensions on lawns of different strains. 105 *K. pneumoniae*, and 166 *S. aureus* isolates were tested. Susceptible

isolates were further tested by titer determination and efficiency of plating (EOP) measurement.

To test if resistant colonies are *K. pneumoniae,* they were tested by MALDI-TOF MS (matrix-assisted laser desorption/ionization–time of flight mass spectrometry) (Vitek MS, Biomerieux, Marcy-l'Étoile, France) (Maasz *et al*., 2020). To investigate the resistance-gaining, a previously described method was used (D'Andrea *et al*., 2014).

We detected the lipopolysaccharide-capsular polysaccharide (LPS-CPS) amount of the four 52145 *K. pneumoniae* mutants by LPS isolation, and processed the samples by 1-dimension protein gel electrophoresis (SDS-PAGE, sodium dodecylsulfate-polyacrylamide gel electrophoresis), which was stained by silver-nitrate treatment.

7. Investigating biofilm formation and biofilm degradation

Biofilm growth, biofilm staining, phage effect on biofilms: Both *K. pneumoniae* and *S. aureus* strains were grown in 96-well tissue culture polystirol plates (Sarstedt, Nümbrecht, Germany) to test biofilm formation or absence. For this, overnight (ON) cultures grown in the wells were washed with PBS and fixed with 2% formaline, subsequently stained with crystal violet. Optical density (OD) was measured with ELISA reader (λ=595 nm).

Phage effect on biofilm of different strains were examined as follows: tissue culture plates with grown cultures were washed with PBS, then 100-200 µl concentrated or diluted phage suspensions were added. After ON incubation, phages were removed and the plate was stained by the above mentioned method.

Biofilm-adapted A1 phage (A1-BA) preparation: *S. aureus* 53. isolate was selected due to its ideal biofilm-growing properties. One A1 phage clone was propagated on the biofilm of this isolate in a 6-well plate, in many cycles. The phage was removed and added to the biofilm in a following step. This is how we gained A1-BA phage. Original A1 phage was termed A1-E (as „eredeti") after, in the comparison experiments.

Phage A1-E and A1-BA were compared in different ways. Confocal laser scanning microscopy (CLSM) and scanning electronmicroscopy (SEM) was performed for qualitative and quantitative analysis of the level of biofilm eradication. The two phage variations were also compared by testing their host range and adaptation limits.

8. Producing and testing recombinant phage proteins

We wanted to express different bacteria-degrading enzymes of the Klebsiella and Staphylococcus phages. From Klebsiella phage B1, one K2 capsule-specific depolymerase, from phage 731 two putative depolymerase were obtained, while from Staphylococcus phage A1, we selected two cell wall-degrading enzymes, an endolysin and a VAL.

Homology findings, conserved region predictions, 3D model and secondary structure predictions of the selected proteins were performed. Using phage DNA as template, selected genes were amplified,

cloned and expressed. Proteins were purified by polyhistidine-tag based affinity chromatography, and for more effective detection, they were concentrated. Protein presence was detected on an SDS gel. After separation, protein bands were visualized by Coomassie Brillant Blue or silver-nitrate staining. Enzymatic activity of the expressed proteins was detected by spot testing on different strains.

Activity of B1dep depolymerase on a gel was presented by isolation of capsular polysaccharides (CPS) of *Klebsiella* strains by the hot water-phenol extraction method using two different protocols (Hsieh *et al*., 2017; Dunstan *et al*., 2021), subsequently treated with the B1dep depolymerase, followed by the running on SDS gel.

9. *In vivo* experiments: nasal colonization infection murine model, B1 phage rescue

In vivo experiments were performed as previously described (Horváth et al., 2020) with some modifications. Animals were cared for in accordance with the guidelines of the European Federation for Laboratory Animal Science Associations (FELASA) and all procedures, care and handling of the animals were ap-proved by the Animal Welfare Committee of University of Pécs (Permit number: BA02/2000-37/2015). For the experiments, 6-7 weeks-old (16-22 g) BALB/c mice were used. Mice were anesthetized with COMBO solution (1:1:0.015 v/v/v physiological saline: Calypsol (Richter Gedeon, Hungary): 2% Primazin (Alfasan, Woerden, Holland)). Seven groups were treated with *K. pneumoniae* 52145 WT by injecting into the nose. Six groups were treated with B1 phage at different time points pre/post-infection. One group was not treated with the phage and served as a positive control. General conditions (mass and vitality) and survival of the mice were monitored for 16 days. At the end, tissue homogenates were processed for bacterial and phage presence verification.

RESULTS

I. Properties of Klebsiella phages

1. General traits of phage B1 and 731

Both B1 and 731 phages have a ≈50-60 nm diameter head and a ≈150-200 nm long, flexible tail, these traits make them belong into the former *Siphoviridae* family. They both form 1.5-2.5 mm individual plaques on their host bacterial lawn. In case of B1, on the 52145 WT lawn, plaques are surrounded by semi-translucent, relatively big (6-10 mm) bulls-eye clearing rings, so-called halos, which are growing in diameter after time. This indicates no lysis, but degradation of capsular polysaccharides by the soluble phage depolymerases, which can diffuse freely in the agar. Plaques of phage 731 on the lawn of 52145-Δ*wca*$_{K2}$ have no halo zones, since this mutant has no capsule. Phage 731 did not produce halo even on the strain 53.8 with K33 capsule; plaques on this lawn are smaller, 0.2–0.5 mm in diameter.

Determining latency period and burst size, the two phages were propagated on their hosts and drew a one-step growth curve, which was triphasic in all cases, with a latency, a logarithmic and a plateau phase. These, and more precise values of burst sizes are in accordance with values of different Klebsiella phages in the literature. Phage 731 on its capsule-less host reached a less-dynamic growth phase and a smaller burst size than on the strain with K33 serotype. All phages had bigger adsorption values than 97-99%.

The genetic analysis is the most important part of the characterization of a bacteriophage. Both phages have a double-stranded, linear DNA, and have minor differences in their restriction pattern. All Klebsiella phages that are homologous with B1 and 731 are members of the *Webervirus* genus, which is classified into *Drexlerviridae* < *Caudoviricetes* < *Uroviricota* < *Heunggongvirae* < *Duplodnaviria* (ICTV taxonomy release, March of 2022).

2. Host range and receptors of phage B1, B1dep protein, and phage 731.

For host range testing, 105 isolates were used, altogether with 37 different capsule serotypes. This examination was useful to determine the capsule specificity of the phages.

Phage B1 has a narrow spectrum, it only evoked full, clear lysis on its host strain, 52145 WT with high efficiency. It was also detected on the LPS mutant 52145-Δ*waaL* (O-:K2), showing also halo formation. On the rest of the susceptible isolates, phage B1 only resulted a halo spot without lysis. This was fully covered the spectrum of B1dep. Halo spots were growing by time.

In the case of **phage 731,** even though it was isolated on the capsule-less 52145-Δ*wca*$_{K2}$ mutant, a **broader host range** was observed than expected. Altogether 8 serotypes were susceptible on different levels. Halo-less, clear lysis was evoked with high efficiency on strains with K21, K24 and K33 capsule. High efficiency was also measured on double mutant 52145-Δ*wca*$_{K2}$Δ*waaL* (O-:K-). The phage

presented more temperate lytic activity on strains with serotype K11, K20, K21, K27, K27, K51 and K64. Spots were less translucent ('veiled'), did not grow by time, and produced individual plaques upon dilution, which mean incomplete lysis.

Effect of the Klebsiella phages on the 52145 variants was useful for resistance and phage receptor mapping.

Phages are not active on each other's host: **B1 had no effect on capsule-less mutant, and 731 had none on the wild type**. First, this lead to the conclusion that the capsule enables the effect of B1 phage and blocks the attachment of 731. B1 resistant wild type colonies analyzed by MALDI-TOF MS proved that these are *K. pneumoniae*, 40 selected and cultured colonies were all sensitive to phage 731, suggesting that they gained resistance by the loss of capsule, thus leaving the membrane receptor of phage 731 unprotected.

Phage B1, however, was active on the double mutant 52145-Δ*wca*$_{K2}$Δ*waaL* (O-:K-) variant with lower efficiency, but full, clear lysis. Adsorption was not much lower than on WT strain. This meant that for phage B1 infection, the K2 capsule is not necessary, capsule is not the exceptional, rather a secondary (reversible) receptor of the phage, and the primary (irreversible) receptor is rather a membrane protein.

By isolating the LPS from the four 52145 variants, the capsule mutant (O1:K-) has much more dense bands representing the O side chains. This indicates a rise in the O-antigen amount due to the lack of capsular polysaccharides. The LPS mutant (O-:K2) and double mutant (O-:K-) had no such bands at all, meaning the total absence of the O side chains. The **ineffectiveness of phage B1 on the capsule-less mutant** (O1:K-) is not because the capsule is not present as a receptor, rather due to the fact that long O chains are blocking the phage's path to bind to the primary membrane receptors.

Phage B1 was propagated on the double mutant (DM) variant with different conditions (lawn and culture), and we observed that the originally low titer of the phage was enhanced on the double mutant, while the halo size decreased on the WT strain. This latter means the decrease of depolymerase production and yield. We created many phage-variants, termed B1-dm phages. We concluded that phage B1 has two morphotypes: in culture and on lawn, co-incubated with DM is more beneficial for morphotype 2. If DM is present in the propagation, morphotype 2 becomes dominant, which has bigger plaque size but smaller halo than morphotype 1.

3. Depolymerases of phage B1 and 731

In case of the Klebsiella phages, identified and expressed proteins were capsule depolymerases, simply, because we wanted to target the capsule, the most significant virulence factor, biofilm component and first line of defense of *K. pneumoniae*. Depolymerase of phage B1 was identified, its expression was performed, the activity and host spectrum of the recombinant protein was tested. Depolymerase of phage 731 was identified, however, successful expression was not yet concluded. Instead, we published its molecular properties by bioinformatic methods, and mapped the potential

depolymerase coding regions of the homologous phages. In the future, we want to express and test the depolymerases of phage 731 as well.

Depolymerase of phage B1. Activity of the recombinant B1dep protein on the 52145 WT lawn is limited to the degradation of the K2 capsule, the enzyme itself is not lytic. Capsule degradation is also detectable after the treatment of the isolated CPS with B1dep on SDS gel, where the depolymerase visibly reduced CPS amount compared to the positive control WT strain.

The spread and growth of the halo spots was also observable. Translucency decreased when diluted protein samples were spotted, which also reduced spreading rate over the lawn. B1dep is a soluble protein, which diffuses freely in the agar, unlike the whole phage particles.

B1dep also showed effectivity when it was spotted on a 1-day-old 52145 lawn, and it visibly starts to degrade capsule in 5 minutes on room temperature. This was observable on every strains susceptible to B1dep.

The protein had a supportive effect with phages, originally lacking capsule degrading capabilities. On 52145 WT lawn, B1dep results in a halo spot, so phage 731 is able to lyse the capsule-deprived 52145 lawn, just as in the case of the capsule-less mutant or the B1 resistant colonies. B1dep also supported the lytic effect of a different phage, *Webervirus 13*, which is active on 40 different strains with K24 (Horváth *et al*., 2020). Phage 13 was not active on the 52145, but produced clear lysis when it was co-spotted with B1dep. Supplemented activity of phage 731 and 13 on the 52145 strain means that by degrading capsule *'a',* lytic spectrum of phages with depolymerases specific for *'b'* and '*c*' but not for *'a'* capsule, can be expanded to '*a*+*b*+*c*', since phages will have their way to susceptible membrane receptors, which were masked by capsule *'a'* before.

Putative depolymerases of phage 731. Polyvalence of phage 731 on different capsular serotypes lead to the assumption that it embarks more depolymerases. The mapping of putative depolymerases in the homologous Klebsiella phages of the *Webervirus* genus lead to assume *orf22* and *orf17* of phage 731 to be depolymerases. *Orf22* is a 2919 bp long gene, coding for a 102.9 kDa, 972 aa long protein, annotated as a phage tail fiber, and has a conserved peptidase domain. *Orf17* is a 738 bp long gene, coding for a 28.4 kDa, 245 aa long protein, embarking conserved domains with catalytic isopeptidase and hydrolase activity.

4. Effect of phage 731 on the biofilm of *K. pneumoniae* strains

Degrading activity of phages on biofilm is a significant trait, which we wanted to examine. Phage 731 showed no degrading effect on the grown biofilms of strains with K21, K24 and K33 serotype. It did, however, inhibited biofilm formation of K33, just like in case of 52145 K-, in which case the eradication of the grown biofilm is also significant.

5. Adaptation limits of phage B1 to environmental effects

From the practical point-of-view, adaptation limits of phage B1 are important to investigate.

Phage B1 can endure 50 and 60°C for 1 hour, showing no titer loss (10^9 PFU/ml). Long-term experiments showed that there is a gradual titer drop at 42 and 37°C, losing activity totally after 9 months. This time interval did not, but 3 years at 23 and -20°C demolished the activity, while at -80°C and 4°C the titer did not drop at all.

6. *In vivo* results: nasal colonization and phage-rescue

Therapeutic potential of phage B1 was revealed in a murine nasal cavity colonization model. The effect of the phage strongly depended on the time that had passed between bacterial infection and phage administration.

K. pneumoniae 52145 infection resulted the mice in the positive control group to die within 3 days (LD_{50}=36 h), and the survival rate drastically dropped in other where the phage suspension was injected post-infection. All these mice died within 96 h.

Survival rate of the pre-treated group was 100% after 9 days and dropped to 50% by day 13. Sixteen days after bacterial challenge, the nasal cavity, lungs, spleen, kidneys and the brain of the two surviving mice contained bacteria in relatively high numbers. The nasal cavity was colonized by the 52145 strain, which caused a systemic infection, and the bacteria were still present in the distant organs of the surviving mice. No phage activity was observed in any of the processed organs, neither capsule-less 52145 mutants, which latter means that the capsule is necessary for the bacteria to survive and colonize in the body.

II. Properties of Staphylococcus phages

1. General traits of phage A1 and R4

Phage A1 has a 150-200 nm long, contractile tail; this is a property of the former *Myoviridae* family. It has an icosahedral head with a diameter of 80-90 nm. Phage plaques are clear, 0.5-1 mm wide, halo is not observable around them. Phage R4 has the traits of the former *Siphoviridae* family: a flexible, non-contractile, 300 nm long tail, and a 100×50 nm, prolate head. Plaques are clear, 0.5 mm in diameter.

Concerning one-step growth, A1 has a burst between 20 and 30 minutes, the burst size is ≈1000, latency is 20 minutes, plateau phase starts at 30 min.

Both phages have double-stranded, linear DNA, but they differ in size: A1 has a 141 kbp, R4 has a 45 kbp long genome, number of coding regions are 250 and 69, respectively. From only this information, one can conclude that A1 is a myovirus, while R4 is a siphovirus (II. and III. class Staphylococcal phages). The two phages are also differing in their restriction pattern. R4 genome has a lysogeny module, with genes (integrase) responsible for integration into the bacterial chromosome, so the phage is

probably temperate and can follow lysogenic cycle. This trait is characteristic for Stahylococcal siphoviruses, which implies concerns with their therapeutic use. By contrast, Staphylococcal myoviruses are generally obligate lytic (virulent) – by this mean, they are more suitable for therapeutic purposes. The genome of A1 does not embark an integrase gene.

Phage R4 shows homology with phages of the *Triavirus* genus, classified into *Duplodnaviria › Heunggongvirae › Uroviricota › Caudoviricetes › Triavirus* (ICTV 2022). Phage A1 is a *Kayvirus*, classified into *Duplodnaviria › Heunggongvirae › Uroviricota › Caudoviricetes › Herelleviridae › Twortvirinae › Kayvirus* (ICTV 2022).

2. Lysins of phages A1 and R4

With the Staphylococcus phages, our goal was to produce phage proteins, which are able to specifically target the cell wall peptidoglycan of *S. aureus*, thus allowing the immediate lysis from without and their direct application as a lytic agent. The cloned and expressed two lysins of phage A1 was not successfully detected yet.

Endolysins were coded by *orf27* of phage R4, and *orf183* of phage A1. A virion-associated lysin (VAL) was coded by *orf4* of phage A1. All three proteins possess conserved domains: one or more enzymatically active amidase, and cell-wall binding domains. Staphylococcal phage lysins are typically modular, which was observed at our three proteins as well. Combining different enzymatically active domains often results in synergy, lysins can also be combined with antibiotics (Nelson *et al*., 2012).

3. Adaptation of Staphylococcus A1 phage to biofilm (A1-E vs. A1-BA)

One of the main aim with phage A1 was to examine if it is able to be propagated specifically on biofilm, does it result in any difference between the original and propagated phage versions, and is it possible to 'train' the biofilm-degrading ability by this way. For this, first an optimally biofilm-producing strain had to be selected, then the phage-growth on biofilm had to be optimized, then comparison of the original (A1-E) and biofilm-adapted (A1-BA) phage had to be performed by different methods.

The simplest biofilm-measuring method is crystal violet staining and absorbance measurement. Due to insignificant results, we focused on other methods to detect and present the difference between the A1-E and A1-BA phages. The most spectacular results of the different levels of biofilm degradation capabilities were gained via confocal laser-scanning microscopic images, and from the data originated from these measurements. Scanning electronmicroscopic pictures supported these phenomena. Biofilm of the 53. *S. aureus* isolate forms a highly structured matrix. Treating this with phage A1-BA results a disintegrated, highly dispersed structure, indicating the collapse of biofilm. Between this and control biofilm, treatment with phage A1-E takes an interim condition: the biofilm is not-that-much disintegrated, it's aggregates are bigger, however, still more fractured and less homogenous than the control biofilm. Phage A1-E executes a smaller eradicating activity than A1-BA. On SEM images, it is

also observable, that phage-treated samples have fewer intact bacterial cells. Biofilm parameters (mean thickness, biomass, maximal thickness, biovolume) also show quantitatively the gradual decrease in the control – A1-E – A1-BA direction, which, on one hand, means the biofilm-degrading activity of both phages, on the other hand, higher level of eradication by phage A1-BA.

Difference between the two phages was also presented by the host range differences, which was tested by spotting the phages on 166 isolates. Ratio of susceptible and resistant isolates was the indicator of the change in effectiveness of the A1 phage. There were many different lytic patterns between clear lysis and no lysis, thus we constructed a system to standardize the results of spot testing of Staphylococcus phages.

Phage A1-E was effective on 25/165 strains (14,5%), ineffective on 59/165 strains (35,8%). The remaining 81 strains (≈50%) showed partial lysis. Phage A1-BA was effective on 74/165 (44,8%), ineffective on 32/165 (19,4%). The remaining 59 strain (≈35%) was partially susceptible. This showed that phage A1-BA is altogether more effective, hence number of clear lyses increased, number of ineffective strains decreased, compared to A1-E.

The two host ranges were also examined in a more delicate manner. Effectivity was compared in the isolate level, the differences were summed up. There is a shift in host ranges towards A1-BA, which means greater lytic clearance.

Changes in phage robustness can be best demonstrated by comparing adaptation limits. Heat tolerance experiments showed that A1-E is less resistant to different temperatures than A1-BA. After 2 years, both are totally losing activity at all examined temperatures. A1-E phage was tested at 60°C and 50°C for 1 hour, and it turned out that it can tolerate this latter without a titer drop, but it gradually decreases at 60°C.

New results achieved during my PhD work

1. Detailed pheno- and genotypic characterization of two lytic bacteriophages was performed: one active on *Klebsiella pneumoniae* strains with K2 serotype (*Webervirus B1*) with a narrow spectrum, and one not active on K2 strains (*Webervirus 731*) with a broader host range.

2. Capsule depolymerase of phage B1 (B1dep) was characterized, it's molecular properties and activity was presented. The synergistic effect of the enzyme on a K2 strain with other, originally not-K2-specific phages was demonstrated. Two putative depolymerases of phage 731 were also identified and characterized by bioinformatic methods.

3. With the help of the two phages and the four 52145 strain variants, we mapped the phage receptor-host interactions: we showed that the capsule can act as a barrier or have a recruiting function, and it is not necessary for phage B1 adsorption.

4. We provided detailed pheno- and genotypic characterization of two bacteriophages, active on different *Staphylococcus aureus* strains (*Triavirus R4, Kayvirus A1*).

5. Lysins of the two Staphylococcus phages were structurally characterized.

6. We experimentally proved that the A1 phage adapted to *S. aureus* biofilm (A1-BA) has a broader host range, better heat tolerance and stronger biofilm-degrading ability than the original phage (A1-E).

Our results indicate that our phages and the already expressed or identified phage enzymes are promising candidates for expanding the rich field of global phage research in the future.

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Publications on which the PhD thesis is based

Pertics, B.Z.; Kovács, T.; Schneider, G. Characterization of a Lytic Bacteriophage and Demonstration of Its Combined Lytic Effect with a K2 Depolymerase on the Hypervirulent *Klebsiella pneumoniae* Strain 52145. *Microorganisms* **2023**, *11*(3), 669. Published 2023 Mar 6. doi:10.3390/microorganisms11030669 ***IF=4,5***

Pertics, B. Z.; Cox, A.; Nyúl, A.; Szamek, N.; Kovács, T.; Schneider, G. Isolation and Characterization of a Novel Lytic Bacteriophage against the K2 Capsule-Expressing Hypervirulent *Klebsiella pneumoniae* Strain 52145, and Identification of Its Functional Depolymerase. *Microorganisms* **2021**, *9*(3), 650. https://doi.org/10.3390/microorganisms9030650 ***IF=4,8***

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List of other publications

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